Journal of Cellular Biochemistry 90:1128–1139 (2003)

Induction of *GADD* Gene Expression by Phenethylisothiocyanate in Human Colon Adenocarcinoma Cells

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Abstract Phenethylisothiocyanate (PEITC), a potential cancer chemopreventive agent, induces colon cancer cell death, but the mechanism is not entirely clear. Therefore, the aim of this study was to further clarify the molecular effects of PEITC in causing death of human colon adenocarcinoma cells. When incubated with PEITC, HCT-116 colonocytes showed morphological features characteristic of apoptosis, such as irregular cell shape, translocation of plasma membrane phosphatidylserine, and also chromatin condensation and fragmentation. These changes occurred after single-strand breaks in DNA were detected, suggesting that PEITC induced irreparable DNA damage, which in turn triggered the process of apoptosis. DNA macroarray analysis of a selected small cluster of apoptosis-related genes revealed noticeably higher expression of only GADD45, which was confirmed by gene-specific relative RT-PCR analysis. This led to investigation of other *GADD* gene members possibly affected by PEITC. Whereas GADD34 mRNA expression increased just slightly, there was an appreciable elevation of the mRNA for GADD153, which is recognized as a pro-apoptotic gene. The effect of PEITC on GADD153 was attenuated by either actinomycin D or *N*-acetylcysteine, suggesting that PEITC-induced upregulation of GADD153 mRNA expression was partly at the level of transcriptional activation involving reactive oxygen species. Additionally, PEITC-induced upregulation of GADD153 mRNA expression did not appear to require p53, based on the observation that PEITC also increased GADD153 mRNA expression in HCT-15 colonocytes, which are known to express mutant p53. These findings suggest that PEITC creates an oxidative cellular environment that induces DNA damage and *GADD153* gene activation, which in turn helps trigger apoptosis. J. Cell. Biochem. 90: 1128–1139, 2003.

Key words: apoptosis; GADD153; isothiocyanates; phenethylisothiocyanate

Colorectal cancer is a major health concern in the US. Sensible changes in lifestyle may reduce the chances of developing colorectal cancer. Good dietary habits, such as the ingestion of certain phytochemicals in plant products, may help lower the risk of developing the disease [Block et al., 1992]. In particular, eating *Cruciferae brassica* vegetables (e.g., broccoli, brussels sprouts, cabbage, and cauliflower) provide glucosinolates, which are precursors of isothiocyanates that are thought to be bioactive as anti-cancer agents [Hecht, 2000; Talalay and Fahe, 2001].

At the cellular level, isothiocyanates inhibit cancer cell proliferation. Allyl isothiocyanate had cytostatic and cytotoxic effects on HT29 human colon cancer cells [Musk and Johnson, 1993]. Intriguingly, detransformation of the HT29 cells made them less susceptible to the effects of allyl isothiocyanate. Based on this particular finding, it was hypothesized that ingestion of allyl isothiocyanate may selectively inhibit the growth of transformed cell clones, while not affecting normal cells, within the colonic mucosa and thus prevent colon tumorigenesis. Another isothiocyanate, sulforaphane, caused cell cycle arrest at the G_2/M-phase in HT-29 cells, which was followed later by apoptosis [Gamet-Payrastre et al., 2000].

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Received 11 September 2003; Accepted 12 September 2003
DOI 10.1002/jcb.10733

Similarly, phenethylisothiocyanate (PEITC) induced apoptosis in HeLa human cervical carcinoma cells [Yu et al., 1998], and it was concluded that caspases were activated as a pivotal event for the execution phase of apoptosis. Isothiocyanate-induced apoptosis can occur via the mitochondrial cell death pathway [Nakamura et al., 2002].

The precise molecular mechanism of isothiocyanates in inducing apoptosis is not entirely known, although changes in the expression of certain p53-responsive genes has been suggested to be implicated. It was concluded that elevation of p53 is required in order for PEITC to induce apoptosis [Huang et al., 1998]. The apoptosis-related genes, *Bax* and *Bcl-2*, are upregulated [Miyashita and Reed, 1995] and downregulated [Miyashita et al., 1994], respectively, by p53. Subsequently, it was found that sulforaphane caused apoptosis in Jurkat T-leukemia cells, which was accompanied by changes in expression of the pro-apoptotic *Bax* gene and the anti-apoptotic *Bcl-2* gene [Fimognari et al., 2002]. Thus, these studies would imply that isothiocyanate-induced apoptosis involves p53-mediated modulation of *Bax*/*Bcl-2* gene expression. On the other hand, it is possible that other relevant genes, which may not necessarily require p53 for activation, could be activated in cells treated with isothiocyanates to induce apoptosis.

To consider the possibility that isothiocyanates cause activation of other apoptosis-related genes, the objective of the present study was to ascertain if certain growth arrest and DNA damage-inducible genes (GADD) are upregulated during PEITC-induced apoptosis of human colon adenocarcinoma cells. GADD34, GADD45, and GADD153 were targeted for investigation because they are known to be involved in mediating growth arrest and/or apoptosis [Zhan et al., 1994], which are recognized as cellular events ultimately caused by PEITC. Furthermore, HCT-116 and HCT-15 colonocytes, which express wild-type and mutant p53 [Chinery et al., 1997], respectively, were selected as cellular models with the aim of assessing the requirement of p53 in mediating the effect of PEITC on *GADD* gene expression. We report that PEITC has the capability to increase GADD mRNA expression, which appears to be largely via a redox-sensitive, p53-independent pathway.

MATERIALS AND METHODS

Materials

HCT-116 and HCT-15 human colon adenocarcinoma cells were both purchased from ATCC (Manassas, VA). PEITC and all other common reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Culture and Treatment of Cells

HCT-116 and HCT-15 colonocytes were propagated in McCoy's 5A and RPMI-1640 media (Sigma Chemical Co.), respectively, that were supplemented with 100 ml/L fetal bovine serum (BioWhittaker, Inc., Walkersville, MD), 2 mmol/L glutamine, 0.54 μmol/L fungizone, 100,000 U/L penicillin, and 100 mg/L streptomycin (last four items from Atlanta Biologicals, Atlanta, GA). Upon reaching 70–80% confluency, the cells were exposed to 0–100 μM PEITC for 0–48 h depending on the experiment. In some experiments, the cells were co-incubated with actinomycin D (act-D) (5 μg/ml). In other experiments, cells were pre-treated with 20 mM *N*-acetylcysteine (NAC) for 2 h, prior to exposing the cells to PEITC.

Examination of Cells for Morphological Evidence of Apoptosis

To detect chromatin condensation and fragmentation, cells were fixed with 4% formaldehyde and stained with 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI). The cells were examined using an Olympus BX-60 fluorescence microscope equipped with a UV filter. To detect translocation of plasma membrane phospholipid, cells were labeled with annexin V-alexa fluor 488 (Molecular Probes, Inc., Eugene, OR) following the protocol of the manufacturer. The cells were examined by fluorescence microscopy using a fluorescein filter.

Assessment of DNA Damage

Single-strand breaks in DNA were assessed in the cells by the comet assay, as previously described [Powolny et al., 2001]. Essentially, cells were suspended in agarose gel, and the suspension pipetted onto glass slides. Following treatment of the supported cells with alkaline detergent to remove cellular membranes, the resulting nucleoids were subjected to electrophoresis. The DNA of the nucleoids were stained

with ethidium bromide to permit visualization using an Olympus BX-60 fluorescence microscope.

DNA Macroarray Analysis of Gene Expression

Briefly, total RNA was isolated with the QIAGEN RNeasy system. Following the instructions provided with the Human Stress and Apoptosis GEArray Kit (Superarray, Inc., Bethesda, MD), the isolated RNA was then used to generate biotin-labeled cDNA probes by reverse transcription. After hybridizing the biotinylated c-DNA samples to the gene-specific oligomers pre-spotted on the nylon membrane, the chemiluminescence signal was generated with the streptavidin–alkaline phosphatase system and detected using Kodak X-ray film.

Determination of GADD mRNA Expression

Total RNA was isolated from the cells using a Qiagen RNeasy Mini kit. The expression levels of GADD34, GADD45, and GADD153 mRNA were determined by multiplex relative RT-PCR analysis of total RNA using a Qiagen OneStep RT-PCR kit and gene-specific primers. The PCR primer base sequences for GADD34 and GADD45 [Oh-Hashi et al., 2001] were: GADD34 sense primer, 5′-ATG TAT GGT GAG CGA GAG GC-3′; GADD34 antisense primer, 5′-GCA GTG TCC TTA TCA GAA GGC-3′; GADD45 sense primer, 5′-AGA ACG ACA TCA ACA TCC TGC-3′; GADD45 antisense primer, 5′-AAT GTG GAT TCG TCA CCA GA-3′. The GADD153 PCR primer sequences [Mertani et al., 2001] were: sense, 5′-GCACCTCCCAGAGCCCTCA-CTCTCC-3′ and antisense, 5′-GTCTACTCCA-AGCCTTCCCCCTGCG-3′. Either the β-actin or 18S rRNA QuantumRNA primer/competimer sets (Ambion, Inc., Austin, TX) were utilized to generate the internal standards. The multiplex RT-PCR conditions were 30 min at 50°C followed by 15 min at 95°C (RT), then 0.5 min at 95°C, 0.5 min at 60°C, and 1 min at 72°C (PCR) for 24 cycles (GADD34), 25 cycles (GADD153), or 28 cycles (GADD45). The resulting cDNA products were separated by 2% agarose gel electrophoresis with ethidium bromide staining. For the target genes, the PCR product sizes were 110 bp (est), 150 bp (est), and 422 bp for GADD34, GADD45, and GADD153, respectively. For the internal control genes, the PCR product sizes were 294 and 495 bp for β-actin or 18S rRNA, respectively. Thus, to observe the greatest electrophoretic separation between the targets and internal controls, GADD153 was multiplexed with β-actin, whereas GADD34 and GADD45 were each multiplexed with 18S rRNA, during multiplex RT-PCR.

RESULTS

Morphological Features Characteristic of Apoptosis in PEITC-Treated HCT-116 Colonocytes

Examination of cells by bright-field microscopy (Fig. 1A) revealed that control cells were whole and adherent to the culture dish. After 2 h of incubation with 25 μM PEITC, many of the cells began losing their oblong, jagged shape and began rounding up. Much later (after 48 h), some membrane blebbing and presence of small apoptotic bodies were observable. Translocation of phosphatidylserine (PS) from the inner to outer leaflet of the plasma membrane is known to occur during the early stages of apoptosis. To determine if PS translocation occurred in PEITC-treated HCT-116 colonocytes, cells were exposed to 25 μM PEITC for 12 h, and then the standard annexin V-alexa fluor 488 binding assay was performed to detect possible PS translocation by fluorescence microscopy (Fig. 1B). As can be seen, a greater number of PEITC-treated cells than control cells bound the annexin probe, giving them the brilliant green fluorescence at the cell surface. The number of cells positive for annexin V-binding (per randomly selected, microscopic view) from three different experiments was 16.00 ± 2.09 for PEITC-treated cells and 1.67 ± 1.20 for control cells (mean ± SEM). Chromatin condensation and fragmentation are known to commonly occur during the later "degradative" stages of apoptosis. To determine if chromatin condensation and fragmentation occurred in PEITC-treated HCT-116 colonocytes, we exposed the cells to 25 μM PEITC for 20 h and then examined the cells for signs of nuclear abnormality by fluorescence microscopy (Fig. 1C). As can be seen, many of the PEITC-treated cells had nuclei with condensed and fragmented chromatin, in contrast to the nuclei of control cells. The percentage of cells with changes to chromatin (per 100 randomly scored cells) from three different experiments was 25.0 ± 7.8 for PEITC-treated cells and 3.0 ± 2.6 for control cells (mean ± SEM).

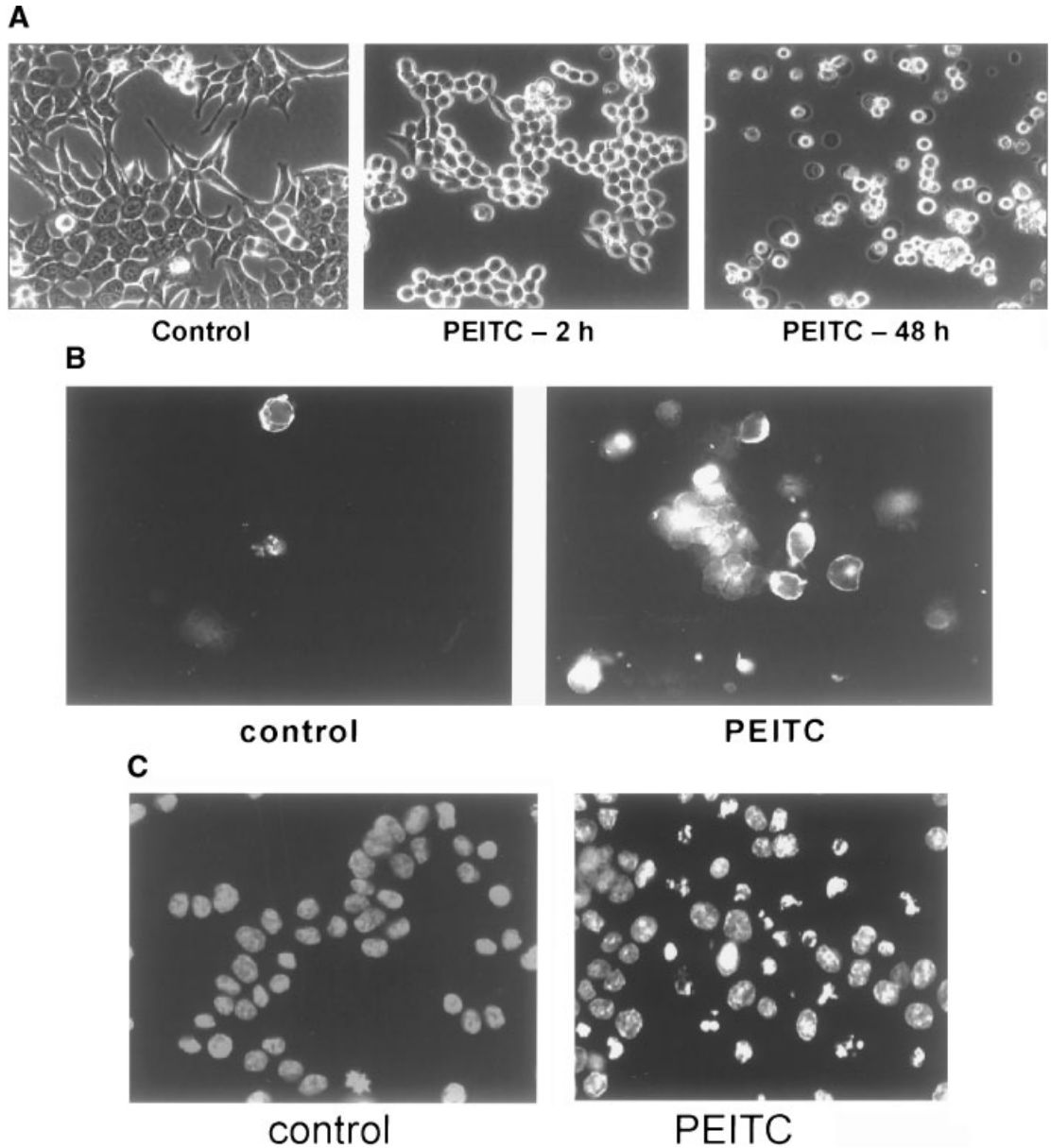


Fig. 1. Evidence of apoptosis in HCT-116 colonocytes exposed to PEITC. **A**: Morphological changes caused by PEITC in HCT-116 colonocytes. The cells were incubated with 25 μM PEITC for 2 and 48 h. They were then examined by light microscopy. **B**: Greater binding of annexin V-alexa fluor 488 to PEITC-treated versus untreated HCT116 colonocytes. The cells were incubated in the presence and absence of 25 μM PEITC for 12 h. They were then labeled with annexin V-alexa fluor 488 and examined by fluorescence microscopy using a fluorescein filter. **C**: Chromatin condensation and fragmentation in HCT116 colonocytes after exposure to PEITC. The cells were incubated without (left photo) or with (right photo) 25 μM PEITC for 20 h. After fixation, they were stained with DAPI and examined by fluorescence microscopy using a UV filter. Similar results as in A, B, and C were produced by two additional different experiments.

DNA Damage in PEITC-Treated HCT-116 Colonocytes

To determine if exposing HCT-116 colonocytes to PEITC induces DNA damage as an indicator of oxidative stress, cells were exposed to 0–100 μM PEITC for 2 h, which is a time that preceded any noticeable effects on GADD153 mRNA levels or signs of cell death. The comet assay was then performed to detect any single-strand breaks in DNA (Fig. 2). As can be seen in the representative photos from fluorescence microscopy, the nucleoids of control cells (0 μM PEITC) were uniformly spherical in shape,

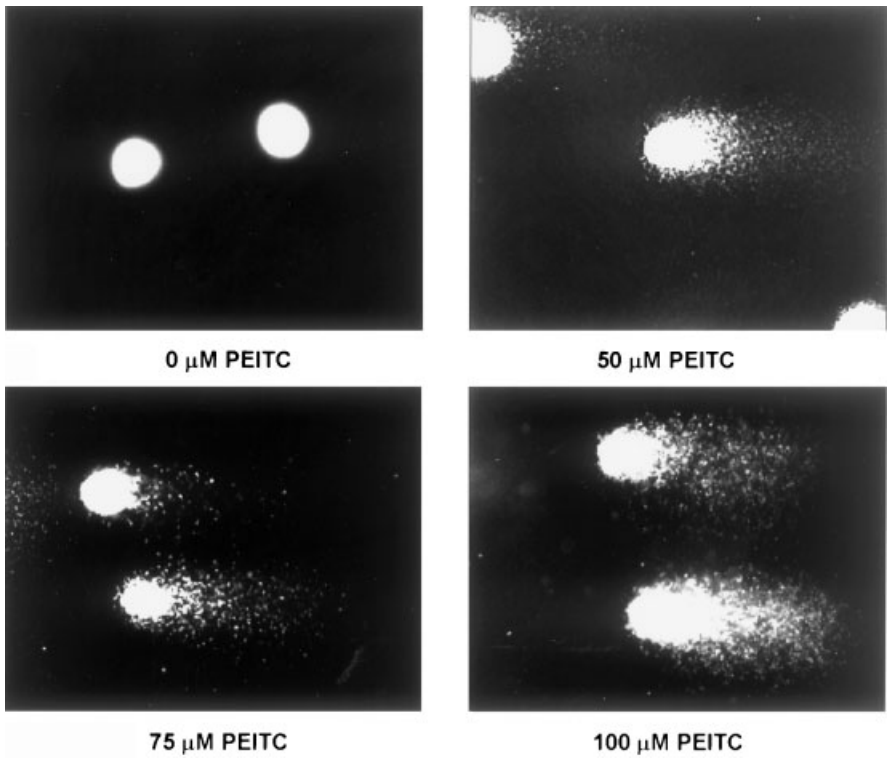
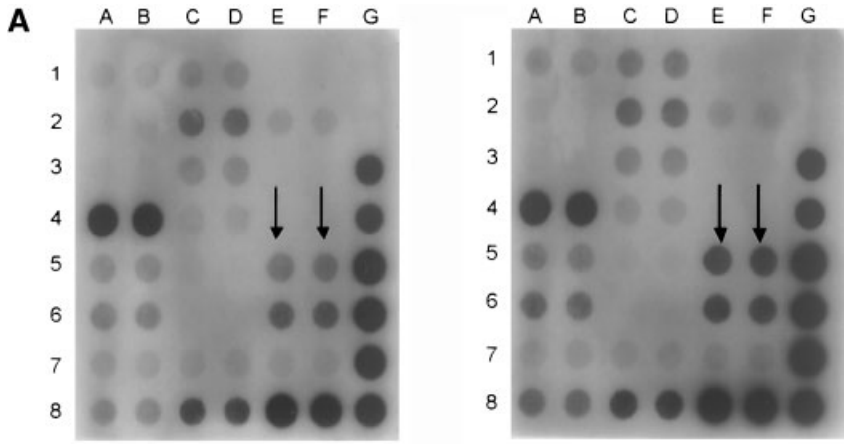


Fig. 2. Greater single-strand breaks in DNA of HCT-116 colonocytes exposed to increasing concentrations of PEITC for 2 h. The cells were exposed to 0–100 μM PEITC for 2 h. The comet assay was then performed as described under Materials and Methods to assess the extent of DNA damage. Similar results were produced by two additional different experiments.

reflecting the absence of any DNA damage. In contrast, the nucleoids of PEITC-treated cells appeared as "comets," reflecting the presence of significant DNA damage as evidenced by the typical "comet tail" that shows up when DNA single-strand breaks exist. Note that the span of the comet tail is proportional to the extent of DNA damage caused by increasing concentrations of PEITC.

Increased GADD45 mRNA Expression in PEITC-Treated HCT-116 Colonocytes

Initially, differential gene expression was assessed in PEITC-treated and untreated cells using a dedicated DNA macroarray system, which was limited to a relatively small cluster of stress/apoptosis-related genes that included GADD45. The exclusive aim of this experiment was to determine if there is a single gene most upregulated in HCT-116 colonocytes by PEITC. As shown by the data (Fig. 3A), the most striking difference can be seen in comparing the duplicate 5E & 5F dot signal intensities (i.e., *GADD45* gene) for control cells (left autorad) versus PEITC-treated cells (right autorad). Such a comparison indicates greater *GADD45* gene expression in PEITC-treated cells, although precise interpretation is somewhat obscured by inadvertent overexposure of the X-ray films. Accordingly, to verify that PEITC actually increased *GADD45* gene expression, the total RNA samples from above were subjected to relative RT-PCR analysis, using gene-specific primers for GADD45 and also 18S rRNA as the internal control (Fig. 3B). The representative agarose gel shows separation of the two PCR products, namely, that corresponding to the mRNA of GADD45 and 18S rRNA. Control cells constitutively expressed low GADD45 mRNA levels. However, PEITC-treated cells expressed noticeably higher GADD45 mRNA levels. Densitometrically, the GADD45:18S rRNA relative fluorescence intensity ratios ($n = 3$) were 0.825 ± 0.111 (mean $\pm$ SEM) for control cells and 3.112 ± 0.309 for PEITC-treated cells, reflecting a 3.8-fold increase in GADD45 mRNA expression. Thus, these findings prompted subsequent efforts to determine the effect of PEITC on other *GADD* gene members.



Gene	Location
Bad	1A,1B
Bax	1C,1D
Bcl-2	1E,1F
Bcl-w	2A,2B
Bcl-x	2C,2D
Casp-1	2E,2F
Casp-10	3A,3B
Casp-3	3C,3D
Casp-5	3E,3F
Casp-6	4A,4B
Casp-7	4C,4D
C-myc	4E,4F
Fas	5A,5B
FasL	5C,5D

Gene	Location
Gadd45	5E,5F
Gadd45b	6A,6B
iNOS	6C,6D
Mdm2	6E,6F
NFkB	7A,7B
P21	7C,7D
P53	7E,7F
DR5	8A,8B
TRAIL	8C,8D
B-actin	3G,4G
GAPDH	5G,6G,7G
	8E,8F,8G
pUC18	1G,2G

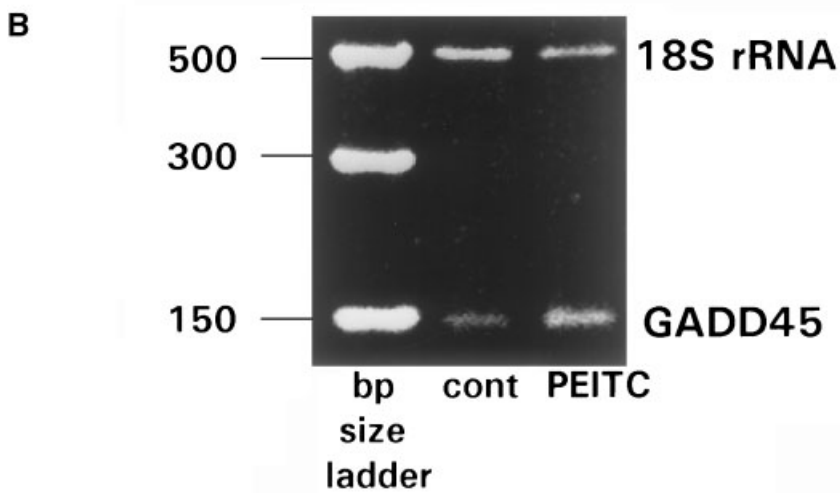


Fig. 3. Effect of PEITC on *GADD45* gene expression in HCT-116 colonocytes. Initial screening was performed by DNA macroarray analysis (**A**). The cells were incubated with 25 μM PEITC for 6 h. Total RNA was isolated for subsequent analysis utilizing a dedicated DNA macroarray system with chemiluminescence detection. The small arrows in each autorad of the macroarrays point to duplicate dots (5E & 5F) representing GADD45. The results are from a single determination. Verification of an effect of PEITC on GADD45 was achieved by relative RT-PCR analysis (**B**) using gene specific primers for the target gene *GADD45* and the internal control gene 18S rRNA. The PCR products were separated on 2% agarose gel and stained with ethidium bromide. The results are representational of three different experiments.

Effect of PEITC on GADD34 and GADD153 mRNA Expression in HCT-116 Colonocytes

To examine the possibility that PEITC increased the expression of other *GADD* genes, GADD34 mRNA expression was examined next because *GADD34* gene expression is known to be important in suppressing cell growth [Zhan et al., 1994] and contributing to apoptosis [Hollander et al., 2001]. Exposing HCT-116 colonocytes to PEITC resulted in only a slight increase in GADD34 mRNA expression (Fig. 4A). The GADD34:18S rRNA relative fluorescence intensity ratios ($n = 3$) were 0.899 ± 0.377 (mean $\pm$ SEM) for control cells and 1.955 ± 0.401 for PEITC-treated cells, reflecting only a 2.2-fold increase in GADD34 mRNA expression. To complement this minor finding, GADD153 mRNA expression was subsequently examined because *GADD153* gene expression is known to be important in directly mediating apoptosis [Maytin et al., 2001]. As can be seen (Fig. 4B), exposing HCT-116 colonocytes to PEITC resulted in an appreciable increase in GADD153 mRNA expression. The GADD153:β-actin relative fluorescence intensity ratios ($n = 3$) were 1.086 ± 0.216 (mean $\pm$ SEM) for control cells and 5.891 ± 0.777 for PEITC-treated cells, reflecting a 5.4-fold increase in GADD153 mRNA expression.

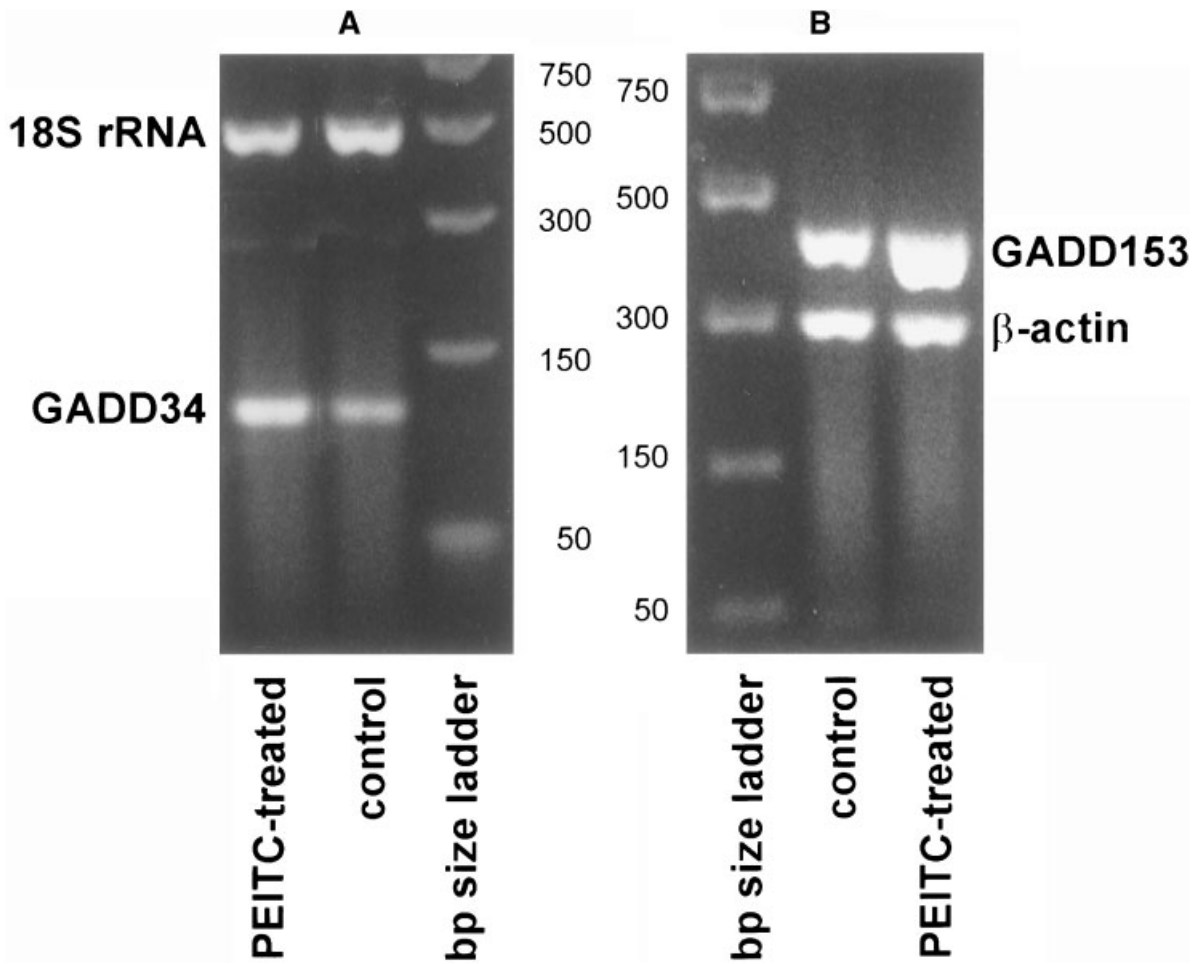


Fig. 4. Effect of PEITC on mRNA expression of GADD34 (**A**) and GADD153 (**B**) in HCT-116 colonocytes. The cells were incubated with 25 μM PEITC for 6 h. Total RNA was isolated for subsequent relative RT-PCR analysis using gene specific primers for the target genes *GADD34* and *GADD153,* and also the internal control genes 18S rRNA and β-actin. The PCR products were separated on 2% agarose gel and stained with ethidium bromide. The results are representational of three different experiments.

Sensitivity of PEITC-Induced Upregulation of GADD153 mRNA Expression to Actinomycin D and *N*-Acetylcysteine

Because GADD153 was affected the most by PEITC of the three GADD genes investigated, further experiments were dedicated to generally characterize the increase in GADD153 mRNA expression in PEITC-treated HCT-116 colonocytes. A standard experiment was initially done using the RNA synthesis inhibitor, act-D, to block transcription (Fig. 5A). As before, PEITC increased the amount of PCR product corresponding to GADD153 mRNA in the cells (lane 2), when compared to the PCR product corresponding to GADD153 mRNA in control cells (lane 1). However, when cells were co-treated with PEITC and act-D, the increased expression of GADD153 mRNA was attentuated to a noticeable extent (lane 4). Therefore, the data suggest that the increased expression of GADD153 mRNA caused by PEITC could be attributed partly to increased synthesis, but also stability, of the transcript. It is known that *GADD* genes are activated when oxidative stress exists in the cellular environment [Oh-Hashi et al., 2001], suggesting that reactive oxygen species could be involved in *GADD* gene activation. Furthermore, there is evidence to support the concept that exposing cells to PEITC promotes the formation of reactive oxygen species [Kassie et al., 1999; Murata et al., 2000]. Therefore, an experiment was performed next to determine if an antioxidant (NAC), which would be expected to scavenge reactive oxygen species, prevents PEITC-induced upregulation of GADD153 mRNA expression (Fig. 5B). NAC alone did not change GADD153 mRNA expression in the cells (lane 2), when compared to the control cells (lane 1). Treatment of cells with PEITC increased GADD153 mRNA expression (lane 3), but this increase was essentially prevented by pretreatment of the cells with NAC (lane 4).

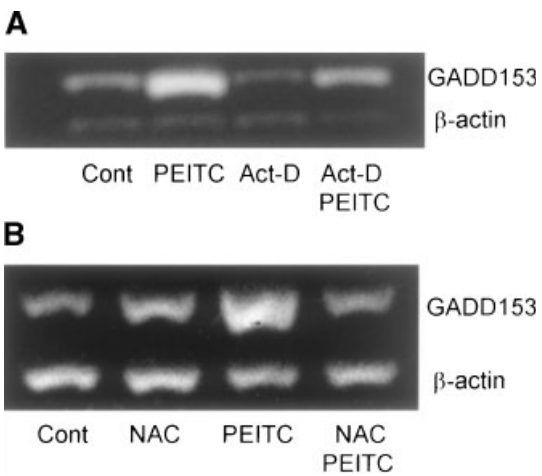


Fig. 5. Effects of actinomycin D (act-D) (**A**) and *N*-acetylcysteine (NAC) (**B**) on GADD153 mRNA expression in HCT-116 colonocytes. In (A), cells samples were co-treated for 6 h with 25 μM PEITC and 5 μg/ml act-D. In (B), cell samples were pretreated with 20 mM NAC for 2 h, and then treated with 25 μM PEITC for 6 h. Total RNA was isolated for subsequent relative RT-PCR analysis using gene specific primers for the target gene *GADD153* and the internal control gene β-actin. PCR products were separated on 2% agarose gel and stained with ethidium bromide. The results are representational of three different experiments.

Importance of p53 for the Effects of PEITC on GADD mRNA Expression

Whereas HCT-116 colonocytes express wild-type p53, HCT-15 colonocytes express mutant (non-functional) p53 [Chinery et al., 1997]. Because of this cellular difference in *p53* gene status, the two distinct cell lines were utilized to evaluate the possible requirement for p53 in mediating PEITC-induced GADD gene expression. As shown by the representative data (Fig. 6), the conclusion is that mRNA expression of GADD34 (left gel) and GADD153 (right gel), but not GADD45 (center gel), was increased by PEITC in HCT-15 colonocytes. The GADD34: 18S rRNA relative fluorescence intensity ratios ($n = 3$) were 0.903 ± 0.255 (mean ± SEM) for control cells and 2.534 ± 0.169 for PEITC-treated cells, reflecting a 2.8-fold increase in GADD34 mRNA expression. Moreover, the GADD153:β-actin relative fluorescence intensity ratios were 1.208 ± 0.094 (mean ± SEM) for control cells and 6.182 ± 1.290 for PEITC-treated cells, reflecting a 5.1-fold increase in GADD153 mRNA expression. Hence, these findings suggest that p53 is required for activating the *GADD45* gene, but not the *GADD153* gene, when the above results with HCT-116 colonocytes are considered.

DISCUSSION

Previous studies [Yu et al., 1998; Gamet-Payrastre et al., 2000; Nakamura et al., 2002] have shown that isothiocyanates induce cancer cell death, which was suggested to have occurred by way of apoptosis based on the observation of a few features characteristic of

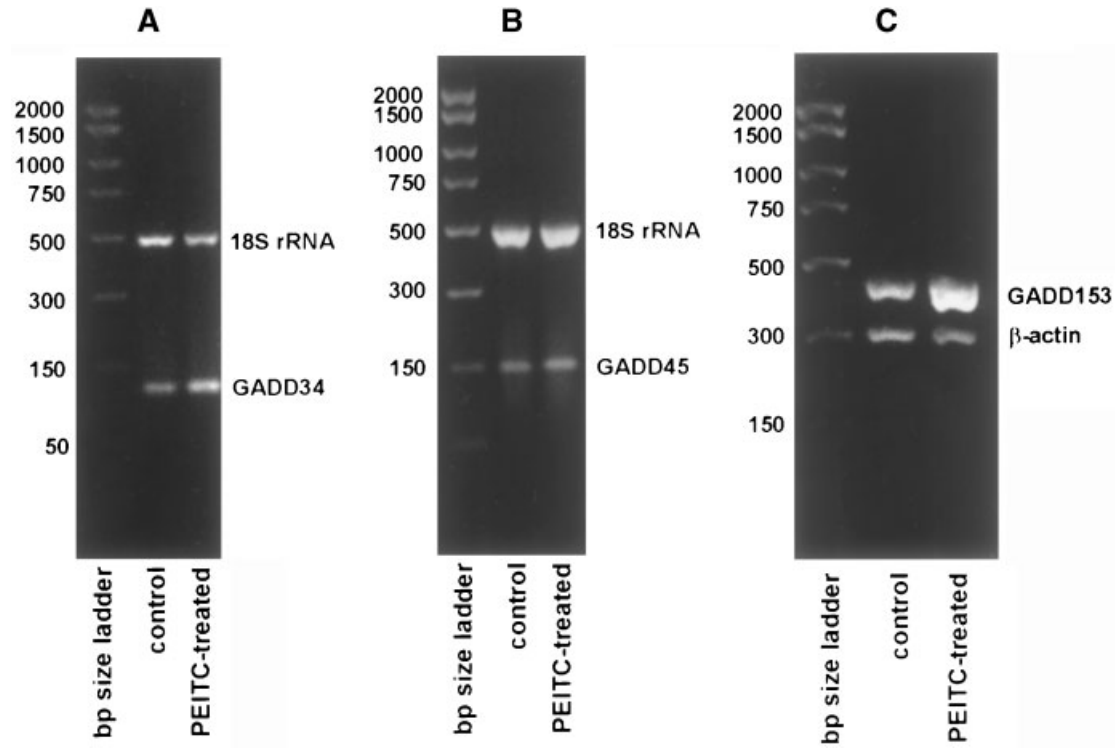


Fig. 6. Effect of PEITC on mRNA expression of GADD34 (**A**), GADD45 (**B**), and GADD153 (**C**) in HCT15 colonocytes. Cells were incubated with 25 μM PEITC for 6 h. Total RNA was isolated for subsequent relative RT-PCR analysis using gene specific primers for the target genes (GADD34, GADD45, and GADD153) and the internal control genes (18S rRNA and β-actin). PCR products were separated on 2% agarose gel and stained with ethidium bromide. The results are representational of three different experiments.

apoptosis in isothiocyanate-treated cells. In particular, PEITC killed HeLa human cervical carcinoma cells [Yu et al., 1998]. In support of this previous finding, the present study found that PEITC caused morphological features characteristic of apoptosis to appear in HCT-116 human colon adenocarcinoma cells. More specifically, membrane blebbing, apoptotic bodies, phospholipid translocation, and also chromatin condensation and fragmentation were all detected in PEITC-treated HCT-116 colonocytes.

A possible fundamental explanation for why isothiocyanates such as PEITC cause apoptosis could be that they induce oxidative DNA damage, as suggested by previous studies. For example, when incubated with calf thymus DNA in the presence of cupric ions, allyl isothiocyanate, benzyl isothiocyanate, and PEITC each caused the formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) [Murata et al., 2000], which is an oxidized DNA base product. Furthermore, allyl isothiocyanate, which was found to be the most potent, induced formation of 8-oxodG in HL-60 human leukemia cells. In another study [Kassie et al., 1999], increased DNA damage was found in HepG2 human hepatoma cells exposed to benzyl isothiocyanate, which also promoted lipid peroxidation. Such DNA damage was diminished in cells pretreated with antioxidants before subsequently being exposed to benzyl isothiocyanate. These earlier findings have been expanded by the comet assay results of the present study. It was found that PEITC caused single-strand breaks in the DNA of HCT-116 colonocytes. Thus, if such cellular DNA damage were irreparable, apoptosis would be expected, as was found to be the case with PEITC-treated HCT-116 colonocytes. It is well known that various other agents that substantially damage DNA, like PEITC did, typically cause cell death by apoptosis [Coultas and Strasser, 2000].

The molecular events that occur before the clear onset of apoptosis in PEITC-treated HCT-116 colonocytes are largely unknown. To our knowledge, the present study is the first to show that the potential cancer chemopreventive agent, PEITC, creates a cellular environment

that promotes increased expression of the *GADD* genes, which may help further explain the ability of PEITC to kill cancer cells. More specifically, mRNA expression of GADD34, GADD45, and GADD153 was increased as a result of exposing cells to PEITC. Although the exact significance of upregulation of *GADD* gene expression in PEITC-treated cells is unclear, it is suggested that this effect by PEITC contributes to the capacity of PEITC to inhibit cellular proliferation and induce apoptosis. GADD34 may help mediate apoptosis, based on the finding that over 30% of GADD34-transfected cells showed nuclear fragmentation by 48 h [Hollander et al., 2001]. Although the exact function of the *GADD45* gene product is not completely clear [Sheikh et al., 2000], it may have a role in regulating cell growth because cells were arrested in the G_2-phase of the cell cycle after being microinjected with a GADD45 expression vector [Wang et al., 1999]. The *GADD153* gene product belongs to the CCAAT/enhancer binding protein (C/EBP) family of transcription factors. There is evidence to support the concept that GADD153 is directly involved in apoptosis, based on the finding that targeted overexpression of a GADD153 vector in several different cell lines resulted in apoptosis [Maytin et al., 2001]. However, it is unclear how GADD153 protein induces cellular apoptosis. Furthermore, several recent studies [Conn et al., 2002; Kim et al., 2002; Lengwehasatit and Dickson, 2002; Xia et al., 2002] have suggested that the increased *GADD153* gene expression caused by various inducers of apoptosis somehow triggers the critical early events leading to the initiation of apoptosis. Therefore, because there is substantial evidence that GADD153 has a role in triggering apoptosis, it is conceivable that the increased *GADD153* gene expression in PEITC-treated HCT-116 colonocytes could be involved in triggering apoptosis in these cells. This is not to say, however, that other apoptosis-related genes are unimportant in the apoptosis induced by PEITC. It is known that other isothiocyanates, such as sulforaphane, can increase the expression of the pro-apoptotic gene, *Bax*, in inducing apoptosis [Gamet-Payrastre et al., 2000; Fimognari et al., 2002].

GADD genes are often activated when cells are subjected to a stressful environment, such as one deficient in essential nutrients such as leucine [Bruhat et al., 1997], glutamine [Abcouwer et al., 1999], zinc [Fanzo et al., 2001], and glucose [Carlson et al., 1993]. Additionally, exposing cells to DNA-damaging agents such as peroxynitrite [Oh-Hashi et al., 2001], UV radiation [Tong et al., 2001], and anticancer drugs [Kim et al., 2002] increase *GADD* gene expression. Therefore, it is possible that the effects of PEITC on GADD mRNA expression in PEITC-treated HCT-116 colonocytes could be an early response to the cellular DNA damage caused by PEITC. In particular, the increase in GADD153 mRNA expression was probably due to a combination of increased transcription and greater mRNA stability. Support for the former possibility comes from the experiment where act-D partially prevented the increased GADD153 mRNA expression caused by PEITC in HCT-116 colonocytes. Other inducers of *GADD153* gene expression have been shown to exert similar effects. For example, depriving cells of leucine [Bruhat et al., 1997] increased GADD153 mRNA expression, which was attributed to both increased transcription and mRNA stability. However, glutamine deprivation also induced GADD153 mRNA expression [Abcouwer et al., 1999], but the primary mechanism was through mRNA stabilization.

On the other hand, upregulation of *GADD* gene expression may not necessarily occur as a direct consequence of growth arrest and/or DNA damage in PEITC-treated HCT-116 colonocytes. Because isothiocyanates induce oxidative stress in cells [Kassie et al., 1999; Murata et al., 2000], it is conceivable that PEITC might stimulate redox-sensitive signaling events leading to transcriptional activation of *GADD* genes. This notion is supported by the results of the present study showing that the antioxidant, NAC, prevented PEITC-induced upregulation of GADD153 mRNA expression. The molecular cascade leading to *GADD* gene activation may directly involve mitogen-activated protein kinases (MAPK). For example, when p38/SAPK2 was activated by anisomycin in Jurkat T-lymphocytes, the levels of GADD153 transcript increased, but this effect was nullified with a selective chemical inhibitor of p38/SAPK2 [Rolli-Derkinderen and Gaestel, 2000]. Exposing HCT-116 human colon adenocarcinoma cells to UV radiation, which promoted the formation of reactive oxygen species, induced transcriptional activation of GADD45 that involved ERK and JNK signaling based on the

findings that a selective chemical inhibitor of ERK and a dominant negative mutant JNK1 expression vector blunted reporter construct activation [Tong et al., 2001]. In some cases of apoptosis, MAPK are required to increase gene expression of *GADD* genes, including GADD153, and to mediate apoptosis [Sarkar et al., 2002]. Exposing Jurkat T-lymphocytes to isothiocyanates, including PEITC, led to activation of JNK, which was concluded to be required for subsequent induction of apoptosis [Chen et al., 1998]. There is additional evidence to support the concept that PEITC causes activation of the MAPK pathway, which in turn triggers apoptosis, as reviewed in greater depth [Owuor and Kong, 2002].

Previously, it was concluded that the *p53* gene is required for induction of apoptosis by PEITC [Huang et al., 1998]. On the other hand, it has been reported more recently that p53 is not required for PEITC-induced apoptosis [Xiao and Singh, 2002]. In other experiments conducted in the present study (data not shown), PEITC-treated HCT-15 colonocytes that express mutant p53 also showed features characteristic of apoptosis, similar to what was observed with PEITC-treated HCT-116 colonocytes that express wild-type p53. Therefore, these particular results support the previous conclusion [Xiao and Singh, 2002] that p53 is not required for the induction of apoptosis by PEITC. However, the most revealing new information in utilizing HCT-15 colonocytes as a comparative model was the result suggesting that the effect of PEITC on expression of certain *GADD* genes was not dependent on p53. That is, whereas PEITC increased GADD34, GADD45, and GADD153 mRNA expression in HCT-116 colonocytes, only GADD34 and GADD153 mRNA expression was increased by PEITC in HCT-15 colonocytes. Therefore, these particular results support the notion that p53 is required for transcriptionally activating the *GADD45* gene, but not the *GADD34* and *GADD153* genes.

In summary, PEITC induced single-strand breaks in DNA of HCT-116 colonocytes. Such DNA damage was followed by the appearance of cellular features characteristic of apoptosis. However, before clear signs of apoptosis became evident, there was increased mRNA expression of the *GADD* genes, but particularly GAIDD153 that is also considered a pro-apoptotic gene. PEITC-induced upregulation of GADD153 mRNA expression seemed to be due partly to increased transcription. Furthermore, increased GADD153 mRNA expression was essentially prevented by NAC, suggesting that a redox mechanism was involved in increasing GADD153 mRNA expression.

The effect of PEITC on *GADD* gene expression did not seem to be totally dependent on p53, since PEITC also increased *GADD* gene expression in HCT-15 colonocytes that express mutant p53 whereas HCT-116 express wild-type p53. A possible role for GADD153 in triggering apoptosis caused by PEITC in human colon adenocarcinoma cells is under investigation.

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