Phenethyl Isothiocyanate, a Cancer Chemopreventive Constituent of Cruciferous Vegetables, Inhibits Cap-Dependent Translation by Regulating the Level and Phosphorylation of 4E-BP1

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Abstract

Phenethyl isothiocyanate (PEITC), a constituent of many edible cruciferous vegetables, exerts significant protection against chemically induced cancer in animal models and inhibits growth of cancer cells in culture and in vivo by causing cell cycle arrest and apoptosis induction. In this study, we report a novel response to PEITC involving the regulation of translation initiation at pharmacologically achievable concentrations. Treatment of human colorectal cancer HCT-116 cells and human prostate cancer PC-3 cells, but not a normal prostate epithelial cell line (PrEC), with PEITC caused an increase in expression of the eukaryotic translation initiation factor 4E (eIF4E) binding protein (4E-BP1) and inhibition of 4E-BP1 phosphorylation. Results from pull-down assay using 7-methyl-GTP Sepharose 4B beads indicated that PEITC treatment reduced cap-bound eIF4E, confirming that increased 4E-BP1 expression and inhibition of 4E-BP1 phosphorylation indeed reduced the availability of eIF4E for translation initiation. Accordingly, results from in vivo translation using luciferase reporter assay indicated that PEITC treatment inhibited cap-dependent translation, in particular the translation of mRNA with secondary structure (stem-loop structure). Ectopic expression of eIF4E prevented PEITC-induced translation inhibition and conferred significant protection against PEITC-induced apoptosis. These results indicate that PEITC modulates availability of eIF4E for translation initiation leading to inhibition of capdependent translation. The present study also suggests that inhibition of cap-dependent translation may be an important mechanism in PEITC-induced apoptosis. [Cancer Res 2007; 67(8):3569-73]

Introduction

Epidemiologic studies continue to support the premise that dietary intake of cruciferous vegetables may be protective against the risk of various types of malignancies (1). The anticarcinogenic effect of cruciferous vegetables is attributed to organic isothiocyanates (ITC), which are present in a variety of edible cruciferous vegetables such as broccoli (1). Phenethyl ITC (PEITC) is one of the best studied members of the ITC family of compounds that has

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generated a great deal of research interest due to its cancer chemopreventive activity (2).

Very little information currently exists regarding the involvement of translational control in chemoprotective effects of dietary agents. The exclusive emphasis on transcriptional regulation has overlooked the molecular events and pathways involved in translational control in cancer chemoprevention. However, evidence is accumulating to indicate that targeting protein translation represents one of the most promising approaches for cancer intervention (3). Therefore, identification of natural compounds that modulate translational control is considered a highly promising strategy for the development of anticancer agents.

In eukaryotes, about 90% of protein is synthesized through capdependent translation. It is estimated that up to 10% of all mRNAs are translated by an alternative initiation mechanism that involves a complex RNA structural element termed an internal ribosome entry segment (IRES). A key step of cap-dependent mRNA translation is the binding of the eukaryotic translation initiation factor 4E (eIF4E) to mRNA molecules with a 5'-terminal 7-methyl-GTP cap. The availability of eIF4E for binding to the cap structure of mRNA is regulated by the phosphorylation of a small family of eIF4E-binding proteins (4E-BP). 4E-BP1, the most abundant member of the 4E-BP family, can be phosphorylated at multiple sites in a sequential order, and a combination of phosphorylation events is necessary to dissociate 4E-BP1 from eIF4E (4). Phosphorylation of 4E-BP1 decreases the affinity of the protein for eIF4E, which facilitates the formation of eIF4F complex (consisting of eIF4A, eIF4G, and eIF4E) for the initiation of capdependent translation.

Both 4E-BP1 and eIF4E are involved in the regulation of apoptosis. For instance, increased expression of eIF4E was sufficient to inhibit apoptosis in serum-restricted primary fibroblasts (5), whereas ectopic expression of 4E-BP1 activates apoptosis (6). The ability of 4E-BP1 to induce apoptosis is governed by its phosphorylation status (6). This finding, along with the observation that enforced expression of 4E-BP1 sensitizes fibroblasts to apoptosis in a manner strictly dependent on its ability to sequester eIF4E from a translationally active complex (7), strongly suggests that 4E-BP1 and eIF4E's regulation of translation initiation account for their apoptosis modulation.

We now show that PEITC effectively induces 4E-BP1 expression and inhibits 4E-BP1 phosphorylation leading to reduced availability of eIF4E for cap-dependent translation. Reduced eIF4E availability not only leads to inhibition of translation but also plays a crucial role in PEITC-induced apoptosis. We propose that inhibition of protein translation may be an important event in the overall scheme of cancer chemoprevention by PEITC.

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Table 1. Treatment with PEITC induces apoptosis but not cell cycle arrest in HCT-116 cells

Treatment	% Cells in			
	Sub-G ₀ /G ₁	G ₀ /G ₁	S	G ₂ -M
Control (DMSO)	1.81 ± 0.10	38.25 ± 0.73	26.31 ± 2.86	33.41 ± 2.50
1 µmol/L PEITC (24 h)	$4.42 \pm 0.76^{*}$	38.29 ± 2.72	25.34 ± 0.76	32.26 ± 2.43
2.5 μmol/L PEITC (24 h)	$9.14 \pm 0.42^{*}$	31.83 ± 1.97	25.62 ± 1.00	33.61 ± 0.81
1 µmol/L PEITC (48 h)	$8.55 \pm 0.23^{*}$	37.08 ± 2.67	26.16 ± 1.24	28.29 ± 3.61
2.5 μ mol/L PEITC (48 h)	$28.29 \pm 2.60^*$	29.89 ± 0.95	21.37 ± 1.07	20.92 ± 1.44

NOTE: Results are mean \pm SE (n = 3).

*Significantly different compared with control by one-way ANOVA followed by Dunnett's test.

Materials and Methods

Reagents and antibodies. PEITC was purchased from Aldrich (St. Louis, MO). Antibodies against eIF4E, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and β -actin were from Santa Cruz Biotechnology (Santa Cruz,

CA). Antibody-recognizing epitope influenza hemagglutin (HA) tag was from Abgent (San Diego, CA). All other antibodies were purchased from Cell Signaling Technology (Beverly, MA). The expression plasmid of HA-tagged wild-type eIF4E and the luciferase and stem-loop luciferase cDNA plasmids (pcDNA-LUC and pcDNA-SL-LUC) have been described previously (8, 9).

Α	
Fold	1.0 2.5 3.6 2.3 1.0 3.2 3.1 2.9
	4E-BP1
	1.0 1.8 1.4 1.1 1.0 1.1 1.0 0.9
	p-4E-BP1-Thr ^{37/46}
	1.0 1.1 0.7 0.6 1.0 0.7 0.6 0.4
	p-4E-BP1-Thr ⁷⁰
	1.0 1.2 0.7 0.6 1.0 1.0 0.6 0.3
	p-4E-BP1-S65
	CONTRACTOR POLICY PRODUCT AND
	GAPDH
PEITC (µmol/L)	0 1 2.5 5 0 1 2.5 5
	24 hour 48 hour
_	
B Fold	1.0 1.5 1.4 1.3 1.0 0.8 0.3
	4E-BP1
	1.0 1.4 1.2 1.7 1.0 0.9 1.8
	p-4E-BP1-Thr ^{37/46}
	1.0 0.4 0.3 0.3 1.0 1.2 1.0
	p-4E-BP1-Thr ⁷⁰
	1.0 0.7 0.5 0.4 1.0 3.5 4.6
	p-4E-BP1-S65
	Actin
PEITC (µmol/L)	0 1 2.5 5 0 2.5 5
	PC3 PrEC

Figure 1. PEITC treatment induces 4E-BP1 expression and inhibits its phosphorylation in cancer cells but not in normal epithelial cells. A, immunoblotting analysis of 4E-BP1 phosphorylation status. The colon cancer HCT-116 cells were treated with PEITC at the indicated concentration for 24 and 48 h. The whole cell lysates were used for immunoblotting. Phosphorylation of 4E-BP1 was evaluated by antibodies recognizing phosphorylated 4E-BP1 at individual phosphorylation sites. The cellular level of GAPDH shows the equal loading of the sample. B, prostate cancer PC-3 cells and normal prostate epithelial PrEC cells were treated with PEITC at the indicated concentration for 24 h. Whole cell lysates were used for immunoblotting, and the cellular actin level shows the equal loading of the sample.

Cell culture and PEITC treatment. HCT-116 were cultured in McCoy's 5a modified medium (Mediatech Inc., Herndon, VA) supplemented with 10% fetal bovine serum (FBS; Sigma, St. Louis, MO) and 1.5 mmol/L L-glutamine (Sigma). Culture of prostate cancer cell PC-3 cells and normal prostate epithelial cell line PrEC (Clonetics, San Diego, CA) has been described previously (10, 11). All other cell culture reagents were purchased from Mediatech. For PEITC treatment, the cells were exposed to PEITC (1, 2.5, and 5 μ mol/L) or DMSO (final concentration 0.05% was added to the controls) for the indicated time. After PEITC treatment, the cells were harvested for further analysis.

Immunoprecipitation and immunoblotting. The assays were done as described previously (12).

7-Methyl-GTP Sepharose 4B pull-down assay. After PEITC treatment, the HCT-116 cells were lysed with immunoprecipitation lysis buffer, and the whole cell lysates were used for the pull-down assay with 7-methyl-GTP Sepharose 4B beads (Amersham, Buckinghamshire, United Kingdom). Precipitated proteins were separated on 12% SDS-PAGE gels and analyzed by immunoblotting for eIF4E.

Transient transfection assays of translation. An *in vivo* translation assay of the reporter system was done as described previously (9). Protein concentration of each well was measured and used for normalizing luciferase activity.

Reverse transcription-PCR. The total RNA was isolated by TRIzol reagent (Invitrogen, Cincinnati, OH) according to the protocol provided by the manufacturer. The primers used to amplify 200 bases of the firefly luciferase gene were 5'-CTG CAT CCG GCT ATG AAG AGA TAC G-3' and 5'-CCC AAC TGC AAC TCC GAT AAA TAA CGC-3'. The primers used to amplify 146 bp of the housekeeping gene β -actin were 5'-CATGGAGTCCTGTGGG-CATCCACGAAACT-3' and 5'-ATCTCCTTCTGCATCCTGTCGGCAAT-3'.

Detection of apoptosis and cell proliferation analysis. The PEITCinduced apoptosis was assessed by (*a*) flow-cytometric analysis of subdiploid cells (sub- G_0/G_1 cells with DNA fragmentation) after staining with propidium iodide and (*b*) ELISA-based quantitation of cytoplasmic histone-associated DNA fragmentation using a commercially available kit from Roche Diagnostics (Mannheim, Germany) according to the manufacturer's recommendations. The experimental procedure was as we described previously (13, 14). The effect of PEITC on cell growth was evaluated by trypan blue dye exclusion assays as described previously (11).

Results and Discussion

PEITC treatment caused apoptosis in HCT-116 cells. It is known that PEITC suppresses cancer cell proliferation by causing cell cycle arrest and/or apoptosis (11, 15, 16). In colorectal cancer HT-29 cells, treatment with PEITC at a relative high dose (10–50 μ mol/L) induces apoptosis at the shortest time point of 3-h exposure (17). In this study, we tested whether chronic exposure (24–48 h) to PEITC at pharmacologically relevant concentrations exerts a similar effect in colorectal cancer HCT-116 cells. Because the maximal plasma concentration of PEITC following ingestion of 100 g watercress was shown to vary between 673 and 1,155 nmol/L (18), we decided to focus on the effect of PEITC treatment at dose range of 1–5 μ mol/L.

As shown in Table 1, the fluorescence-activated cell sorting analysis results showed that statistically significant induction of apoptosis by PEITC was evident at concentrations within the pharmacologically achievable range. For instance, the percentage of



Figure 2. Treatment with PEITC preferentially inhibits translation of 5' UTR structured mRNA *in vivo. A*, pull-down assay with 7-methyl-GTP Sepharose 4B beads. The whole cell lysates were used for the pull-down assay with 7-methyl-GTP Sepharose 4B beads (Amersham). The cap-bound elF4E was evaluated by immunoblotting with elF4E antibody. The input elF4E was evaluated by immunoprecipitation with elF4E and followed by immunoblotting with elF4E. B and *C, in vivo* translation assay of luciferase gene with stem loop (*B*) and without secondary structure (*C*). About 2.4×10^4 HCT-116 cells were treated with PEITC at the indicated concentration for 24 h. The cells were lysed, and the luciferase activity was measured using Luciferase Assay Kit (Promega, Madison, WI). The luciferase activity of each sample was normalized by protein concentration. These experiments were repeated thrice with followed by Dunnet's test. *D*, RT-PCR amplification of luciferase gene. The HCT-116 cells were transfected with pEITC (7.5 µmol/L) con a pcDNA-LUC; 24 h after transfection, the transfected cells were treated with PEITC (7.5 µmol/L) for an apple was extracted for RT-PCR analysis of luciferase mRNA expression.



Figure 3. Overexpression of eIF4E renders resistance to PEITC-induced apoptosis. *A* and *B*, apoptosis assay of HCT-116 cells treated with 1 µmol/L of PEITC (*A*) and 2.5 µmol/L of PEITC (*B*). The HCT-16 cells were transfected with vector control and wild-type eIF4E. Twenty-four hours after transfection, the transfected cells were treated with PEITC for an additional 24 h and then harvested for apoptosis evaluation. Apoptosis was measured by analysis of cytoplasmic histone–associated DNA fragmentation using a commercially available kit (Roche Diagnostics) according to the manufacturer's recommendations. *Columns*, mean; *bars*, SE (*n* = 4). *, *P* < 0.01, significantly different by one-way ANOVA followed by Tukey's multiple comparison test. Ectopic expression of HA-tagged eIF4E was confirmed by immunoblotting with anti-HA.

cells undergoing apoptosis (sub- G_1/G_0) increased from 1.81% in control cells to 4.42% in cells treated with 1 µmol/L PEITC for 24 h and 8.55% in cells treated with 1 µmol/L PEITC for 48 h. In addition, these results clearly show that PEITC induces apoptosis but does not cause G_1 or G_2 -M arrest, suggesting that induction of apoptosis is the major mechanism of PEITC-induced inhibition of cell proliferation in HCT-116 cells.

PEITC treatment increased 4E-BP1 expression and inhibited phosphorylation of 4E-BP1. Apoptosis induction is often associated with reduced eIF4E availability due to increased binding of eIF4E with 4E-BP1 (19). The findings that enforced expression of 4E-BP1 promotes apoptosis and the phosphorylation status of 4E-BP1 governs its proapoptotic potency (6) prompted us to test whether PEITC-induced apoptosis involved 4E-BP1 modulation. Indeed, treatment of HCT-116 cells with 1 µmol/L PEITC markedly increased cellular level of 4E-BP1 protein (Fig. 1A). In addition, PEITC treatment inhibited phosphorylation of 4E-BP1 at S65 and at Thr⁷⁰ especially at 2.5 and 5 µmol/L concentrations. Although it

seemed that PEITC treatment failed to inhibit 4E-BP1 phosphorylation at Thr^{37/46} sites, we propose that this might be due to the increased amount of 4E-BP1 by PEITC treatment. It is also possible that the lack of inhibition of 4E-BP1 at Thr^{37/46} resulted from the fact that specific signaling pathways involved in Thr^{37/46} were not affected by PEITC. The PEITC-inhibited 4E-BP1 phosphorylation was more pronounced at 48-h time point relative to 24 h of exposure. Together, these data showed modulation of 4E-BP1 protein level and phosphorylation by PEITC even at pharmacologically achievable concentration.

Next, we tested whether the effect of PEITC on 4E-BP1 was restricted to HCT-116 cells due to its unique genetic background by examining the effect of PEITC on 4E-BP1 protein level/phosphorvlation in PC-3 human prostate cancer cells. Similar to HCT-116 cells, PEITC treatment not only increased the expression of 4E-BP1 protein, but also inhibited 4E-BP1 phosphorylation at S65 and Thr⁷⁰ but not at Thr^{37/46} in PC-3 cells in a dose-dependent manner (Fig. 1B). To determine if the effect of PEITC on 4E-BP1 was cancer cell specific, we used a normal prostate epithelial cell line PrEC, which is resistant to growth inhibition and apoptosis induction by PEITC (11). Interestingly, PEITC treatment neither caused induction of 4E-BP1 expression nor inhibited 4E-BP1 phosphorylation in PrEC cells. The induction of 4E-BP1 phosphorylation at Thr^{37/46} and S65 in PrEC cells may result from the fact that PEITC differentially targets signaling pathways in cancer cells versus normal cells. It is also possible that certain signaling events are affected differently by PEITC in cancer cells and normal epithelial cells. Two conclusions can be drawn from these experiments: (a) the effect of PEITC on 4E-BP1 protein level and phosphorylation is not cancer cell type specific and (b) resistance of PrEC cells to growth inhibition and cell death may be partly attributable to the lack of 4E-BP1 induction and reduced phosphorylation by PEITC.

PEITC treatment reduced the eIF4E availability and inhibited cap-dependent translation of the luciferase reporter gene. Because a combination of 4E-BP1 phosphorylation events is necessary to dissociate 4E-BP1 from eIF4E (4) and binding of eIF4E to mRNA cap structure (M7GppX) is essential for initiating cap-dependent protein translation, we hypothesized that the induction of 4E-BP1 protein expression and inhibition of its phosphorylation may reduce the availability of eIF4E for translation initiation. The results from pull-down with 7-methyl-GTP Sepharose 4B beads confirmed that the binding of eIF4E to mRNA molecules with a 5'-terminal 7-methyl-GTP cap was remarkably reduced (Fig. 2*A*), demonstrating that PEITC treatment reduced the availability of eIF4E for cap-dependent translation initiation.

Next, we examined if reduction of eIF4E availability for translation initiation indeed caused inhibition of cap-dependent translation, a major mechanism by which mRNAs are translated to proteins. To evaluate the cap-dependent translation, we used the luciferase cDNA reporter with or without stable secondary structure (stem-loop) introduced in the 5' leader sequence (pcDNA-SL-LUC versus pcDNA-LUC; ref. 9). As shown in Fig. 2B, treatment with PEITC significantly suppressed the translation of the luciferase reporter gene with stem-loop structure (pcDNA-SL-LUC). To a lesser extent, PEITC treatment at 7.5 µmol/L concentration suppressed the translation of the luciferase reporter gene without the stem-loop structure (pcDNA-LUC; Fig. 2C). The PEITCmediated inhibition of the luciferase reporter gene expression with stem-loop structure was not due to a decrease in mRNA level of the luciferase gene as judged by reverse transcription-PCR (RT-PCR; Fig. 2D), confirming that the inhibition of the luciferase gene

was at the translational level. To rule out the possibility of cell type–specific effect, we determined the effect of PEITC on cap-dependent translation using PC-3 cells. Similar to HCT-116 cells, PEITC treatment decreased the translation of pcDNA-SL-LUC, but did not have any appreciable effect on the translation of pcDNA-LUC (data not shown). Although it remains to be determined whether PEITC treatment affects mRNA translation through IRES mechanism, our data indicated that PEITC treatment inhibited cap-dependent translation, and PEITC preferentially inhibited cap-dependent translation for this phenomenon is that translation of mRNA with a stable secondary structure at the 5' untranslated region (UTR) would be more susceptible to the changes of formation of translation.

Ectopic expression of eIF4E confers significant protection against PEITC-induced apoptosis. Stress-induced apoptosis is often associated with the down-regulation of protein translation (19) and the down-regulation of protein translation can result in apoptosis as well (20). However, the down-regulation of protein synthesis is not always sufficient to cause apoptosis (20). To determine whether translation inhibition is simply a consequence of PEITC-induced apoptosis or translation inhibition is crucial for PEITC-induced apoptosis, we tested if overexpression of eIF4E prevents PEITC-induced apoptosis. As expected, enforced expression of eIF4E not only prevented PEITC-mediated translation inhibition (data not shown), but also significantly blocked PEITC-induced apoptosis (Fig. 3A and B) in HCT116 cells. Moreover, enforced expression of eIF4E also blocked PEITC-caused inhibition of cell proliferation (data not shown). Similarly, overexpression of eIF4E prevented PEITC-induced inhibition of translation and apoptosis in PC-3 cells (data not shown). Together, these data strongly suggest that inhibition of translation is the cause but not a consequence of PEITC-induced apoptosis.

In conclusion, the present study shows that PEITC treatment modulates translation regulators, and PEITC is a potent inhibitor of cap-dependent translation. It is important to note that the PEITC-mediated inhibition of protein translation occurs at concentrations achievable by dietary intervention or pharmacologic administration and seems selective for cancer cells. We speculate that inhibition of protein translation may be an important mechanism in the overall scheme of PEITC-mediated cancer prevention.

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