RESEARCH ARTICLE

Effect of orally administered phenethyl isothiocyanate on hepatic gene expression in rats

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Received: December 21, 2009
Revised: May 15, 2010
Accepted: May 17, 2010

Scope: Phenethyl isothiocyanate (PEITC) is a constituent of cruciferous vegetables that has demonstrated cancer preventive activity in a number of cancer models including lung, prostate, and breast cancer. Our objective was to examine the effects of the oral administration of PEITC for 7 days on the hepatic expression of genes important in drug metabolism and toxicity in Sprague Dawley rats. The liver is the major site for the metabolism of various xenobiotics and carcinogens, and determining the effects of PEITC on the gene expression of hepatic enzymes may provide insight into mechanisms underlying the cancer preventive activity of PEITC.

Methods and results: Using a microarray containing 282 genes, we observed that PEITC significantly up-regulated UDP-glucuronosyltransferase UGT1A6 and strongly down-regulated nicotinamide N-methyltransferase (NNMT). We also confirmed the down-regulation of NNMT by real-time quantitative RT-PCR. Other genes that were significantly up-regulated were the drug metabolizing enzyme cyp2b15, the anti-apoptotic gene bcl2l2, and the stress regulators Gadd45b, Dnajb9, Dnajb5 and Hspb1.

Conclusion: Our results indicate new targets that may be important in the mechanisms of the anticancer effects of PEITC. Of particular significance was the down-regulation of NNMT which may represent a new target for the treatment of a variety of cancers.

Keywords:
Cancer prevention / Gene expression / Nicotinamide *N*-methyltransferase / Phenethyl isothiocyanate / UDP-glucuronosyltransferase

1 Introduction

Brassica vegetables of the family Cruciferae (*e.g.* cabbage, watercress, and broccoli) and the genus *Raphanus* (radishes and daikons) contain isothiocyanates and indoles that have been implicated in the reduction of cancer risk [1]. Intake of broccoli and watercress may reduce the risk for lung cancer through the inhibition of CYP450 enzymes that are responsible for the activation of procarcinogens [2]. In addition to CYP450 inhibition, isothiocyanates are inducers of phase II metabolic enzymes that play a role in the detoxification of activated carcinogens [3]. Induction of apoptosis also represents an important mechanism by which isothiocyanates exert their anticancer effects [4, 5].

Phenethyl isothiocyanate (PEITC) is derived from gluconasturtiin, a glucosinolate of PEITC that occurs naturally in cruciferous vegetables [6]. The action of the enzyme myrosinase, present in cruciferous vegetables, converts gluconasturtiin to PEITC once the vegetable is cut or ingested. Significant plasma concentrations of PEITC can be achieved with dietary consumption; ingestion of a 100-g dose of watercress results in a maximal plasma concentration of 1 μM in humans [6, 7]. PEITC has been shown to induce several phase II enzymes *in vivo*. When 1 mmol/kg PEITC was administered to F344 rats orally, induction of

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Abbreviations: NNMT, nicotinamide *N*-methyltransferase; **PEITC,** phenethyl isothiocyanate; **RTQ,** real-time quantitative

NAD(P)H:quinone oxidoreductase and glutathione *S*-transferase activities were observed in the liver. Additionally, sulfotransferase activity was induced in the nasal mucosa, while increased UDP glucuronosyl transferase activity was observed in the liver [8]. A diet containing 816 mg/kg PEITC administered to young male rats also significantly induced UDP glucuronosyl transferase activity 1.2-fold and NAD(P)H:quinone oxidoreductase and glutathione *S*-transferase activities 1.8- and 1.5-fold, respectively [9]. To investigate the mechanisms of action of PEITC as a chemopreventive/therapeutic agent and identify other drug metabolizing enzymes that may be targets of PEITC action, we studied the effect of PEITC on gene expression of drug metabolizing enzymes in rat livers. We hypothesized that PEITC alters the gene expression of enzymes that are involved in the metabolism of xenobiotic and endogenous carcinogens. To evaluate this hypothesis, we used an oligomicro array, which has 282 genes that represent phase I and phase II enzymes, as well as some drug transporters. Additionally, these arrays also contain genes that are involved in apoptosis and cell cycle signaling.

2 Materials and methods

2.1 Materials

Female Sprague Dawley rats were obtained from Harlan (Indianapolis, IN). PEITC and corn oil were obtained from Sigma Aldrich (St. Louis, MO). SV RNA isolation kit was purchased from Promega (Madison, WI). Oligo GEArray® Rat Toxicology & Drug Resistance Microarrays and True-Labeling-AMP 2.0 kit, and related supplies were obtained from SA Biosciences (Frederick, MD).

2.2 Animal studies

Female Sprague Dawley rats (3–4 animals *per* group), weighing 170–190 g, were housed in a room with controlled lighting and temperature. The animals were fed with a phytoestrogen free diet (Tekland 2016S) obtained from Harlan. Animals had free access to food and water throughout the study. Rats were acclimated for 1 wk before the start of the study. The research protocol for the study was approved by the Institutional Animal Care and Use Committee at the University at Buffalo, Amherst, NY. PEITC (150 μmol/kg) in 0.5 mL corn oil was administered to animals once daily for 7 days. Oral gavage was performed using 20-gauge curved stainless steel gavage needles. Control animals received 0.5 mL corn oil once daily for 7 days. At the end of 7 days, the animals were sacrificed by exsanguination of anesthetized animals. The rat livers were washed in PBS, snap frozen in liquid nitrogen, and stored at −80°C until further processing.

2.3 Gene array

RNA was isolated from the livers using the SV RNA isolation system from Promega. Biotinylated cRNA was produced using the TrueLabeling-AMP 2.0 kit from SuperArray. The cRNA was then hybridized on the array membrane under specified conditions, as *per* the manufacturer's instructions. After washing, the net intensity of each gene spot on the array was determined using a Kodak Image Station 2000MM.

2.4 Data analysis and normalization

A number of sequences, such as sod1, ldha, GAPDH, and BAS2C, were available on the array for normalization of the data. The net intensities of all these genes available for normalization of the array genes were compared for the treated and control groups. We determined that among all of these genes, only BAS2C and GAPDH did not change with treatment. BAS2C is an artificial sequence that does not change between treatments and assesses experimental variation, whereas GAPDH is frequently used as a housekeeping gene. The natural log of the net intensity of each spot was normalized by the average of the log of the net intensities of the housekeeping genes, BAS2C and GAPDH. Genes with significant changes in expression were identified using the unpaired Student's *t*-test, with the level of statistical significance set at $p < 0.05$. Significance Analysis of Microarrays was also used to analyze the data, which accounts for errors arising from repeated measurements [10]. While using significance analysis of microarrays, the delta value was set such that the false discovery rate for each array was minimized. The false detection rate for comparisons ranged from 0 to 1%. Results from both tests were compared, and genes that were significant by both tests are reported.

2.5 Real-time quantitative RT-PCR (RTQ RT-PCR)

From the genes that were shown to be upregulated, we analyzed nicotinamide *N*-methyltransferase (NNMT) in another group of rats by RTQ RT-PCR ($n = 4$ *per* group). RTQ RT-PCR was performed on Alien RNA (Stratagene, La Jolla, CA) (for normalization) and NNMT using the Stratagene Mx4000TM Multiplex Quantitative PCR System (Stratagene). RTQ RT-PCR reactions were carried out by mixing 4 μL of cDNA, 4 μL of 10 × PCR buffer, 2 μL of deoxynucleoside triphosphate mix (1 mM each dATP, dCTP, dGTP, and dTTP), 2 μL each of 10 μM primer, 0.4 μL reference dye rhodamine-X (1/500 dilution, Molecular Probes, Eugene, OR), 0.4 μL SYBR green I (1/750 dilution, Molecular Probes), 0.25 μL of 2 U Taq polymerase (Eppendorf, Westbury, NY), and 25.35 μL H_2O and amplified for 40 cycles. The thermal cycling conditions consisted of an initial denaturation step at 95°C for 10 min, then 40 cycles denaturing at 95°C for 30 s, and an annealing temperature of 57°C for 30 s and polymerization for 30 s at 72°C.

For the standard curve for NNMT, the PCR product for NNMT was cloned into a pCR® 2.1 TOPO® vector (Invitrogen, Carlsbad, CA) and transformed into One Shot chemically competent *Escherichia coli* cells (Invitrogen). Plasmid containing the PCR product sequence was extracted from *E. coli* cells using a Wizard Plus DNA purification kit (Promega). The product was resolved by electrophoresis through a 1.2% agarose gel to confirm target size and the presence of a single PCR product. The standard curves using dilution of dSDNA were run in duplicate along with the unknown samples, also in duplicate on the same plate. The reported copy number was estimated from the linear regression of the standard curve on the same plate. Statistical analysis using an unpaired *t*-test ($p<0.05$) was performed on the copy numbers, corrected using Ct values from alien RNA runs.

3 Results

3.1 Gene array

As shown in Table 1, a total of six genes, namely, Cyp2b15, ugt1a6, bcl2l2, Dnajb9, Dnajb5, and Hspb1, were significantly upregulated by PEITC treatment. The variability in the gene array results, as determined in 3–4 animals, is presented in Fig. 1. One gene NNMT was significantly downregulated. The expression of NNMT in the treated animals was completely abolished in the treated animals, leading to a normalized intensity of zero and therefore, the absence of a black (closed) bar for NNMT in Fig. 1.

A list of the genes present in the gene array can be found in the Supporting Information.

3.2 RTQ RT-PCR

There were no significant differences in the Ct values for Alien RNA amplification, and values were in a narrow range of 8.0–9.9. The standard curve for NNMT was $Y = 2.99 \times \mathrm{Log}(X)3.95$ ($R^2 = 0.999$) with the *X*-axis representing the dilution factor and the *Y*-axis representing threshold cycle (Ct) number. We confirmed our results in an independent group of rats ($n = 4$ *per* group) and found a 77% downregulation in NNMT, which was consistent with our gene array results.

4 Discussion

Isothiocyanates are compounds derived from cruciferous vegetables, such as broccoli, cabbage, and watercress. Based on epidemiological studies, isothiocyanates are widely recommended as preventive agents, and commercially available in herbal supplements [11–13]. The purpose of this study was to evaluate potential mechanisms underlying the effect of PEITC, a component of watercress, as a cancer preventive agent. The liver is the major site of detoxification of endogenous compounds and xenobiotics, including carcinogens. Therefore, to gain insight on the effects of PEITC on metabolism related genes, we examined the effect of the oral administration of PEITC, for 7 days, on hepatic gene expression. The genes we studied were related to drug transport, phase I and phase II metabolism, apoptosis, and cell cycle signaling. Based on our previous pharmacokinetic studies [6, 7], we selected a dose of 150 μmol/kg which yields plasma concentration values similar to those obtained by oral exposure to PEITC from 100 g of watercress in humans.

A significant novel finding from our gene array experiment was the downregulation of NNMT by oral PEITC administration, which was further confirmed by RTQ RT-PCR. NNMT is a cytosolic enzyme that catalyzes the *N*-methylation of nicotinamide and other pyridines to form

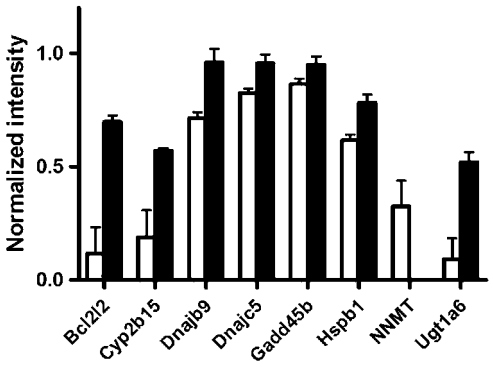

Figure 1. Effect of PEITC on hepatic gene expression of significantly altered genes in Sprague Dawley rats, as measured using a gene array. Animals ($n = 3$–4 *per* group) were administered corn oil (white bar, controls) or 150 μmol/kg PEITC (black bar) for 7 days. Data are expressed as net intensity normalized to GAPDH and BAS2C. Error bars represent standard error. All genes represented were statistically different from controls ($p<0.05$).

Table 1. Effect of PEITC on hepatic gene expression in Sprague Dawley rats ($n = 3$–4 *per* group)

Role	Gene name	Ratio (treatment/control)	*p*-Value (*t*-test)
Phase I metabolism	Cyp2b15	3.01	0.047
Phase II metabolism	Ugt1a6	5.67	0.015
Apoptosis	Bcl2l2	6.01	0.014
Stress regulator	Dnajb9	1.34	<0.005
Stress regulator	Dnajc5	1.16	0.01
Stress regulator	Hspb1	1.27	<0.005
Cancer marker	NNMT	0.00	<0.005

pyridinium ions [14]. Recently, the upregulation of NNMT was implicated in the pathogenesis of neurological diseases, as well as in the increased incidence of certain cancers [15, 16]. NNMT has been shown to be a good diagnostic tumor marker in hepatic, thyroid, lung, and colorectal cancer [17–20]. Although the relationship between the increased serum levels of NNMT in cancer is not fully understood, Dostalek *et al.* [21] have hypothesized that an increase in NNMT activity leads to a reduction of pyridine nucleotides available for defense against reactive oxygen species. NNMT gene depletion using siRNA in bladder cancer cells resulted in the reduced cellular proliferation and decreased cell migration, indicating that there is a functional role of NNMT in the progression of cancer [22]. In such a scenario, the downregulation of NNMT may play a role in the anticancer activity of PEITC [21]. It is reported that NNMT is regulated by HNF-1 and STAT3 [15, 23]. STAT3 has been shown to be significantly reduced through the administration of benzyl isothiocyanate, indicating a potential mechanism underlying the effects of isothiocyanates in the downregulation of NNMT [24]. NNMT has been shown to be downregulated by other dietary compounds as well, although all studies were performed in cancer cell lines. Curcumin treatment for 14 h, at a concentration of 50 μM, significantly reduced the expression of NNMT in MBA-MB-460 breast cancer cells [15]. In our laboratory, 1 μM PEITC and 1 and 10 μM indole-3-carbinol treatments, for 72 h, significantly downregulated NNMT in MCF7 breast cancer cells (Leuko, Telang, and Morris, unpublished work).

Another significant finding we observed was an upregulation of UGT1A6, which is a member of the family of UDP glucuronosyl transferases, a class of phase II enzymes in our gene array. This gene represents a phase II detoxification enzyme that is involved in the attachment of a glucuronide moiety to a compound. Carcinogens, including benzo(a)pyrene, are conjugated by UGT1A6, and increased glucuronidation would result in their increased elimination, and potentially decreased toxicity [25]. Therefore, the upregulation of this enzyme by PEITC may also represent a cancer preventive mechanism of PEITC. Similar upregulation of members of the UGT family by isothiocyanates has been previously reported. A 16-h incubation of liver cancer HepG2 cells with 30 μM sulforaphane caused a 2.8-fold increase in the glucuronidation of bilirubin, suggesting increased activity of UDP glucuronosyl transferase activity and increased elimination of bilirubin [3].

Cyp2b15 was the only phase I enzyme that was significantly upregulated by PEITC. Little information exists on the modulation of this enzyme by xenobiotics. However, it is reported that in the rat colon, co-incubation with 4 mM phenobarbital and 1 μM dexamethasone, known constitutive androstane receptor, and pregnane X receptor agonists, caused a 72-fold induction in cyp2b15 mRNA. However, isothiocyanates are known to chemically inhibit a number of CYP450 enzymes, including members of the CYP2B family. While confirmation is needed, it is likely that the increase in gene expression observed in our experiments may not translate into increased activity as a result of concomitant chemical inhibition.

Another gene that was significantly altered was the anti-apoptotic gene bcl2l2. In our experiments, we observed a 6.01-fold upregulation of bcl2l2. Bcl2l2, also known as Bcl-w, is upregulated in colon and gastric cancers [26]. In colorectal cancers, Bcl2l2 (bcl-w) was reported to be upregulated in 92% of adenocarcinomas, compared with 6% adenomas, suggesting a role for the protein in the progression of the cancer [27]. In SNU-16 gastric cancer cells overexpressing Bcl-w, activation of stress-activated protein kinase by agents, like etoposide, was inhibited, leading to reduced cell death. A 40-μM etoposide treatment (40-h treatment) produced 20% less cell death in bcl-w overexpressing cells compared with wild-type cells, suggesting an anti-apoptotic effect [28]. Overall, these results suggest that the upregulation of bcl-w by PEITC may represent a deleterious effect in hepatic cells.

Among other genes altered, Gadd45b is involved in hepatic regeneration in mice [29]. Heat shock proteins were also upregulated in the livers of rats that were treated with PEITC. Heat shock proteins are upregulated in events of stress, such as increased temperature or cold. Heat shock proteins and related binding proteins play a role in oxidative stress and apoptosis [30]. The consequences of the upregulation of these genes by PEITC need to be further investigated.

In conclusion, PEITC, a dietary agent found in cruciferous vegetables, altered the hepatic gene expression of enzymes that are responsible for the metabolism of carcinogens *in vivo*. Of the genes altered, the downregulation of NNMT was confirmed by RTQ RT-PCR methods. Upregulation of ugt1a6 represents another significant effect of oral administration of PEITC, as this may contribute to increased elimination of carcinogens. Other significant changes included the upregulation of the drug metabolizing enzyme cyp2b15 and the anti-apoptotic gene bcl2l2. The downregulation of NNMT by PEITC is a novel finding that may represent an important mechanism for the cancer preventive effects of dietary PEITC.

This study was financially supported by National Institutes of Health grant NIH CA121404. U. T. was supported in part by a fellowship from Daiichi Sankyo.

The authors have declared no conflict of interest.

5 References

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