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RESEARCH PAPER

Differential Response of Normal (PrEC) and Cancerous Human Prostate Cells (PC-3) to Phenethyl Isothiocyanate-Mediated Changes in Expression of Antioxidant Defense Genes

Anna A. Powolny • Shivendra V. Singh

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ABSTRACT

Purpose The present study was undertaken to test a hypothesis that differential sensitivity of normal and cancerous human prostate cells to prooxidant effect of phenethyl isothiocyanate (PEITC) is determined by altered expression of antioxidant defense genes.

Methods Prooxidant effect of PEITC was assessed by flow cytometry using a chemical probe and measurement of hydrogen peroxide production. Gene expression was determined by real-time PCR using Human Oxidative Stress and Antioxidant Defense RT^2 Profiler™. Protein expression was determined by Western blotting.

Results The PEITC treatment resulted in generation of reactive oxygen species and hydrogen peroxide production in PC-3 human prostate cancer cells but not in a representative normal human prostate epithelial cell line (PrEC). Basal oxidative stress-antioxidant defense gene expression signature was strikingly different between PC-3 and PrEC cells. The PEITC treatment (2.5 μM, 6 h) caused up-regulation of 29 genes and down-regulation of 2 genes in PC-3 cells. Conversely, 4 genes were up-regulated, and 10 genes were down-regulated by a similar PEITC treatment in the PrEC cell line.

Conclusions Differential sensitivity of PC-3 *versus* PrEC cells to prooxidant effect of PEITC is likely attributable to difference in basal as well as altered expression of antioxidant defense genes.

KEY WORDS antioxidant defense genes · chemoprevention · gene expression · oxidative stress · phenethyl isothiocyanate · reactive oxygen species

INTRODUCTION

Population-based case-control studies suggest that dietary intake of cruciferous vegetables may be protective against the risk of different malignancies, including cancer of the prostate (1–3), which is one of the leading causes of cancer-related death in American men (4). For example, a multicenter case-control study involving African-American, white, Japanese, and Chinese men (n=1,619) with histologically confirmed prostate cancer and matched controls (n=1,618; matched by ethnicity, age, region of residence) showed an inverse association between intake of cruciferous vegetables and the risk of prostate cancer (3). Anticancer effect of cruciferous vegetables is credited to chemicals with –N=C=S (isothiocyanate; ITC) functional group, which are produced upon cutting or chewing of these vegetables due to myrosinase-mediated hydrolysis of corresponding glucosinolates (5). Studies conducted in our laboratory and by others have shown that ITCs, including phenethyl-ITC (PEITC), benzyl-ITC (BITC), and sulforaphane (SFN), not only confer protection against chemically-induced cancer but also inhibit cancer development in transgenic mouse models (6–11). For example, oral gavage of SFN thrice per week reduced the incidence of prostatic intraepithelial neoplasia and well-differentiated carcinoma by ~23–28% (P<0.05 compared

A. A. Powolny • S. V. Singh
Department of Pharmacology & Chemical Biology
University of Pittsburgh Cancer Institute
University of Pittsburgh School of Medicine
Pittsburgh, Pennsylvania, USA

S. V. Singh (✉)
2.32A Hillman Cancer Center Research Pavilion
5117 Centre Avenue
Pittsburgh, Pennsylvania 15213, USA
e-mail: singhs@upmc.edu

with control) in the dorsolateral prostate, which was not due to the suppression of T-antigen expression (9). Moreover, the area occupied by the well-differentiated carcinoma was ~44% lower in the dorsolateral prostate of SFN-treated mice relative to that of control mice (9). Strikingly, the SFN-treated mice exhibited an approximate 50% and 63% decrease, respectively, in pulmonary metastasis incidence and multiplicity compared with control mice ($P<0.05$ (9)). In a separate study, we showed that dietary administration of BITC (3 mmol/kg diet) for 25 weeks markedly suppressed the incidence and/or burden of mammary hyperplasia and carcinoma in female MMTV-neu mice without causing weight loss or affecting neu protein level (10). The BITC-mediated prevention of mammary cancer development in MMTV-neu mice correlated with reduced cellular proliferation, increased apoptosis, and tumor infiltration of T-cells (10).

Though the exact mechanisms responsible for the beneficial effects of ITCs are not fully understood, cancer prevention by this class of compounds *in vivo* correlates with apoptosis induction (10). *In vitro* cellular studies have revealed that ITCs can selectively kill cancer cells by causing apoptotic and/or autophagic cell death (12–21). We have shown recently that different ITCs, including PEITC, BITC, and SFN, target mitochondrial respiratory chain complexes to trigger generation of reactive oxygen species (ROS), and both apoptotic and autophagic responses to ITC treatment are intimately linked to the ROS production (15,17,19–22). Interestingly, normal epithelial cells (a spontaneously immortalized and non-tumorigenic MCF-10A normal mammary epithelial cell line and PrEC normal human prostate epithelial cell line) are significantly more resistant to the proapoptotic and prooxidant effect of ITCs compared with cancer cells (16,17,21,22). Despite these advances, however, the mechanism behind selectivity of ITCs for cancer cells with regards to the apoptosis induction and ROS production remains elusive.

The present study was undertaken to test a hypothesis that differential sensitivity of normal (PrEC) and cancerous human prostate cells (PC-3) to prooxidant effect of PEITC is determined by differences in basal and/or altered expression of antioxidant defense genes. We found that basal oxidative stress-antioxidant defense gene expression signature is strikingly different between PC-3 and PrEC cells. Furthermore, the PC-3 and PrEC cells respond differentially to the PEITC-mediated changes in expression of oxidative stress-antioxidative defense genes.

MATERIALS AND METHODS

Reagents

PEITC (purity >99%) was purchased from Sigma-Aldrich (St. Louis, MO). Reagents for cell culture were purchased from GIBCO-Invitrogen (Carlsbad, CA). The hydroethidine (HE) and 5-(and-6)-carboxy-2′,7′-dichlorofluorescein

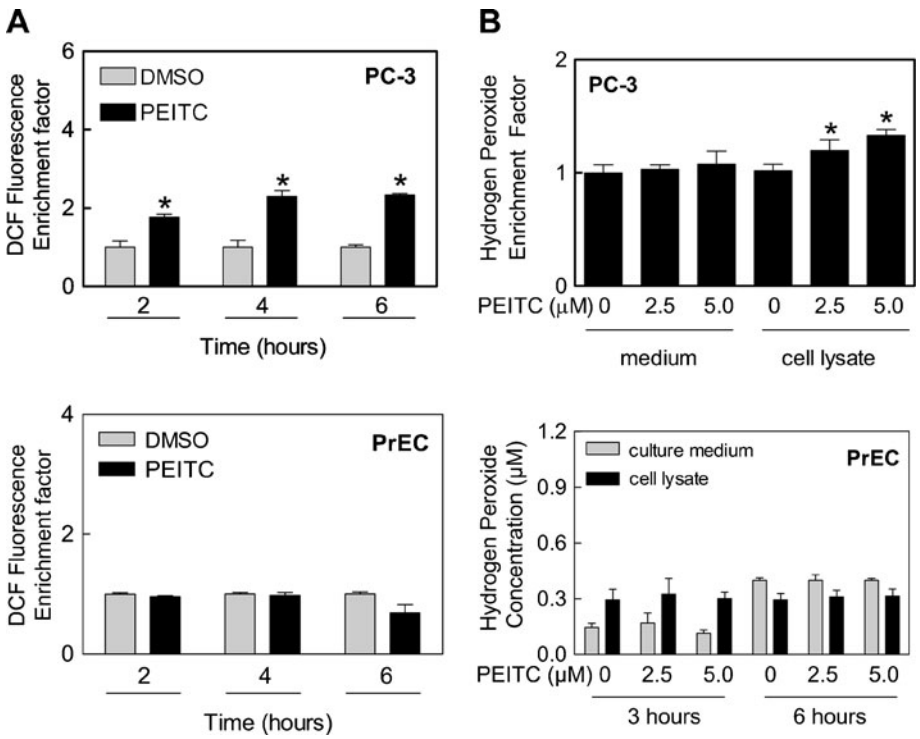

Fig. 1 Generation of reactive oxygen species by PEITC treatment in PC-3 cells. **A** Flow cytometric analysis of DCF fluorescence (a measure of ROS production) in PC-3 and PrEC cells treated with DMSO (control) or 5 μM PEITC for the indicated time periods. **B** Hydrogen peroxide production in medium and lysate of PC-3 and PrEC cells treated with the indicated concentrations of PEITC for 6 h (PC-3) or 3 and 6 h (PrEC). Data represent mean±SD ($n=3$). *Significantly different compared with corresponding DMSO-treated control by Student's *t*-test.

diacetate, succinimidyl ester (CDCFDA) were purchased from Molecular Probes (Eugene, OR). The antibodies against NADPH oxidase, EF hand calcium-binding domain 5 (NOX5) and Forkhead box protein M1 (FOXM1) were from Santa Cruz Biotechnology (Santa Cruz, CA). Human Oxidative Stress and Antioxidant Defense RT^2 Profiler™ was obtained from SuperArray Biosciences, a QIAGEN company (Frederick, MD).

Cell Lines and Cell Culture

The PC-3 cell line was procured from the American Type Culture Collection (Manassas, VA). Monolayer cultures of PC-3 cells were maintained in F-12K Nutrient Mixture supplemented with 7% non-heat-inactivated fetal bovine serum and antibiotics. The PrEC normal prostate epithelial cell line was purchased from Clonetics (now part of Lonza) and maintained in prostate epithelial basal medium (Cambrex, Walkersville, MD). Each cell line was maintained at 37°C in an atmosphere of 5% CO_2 and 95% air.

Measurement of ROS Generation and Hydrogen Peroxide (H_2O_2) Production

Stock solution of PEITC was prepared in dimethyl sulfoxide (DMSO) and diluted with complete medium

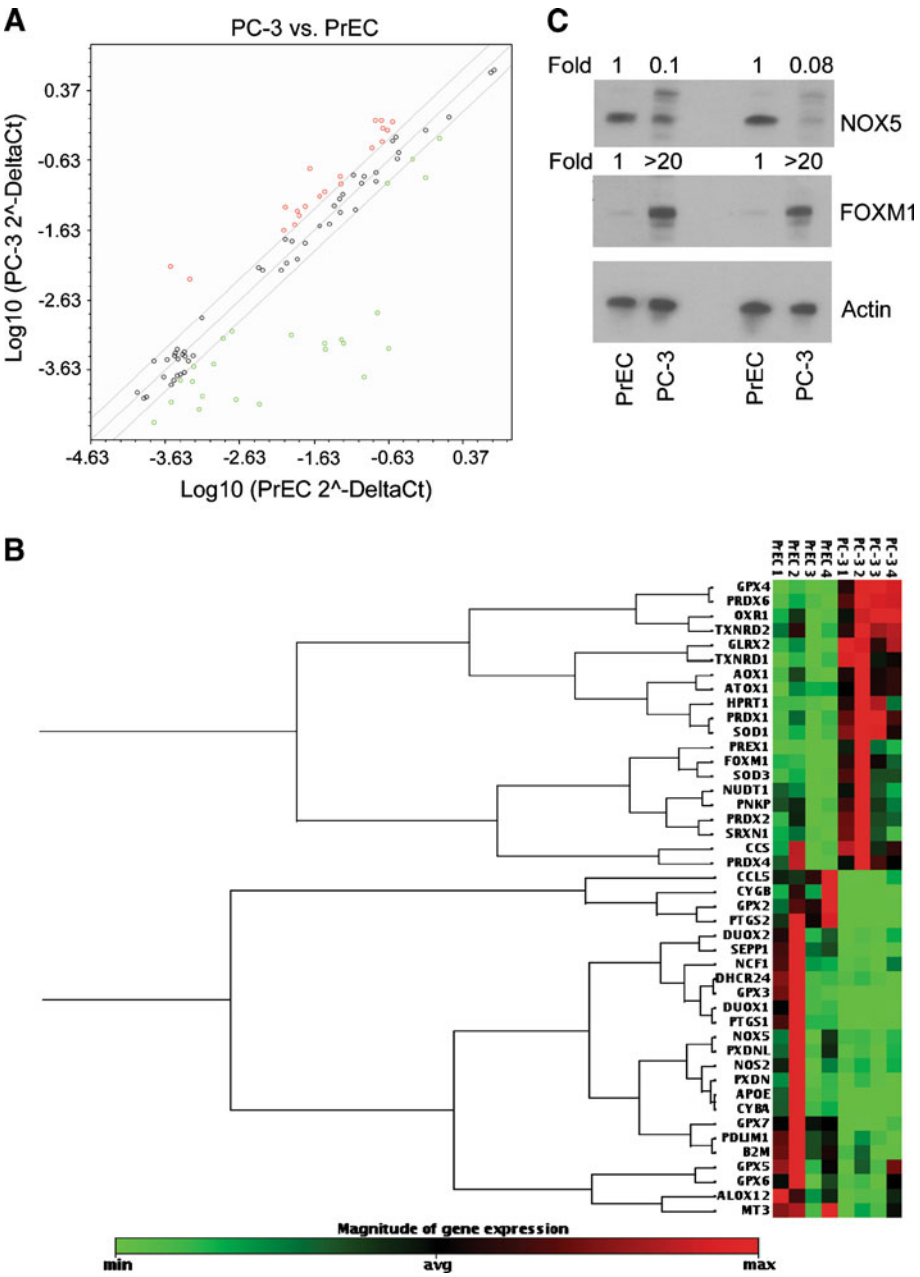

Fig. 2 Comparative expression of oxidative stress response and antioxidant defense genes in PC-3 and PrEC cells. **A** Relative expression of genes involved in oxidative stress response and antioxidant defense between PrEC and PC-3 cells. Genes up- and down-regulated are represented by red and green dots, respectively. Scatter plot shows a log transformation of the relative expression level of each gene between PC-3 and PrEC cells. **B** Cluster analysis demonstrating differences in gene expression between PC-3 and PrEC cells. Genes represented in the gene cluster analysis are limited to those whose expression differs between the cells by at least two-fold. Two independently prepared samples of each cell line in duplicate were used for gene expression profiling ($n = 4$). **C** Immunoblotting for NOX5 and FOXM1 using two lysates from PrEC and PC-3 cells. Membranes were stripped and re-probed with anti-actin antibody to ensure equal protein loading.

Table 1 Gene Expression in Prostate Cancer Cells (PC-3) and in Normal Prostate Epithelial Cells (PrEC)

Gene name	Symbol	UniGene	PC-3/PrEC	*P*- value
Aldehyde oxidase 1	AOX1	Hs.406238	5.233	0.00639
ATX1 antioxidant protein 1 homolog (yeast)	ATOX1	Hs.125213	2.491	0.00602
Copper chaperone for superoxide dismutase	CCS	Hs.502917	2.241	0.11227
Forkhead box M1	FOXM1	Hs.239	8.622	0.01064
Glutaredoxin 2	GLRX2	Hs.458283	2.554	0.00029
Glutathione peroxidase 4 (phospholipid hydroperoxidase)	GPX4	Hs.433951	2.837	0.00017
Nudix (nucleoside diphosphate linked moiety X)-type motif 1	NUDT1	Hs.534331	2.535	0.12080
Oxidation resistance 1	OXR1	Hs.148778	3.199	0.00400
Polynucleotide kinase 3′-phosphatase	PNKP	Hs.78016	2.870	0.11285
Peroxiredoxin 1	PRDX1	Hs.180909	2.005	0.00075
Peroxiredoxin 2	PRDX2	Hs.432121	3.516	0.05590
Peroxiredoxin 4	PRDX4	Hs.83383	2.266	0.18258
Peroxiredoxin 6	PRDX6	Hs.120	5.692	0.00005
Phosphatidylinositol-3,4,5-trisphosphate-dependent Rac exchange factor 1	PREX1	Hs.153310	25.880	0.05518
Superoxide dismutase 1, soluble	SOD1	Hs.443914	3.166	0.00044
Superoxide dismutase 2, mitochondrial	SOD3	Hs.487046	9.249	0.01570
Sulfiredoxin 1 homolog (S. cerevisiae)	SRXN1	Hs.516830	2.595	0.09924
Thioredoxin reductase 1	TXNRD1	Hs.708065	4.641	0.00324
Thioredoxin reductase 2	TXNRD2	Hs.443430	2.562	0.01016
Hypoxanthine phosphoribosyltransferase 1	HPRT1	Hs.412707	2.647	0.00857
Arachidonate 12-lipoxygenase	ALOX12	Hs.654431	0.4583	0.14090
Apolipoprotein E	APOE	Hs.654439	0.0116	0.00794
Chemokine (C-C motif) ligand 5	CCL5	Hs.514821	0.1343	0.10734
Cytochrome b-245, alpha polypeptide	CYBA	Hs.513803	0.0017	0.05172
Cytoglobin	CYGB	Hs.95120	0.0139	0.11042
24-dehydrocholesterol reductase	DHCR24	Hs.498727	0.1795	0.10203
Dual oxidase 1	DUOX1	Hs.272813	0.0169	0.05656
Dual oxidase 2	DUOX2	Hs.71377	0.0971	0.00523
Glutathione peroxidase 2 (gastrointestinal)	GPX2	Hs.2704	0.0097	0.10246
Glutathione peroxidase 3 (plasma)	GPX3	Hs.386793	0.0094	0.23506
Glutathione peroxidase 5 (epididymal androgen-related protein)	GPX5	Hs.248129	0.2942	0.24582
Glutathione peroxidase 6 (olfactory)	GPX6	Hs.448570	0.4428	0.00320
Glutathione peroxidase 7	GPX7	Hs.43728	0.0421	0.07772
Metallothionein 3	MT3	Hs.73133	0.2954	0.09047
Neutrophil cytosolic factor 1	NCF1	Hs.647047	0.2756	0.18396
Nitric oxide synthase 2, inducible	NOS2	Hs.709191	0.4796	0.17236
NADPH oxidase, EF-hand Ca binding domain 5	NOX5	Hs.657932	0.4679	0.14090
PDZ and LIM domain 1	PDLIM1	Hs.368525	0.4975	0.01117
Prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)	PTGS1	Hs.201978	0.0176	0.06976
Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	PTGS2	Hs.196384	0.002	0.00293
Peroxidasin homolog (Drosophila)	PXDN	Hs.332197	0.4788	0.22395
Peroxidasin homolog (Drosophila)-like	PXDNL	Hs.444882	0.2552	0.10323
Selenoprotein P, plasma, 1	SEPP1	Hs.275775	0.0646	0.01386
Beta-2-microglobulin	B2M	Hs.534255	0.4284	0.01100

immediately before use. An equal volume of DMSO (final concentration <0.1%) was added to the controls. ROS generation was assessed by flow cytometry after staining the cells with HE and CDCFDA and colorimetric analysis of H_2O_2 production. Flow cytometric analysis of ROS production using chemical probes HE and CDCFDA was performed essentially as described by us previously (15,22). The H_2O_2 production was monitored by a colorimetric assay using a kit from BioVision (Mountain View, CA). The chemical probe reacts with H_2O_2 to produce a byproduct with absorption maximum at 570 nm. Briefly, PC-3 or PrEC cells (3×10^5) were plated and allowed to attach by overnight incubation. The cells were then treated with DMSO (control) or desired concentrations of PEITC for specified time periods. The level of H_2O_2 in the culture medium and cell lysate was determined by following the manufacturer's instructions.

Gene Expression Analysis

The PC-3 and PrEC cells were plated at a density of 1×10^6, allowed to attach by overnight incubation, and then treated with 2.5 μM PEITC or DMSO (control) for 6 h, or left untreated to determine basal gene expression. Cells were harvested by scraping, and total RNA was extracted using the RNeasy Kit (Qiagen, Valencia, CA). Reverse transcription was performed using 3 μg of total RNA and SuperScript III First-Strand Synthesis System using standard protocol. In order to evaluate the effect of PEITC treatment on the levels of genes involved in oxidative stress and antioxidant defense and for comparison of basal expression of these genes between PrEC and PC-3 cells, the Human Oxidative Stress and Antioxidant Defense RT^2 Profiler™ was used. Experimental cocktail mixture was prepared immediately before the real-time analyses and contained

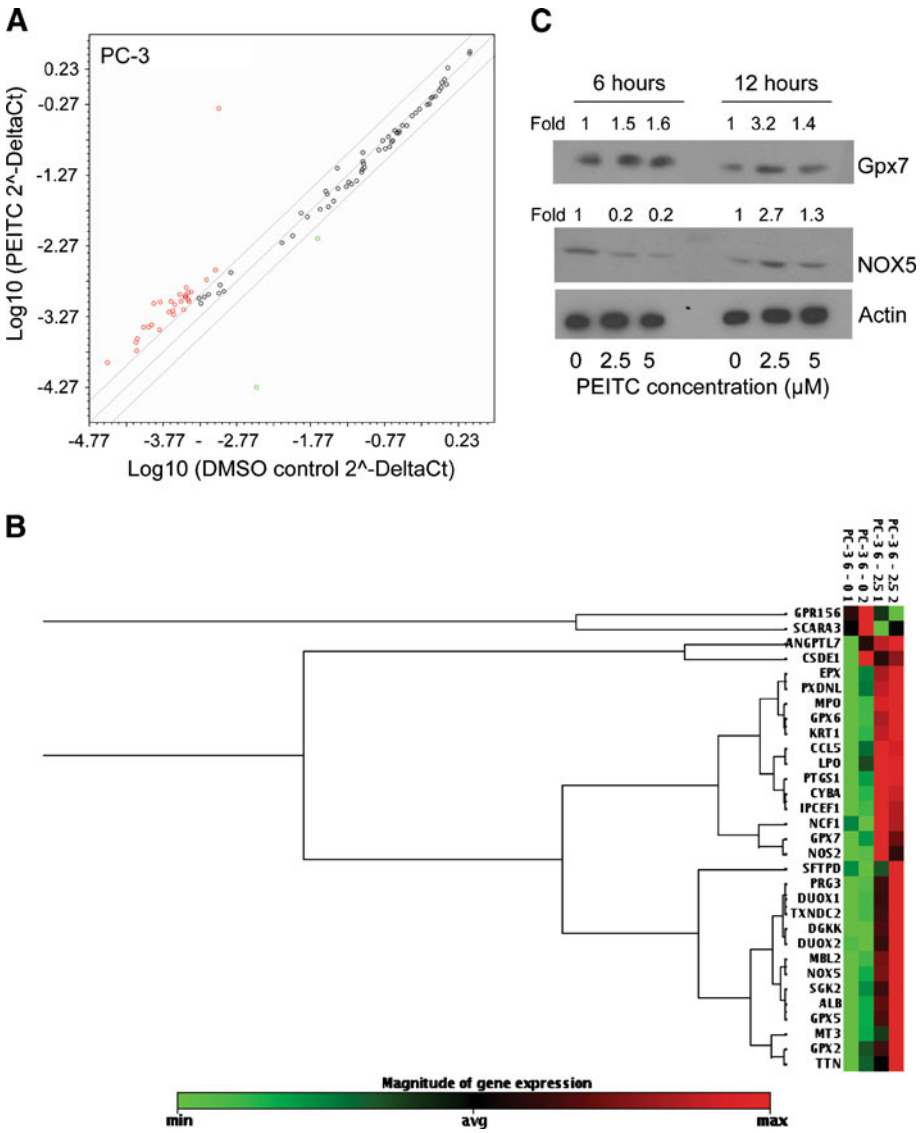

Fig. 3 Effect of PEITC treatment on expression of oxidative stress response and antioxidant defense genes in PC-3 cells. **A** Scatter plot shows a log transformation of the relative expression level of each gene between PC-3 cells treated with DMSO (control) and 2.5 μM PEITC for 6 h. **B** Cluster analysis demonstrating differences in gene expression in PC-3 cells in response to PEITC treatment. Cluster analysis shows only those genes which have a minimum of two-fold change in expression in response to PEITC treatment. The PC-3 cells were treated with DMSO or 2.5 μM PEITC for 6 h. Data are from duplicate measurements ($n=2$). **C** Immunoblotting for glutathione peroxidase 7 and NOX5 using lysates from PC-3 cells treated with 2.5 and 5 μM PEITC or DMSO (control) for 6 or 12 h. In order to ensure equal lysate protein loading, membranes were stripped and re-probed with anti-actin antibody. Change in protein level is expressed relative to DMSO-treated control.

cDNA and SYBR green probe. Twenty-five μL of the mixture was transferred into each well of the 96-well plate provided by the manufacturer. Two-step cycling protocol was employed using an ABI 700 cycler: 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. The data were analyzed using the web-based software provided by the manufacturer. The threshold cycle (C*t*) values were calculated, and the genes with C*t* values above 35 were considered undetected. Baseline and threshold values were set manually at the same level for all the samples to allow the comparison of multiple plates. The C*t* value of each gene was adjusted for the average C*t* of the housekeeping genes to generate ΔC*t* values. The ΔΔ*Ct* values were calculated as the difference in Δ*Ct* between the control and treated samples, or Δ*Ct* in PrEC *versus* PC-3 cells for the basal gene expression levels.

Immunoblotting

Immunoblotting was performed essentially as described by us previously (13–15). Briefly, PC-3 and PrEC cells were plated at a density of 1×10^6 cells in 100-mm culture dishes, allowed to attach by overnight incubation, and then treated with DMSO or desired concentrations of PEITC for specified time period. To examine basal levels of protein, PrEC and PC-3 cells were plated at a density of 1×10^6 cells in 100-mm cultured dishes, allowed to attach overnight, and then harvested without any treatment. Immunoreactive bands were visualized by enhanced chemiluminescence method. Densitometric analysis was performed to determine change in protein expression. Actin was used as a loading control.

RESULTS AND DISCUSSION

Effect of PEITC Treatment on ROS Production

We have shown previously that the PrEC normal human prostate epithelial cell line is significantly more resistant to growth inhibition by PEITC compared with prostate cancer cells (16), and growth suppression by PEITC against prostate cancer cells is intimately linked to ROS generation (20,23). In a recently published study complementing the results shown herein, we have demonstrated that the PEITC-mediated ROS are mitochondria-derived in prostate cancer cells, including PC-3 (21). This conclusion is based on the following observations: (a) the PEITC treatment inhibits mitochondrial respiratory chain complex III and oxidative phosphorylation in PC-3 and LNCaP cells, (b) the PEITC-induced ROS production as well as histone-associated apoptotic DNA fragmentation are significantly attenuated by ectopic expression of superoxide dismutase, and (c) mitochondrial DNA-deficient Rho-0 variants of PC-3 and LNCaP, which lack oxidative phosphorylation but rely on anaerobic glycolysis for survival, are significantly more resistant to PEITC-mediated ROS production, apoptotic DNA fragmentation, collapse of mitochondrial membrane potential, Bax activation, and caspse-3 activation compared with respective wild-type cells (21). In the present study, we questioned if the differential sensitivity of cancerous *versus* normal prostate epithelial cells to prooxidant effect of PEITC was

Table II Gene Expression Changes in PC-3 Cells Treated with 2.5 μM PEITC for 6 h

Gene name	Symbol	UniGene	PEITC/ Control
Albumin	ALB	Hs.418167	3.9945
Angiopoietin-like 7	ANGPTL7	Hs.146559	2.0677
Chemokine (C-C motif) ligand 5	CCL5	Hs.514821	2.2548
Cold shock domain containing E1, RNA-binding	CSDE1	Hs.69855	492.1656
Cytochrome b-245, alpha polypeptide	CYBA	Hs.513803	3.2221
Diacylglycerol kinase, kappa	DGKK	Hs.631770	4.1068
Dual oxidase 1	DUOX1	Hs.272813	2.9445
Dual oxidase 2	DUOX2	Hs.71377	3.4895
Eosinophil peroxidase	EPX	Hs.279259	2.8939
Glutathione peroxidase 2 (gastrointestinal)	GPX2	Hs.2704	2.5193
Glutathione peroxidase 5 (epididymal androgen-related protein)	GPX5	Hs.248129	3.0483
Glutathione peroxidase 6 (olfactory)	GPX6	Hs.448570	4.0222
Glutathione peroxidase 7	GPX7	Hs.43728	3.1667
Keratin 1 (epidermolytic hyperkeratosis)	KRT1	Hs.80828	3.8053
Lactoperoxidase	LPO	Hs.234742	6.4666
Mannose-binding lectin (protein C) 2, soluble	MBL2	Hs.499674	3.1777
Myeloperoxidase	MPO	Hs.458272	3.394
Metallothionein 3	MT3	Hs.73133	2.8343
Neutrophil cytosolic factor 1, (chronic granulomatous disease, autosomal 1)	NCF1	Hs.647047	5.4945
Nitric oxide synthase 2A (inducible, hepatocytes)	NOS2	Hs.706746	2.7856
NADPH oxidase, EF-hand calcium binding domain 5	NOX5	Hs.657932	2.3506
Interaction protein for cytohesin exchange factors 1	IPCEF1	Hs.146100	3.1998
Proteoglycan 3	PRG3	Hs.251386	3.0168
Prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)	PTGS1	Hs.201978	3.4895
Peroxidasin homolog (Drosophila)-like	PXDNL	Hs.444882	4.0362
Surfactant, pulmonary-associated protein D	SFTPD	Hs.253495	2.3751
Serum/glucocorticoid regulated kinase 2	SGK2	Hs.472793	2.6445
Titin	TTN	Hs.654592	2.4334
Thioredoxin domain-containing 2 (spermatozoa)	TXNDC2	Hs.98712	2.9241
G protein-coupled receptor 156	GPR156	Hs.333358	0.0171
Scavenger receptor class A, member 3	SCARA3	Hs.128856	0.3226

related to differences in their antioxidant defense capacity. As shown in Fig. 1A, exposure of PC-3 cells to 5 μM PEITC resulted in ROS production, as evidenced by flow cytometric analysis of CDCFDA oxidation, in a time-dependent manner. The PEITC-mediated oxidation of CDCFDA was not observed in the PrEC cell line (Fig. 1A). Consistent with these results, 6 h treatment with PEITC increased H_2O_2 levels in PC-3 cells but not in the PrEC cell line (Fig. 1B).

Basal Expression of Redox Genes in PC-3 and PrEC Cells

Next, we explored the possibility whether resistance of PrEC cells to prooxidant effect of PEITC compared with PC-3 was due to differences in basal expression of antioxidant defense genes. We tested this possibility by real-time PCR for a set of 84 selected genes involved in the oxidative stress and antioxidant defense. This analysis revealed that 44 genes were differentially expressed between PC-3 and PrEC cells (Fig. 2A, B). Of the 84 genes, 20 were up-regulated and 24 were down-regulated in PC-3 cells compared with PrEC (Table 1). Genes statistically significantly down-regulated in PC-3 cells included Apolipoprotein E, dual oxidase 1 and 2, and some members of the glutathione peroxidase family, to name a few. On the other hand, genes that were up-regulated in the PC-3 cell line compared with PrEC included FOXM1, superoxide dismutase 1 (soluble) and 2 (mitochondrial), among others (Table 1). Gene expression differences were confirmed by Western blotting for selected proteins. In agreement with real-time PCR results, basal expression of NOX5 protein was significantly higher in the PrEC cell line compared with PC-3 (Fig. 2C). Conversely, higher constitutive expression of FOXM1 protein was clearly visible in the PC-3 relative to the PrEC cells (Fig. 2C).

Cancer cells are characterized by increased level of ROS and reduced ability to remove these deleterious species (24,25). It has been postulated that persistent oxidative stress promotes tumor cell survival, proliferation, migration/invasion, and angiogenesis and inhibits apoptosis by activating certain redox-sensitive transcription factors such as NF-κB and AP-1 (26–28). The present study reveals that the basal expression of glutathione peroxidases and NOX5

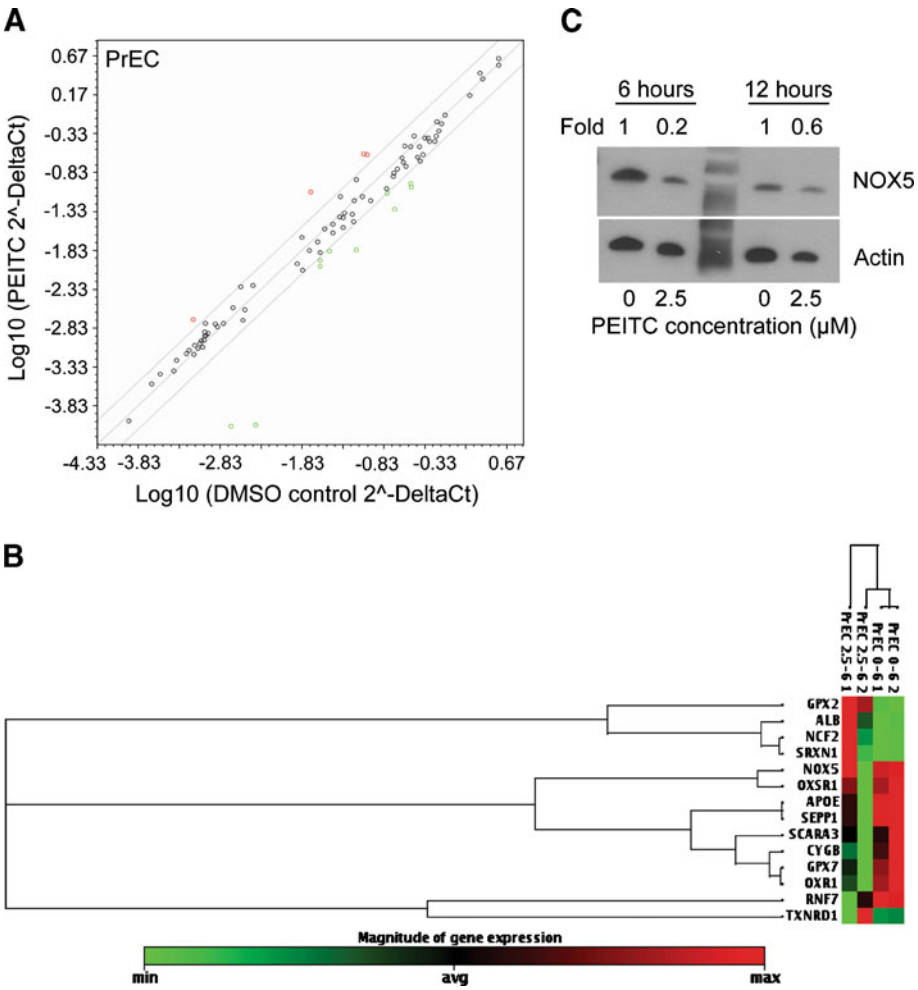

Fig. 4 Effect of PEITC treatment on expression of oxidative stress response and antioxidant defense genes in PrEC cells. **A** Scatter plot shows a log transformation of the relative expression level of each gene between the PrEC cells treated with DMSO and 2.5 μM PEITC for 6 h. **B** Cluster analysis demonstrating differences in gene expression in PrEC cells in response to PEITC treatment. Cluster analysis shows only those genes whose expression was changed by a minimum of two-fold in response to PEITC treatment. **C** Immunoblotting for NOX5 using lysates from PrEC cells treated with DMSO or the indicated concentrations of PEITC for 6 or 12 h. Membranes were stripped and re-probed with anti-actin antibody to ensure equal protein loading. Change in the protein level is expressed relative to DMSO-treated control.

is significantly lower in PC-3 cells than in the PrEC cell line. Glutathione peroxidases are responsible for reduction of H_2O_2, as well as soluble fatty acid hydroperoxides (29). The glutathione peroxidases function as antioxidant enzymes, and their activity has been inversely associated with the development of various types of malignancies, including prostate cancer. This hypothesis is supported by the findings from several clinical studies which reveal that prostate cancer patients exhibit lower levels of glutathione peroxide activity in the plasma as well as in prostate tissue when compared to men with benign hyperplasia or healthy controls (30–32). Levels of glutathione peroxidase 1 were 5-fold lower in PC-3 (R), a variant that is more resistant to several anticancer drugs as compared to the wild-type PC-3 cells (33). In accordance with our findings, glutathione peroxidase 2 was previously shown to be down-regulated in Nkx3.1 mutant mice, which demonstrated prostatic intra-epithelial neoplasia (34). Additionally, Nkx3.1 and PTEN double knock-out mice showed down-regulation of both glutathione peroxidase 2 and glutathione peroxidase 3, which would indicate that in this model glutathione peroxidases were being progressively down-regulated as the disease developed. The glutathione peroxidase 3 was also shown to be affecting metastatic ability of prostate cancer cells, and its down-regulation correlated with CpG methylation (35,36). The glutathione peroxidase 5, which is expressed in the male reproductive organs, was previously shown to protect immature spermatozoa from oxidative damage (37). Even though this isoform has not been directly linked to prostate cancer, its down-regulation in our model may further contribute to an environment promoting oxidative stress and prostate carcinogenesis. Glutathione peroxidase 6 (expressed in olfactory epithelium) and glutathione peroxidase 7 (putative) have not directly been linked to prostate cancer development; however, it is of interest that glutathione peroxidase 7 is hypermethylated in Barrett's adenocarcinoma (38).

Another gene down-regulated in PC-3 cells was the NOX5, which has been shown to regulate growth and apoptosis in DU145 human prostate cancer cells (39). The NOX5 has been shown to contribute to the ROS production by generating large amounts of superoxide anion (40). It would seem counterintuitive for prostate cancer cells in our system to down-regulate expression of this enzyme. However, it is possible that PC-3 cells rely mostly on mitochondria for generation of ROS, and additional superoxide production by NOX5 would actually lead to dangerous increases in ROS and to apoptosis. This speculation is plausible considering hydrogen peroxide-mediated down-regulation of NOX5 has been observed in MCF-7 human breast cancer cells (41).

Interestingly, expression of the FOXM1 is markedly higher in the PC-3 cell line compared with PrEC (Table 1). FOXM1 is expressed in a variety of cancers, including prostate (42–44). It was previously shown that increased levels of FOXM1 accelerated prostate cancer development and progression in TRAMP and LADY mouse models (44). Additionally, inhibition of FOXM1 expression with FOXM1-specific siRNA in DU145, PC-3 and LNCaP prostate cancer cells led to reduction in proliferation in association with reduction in cyclinA2 and cyclinB1 proteins (44). The FOXM1 was also up-regulated in metastatic prostate caner tissue samples, which suggests that FOXM1 is involved in regulation of tumor progression and the development of metastasis (45). The idea that FOXM1 regulates metastasis was supported by a study in breast cancer which revealed that down-regulation of FOXM1 attenuated not only cell proliferation but also migration and invasion of MDA-MB-231 breast cancer cell (46). Simultaneously, reduced FOXM1 levels cause reduced secretion of factors such as MMP-2, MMP-9, and VEGF, all of which promote extracellular matrix remodeling and angiogenesis (46). The FOXM1 was also implicated in oxidative stress response (47). Specifically, Ras-induced ROS overexpressed FOXM1 which in turn modulated the ROS levels by inducing expression of genes such as Mn-SOD (47). This may explain why we saw moderate up-regulation of SOD in our system and provide a mechanism by which prostate cancer cells modulate ROS levels to escape cell death.

Table III Gene Expression Changes in PrEC Cells Treated with 2.5 μM PEITC for 6 h

Gene name	Symbol	UniGene	PEITC/ Control
Albumin	ALB	Hs.418167	2.7435
Glutathione peroxidase 2 (gastrointestinal)	GPX2	Hs.2704	3.108
Neutrophil cytosolic factor 2 (65 kDa, chronic granulomatous disease, autosomal 2)	NCF2	Hs.587558	4.426
Sulfiredoxin 1 homolog (S. cerevisiae)	SRXN1	Hs.516830	2.7151
Apolipoprotein E	APOE	Hs.654439	0.2996
Cytoglobin	CYGB	Hs.95120	0.2208
Glutathione peroxidase 7	GPX7	Hs.43728	0.0207
NADPH oxidase, EF-hand calcium binding domain 5	NOX5	Hs.657932	0.0403
Oxidation resistance 1	OXR1	Hs.148778	0.4478
Oxidative-stress responsive 1	OXSR1	Hs.475970	0.4969
Ring finger protein 7	RNF7	Hs.134623	0.2537
Scavenger receptor class A, member 3	SCARA3	Hs.128856	0.374
Selenoprotein P, plasma, 1	SEPP1	Hs.275775	0.4417
Thioredoxin reductase 1	TXNRD1	Hs.680369	0.3441

PEITC Treatment Differentially Altered Expression of Genes Involved in Oxidative Stress Response and Antioxidant Defense in PC-3 and PrEC Cells

Next, we proceeded to determine the effect of PEITC treatment on genes associated with oxidative stress response and antioxidant defense in these cells. Exposure of PC-3 cells to growth-suppressive and proapoptotic concentration of PEITC (2.5 μM for 6 h) resulted in up-regulation of 29 genes and down-regulation of only two genes at a 2-fold level (Fig. 3A, B). Genes up-regulated in response to the PEITC treatment in PC-3 cells included some glutathione peroxidases (2,5–7), myeloperoxidase, and lactoperoxidase, to name a few. The two genes down-regulated included G protein-coupled receptor 156 and scavenger receptor class A, member 3 (Table 2). We selected a few proteins to confirm our initial findings. As can be seen in Fig. 3C, we observed increased protein levels of glutathione peroxidase 7 at 6 and 12 h time points in response to 2.5 and 5 μM PEITC treatments. Additionally, NOX5 protein level was slightly up-regulated after 12 h exposure to PEITC (Fig. 3C).

We have previously described that PEITC can induce cell cycle arrest as well as apoptotic and autophagic cell death in prostate cancer cells (14,16,18,21,23). Results of the present study indicate that prostate cancer cells do attempt to counteract this additional ROS threat by up-regulating a variety of peroxidases, including glutathione peroxidases 6 and 7, lactoperoxiadase, and myeloperoxidase. All of these enzymes are able to reduce oxidative stress and may function to protect the cancer cell from apoptosis. Simultaneously though, PEITC increases expression of dual oxidase 2, which is responsible for generation of hydrogen peroxide, and down-regulates scavenger receptor class A, member 3, which in turn functions to protect from ROS-induced damage.

Strikingly, a similar PEITC treatment produced a different response in the PrEC cell line (Fig. 4A, B). Specifically, treatment of PrEC cells with 2.5 μM PEITC for 6 h resulted in change in expression of only 14 genes (Table 3). In contrast to PC-3 cells, more genes were down-regulated by PEITC treatment in the PrEC cell line (Table 3). Among the genes affected by the PEITC treatment, ten were down-regulated and four were up-regulated. These results indicate that the normal prostate epithelial cells respond by changing expression of several genes. In fact, it is possible that this state of readiness exhibited by untreated PrEC cells is the cause of the resistance of these cells to PEITC-induced ROS generation. Specifically, in our system, PrEC cells responded by up-regulating glutathione peroxidase 2 and down-regulating genes such as NOX5. Our results suggest that PEITC does not induce a dramatic change in PrEC cells, and these cells only require modest change in their status to balance their redox status. It is not clear why Apolipoprotein E and glutathione peroxidase 7 were down-regulated in our system. It is possible that these proteins do not play a major role in the regulation of oxidative stress in normal prostate epithelium, or that their levels are high enough in the basal state and no additional gene expression is required to protect the cells.

CONCLUSIONS

Based on the results shown herein we speculate that the differences in basal as well as altered expression of oxidative stress-related and antioxidant defense genes result in increased susceptibility of PC-3 cells to PEITC-induced ROS generation and apoptosis compared to normal prostate epithelial cell line PrEC. However, as a note of caution, our conclusion is based on a single pair cell line. Additional studies with multiple normal and cancerous cell pairs are needed to firmly establish the validity of our conclusions.

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