Epigenetic Mechanism of Growth Inhibition Induced by Phenylhexyl Isothiocyanate in Prostate Cancer Cells

ANASTASIA A. BEKLEMISHEVA1, YUQIANG FANG1, JINGYANG FENG1, XUDONG MA1,2, WEI DAI1 and JEN WEI CHIAO1

1Department of Medicine, New York Medical College, Valhalla, NY 10595, U.S.A.; 2Zhangzhon Municipal Hospital, Zhangzhon, Fujian, 363000, China

Abstract. Background: Isothiocyanates, the constituents of cruciferous vegetables, may be able to prevent prostate cancer. The hypothesis that they could remodel chromatin and activate cell cycle inhibitors, such as p21 for growth inhibition, was tested. Materials and Methods: Prostate cancer LNCaP cells were exposed to phenylhexyl isothiocyanate (PHI). The status of histone acetylation and the activity of histone deacetylases (HDAC) were investigated. The association of p21 with hyperacetylated histones was examined by chromatin immunoprecipitation. Results: The PHI-exposed LNCaP cells had diminished activity of HDAC 1 and 2. Global and selective histone acetylation was enhanced, consistent with the signs of chromatin unfolding. The hyperacetylated histones increased accessibility to the p21 promoter for transcription, leading to G1 arrest and apoptosis. Conclusion: PHI inhibited the activity of HDAC and remodeled chromatin to activate p21 for cell cycle arrest, underlying an epigenetic mechanism regulating the growth of prostate cancer cells.

The epidemiological literature has provided modest to strong support to the hypothesis that the intake of cruciferous vegetables, such as broccoli, cabbages, Brussels sprouts, watercress and cauliflower, reduces prostate cancer risk (1). These studies, however, did not identify the responsible dietary constituents or the mechanisms of action. We have examined the role of the isothiocyanates, released from glucosinolates upon enzyme hydrolysis when the cruciferous vegetables are masticated (2). Natural and synthetic isothiocyanates are potent chemopreventive agents that block carcinogen-initiated tumors in rodents. The major mechanism is cytoprotection, i.e. inhibiting P450s and the metabolism of procarcinogens, and inducing phase 2 enzymes to remove carcinogens (3). Our laboratory has investigated the phenethyl isothiocyanate, sulforaphane, and its major metabolites, and demonstrated that they induce growth arrest and apoptosis in prostate cancer cells, in culture as well as in xenografted tumors in immunodeficient mice (4, 5). These observations, together with reports by other investigators (6, 7), indicated that the isothiocyanates can also act at the level of post-initiation progression of prostate carcinogenesis. Studies indicate that one of the mechanisms is targeting of the cell cycle machinery, with the up-regulation of cell cycle inhibitors such as p21WAF1 (p21) to mediate growth arrest (8). The upstream mechanism that leads to the cell cycle arrest, however, has not been clearly deciphered.

We have since investigated the potential effects of the isothiocyanates on chromatin remodeling for transcriptional regulation of the cell cycle inhibitors. In this study, the effects of a synthetic phenylhexyl isothiocyanate (PHI) on chromatin remodeling were examined, since PHI is among the most potent of the isothiocyanates in blocking carcinogen-initiated lung tumors (9). Chromatin remodeling is influenced by enzymatic modifications of amino acids at the histone tails, which cause nucleosome movement, orchestrating epigenetic events. Covalent modifications, such as acetylation, neutralize the charges on histones and provide a more open conformation with the DNA. The balance of acetylation is regulated by pairs of opposing enzymes, the acetyl transferases and deacetylases (HDAC) (10). The inhibitors of HDAC represent a new class of targeted anticancer agents that mediate gene expression, such as p21, to induce growth arrest and apoptosis in malignant cells (11). In this study, it was demonstrated that PHI is an inhibitor of HDAC and initiates histone modifications and chromatin remodeling to activate the inhibitor of cell cycle progression, p21, leading to proliferation cessation and apoptosis in prostate cancer cells.

Materials and Methods

Cell culture. PHI of more than 98% purity was purchased from LKT Labs (St. Paul, MN, USA). The PHI was prepared in 75%
methanol and phosphate buffer, pH 5. The LNCaP prostate cancer cells, in exponential growth, were seeded at 0.3x10^6 per ml of RPMI-1640 medium supplemented with 15% heat-inactivated fetal bovine serum and antibiotics. Some cultures were exposed to PHI at various concentrations, while the controls were supplemented with the methanol medium. Cell viability was determined by the trypan blue exclusion method and the cell density was calculated by the viable cell counts per ml.

**Apoptosis and cell cycle phases.** The cell cycle phases were determined with a Becton-Dickinson FACScan flow cytometer, according to the methods described previously (12). The cells were incubated on ice before the DNA was stained with propidium iodide (50 μg/ml). The apoptotic cells were determined by the presence of DNA strand breaks with terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick-end labelling (TUNEL). Detection of apoptosis in situ was performed with a detection kit (Roche Molecular Biochemicals, Indianapolis, IN, USA), according to the manufacturer’s directions. The apoptotic cell percentages were calculated by counting 500 cells from multiple fields.

**Protein expression.** The protein levels of the LNCaP cells were determined by Western blotting, according to the standard procedure (12). Total cellular proteins were obtained with a lysis buffer containing freshly prepared protease inhibitors, and the lysates collected after centrifugation at 4°C. The histone proteins were isolated with an acid extraction procedure (13). Rabbit antibodies against the acetylated histones H3, H4 or H3 lysine 14 were purchased from Upstate Biotechnology (Lake Placid, NY, USA). Monoclonal antibodies against PARP, bel-2, caspases-8 or -9, or HDAC 1 were purchased from Santa Cruz (Santa Cruz, CA, USA), and antibody for p21 from Dako (Carpinteria, CA, USA). Densitometric analysis was performed to calculate the % alteration of the protein levels.

**Histone deacetylase (HDAC) activity.** To evaluate the inhibition of HDAC activity by PHI, a cell-free assay detecting HDAC 1 and 2 (Color de Lys, Biomol, Plymouth Meeting, PA, USA) was used according to the manufacturer’s protocol. A nuclear extract of HeLa cells containing HDAC 1 and 2 was incubated with PHI at various concentrations for 10 min at 37°C. A separate test was performed by adding PHI to a standard HDAC activity to evaluate any interference between PHI and the developers. Incubation with trichostatin A (TSA) (50 nM) was used as a positive control for inhibition. The methanol medium was used as the vehicle control. For detecting HDAC activity in the LNCaP cells, cellular lysates of LNCaP cells, that had been exposed to 2 mM sodium butyrate, or PHI, were compared to the lysate from LNCaP cells exposed to the vehicle control of PHI. The HDAC reaction was initiated by adding a substrate of acetylated peptides and incubating at 37°C for 30 min, followed by adding a color developer. The absorbance of triplicate samples was determined at 405 nm with a Bio-Tec microtiter-plate reader. The Student’s t-test was used for statistical analyses with p<0.05 considered to be significant.

**Chromatin immunoprecipitation (ChIP) assay.** The LNCaP cells were cultured for 24 h with PHI at 20 μM or with vehicle control medium. ChIP analyses were performed according to the protocol of the ChIP assay kit (Upstate Biotechnology). Briefly, the DNA and histones were cross-linked by adding formaldehyde to the culture to a final concentration of 1%. After incubation for 10 min, the cell lysates were prepared. After sonication, the cell lysates (200 μl) in a ChIP dilution buffer were incubated with a rabbit antibody against acetylated histone H3, or with a non-specific control Ig overnight. The mixtures were incubated with salmon sperm DNA/protein A agarose slurry for 1 h, the beads were washed and the bound molecules eluted. After de-cross-linking, the DNA molecules in the precipitates were recovered. The p21 gene fragments TATA area (~33 to +47), down TATA area (+43 to +122) and D (+3267–+3366) were selected for amplification by PCR as described (8).

**Results**

**Induction of growth inhibition and apoptosis.** To evaluate the effects of PHI on the growth of prostate cancer cells, the prostate cancer cells LNCaP were exposed to PHI at various concentrations. Floating cells appeared in the PHI-exposed cultures along with a regular decrease in cell density. Concentration-dependent growth inhibitory effects could be detected after 2-day PHI exposure, at a minimal concentration of 1 μM. Approximately a 17% and 23% decrease in cell density was observed at 5 and 10 μM, respectively (Figure 1A). Figure 1A also depicts a gradual decrease of cell viability in these cultures. The effects of PHI on cell proliferation were evaluated by cell cycle phase progression, with the DNA frequency histogram using a flow cytometric method. After 1 day of PHI exposure, the S-phase cell proportion was reduced in a concentration-related manner, with minimal effects seen at 1 μM. The level of S-phase cells was reduced by about 45%, from 33% to 18%, after exposure to 10 μM PHI. An accumulation of G1 cells was observed concomitantly, indicating a blocking of cell cycle progression from G1- to S-phase (Figure 1B).

Many PHI-exposed cells displayed cell shrinkage and condensed chromatin, characteristic of apoptosis. The presence of apoptotic cells was detected by DNA strand breaks with the TUNEL assay. The quantity of apoptotic cells was concentration-dependent; a minimal amount was detected with 1-5 μM, increasing to approximately 26% at 20 μM (Figure 1C). Proteolytic cleavage of PARP, a hallmark of apoptosis, was detected in PHI-exposed cells (Figure 1D). A minimal reduction of PARP could be detected at 5 μM PHI, but in excess of 50% at 20 μM. In addition, the bel-2 protein, an inhibitor of apoptosis, was significantly diminished by PHI at 5 μM or more. The relationship of PHI to the caspases, intracellular cysteine proteases in the initiation and execution of apoptosis, was investigated. Figure 1D depicts an increased expression of caspase-9, but the level of caspase-8 was not altered.

**Inhibition of HDAC and histone modification.** To investigate the effects of PHI on chromatin remodeling as a mechanism of growth arrest, the expression of HDAC was measured. The Western blot analyses, shown in Figure 2A, revealed...
that reduction of the HDAC1 level could be detected with higher PHI concentrations, with an approximately 15% decrease at 20 μM after 6 h of exposure. A similar magnitude of decrease was also observed at 48 h. Experiments were then performed to determine whether PHI inhibited the activity of HDAC. A nuclear extract of HeLa cells containing HDAC was supplemented with PHI, and the HDAC activity determined with a cell-free HDAC activity assay. The concentrations of PHI used were predetermined so that they did not interfere with the reagents of the assay. As demonstrated in Figure 2B, PHI inhibited the activity of HDAC 1 and 2 in a concentration-dependent manner, while the vehicle control of PHI showed no effect. Statistically significant inhibition could be achieved with 50 nM or more PHI.

To further evaluate whether the activity of HDAC was actually lowered in PHI-exposed LNCaP cells, the cellular extracts of LNCaP cells were evaluated for HDAC activity. Figure 2C demonstrates that LNCaP cells exposed for 6 h to PHI had diminished HDAC activity as compared to control LNCaP cells without PHI. A statistically significant reduction, of approximately 25% and 43%, could be achieved, respectively, with 5 and 20 μM PHI. A known HDAC inhibitor, sodium butyrate, used as a control showed an approximately 50% reduction. The results indicated that PHI reduced the HDAC activity in LNCaP cells.

With the finding that PHI inhibited the activity and level of HDAC, experiments were performed to examine the status of acetylation of core histones. Enhancement of histone acetylation could be detected after 6-h exposure to
PHI, with the magnitude of enhancement increasing with longer exposure time. By 24 h, a clear concentration-dependent enhancement of acetylation of the histones H3, H4 and H3 lysine 14 was demonstrated (Figure 3A). At 5 μM, approximately 60% and 110% increases were seen with histones H3 and H4, respectively.

**Induction of p21.** The cell cycle regulators were among the most common genes to be activated as a result of HDAC inhibition (8, 14). To examine the relationship between enhanced histone acetylation and cell growth, the expression of the cdk kinase inhibitor p21 was evaluated. Figure 3B shows that exposure to PHI for 6 h resulted in a significant induction of p21 expression. A minimal increase could be detected in cells after exposure to 1 μM PHI, and an approximately 40% increase with 5 μM.

To further determine the relationship between p21 activation and acetylated histones, the ChiP assay was performed. The DNA from the immunoprecipitates was amplified according to four primers complementary to p21 gene fragments, including the promoter region. As demonstrated in Figure 4, the chromatin from cells exposed to PHI clearly contained the p21 DNA after precipitation with the antibody, as compared to cells exposed to the control.
medium without PHI (Figure 4). Immunoprecipitations using a non-specific Ig were employed as controls to show the negative background (Figure 4). The p21 sequence was detected in the promoter areas TATA, down TATA and D as compared with cells not exposed to PHI where p21 was nearly undetectable. The results showed that more p21 DNA was present in the precipitates of hyperacetylated histones, indicating the association of the p21 gene with the hyperacetylated histones.

**Discussion**

Evidence is presented that a synthetic isothiocyanate, PHI, is capable of inducing G1 arrest and apoptosis in prostate cancer cells. We have demonstrated that there is complex cross-talk between chromatins and the DNA that up-regulates p21 for cell cycle arrest. The transcriptional activation of p21 is revealed as cumulative, occurring as a result of HDAC inhibition, hyperacetylation of histones and the regulation of enzymes controlling the balance of acetylation. Parallel to growth arrest, apoptosis was induced with caspase-9 up-regulation and PARP degradation, suggesting that the mediation of apoptosis is through the mitochondrial pathway, similarly to some of the HDAC inhibitors such as suberoylanilide hydroxamic acid and trichostatin A (15). PHI as an inhibitor of HDAC was supported by the direct inhibition of HDAC by PHI in a cell-free assay. Additionally, the activity of HDAC from PHI-exposed LNCaP cells was diminished, with the extent of reduction close to that mediated by a known HDAC inhibitor, sodium butyrate. The experimental results also demonstrated that, at high PHI concentrations, the HDAC level was reduced, which could also contribute to increased acetylation. However, about 5 μM of PHI was sufficient to inhibit the activity of HDAC in LNCaP cells (25% decrease), while it required 20 μM to reduce the HDAC expression (15% decrease). This supports the interpretation that inhibiting HDAC activity is the primary action to induce histone acetylation. Our conclusion that PHI is an inhibitor of HDAC is in line with the observation that the N-acetylcysteine conjugate of sulforaphane, the metabolite of an isothiocyanate found in broccoli, is also an inhibitor of HDAC (16). The inhibitory activity of PHI, in comparison, was similar to that of the sulforaphane conjugate.

Hyperacetylation of histone allows chromatin unfolding and more accessibility of regulators to the DNA for transcription activation. Hypoacetylation of histone, on the
other hand, is associated with the formation of heterochromatin and gene silencing (17, 18). Our study demonstrated that PHI enhanced acetylation of histones H3, H4 and H3 lysine 14, which is consistent with the signs of transcriptionally competent chromatin (19). Hyperacetylation of histones by PHI may increase accessibility of the transcriptional machinery in the p21 promoter, with increase of the p21 expression as a result. The ChIP analyses clearly demonstrated the association of the p21 gene with the highly acetylated histones. The results support the hypothesis that there is a relationship between growth regulatory genes, like p21, and chromatin remodeling with acetylation by PHI.

The transcriptional activation of p21 and cell cycle arrest mediated by PHI implies a reversal of the aberrant epigenetic events in the prostate cancer cells. Our experimental results indicated that there may be aberrant epigenetic events associated with prostate carcinogenesis. These events may include the local chromatin architecture, the status of histone modifications and the activity of regulatory enzymes, which could represent promising targets for intervention.

Our previous report (5) indicated that isothiocyanates, such as sulforaphane, down-regulate androgen receptor expression as an important growth regulatory mechanism in prostatic cells. Judged together with our present findings, the growth regulation of prostatic cancer cells by isothiocyanates may include a two-pronged mechanism, with one to up-regulate p21 to modulate cell cycle progression, and the other to suppress the androgen receptor and, thereby, the hormonal-driven proliferation. The dual activities could function synergistically to effectively regulate the growth of prostate cancer cells. This has provided an experimental basis for using the isothiocyanates to prevent the development and progression of prostate cancer.

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References


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