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Differential Expression of Thrombospondin (THBS1) in Tumorigenic and Non-Tumorigenic Prostate Epithelial Cells in Response to a Chromatin-Binding Soy Peptide

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Abstract

The chemopreventive properties of the chromatin-binding soy peptide, lunasin, are well documented but its mechanism of action is unclear. To elucidate the mechanism by which lunasin reduces tumor foci formation in cultured mammalian cells, non-tumorigenic (RWPE-1) and tumorigenic (RWPE-2) human prostate epithelial cells were treated with lunasin followed by gene expression profiling and characterization of the chromatin acetylation status for certain chemopreventive genes. The genes, HIF1A, PRKAR1A, TOB1 and THBS1 were upregulated by lunasin in RWPE-1 but not in RWPE-2 cells. Using histone acetyltransferase (HAT) assays with acid-extracted histones as templates, we showed that lunasin specifically inhibited H4K8 acetylation while enhanced H4K16 acetylation catalyzed by HAT enzymes p300, PCAF, and HAT1A. These results suggest a novel mechanism for lunasin-dependent upregulation of gene expression. Chromatin immunoprecipitation (ChIP) revealed hypoacetylation of H4K16 in RWPE-2 cells, specifically at the 5' end of THBS1 containing a CpG island. Moreover, bisulfite PCR (BSP) and subsequent DNA sequencing indicated that this CpG island was hypomethylated in RWPE-1 but hypermethylated in RWPE-2 cells. Histone hypoacetylation and DNA hypermethylation in the 5' region of THBS1 may explain the inability of lunasin to upregulate this gene in RWPE-2 cells.

Keywords

Soy peptide; lunasin; histone acetylation; chemopreventive genes; H4K16

INTRODUCTION

The mechanisms by which dietary factors affect long-term health outcomes are not well understood. Although consumption of plant-rich diets are recommended for reducing risk of

cancer (1, 2), the molecular and cellular changes induced by dietary factors are largely unknown. The chromatin-binding peptide, lunasin, is a bioactive component from soybeans with well-documented chemopreventive properties (3). When applied exogenously to mammalian cells, lunasin suppresses the transformation of normal cells to cancerous foci induced by either chemical carcinogens or oncogenes (5). Consistent with these observations, lunasin has been shown to (a) enter mammalian cells through its RGD cell adhesion motif, (b) colocalize specifically with deacetylated histones, (c) inhibit histone 3 (H3) di-acetylation and H4 tetra-acetylation in sodium butyrate-treated cells and (d) induce apoptosis in E1A-transfected C3H cells (5). These findings suggest a chemopreventive mechanism in which lunasin alters gene expression by modulating histone acetylation status.

There is growing evidence supporting the role of epigenetic modifications in cancer formation as the result of dysregulation of gene expression, especially in the early stages of carcinogenesis (6–9). The objective of this study was to investigate the chemopreventive properties of lunasin at the molecular and cellular levels by examining gene expression patterns and the associated epigenetic changes (i.e., histone acetylation and DNA methylation) in lunasin treated tumorigenic (RWPE-2) and non-tumorigenic (RWPE-1) prostate epithelial cells. We present evidence that supports a mechanism in which genes, such as the pro-apoptosis gene, THBS1, are upregulated as a result of lunasin-induced increase in H4-Lys16 (H4K16) acetylation in RWPE-1 cells. In RWPE-2 cells, however, THBS1 is expressed but not upregulated by lunasin treatment. We believe this differential expression of THBS1 in RWPE-1 and RWPE-2 cells is due to hypoacetylation of H4K16 at the 5'-end of THBS1 and hypermethylation of the CpG island in the THBS1 promoter. The results reported here may provide a molecular mechanism to explain the association between higher soy consumption and lower cancer risk (10–12).

MATERIALS AND METHODS

Cell Culture

NIH 3T3, RWPE-1, and RWPE-2 cells were acquired from American Type Culture Collection (ATCC, Manassas, VA). NIH 3T3 cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA). RWPE-1 and RWPE-2 cells were maintained in Keratinocyte Serum Free Medium (K-SFM) supplemented with 0.05 mg/ml bovine pituitary extract and 5 ng/ml human recombinant epidermal growth factor (Invitrogen). All culture media contained 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen).

Lunasin Immunofluorescence

RWPE-1 and RWPE-2 cells were cultured for 24 h and then treated with 0, 20, or 50 µM lunasin for 3 and 24 h. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.4% saponin after lunasin treatment. Cells were stained with an affinity-purified lunasin antibody (1:500 dilution) (5) followed by an Alexa 488-conjugated goat anti-rabbit antibody (1:250 dilution, Invitrogen). Nuclei were visualized by DAPI staining (Vector Laboratories, Burlingame, CA). Photomicrographs were obtained using a Nikon Eclipse 800 microscope equipped with a digital camera. All images were acquired using the same integration parameters.

Immunoprecipitation

Lunasin (89 pmoles, Soy Labs, Fairfield, CA) was incubated with recombinant H4 (rH4) (89 pmoles, New England BioLabs, Ipswich, MA) in 500 µl of histone binding buffer (1X = 20 mM Tris-HCl pH 7.6, 150 mM KCl, 5 mM MgCl₂, 10 µg/mL BSA, 0.5% NP-40) at 4°C for 1 h. The peptide binding mixture (lunasin/rH4) was immunoprecipitated by adding a

deacetylated H4 (Millipore, Temecula, CA) polyclonal antibody at 1:300 dilution or a lunasin polyclonal antibody (Soy Labs) at 1:1000 dilution and incubating at 4°C for 1 h with gentle rocking. For non-specific binding controls, lunasin or rH4 was also incubated with an affinity-purified GST protein (89 pmoles) at 4°C for 1 h and immunoprecipitated with a GST antibody (1:500 dilution, Stressgen, Ann Arbor, MI). A negative control with no antibody added to the peptide binding mixture was also performed. After incubation, 40 µl of Protein-A/G PLUS-agarose slurry (Santa Cruz Biotechnology, Santa Cruz, CA) was added to the reaction mixture and allowed to incubate at 4°C overnight with gentle rocking. Immunoprecipitates were collected by centrifugation at 1000 g at 4°C for 5 min and washed 4 times with 1 ml 1xPBS, pH 7.4. After final wash, the pellet was resuspended in 40 µl of 1x Laemmli sample buffer containing 1 mM β-mercaptoethanol (Bio-Rad, Hercules, CA), boiled for 1 min and centrifuged at 1000 g at room temperature for 5 min. Supernatant was collected and loaded onto 4–20% Tris-HCl gels (Bio-Rad). The pulled-down peptide was detected using western blot analysis with either the lunasin (1:15,000) or the H4 (1:10,000) primary antibody followed by an HRP-conjugated secondary antibody (1:15,000, Pierce, Rockford, IL) and visualized using a SuperSignal West Femto kit (Pierce) and a ChemiDoc XRS+ Imaging System (Bio-Rad).

Coomassie Blue Staining

Recombinant H4 and GST proteins (8 µg) were separated in a 4–20% Tris-HCl gel. Proteins were stained by submerging the gel in a staining buffer containing: 0.2% (wt/vol) Brilliant Blue G250, 20% ethanol (vol/vol), and 0.5% (vol/vol) methanol, at room temperature for 1 h. The gel was rinsed with water and destained with 30% methanol at 4°C overnight.

In Vitro Histone Acetyl Transferase (HAT) Assay

Two µL lunasin (100 µM) in double distilled sterilized water was mixed with either 10 µg acid-extracted histones from HeLa cells or 1 µg rH4 in a total 25 µl volume. Samples were incubated at room temperature for 5 min, on ice for 5 min, and again at room temperature for 5 min. HAT reactions were initiated by adding 10 µl 5x HAT buffer (Millipore), 2 µg acetyl CoA, HAT enzymes (0.5 µg PCAF, 2 µg p300, or 2 µg HAT1A) into the mixtures (50 µl final reaction volume). HAT reaction mixtures were incubated at 30°C with gentle shaking for 10 min (p300 and HAT1A reactions) or 1 h (PCAF reactions). Reactions were stopped by adding 50 µl Laemmli sample buffer (Bio-Rad), boiling for 5 min, and quenching on ice. Twenty-five µl reaction mixture was loaded in each lane of 10–20% Tricine-HCl gels (Bio-Rad), and electroblotted onto nitrocellulose membranes (Bio-Rad). Blots were immunostained with a primary antibody specific to H4 acetylated at K4 (H4K4), K8 (H4K8), K12 (H4K12), or K16 (H4K16) (Millipore) using manufacturer recommended dilutions. An HRP-conjugated goat anti-rabbit secondary antibody (Amersham Biosciences, Piscataway, NJ) was used at 1:10,000 dilution. Chemiluminescence from an ECL Western detection kit (Amersham) was quantified using an Alpha Innotech digital imaging system (Alpha Innotech San Leandro, CA).

Microarray analysis

RWPE-1 and RWPE-2 cells were grown to 70% confluency in 150 mm plates. Lunasin was added into the medium to a final concentration of 2 µM. For the controls, 1x PBS, pH 7.4, was added to the plates. Cells were incubated for 24 h and lysed with Trizol reagent (Invitrogen) for total RNA purification. cDNA was synthesized from 10 µg of total RNA using a SuperScript Choice system (Invitrogen), then transcribed into cRNA in the presence of biotin-labeled nucleotide triphosphate using T7 RNA polymerase (New England BioLabs). cRNA was purified using an RNeasy mini kit (Qiagen, Valencia, CA) and fragmented at 94°C for 30 min in a buffer containing 0.2 M Tris-acetate, pH 8.1, 0.5 M potassium acetate, and 0.15 M magnesium acetate. Fragmented cRNA was hybridized to

HGU133A GeneChip microarrays (Affymetrix, Santa Clara, CA) at 45°C overnight. Hybridization was detected using a confocal laser scanner (Affymetrix). Gene expression levels were normalized and analyzed using robust multichip average (RMA) (13).

Quantitative RT-PCR

cDNA was synthesized from 3 µg of total RNA using a SuperScript First-Strand Synthesis (Invitrogen). Two µl of cDNA, diluted 4 fold, was added to the PCR reactions containing TaqMan probes (Applied Biosystems, Foster City, CA). Quantitative RT-PCR (qRT-PCR) was performed in triplicate using an ABI 7900HT (Applied Biosystems) and *ACTB* expression was used for normalization. Changes in transcript levels were calculated using relative quantification of gene expression following Applied Biosystems' protocol.

Western blot analysis

RWPE-1 and RWPE-2 were grown to 70% confluency in 100 mm plates. Four hours after seeding, lunasin was added into the medium to a final concentration of 2 µM. For the controls, 1x PBS, pH 7.4 was added to the plates. After 24 h lunasin treatment, cells were lysed with M-PER protein extraction reagent containing 1x Halt Protease Inhibitor cocktail (Pierce). Protein concentrations were determined using Dc protein assay reagents (Bio-Rad). From 25 to 37 µg protein lysate was loaded in each lane of 10–20% Tris-HCl gels with Kaleidoscope markers (Bio-Rad) and electroblotted onto nitrocellulose membranes (Bio-Rad). Blots were immunostained with primary antibodies against THBS1 (1:350) (Abcam, Cambridge, MA), HIF1A (1:500) (Abcam), TOB1 (1:200) (Santa Cruz Biotechnology), PRKAR1A (1:200) (Santa Cruz Biotechnology) and acetylated H4K8 and H4K16 (1:1000) (Millipore) at room temperature for 1 h (THBS1 and HIF1A) or at 4°C overnight (TOB1, PRKAR1A, acetylated H4K8 and H4K16). Blots were also immunostained with a β-ACTIN antibody (1:1000) (Imgenex, San Diego, CA) at room temperature for 1 h for normalization. A HRP-conjugated goat anti-rabbit (Pierce) or a donkey anti-goat secondary antibody (Santa Cruz Biotechnology) at 1:10,000 dilution was incubated with blots at room temperature for 1 h. Chemiluminescence from an ECL western detection kit (Amersham) or a SuperSignal West Femto kit (Pierce) was quantified by using an Alpha Innotech digital imaging system (Alpha Innotech San Leandro, CA).

Chromatin Immunoprecipitation

RWPE-1 and RWPE-2 cells (2×10^7 cells per assay), treated or mock-treated with 2 µM lunasin for 24 h, were incubated with 1% formaldehyde at room temperature for 8 min to crosslink chromatin and quenched with 1 ml 10x glycine (EZ ChIP, Millipore). After washing with ice cold 1x PBS, pH 7.4, cells were collected by centrifugation at 700 g for 4 min and lysed in 1 ml SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris, pH 8.1) containing freshly added protease inhibitor cocktail at 1:5 dilution (Millipore). Lysates were sonicated to generate 300–600 bp DNA fragments and divided into two fractions. The first fraction was incubated with an acetylated H4K16 antibody (1 µg/reaction; Millipore) at 4°C overnight and the second fraction was incubated with 1x TE buffer as the negative control. Protein A-Sepharose beads were added, incubated at 4°C for 1 h, centrifuged at 3000 g for 1 min before washing for 5 min with ice cold buffers (EZ ChIP, Millipore): 1) low salt wash buffer once, 2) high-salt wash buffer once, 3) LiCl wash buffer once, and 4) TE buffer twice. After washing, beads were treated with RNase and then proteinase K at 55°C overnight. DNA was released from crosslinking by heating the samples at 65°C for 6 h, extracted by phenol/chloroform, precipitated by ethanol, and resuspended in water. DNA from fraction 1 and 2 (10–15 ng) were amplified with a THBS1 CpG island primer set (5'CTTTTCGGATGCTTGCTG3' and 5'CAGTCTGGGCTCCTCTCTCC3') and exon 11 primer set (5'CTGAGGCTTCAGTCCCTCTG3' and 5'AGAGTAAGGAAATCTTGGGGTGTC3') designed with the Primer 3 software (14).

Bisulfite-specific PCR and sequencing

Genomic DNA was isolated from RWPE-1 and RWPE-2 cells with a PureLink Genomic DNA Isolation kit (Invitrogen) and subjected to bisulfite conversion (EZ DNA-Methylation-Direct, Zymo Research, Orange, CA). Bisulfite converted unmethylated cytosines to thymines while methylated cytosines remained intact. Bisulfite-converted genomic DNA was subjected to bisulfite-specific PCR (BSP) using primers specific to the 5' end of THBS1. The BSP primers, designed using Methyl-Primer Express v1.0 (Life technologies, Carlsbad, CA), were: methylated primer pair (M), 5'TTATTTTTCGTTTTTCTTCGGTCGTCG3' and 5'GCGCGCTTTTAAAAAACGCTCG3'; unmethylated primer pair (UM), 5'TGTTTTTTGTTTGGTTGTTGTTT3' and 5'CTCACAACCAACTCAAACACAA3'. PCR was carried out in a 25 µl reaction volume using a *Taq* PCR Kit (New England BioLabs). PCR products were run on 1.5% ethidium bromide-stained agarose gels, excised, purified using a Gel Extraction Kit (Qiagen), ligated to pCR4-TOPO vector (Invitrogen), and transformed into DH5α *E. coli* (Zymo Research). Insert-containing plasmids were identified by PCR and *EcoRI* (New England BioLabs) digestion before sequencing (Davis Sequencing, Davis, CA).

Statistical Analysis

Statistical analysis was performed using paired Student t-test with $p < 0.05$ as the significant standard.

RESULTS

Lunasin Upregulated Expression of Chemopreventive Genes

Previous studies showed that lunasin pretreatment of non-tumorigenic mammalian cells, such as NIH 3T3 and C3H 10T1/2, for 24 h is sufficient to inhibit foci formations induced by either chemical carcinogens or by transfection with the E1A oncogene (5, 15). These results are consistent with the finding that adenovirus E1A-induced transformation and global gene expression changes occur within 24 h post-viral infection (16). To investigate the effect of lunasin on global gene expression, we performed microarray analysis of two existing prostate epithelial cell lines, RWPE-1, a non-tumorigenic prostate epithelial cell line, and RWPE-2, a tumorigenic variant of RWPE-1 before and after lunasin treatment. Both cell lines share essentially the same genetic background except for the presence of *Ki-ras* oncogene in RWPE-2 (17). We chose to use prostate epithelial cells because prostate cancer, a common and slow-growing cancer, is a good candidate for dietary intervention with bioactive food components, such as lunasin. Microarray analysis showed that of the 14,500 genes interrogated, 114 had greater than twofold change in expression when exposed to 2 µM lunasin for 24 h (Table 1). Of these genes, 112 were upregulated in RWPE-1 cells and only two genes (*COL5A1* and *LAMP1*) were upregulated in RWPE-2 cells. No genes were downregulated in either cell type treated with lunasin. Genes that were upregulated in RWPE-1 cells included those involved in cycle control, cell proliferation, DNA repair, tumor suppression and pro-apoptotic pathways (see Table 2). To confirm the microarray results, qRT-PCR was performed on the pro-apoptotic genes THBS1 and HIF1A and the anti-proliferative genes PRKAR1A and TOB1 (Fig. 1a). All four genes were shown to be upregulated by lunasin in RWPE-1 cells (Fig. 1b), a finding consistent with the microarray results (Fig. 1a).

Changes in protein levels of the four representative genes were examined using western blot analyses. Protein expression levels of HIF1A, PRKAR1A, TOB1 and THBS, were higher in RWPE-1 cells than RWPE-2 cells with or without lunasin treatment (Fig. 1c). In lunasin-treated RWPE-1 cells, HIF1A showed a 3-fold induction over the lunasin-treated RWPE-2

followed by THBS1, which showed more than a 1.5-fold induction ($p \leq 0.01$) (Fig. 1d). The production of PRKARIA and TOB1 protein were not significantly different in lunasin treated RWPE-1 cells compared lunasin treated RWPR-2 cells (Fig. 1d). Upregulation of both HIF1A and THBS1 have been linked to the suppression of prostate tumors (18) and gastric carcinomas, respectively (19).

Uptake of Lunasin in RWPE-1 and RWPE-2 Cells

To rule out the possibility that the differential effects of lunasin treatment on gene expression in RWPE-1 and RWPE-2 cells were simply due to differences in lunasin uptake, lunasin immunofluorescence experiments were conducted. At a concentration of 20 μ M, lunasin was localized in the perinuclear region of both RWPE-1 and RWPE-2 cells (as identified by nuclear DAPI staining) after 3 h and 24 h lunasin treatments compared to the mock-treated controls (Fig. 2a). However, when RWPE-2 cells were treated with 50 μ M lunasin for 24 h, lunasin immunofluorescent signals could be visualized in both the cytoplasm and the nucleus (Fig. 2b). In the nucleus, lunasin was seen at the edge of the nucleus indicating that lunasin was colocalized to the transcriptionally active territories in chromatin (20). Previous lunasin immunofluorescence studies using NIH3T3 cells showed that lunasin colocalized with chromatin in the nucleus after one cycle of cell division, but was only observed in actively dividing metaphase cells when chromatin was highly condensed (15). Taken together, these results suggest that RWPE-2 cells can take up lunasin from the extracellular space and that the differential effects of lunasin on gene expression (Fig. 1a,b) is not due to the inability of RWPE-2 cells to take up lunasin.

Lunasin Bound Tightly to Deacetylated H4

In addition to its chemopreventive properties, lunasin is known to bind to hypoacetylated chromatin (4, 5). To investigate the ability of lunasin to bind specifically to deacetylated H4, immunoprecipitation (IP) experiments were performed (Fig. 3). The results demonstrated that lunasin interacted with rH4 (Fig. 3a). The interactions between lunasin and rH4 were specific as both lunasin and rH4 could not be immunoprecipitated by a GST antibody when lunasin or rH4 was incubated with an affinity purified GST protein (Fig. 3a). Interestingly, both lunasin and H4 antibodies pulled-down the same two protein complexes migrating at ~23 and ~50 kDa. Compared to the monomer size of lunasin (migrating at ~8 kDa, Fig. 3b) on the western blot and rH4 (migrating at ~15 kDa, Fig. 3b) on the Coomassie Blue stained gel, we believed that the 23 kDa protein complex contained a monomer of both lunasin and rH4 and that the 50 kDa protein complex contained a dimer of lunasin and a dimer of rH4. While this interpretation must be confirmed by more rigorous testing, the results, nonetheless, suggest that lunasin binds tightly to rH4 under these reaction conditions.

Lunasin Inhibited H4K8 Acetylation and Increased H4K16 Acetylation *In Vitro*

Lunasin has been shown to significantly inhibit tetra-acetylation of H4 in C3H 10T1/2 and MCF-7 cells treated with Na butyrate (5). Na butyrate increases histone acetylation by inhibiting histone deacetylases (HDAC). Lunasin, therefore, may bind and mask deacetylated histones and inhibit their acetylation in a manner similar to an endogenous human tumor suppressor, ANP32A (21, 22). Inhibition of histone acetylation has been correlated with silencing and downregulation of gene expression (23). Our gene expression results, however, suggested that lunasin upregulated gene expression in non-tumorigenic prostate epithelial cells (Fig. 1a,b), thus raising the question of how an inhibitor of histone acetylation can also upregulate gene expression. Lunasin has a chromobinding motif that binds more tightly to the deacetylated H4 than lunasin's poly-aspartyl tail (5), suggesting that lunasin may mask a specific H4 lysine residue via its chromobinding domain instead of indiscriminately masking all deacetylated H4 lysines with its negatively charged poly-aspartyl tail. To investigate the specificity of lunasin's potential to mask lysine residues on

the amino terminus of H4 from acetylation, we conducted *in vitro* HAT assays specific for the acetylation of K5, K8, K12, and K16.

Densitometric quantitation of acetylation levels, normalized to the acid-extracted histone template for H4K5, K8, K12, and K16, and tetra-acetylated H4, are shown in Fig. 4. In HAT assays without lunasin, HAT1A preferentially acetylated K5 and K12 and to a lesser extent K8 and K16. This is consistent with HAT1A's role as a cytoplasmic HAT enzyme involved in acetylating newly synthesized histones in the cytoplasm at H4K5 and K12 prior to nuclear transport and deposition in nucleosomes during DNA replication (24). Conversely, the nucleosomal HATs, PCAF and p300 are involved in regulating gene expression. p300 is a global coactivator of gene expression and PCAF is a secondary co-activator that requires association with p300 or CBP to locally acetylate chromatin (25). In the absence of lunasin, both p300 and PCAF acetylated H4K8, consistent with a previous report of their H4 lysine specificity (25). p300 also acetylated H4K16 but to a lesser extent than PCAF which showed preferential acetylation of H4K16, similar to the acetylation specificity of the PCAF yeast homolog GCN5 (26, 27). In the presence of lunasin, p300 and PCAF, and to a lesser extent HAT1A, showed significant decrease in K8 acetylation and increase in K16 acetylation. PCAF showed the most significant increase in K16 acetylation relative to the template, followed by p300 and HAT1A. Tetra-acetylation of H4 was significantly inhibited by lunasin in PCAF HAT reactions but not in the p300 and HAT1A reactions. Both p300 and PCAF acetylated H4K8 while HAT1A preferentially acetylated H4K5 and H4K12. The inhibition of H4 tetra-acetylation by p300 and PCAF but not by HAT1A, reported in this study and in a previous study using Na butyrate-treated cells (5), suggests a specific masking of H4K8 by lunasin.

In the study described above, histones were acid-extracted from HeLa cells and contained heterogeneous states of H4 acetylation. To confirm lunasin's ability to acetylate specific H4 lysine residues, we conducted HAT assays using rH4, a non-acetylated template. When lunasin was present in the HAT reactions, H4K8 acetylation was significantly decreased in the HAT reactions catalyzed by all three HAT enzymes. The increase in H4K16 acetylation was observed in the HAT reactions catalyzed by PCAF and HAT1A but not by p300 (Fig. 5a–c). This suggests that the nuclear HAT enzyme, p300, is unable to acetylate H4K16 when H4 is hypoacetylated regardless of the presence or absence of lunasin.

Chromatin Immunoprecipitation (ChIP) with Acetylated H4K16

As shown in Fig. 6a, lunasin treatment did not significantly change H4K8 acetylation but resulted in an increase in H4K16 acetylation in both RWPE-1 and RWPE-2 cells compared to the mock-treated controls. This increase in H4K16 acetylation was statistically significant in RWPE-1 ($p < 0.005$) and in both cell types combined ($p < 0.01$) (Fig. 6b). If lunasin upregulates genes in RWPE-1 cells by promoting acetylation of H4K16, then chromatin in or around these genes should be enriched for hyperacetylated H4K16. Conversely, corresponding chromatin in tumorigenic RWPE-2 cells should be hypoacetylated at H4K16. To investigate these possibilities, ChIP was performed on chromatin isolated from lunasin-treated and mock-treated RWPE-1 and RWPE-2 cells using an antibody against acetylated H4K16. The resulting ChIP DNA was then used in PCR to detect genomic regions enriched in acetylated H4K16. Primer sets were derived from 5 regions of representative upregulated genes, HIF1A, THBS1, PRKCL2, ANP32A and SP3 (Table 1). Primer combinations were designed to recognize up to 200–300 bp fragments of the upregulated genes in and around the following genomic regions: promoter (including transcription start site), 5' untranslated region, first exon, carboxyl end, and 3' untranslated region. Among the genes interrogated, only the primer set derived from the 5' promoter region of THBS1 produced an amplicon from ChIP DNA that was significantly higher in RWPE-1 than in RWPE-2 cells (Fig. 6c). Fig. 6c also shows that a primer pair flanking a CpG island located at the 5' end of THBS1

produced more amplicons with ChIP DNA from lunasin-treated than mock-treated RWPE-1 cells. However, the same primer pair generated little or no amplicon with ChIP DNA from lunasin-treated or mock-treated RWPE-2 cells. It should be noted that H4K16 acetylation could also be detected in exon 11 of THBS1 as evidenced by a strong amplicon signal with a primer pair flanking this region (Fig. 6c). However, this primer pair produced strong amplicon signals with ChIP DNA from both lunasin-treated and mock-treated RWPE-2 (Fig. 6c).

Methylation Status of the THBS1 CpG Island

Bioinformatic analysis revealed that more than one third of the 112 lunasin-upregulated genes in RWPE-1 cells have CpG islands located within 2,000 bp or approximately 10 nucleosomes from their transcription start sites ($p < 0.005$; compared to randomly selected genes) (data not shown). The failure of lunasin to upregulate THBS1 in RWPE-2 cells suggests that epigenetic changes may have occurred at the 5' end of this gene (e.g., H4K16 hypoacetylation and DNA hypermethylation of CpG islands) that transcriptionally silence it or at least make it refractory to lunasin's affect. To assess the methylation status of this specific CpG island in the THBS1 promoter, bisulfite-specific PCR (BSP) was performed with genomic DNA isolated from RWPE-1 and RWPE-2 cells. Using unmethylated primers specific to the THBS1 CpG island, more THBS1 amplicons were found in RWPE-1 than in RWPE-2 cells (Fig. 7a), indicating that the THBS1 CpG island in RWPE-1 cells was hypomethylated. On the other hand, using methylation-specific primers within the same region, RWPE-2 cells showed significantly more THBS1 amplicons than RWPE-1 cells, indicating that the THBS1 CpG island in RWPE-2 cells was hypermethylated (Fig. 7a). The results of bisulfite DNA sequencing of the THBS1 CpG island confirmed that the DNA was hypomethylated in RWPE-1 cells whereas DNA in the same region was highly methylated in RWPE-2 cells (Fig. 7b). It should be noted that not all methylation sites in the THBS1 CpG island DNA from RWPE-2 cells were revealed by bisulfite sequencing since the sequence is a compilation derived from cloned PCR fragments from different RWPE-2 cells.

DISCUSSION

The objective of this report was to elucidate how lunasin, a 43-amino acid peptide from soybeans, suppressed tumor foci formation and carcinogenesis in mammalian cell culture. Based on the known biochemical and chemopreventive properties of lunasin (3, 5, 15, 28, 29) we conducted a series of experiments showing that lunasin can: (1) upregulate the expression of genes directly or indirectly involved in chemoprevention in the non-tumorigenic prostate epithelial RWPE-1 cells but not in the tumorigenic RWPE-2 cells (Fig. 1); (2) enter both RWPE-1 and RWPE-2 cells in a dose-dependent fashion and colocalize along the inner edge of the nuclear membrane (Fig. 2), a region known to be the site of actively transcribed chromatin (20); (3) bind tightly and specifically to rH4 *in vitro* (Fig. 3); (4) bind to H4 in *in vitro* HAT assays to mask the acetylation of H4K8 while enhancing the acetylation of H4K16 (Fig. 4, 5) and (5) hyperacetylate H4K16 *in vivo* at the 5' end of the pro-apoptotic gene, THBS1, in RWPE-1 cells but not in RWPE-2 cells (Fig. 6). The latter two observations are particularly important for understanding lunasin's mechanism of action since acetylation of H4K16 is known to be a key histone modification involved in chromatin decondensation and subsequent gene expression (30–32). It is believed that H4K16 acetylation in chromatin destabilizes the electrostatic interactions between adjacent nucleosomes thus increasing accessibility of the basal transcription apparatus to the promoter (33–35). Taken together, these observations suggest a two-step epigenetic mechanism in which genes related to chemoprevention are upregulated in non-tumorigenic cells by lunasin first binding to deacetylated H4K8 through its chromobinding domain, followed by an induced conformational change in the N-terminus of H4 making H4K16

more accessible to HAT enzymes. Although the results reported here are consistent with such a model, confirmation will require further studies.

The question remains why the tumorigenic RWPE-2 cells are refractory to lunasin-dependent upregulation of chemopreventive gene expression in spite of the fact that lunasin has a similar effect on global H4K16 acetylation in both RWPE-2 and RWPE-1 cells (Fig. 6). One explanation may be the differential effects of HAT enzymes on H4K16 acetylation. This notion is supported by the results from HAT assays conducted with either acid-extracted histones or rH4. The nuclear HAT enzymes p300 and PCAF were able to acetylate H4K16 in the presence of lunasin using acid-extracted, nuclear histones as the template (Fig. 4), whereas only PCAF, a secondary transcriptional co-activator, was able to acetylate H4K16 when the non-acetylated rH4 was used as the template (Fig. 5). The inability of p300, a ubiquitous and general transcriptional co-activator, to acetylate rH4K16 suggests that its bromodomain (36, 37) requires pre-existing acetylated H4 lysine residues at K5, K8, or K12 for H4 binding and acetylation. Because chromatin hypoacetylation is commonly associated with cancer formation (38–41), it may be that the chromatin in or around chemopreventive genes in RWPE-2 are hypoacetylated at these residues as a result of *Ki-ras*-induced transformation. This particular epigenetic change is associated with carcinogenesis and might explain the loss of p300-mediated acetylation of H4K16 and the inability of lunasin to upregulate chemopreventive genes in RWPE-2 cells. The loss of acetylation at H4K16, albeit in regions of repetitive DNA sequences, has been shown to be a common epigenetic hallmark of many tumors and transformed cells (38).

A third explanation may involve the association of THBS1 expression *in vivo* with DNA hypomethylation of a CpG island in the THBS1 promoter in RWPE-1 cells but not in RWPE-2 cells. In RWPE-2 cells the opposite association (i.e., hypoacetylation of H4K16 and DNA hypermethylation at the 5'-end of THBS1) was observed (Fig 7). Histone hypoacetylation coupled with DNA hypermethylation of CpG islands are commonly found in promoters of tumor suppressor and pro-apoptotic genes and are associated with aberrant gene silencing during carcinogenesis (39–41). We believe that the hypermethylation of the 5' CpG island of THBS1 in RWPE-2 cells may promote the deacetylation of H4K16 by HDACs thus condensing the chromatin in the vicinity of the THBS1 gene and making it inaccessible to the basal transcriptional apparatus. The fact that 30% of the chemopreventive genes upregulated by lunasin in RWPE-1 cells contain at least one CpG island within 2,000 bp of their transcription start sites may explain the difference in upregulation of genes in RWPE-1 and RWPE-2 cells. As a dietary peptide capable of upregulation of chemopreventive gene expression by specific epigenetic modifications of the human genome, we believe lunasin represents a novel regulatory motif and another food bioactive with the potential to reduce cancer risk. This could explain the long-standing inverse correlation between increased soy consumption and risk of various cancers. Future studies will be needed toward test this proposed epigenetic model for lunasin.

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Abbreviations

BSP	bisulfite-specific PCR
GST	glutathione-S-transferase
HAT	histone acetyltransferase
HDAC	histone deacetylase
HRP	horseradish peroxidase
K	lysine
PCAF	p300/CBP-associated factor
rH4	recombinant histone 4
RT-PCR	real time polymerase chain reaction

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FIG. 1.

Expression of HIF1A, PRKAR1A, TOB1, and THBS1 in RWPE-1 and RWPE-2 cells treated with lunasin. *a.* mRNA expression of *HIF1A*, *PRKAR1A*, *TOB1*, and *THBS1* in RWPE-1 and RWPE-2 cells treated with lunasin. Lunasin-induced gene expression was detected by Affymetrix microarrays. *b.* Confirmation of lunasin-induced gene expression of *HIF1A*, *PRKAR1A*, *TOB1*, and *THBS1* using qRT-PCR. The gene expression of each sample was normalized to the housekeeping gene, *ACTB*. All measurements of fold-induction were based on 1xPBS-treated samples of each cell type. *c.* A representative western blot analysis of HIF1A, PRKAR1A, TOB1, and THBS1 expression in lunasin-treated RWPE-1 and RWPE-2 cells. RWPE-1 and RWPE-2 cells were treated with 2 μ M lunasin (+L) or 1xPBS, pH 7.4 (-L), for 24 h before western blot analysis. *d.* Densitometry data of western blot analyses. Each bar represents the average fold induction of 4 western blots generated from two independent experiments. The expression of each sample was normalized to the expression of ACTB.

FIG. 2.

Lunasin uptake in RWPE-1 and RWPE-2 cells. RWPE-1 and RWPE-2 cells were cultured for 24 h before they were treated with 0, 20, or 50 μ M lunasin. After 3 and 24 h treatment, cells were fixed with 4% paraformaldehyde and permeabilized with 0.4% saponin, and incubated with a lunasin polyclonal antibody (1:500) followed by an Alexa 488-conjugated goat anti-rabbit secondary antibody (1:250). Cell nuclei were stained with DAPI. Photomicrographs were obtained by a Nikon Eclipse 800 fluorescence microscope equipped with a digital camera. All images were acquired using the same integration parameters. Arrows indicate the locations of lunasin in the nucleus.

FIG. 3.

Immunoprecipitation of the lunasin-rH4 binding. *a*. Immunoprecipitation and western blot analysis. Lunasin was incubated with either rH4 (lanes 1, 3, 4, and 6) or GST (lanes 2 and 5) and immunoprecipitated with a H4 antibody (lanes 1), a lunasin antibody (lane 4), or a GST antibody (a control antibody, lanes 2 and 5). No antibody was added in the peptide binding reactions (lunasin/H4) in lanes 3 and 6. The bound peptide was detected via western blot analysis using the lunasin antibody (lanes 1–3) or the H4 antibody (lanes 4–6). *b*. Protein/peptide sizes of rH4, GST, and lunasin. rH4 (8 μ g) or GST (8 μ g) was separated in a 4–20% Tris-HCl gel and stained with Coomassie Blue. Lunasin (89 pmoles) was detected by western blot analysis using the lunasin antibody followed by a HRP-conjugated secondary antibody. rH4, recombinant H4; GST, glutathione-S-transferase; IP, immunoprecipitation; WB, western blot assay.

FIG. 4.

Effect of lunasin on acetylation of lysine residues at the H4 N-terminus. Western blot analysis was conducted on products of HAT assays using acid-extracted histones from HeLa cells. HAT reactions were conducted using HAT enzymes p300 (a), PCAF (b), and HAT1A (c) in response to lunasin (+L) or 1x PBS (-L) treatment. Blots were immunostained with antibodies specific to acetylated H4K5, K8, K12, or K16. The Tetra-acetylated H4 antibody only reacts with H4 acetylated at all four lysine residues. Negative template control (-NaB) and positive control (+NaB) correspond to acid-extracted histones from Na butyrate untreated and treated HeLa cells, respectively. Histograms represent densitometric scans of -L and +L bands normalized to their corresponding -NaB template controls. Averages of at least 3-5 replicates were plotted as -L (gray bars) and +L (black bars). Error bars represent S.E.M. values (n=3-5). Statistical significance was calculated using paired Student t-test. *: p<0.05, **: p<0.01, ***: p<0.001.

FIG. 5.

Effect of lunasin on rH4 acetylation. Western blot analysis was conducted on products of HAT assays using rH4 as a template. HAT reactions were performed using HAT enzymes p300 (a), PCAF (b), and HAT1A (c) in response to lunasin (+L) or 1x PBS (-L) treatment. Blots were immunostained with antibodies specific to acetylated H4K5, K8, K12, or K16. Histograms represent densitometric scans of -L and +L bands normalized to their corresponding rH4 template controls. Averages of 3–8 replicates were plotted as -L (gray bars) and +L (black bars). Error bars represent S.E.M. values (n=3–8). Statistical significance was calculated using paired Student t-test. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

FIG. 6.

Effect of lunasin on H4K16 acetylation and THBS1 expression in RWPE-1 and RWPE-2 cells. *a.* Western blot analysis of acetylated H4K8, acetylated H4K16 and THBS1 protein levels in RWPE-1 and RWPE-2 cells treated with 2 μ M lunasin (+L) or 1x PBS (-L). *b.* Densitometric quantification of H4K16 acetylation in lunasin treated (+L) or 1x PBS treated (-L) RWPE-1, RWPE-2 cells, and both cells combined (All). * indicates $p < 0.01$ and ** indicates $p < 0.005$ with a Student t-test based on data normalized to -L controls. *c.* Upregulation of THBS1 was associated with increased H4K16 acetylation. PCR amplicons using template DNA from chromatin immunoprecipitation (ChIP) with an acetylated H4K16 antibody and primers derived from the 5' CpG island and exon 11 of THBS1. Input DNA control represents PCR amplicons using template DNA from unprecipitated chromatin and 5' CpG island primers. *d.* The THBS1 locus with locations of PCR primers in the 5' CpG island and exon 11.

a. **b.** **FIG. 7.**

Methylation status of the THBS1 CpG island in RWPE-1 and RWPE-2 cells. *a.* PCR of bisulfite-treated RWPE-1 (R1) and RWPE-2 (R2) genomic DNA with unmethylated or methylated primers. Methylated genomic DNA (CpG) was used as the template control in PCR using unmethylated (UM) and methylated (M) primers. * indicates CpG methylation at the 5' end of THBS1 in RWPE-2 cells. *b.* DNA methylation mapping of the THBS1 CpG island by bisulfite genomic sequencing. Genomic DNA reference sequence (RefSeq) of the THBS1 CpG island is shown together with bisulfite sequence of the same region in RWPE-1 and RWPE-2 cells. Arrow indicates the transcription start site (TSS). Negative and positive numbers correspond to the base pairs upstream and downstream of TSS, respectively. Bold **T** letters indicate conversion of the unmethylated cytosines in genomic DNA by bisulfite treatment. White **C** letters on the black background correspond to the methylated cytosines present in RWPE-2 cells.

Table 1

Genes upregulated in RWPE-1 cells	
AKAP2	A kinase (PRKA) anchor protein 2
ANP32E	acidic (leucine-rich) nuclear phosphoprotein 32 family, member E
ADAM9	ADAM metalloproteinase domain 9 (meltrin gamma)
AMMECR1	Alport syndrome, mental retardation, midface hypoplasia and elliptocytosis chromosomal region gene 1
AASDHPPT	aminoadipate-semialdehyde dehydrogenase-phosphopantetheinyl transferase
ARGLU1	arginine and glutamate rich 1
ASPM	asp (abnormal spindle) homolog, microcephaly associated (Drosophila)
ATP1B1	ATPase, Na ⁺ /K ⁺ transporting, beta 1 polypeptide
BAT2D1	BAT2 domain containing 1
BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3
BTBD1	BTB (POZ) domain containing 1
BUB1B	budding uninhibited by benzimidazoles 1 homolog beta (yeast)
CD44	CD44 molecule (Indian blood group)
CD46	CD46 molecule, complement regulatory protein
CEP55	centrosomal protein 55kDa
CLCN3	chloride channel 3
CHD1	chromodomain helicase DNA binding protein 1
CHD9	chromodomain helicase DNA binding protein 9
CROP	cisplatin resistance-associated overexpressed protein
CLDND1	claudin domain containing 1
CRIM1	cysteine rich transmembrane BMP regulator 1 (chordin-like)
DDX18	DEAD (Asp-Glu-Ala-Asp) box polypeptide 18
DHX29	DEAH (Asp-Glu-Ala-His) box polypeptide 29
DICER1	dicer 1, ribonuclease type III
DR1	down-regulator of transcription 1, TBP-binding (negative cofactor 2)
DST	dystonin
EFR3A	EFR3 homolog A (S. cerevisiae)
ELK3	ELK3, ETS-domain protein (SRF accessory protein 2)
ERBB2IP	erb2 interacting protein
EIF2AK2	eukaryotic translation initiation factor 2-alpha kinase 2
EIF4E	eukaryotic translation initiation factor 4E
FAM3C	family with sequence similarity 3, member C
FZD6	frizzled homolog 6 (Drosophila)
GJA1	gap junction protein, alpha 1, 43kDa
GOLGA4	golgi autoantigen, golgin subfamily a, 4
HSP90AA1	heat shock protein 90kDa alpha (cytosolic), class A member 1
HELLS	helicase, lymphoid-speci c
HNRPA3	heterogeneous nuclear ribonucleoprotein A3

HNRPH2	heterogeneous nuclear ribonucleoprotein H2
HMGB2	high-mobility group box 2
HIF1A	hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)
IPO7	importin 7
IGF2BP3	insulin-like growth factor 2 mRNA binding protein 3
ITGB1	integrin, beta 1 (bronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)
JAG1	jagged 1 (Alagille syndrome)
KIAA1598	KIAA1598
KTN1	kinectin 1 (kinesin receptor)
KIF5B	kinesin family member 5B
LAMB1	laminin, beta 1
LRRFIP1	leucine rich repeat (in FLII) interacting protein 1
LRRC40	leucine rich repeat containing 40
LRBA	LPS-responsive vesicle trafficking, beach and anchor containing
MAD2L1	MAD2 mitotic arrest deficient-like 1 (yeast)
ME1	malicenzyme 1, NADP(+)-dependent, cytosolic
MATR3	matrin 3
MARCH6	membrane-associated ring finger (C3HC4) 6
MTDH	metadherin
MAP4K4	mitogen-activated protein kinase kinase kinase kinase 4
MCFD2	multiple coagulation factor deficiency 2
NDUFA5	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 5, 13kDa
NAB1	NGFI-A binding protein 1 (EGR1 binding protein 1)
NRIP1	nuclear receptor interacting protein 1
OSBPL8	oxysterol binding protein-like 8
PCNP	PEST proteolytic signal containing nuclear protein
PHF20	PHD finger protein 20
PIK3C2A	phosphoinositide-3-kinase, class 2, alpha polypeptide
PLK2	polo-like kinase 2 (Drosophila)
PAPOLA	poly(A) polymerase alpha
KCTD12	potassium channel tetramerisation domain containing 12
PSMC6	proteasome (prosome, macropain) 26S subunit, ATPase, 6
PKN2	protein kinase N2
PRKAR1A	protein kinase, cAMP-dependent, regulatory, type I, alpha (tissue specific extinguisher 1)
PTPLB	protein tyrosine phosphatase-like (proline instead of catalytic arginine), member b
PRPF40A	PRP40 pre-mRNA processing factor 40 homolog A (S. cerevisiae)
RAB2A	RAB2A, member RAS oncogene family
RANBP2	RAN binding protein 2
RANBP5	RAN binding protein 5
RCN2	reticulocalbin 2, EF-hand calcium binding domain

RBM39	RNA binding motif protein 39
SCFD1	sec1 family domain containing 1
SEMA3C	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3C
SPTLC1	serine palmitoyltransferase, long chain base subunit 1
SMURF2	SMAD specific E3 ubiquitin protein ligase 2
SLC16A1	solute carrier family 16, member 1 (monocarboxylic acid transporter 1)
SP3	Sp3 transcription factor
SMC2	structural maintenance of chromosomes 2
SMC3	structural maintenance of chromosomes 3
SYNCRIP	synaptotagmin binding, cytoplasmic RNA interacting protein
TAX1BP1	Tax1 (human T-cell leukemia virus type I) binding protein 1
TMX1	thioredoxin-related transmembrane protein 1
THBS1	thrombospondin 1
TDG	thymine-DNA glycosylase
TOR1AIP1	torsin A interacting protein 1
TOB1	transducer of ERBB2, 1
TPR	translocated promoter region (to activated MET oncogene)
TRAM1	translocation associated membrane protein 1
TM9SF3	transmembrane 9 superfamily member 3
TMED10	transmembrane emp24-like trafficking protein 10 (yeast)
TMEM123	transmembrane protein 123
TMEM30B	transmembrane protein 30B
TSC22D2	TSC22 domain family, member 2
TTK	TTK protein kinase
TPD52	tumor protein D52
TWF1	twinfilin, actin-binding protein, homolog 1 (Drosophila)
USP1	ubiquitin specific peptidase 1
UBA3	ubiquitin-like modifier activating enzyme 3
WBP5	WW domain binding protein 5
YTHDC1	YTH domain containing 1
ZC3H15	zinc finger CCCH-type containing 15
ZNF146	zinc finger protein 146
ZNF638	zinc finger protein 638
ZZZ3	zinc finger, ZZ-type containing 3
Genes upregulated in RWPE-2 cells	
COL5A1	collagen, type V, alpha 1
LAMP1	lysosomal-associated membrane protein 1

Table 2

Physiologically relevant pathways for upregulated genes

	Symbol	Fold-change
a) Tumor suppressive (anti-cell proliferation) genes		
protein kinase, cAMP-dependent, regulatory, type I, alpha	PRKAR1A	2.45
transducer of ERBB2, 1	TOB1	2.32
erbb2 interacting protein	ERBB2IP	2.23
acidic (leucine-rich) nuclear phosphoprotein 32 family, member E	ANP32E	2.13
b) Genes involved in apoptosis		
protein kinase N2	PKN2	2.33
BCL2/adenovirus E1B 19kDa interacting protein 3	BNIP3	2.05
thrombospondin 1	THBS1	2.05
transmembrane protein 123	TMEM123	2.26
serine palmitoyltransferase, long chain base subunit 1	SPTLC1	2.12
c) Mitotic checkpoint control genes		
budding uninhibited by benzimidazoles 1 homolog beta (yeast)	BUB1B	2.23
TTK protein kinase	TTK	2.08
MAD2 mitotic arrest deficient-like 1 (yeast)	MAD2L1	2.12
d) Protein degradation genes		
proteasome (prosome, macropain) 26S subunit, ATPase, 6	PSMC6	2.58
RAN binding protein 2	RANBP2	2.36
SMAD specific E3 ubiquitin ligase 2	SMURF2	2.29
ubiquitin specific peptidase 1	USP1	2.23
ubiquitin-like modifier activating enzyme 3	UBA3	2.15
e) Cellular communication		
gap junction protein, alpha 1, 43kDa (connexin 43)	GJA1	2.58