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Cholesterol and phytosterols differentially regulate the expression of caveolin 1 and a downstream prostate cell growth-suppressor gene

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Abstract

Background—The purpose of our study was to show the distinction between the apoptotic and anti-proliferative signaling of phytosterols and cholesterol enrichment in prostate cancer cell lines, mediated by the differential transcription of caveolin-1, and *N-myc downstream regulated gene1* (*NDRG1*), a pro-apoptotic androgen-regulated tumor suppressor.

Methods—PC-3 and DU145 cells were treated with sterols (cholesterol and phytosterols) for 72 h, followed by trypan blue dye exclusion measurement of necrosis and cell growth measured with a Coulter counter. Sterol induction of cell growth-suppressor gene expression was evaluated by mRNA transcription using RT-PCR, while cell cycle analysis was performed by FACS analysis. Altered expression of Ndr1 protein was confirmed by Western blot analysis. Apoptosis was evaluated by real time RT-PCR amplification of *P53*, *Bcl-2* gene and its related pro- and anti-apoptotic family members.

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Results—Physiological doses (16 μ M) of cholesterol and phytosterols were not cytotoxic in these cells. Cholesterol enrichment promoted cell growth ($P<0.05$), while phytosterols significantly induced growth-suppression ($P<0.05$) and apoptosis. Cell cycle analysis showed that contrary to cholesterol, phytosterols decreased mitotic subpopulations. We demonstrated for the first time that cholesterol concurred to attenuate the expression of *caveolin-1* (*cav-1*) and *NDRG1* genes in both prostate cancer cell lines. Phytosterols had the opposite effect by inducing overexpression of *cav-1*, a known mediator of androgen-dependent signals that presumably control cell growth or apoptosis.

Conclusions—Cholesterol and phytosterol treatment differentially regulated the growth of prostate cancer cells and the expression of *p53* and *cav-1*, a gene that regulates androgen-regulated signals. These sterols also differentially regulated cell cycle arrest, downstream pro-apoptotic androgen-regulated tumor-suppressor, *NDRG1* suggesting that *cav-1* may mediate pro-apoptotic *NDRG1* signals. Elucidation of the mechanism for sterol modulation of growth and apoptosis signaling may reveal potential targets for cancer prevention and/or chemotherapeutic intervention. Sterol regulation of *NDRG1* transcription suggests its potential as biomarker for prediction of neoplasms that would be responsive to chemoprevention by phytosterols.

Keywords

Prostate cancer; *Caveolin-1*; Sterols; Cholesterol; Phytosterols; *NDRG1*; Apoptosis; growth-suppressor; Biomarkers; Chemoprevention

1. Introduction

Prostate cancer is the most common malignancy and the second cause of cancer death in men [1,2]. Preventive strategies for prostate cancer are achievable by the identification of the populations at risk for developing the neoplasm [2]. Well-established risk factors for prostate cancer include advancing age, race and family history [2,3]. Also, some of the most acknowledged putative risk factors for prostate cancer include androgens and diets, despite their complete role in cancer etiology having not been resolved [3]. Regardless, experimental and epidemiological data still implicate androgens as the main factors controlling the development, maintenance and progression of prostate cancer [4]. Therefore, identification of androgen-regulated genes may be important in elucidating the mechanism of prostate cell proliferation. Interestingly, the search for novel and biologically relevant androgen-regulated genes and processes in the human prostate has led to the identification of several genes implicated in cell growth or growth suppression [5,6]. In this regard, very recent data show ethnic variation in the expression pattern of a prostatic androgen regulated gene namely, *N-myc downstream-regulated gene 1* (*NDRG1*) [5]. The poor prognosis of prostate cancers in populations showing a low expression of this gene [5] highlights its importance in determining the outcome of the disease. The regulation of *NDRG1* and its biomarker status may be investigated from the perspective of the positive or negative regulation of several oncogenes or tumor suppressors by *caveolin1* (*cav-1*) [7,8,9], a marker of aggressive disease in prostate carcinoma [10,11]. *Cav-1* is a membrane-anchored protein enriched on caveolae, a cholesterol-rich plasmalemmal invagination where signal transduction molecules are concentrated [12,13]. Caveolae formation and *cav-1* expression are highly dependent on the availability of cholesterol [14]. It has been clearly demonstrated that enriched dietary cholesterol promotes oxidative stress, which enhances the induction and functional activation of *p53* [15]. Further, *cav-1* gene promoter includes sequences that bind to the oxidative stress-inducible tumor suppressor protein *p53* [16,17], thus explaining why *p53* mediates induction of *cav-1* gene expression [18]. Conversely, it has been found that *cav-1* gene expression can increase the activity of *p53*, indicating that both proteins may act synergistically [18]. Interestingly, phytosterol enriched cells have been reported to

inhibit absorption of cholesterol and replace a part of the cholesterol in cell membranes [19]. Cholesterol depletion is known to disrupt rafts and affect cell function [20], implying that phytosterol replacement of cholesterol may affect signal transduction processes. This is underscored by recent findings which suggest that changes in sterol structure strongly diminish their capacity to mimic cholesterol [21]. Functionally, *cav-1* is involved in signal transduction largely because of the presence of a 20 amino acid microdomain which can bind a variety of signal proteins [22], most often leading to downstream signaling events [23,24]. Typically, *cav-1* co-localizes with androgen receptors within lipid raft domains to mediate these downstream androgen-dependent signals [25,26] that modulate the expression of genes implicated in unregulated cell growth [22]. Unregulated cell growth occurs mostly when cells lose their ability to undergo apoptosis, often leading to cancer. Various studies have confirmed the loss in cell growth control following alteration of apoptotic pathways [27].

Based on evidence that certain sterol derivatives act in an anticholesterol fashion [21], we envisaged a differential modulation of *cav-1* expression by cholesterol [20] or phytosterols, thus promoting cell growth or apoptosis respectively. We therefore hypothesized that; these sterols may regulate prostate cell growth or apoptosis by promoting or repressing the transcription of *cav-1* and its downstream signals. To test this hypothesis, we examined the effects of different sterol-enrichment on *cav-1* expression, cell growth, apoptosis, and on the expression of downstream growth-suppressor *NDRG1*, using PC-3 and DU145 prostate cancer cell lines

2. Materials and Methods

2.1. Cell Culture

Prostate cancer cell lines PC-3 and DU145 were obtained from ATCC (Manassas, VA). The cells were cultured in MEM with 10 % FBS, 1 % penicillin/streptomycin, 1 % glutamine, 1% non essential amino acids, 0.1% gentamicine and fungizone and buffered with 0.75% HEPES at 37 °C in 5 % CO₂ for 24hr. The medium was then changed to 1 % FBS-MEM, and the cells incubated for 24 hr before treatment with sterols. Experimental media were supplemented with sterols (cholesterol or phytosterols; 10% campesterol: 75% β -sitosterol) (Acros organics, NJ) at final concentrations of 16 μ M. The phytosterol combination chosen represent the typical percentage concentration of β -sitosterol (78–83%) relative to phytosterols found in peanuts; which is a reported classic phytosterol source [28]. Optimum sterol concentration of 16 μ M was chosen after dose-response experiments produced results that were consistent with physiological levels for phytosterols, and correspond with previously observed values [29,30]. For all treatments, sterols were mixed in the media with a sterol carrier, (2-hydroxypropyl)- β -cyclodextrin (β -CD) (Sigma-Aldrich, St Louis, MO) and supplied to the cells as complexes. To provide the sterols in an assimilable and nontoxic form, the sterol to cyclodextrin molar ratio was maintained at 1:300 (16 μ M sterol: 5mM β -CD) as previously reported [30,31]. All treatments were performed in triplicates. After stimulation for 72 h, the cells or total RNA were used in various experiments.

2.2. Measurement of sterol effects on cell viability and growth

PC-3 and DU145 cells (5000 cells/cm²) were seeded in triplicates into 6-well plates for 24 h. The medium was replaced with 1% FBS-MEM for 24 h, and the cells supplemented with 16 μ M sterols in vehicle for 72 h. Viability of cells given different sterol treatment, was measured by trypan blue dye exclusion method. After 72 h, 1 $\times$ 10⁶ /ml cell suspension needed for viability assay was prepared by trypsinization, centrifugation and counting with a hemocytometer. A 1: 1 dilution of 200 μ l of the cell suspension was made using 0.4 % trypan blue solution and incubated for 3 min. at room temperature. Replicate samples of

stained and unstained cells from each well were counted with a hemocytometer on a microscope under a 10 × objective. Following analysis of variance, data from all experiments were pooled for further statistical analysis. The calculated percentage of unstained cells represents the percentage of viable cells. In view of the high cell viability obtained after 72h of sterol treatment options, the cells were harvested by trypsinization, and cell growth quantitated with a Coulter counter (Beckman Coulter, Inc.).

To verify whether tested sterols are necrotic to studied cells, we investigated their effects on cell growth using previously described [32] MTT assay, which assesses the metabolic status of the cells. Cell proliferation was determined using the CellTiter 96 Non-Radioactive Cell proliferation Assay (Promega Corp., Madison, WI), which is based on the cellular conversion of a tetrazolium salt into formazan product that is detectable using a 96-well plate reader. Briefly, 100 µl of 1×10^4 prostate cells were seeded into wells of a 96-well plate and incubated for 24 h. Then the medium was changed to another containing 1% FBS for another 24 h and then supplemented with vehicle (β -CD), cholesterol or phytosterols (10% campesterol: 75% β -sitosterol) for 48 h at 37 °C in a humidified atmosphere. After 72 h of culture, a chromogenic dye solution (15 µl) was added to each well and the plate incubated again at 37 °C for 4 h. After incubation, 100 µl of solubilization solution/Stop Mix was added to each well and within 1 h, the contents of the wells were mixed to get a uniformly colored solution. The absorbance of the colored reaction product was recorded at 570 nm wavelength using Synergy 2 multi-detection microplate reader (BioTek Instruments Inc., Winooski, VT).

2.3. Cell cycle analysis by flow cytometry

Cell cycle analysis was performed by propidium iodide (PI) staining. Triplicates of sterol and vehicle treated prostate cancer cells (1×10^{-6}) seeded in six-well cell cultures dishes were harvested after 72 h and washed with ice-cold PBS. The cells were then fixed in ice-cold 70 % ethanol, vortexed and stored at 4 °C for 1 h. This was followed by centrifugation at 3,000 rpm for 5 min. and two times washing in PBS. Fifty microliters of RNase (100 µg/ml) was added to the pelleted cells and incubated at room temperature for 15 min. After addition of 400 µl of PI (50 µg/ml) to the cells, stained nuclei were analyzed for DNA-PI fluorescence using an Accuri C6 flow cytometer (Accuri Cytometers Inc. MI). Resulting DNA distributions were analyzed for the proportion of cells in apoptosis and in the G₀/G₁, S, and G₂-M phases of the cell cycle.

2.4. RNA isolation and RT-PCR

To determine the effects of sterol treatment on the expression of *cav-1* and *NDRG1*, total RNA was extracted from cultured prostate cancer cells using RNA isolation protocol according to manufacturer's instructions (RNeasy kit, Qiagen, Valencia, CA). To synthesize cDNA by reverse transcription, 0.25 µg of total RNA and 1 µl each of oligo-dT and dNTP mix were used as described in the enhanced avian reverse transcriptase-PCR kit protocol (Sigma-Aldrich, St. Louis, MO). A portion (2µl) of each cDNA was used to amplify the fragments of the genes in the presence of Taq DNA polymerase (Sigma, MO). Specific primers for RT-PCR amplification of the various genes were as follows: (a) *NDRG1*: sense; 5'-GCTACAACCCCTCTTCAAC-3' and antisense 5'-GGGTTACGTTGATAAGGAC-3' (b) *Cav-1*: sense; 5'-ACCTCAACGATGACGTGGTCAAGA-3' and antisense 5'-TGGAATAGACACACGGCTGATGCACT-3' (c) GAPDH: sense; 5'-CCACCATGGCAAATTCCATGGCA-3' and antisense 5'-TCTAGAGGGCAGGTCAGGTCCACC-3'.

Reaction conditions were: 1 cycle of 95 °C for 10 min, followed by 40 cycles. This was followed by heating at 95 °C for 1 min. (*cav-1* and *NDRG1*) and 2 min. (GAPDH) for denaturation. Annealing was at 65 °C for 1 min. (*cav-1* and *NDRG1*), and for 2 min. (GAPDH). Extension was at 72 °C for 2 min. and final extension of 1 cycle of 72 °C for 5 min. PCR products were analyzed on 1.5 % agarose gel and visualized by ethidium bromide staining method. The intensity of the bands was quantified using Quantity One software package (Bio-Rad). All reactions were normalized by GAPDH mRNA expression.

Quantitative real-time PCR was used to determine mRNA transcripts in anti-apoptotic genes *Bcl-2*, *Bcl-XL*, pro-apoptotic gene *Bcl-Xs* and tumor suppressor *p53* using the Biorad iCycler Detection system (Bio-rad Laboratories). The specific primers for amplification of these genes by real time RT-PCR were as follows: (a) *Bcl-2* Sense; 5'-CATGCTGGGGCCGTACAG-3' and antisense 5'-GAACCGGCACCTGCACAC-3' (b) *Bcl-XL* Sense; 5'-TGCATTGTTCCCATAGAGTTCCA-3' and antisense 5'-CCTGAATGACCACCTAGAGCCTT-3' (c) *Bcl-Xs* Sense; 5'-ATCCAAACTGCTGCTGTGGC-3' and antisense 5'-TTCGACTTTCTCTCCTACAAGC-3' (d) *p53* Sense; 5'-AAGGAAATCTCACCCATCC-3' and antisense 5'-AAAGCGAGACCCAGTCTCAA-3'. Amplification followed by standard PCR reaction chemistry with the addition of fluorescent DNA-binding dye iQ SYBR Green super mix (Bio-Rad). The reaction was carried out in a volume of 50 µl containing 2 µl cDNA and 0.3 µM of each primer. Quantitative PCR results from each gene/primer pair were normalized to results obtained from housekeeping gene GAPDH. Each reaction comprised 40 cycles with melting at 95 °C for 20 s, annealing at 54 °C for 25 s and extension at 72 °C for 30s. Each sample was amplified in triplicates. Using the $2^{-\Delta\Delta C_T}$ method [33], the data was calculated as the fold change in the different gene expressions normalized to the GAPDH (endogenous control) and relative to the untreated control. For qualitative analysis, the PCR products from each primer pair were subjected agarose to gel electrophoresis. A 15 µl sample was resolved on a 1.5 % agarose gel containing ethidium bromide.

2.5. Colorimetric and immunocytochemical detection of caspase -3

Since the presence of caspase-3 in cells of different lineages suggests its requirement for the execution of apoptosis [34], its increased enzymatic activity in apoptotic cells was determined colorimetrically using the assay kit of BioVision, CA. Sterol-treated and control PC-3 and DU145 cells were collected by centrifugation after 72 h, then counted and pelleted at 1.5×10^6 cells before resuspension in 50 µl of chilled cell lysis buffer provided, and finally incubated on ice for 10 min. The cell suspension was then centrifuged for 1 min at $10,000 \times g$, and the supernatant (cytosolic extract) transferred to a fresh tube and put on ice, and aliquots stored at -80 °C for future use. Protein concentrations in the extract were determined with Bradford solution, using Bio Rad protein assay method (Bio-Rad Labs, Richmond, CA). In all samples, the final protein concentration for each assay was 200 µg/50 µl of cell lysis buffer. To each sample, 50 µl of 2× reaction buffer containing 10 mM DTT provided in the kit was added together with 5 µl of 4 mM caspase-3 colorimetric substrate DEVD-pNA to a 200 µM final concentration and incubated at 37 °C for 2 h. The samples were then read at 405-nm in a Synergy 2 multi-detection microplate reader (BioTek, VT). Fold increase in caspase activity was determined by comparing the absorbance with that of uninduced control.

Immunocytochemical analysis of caspase-3 in sterol-treated and control PC-3 and DU145 cells was performed by briefly washing cells with PBS and fixing with 4 % paraformaldehyde in PBS for 15 min at room temperature. Cells were permeabilized by incubating with PBS containing 0.5 % Triton X-100 for 10 min. The permeabilized cells were immersed in blocking solution containing 10 % normal goat serum in PBS for 1 h. The

cells were then incubated overnight with anti-caspase-3 primary antibody (Cell Signaling Tech, MA) diluted 1: 100 in PBS. After three washes with PBS, cells were incubated with secondary antibody (anti-rabbit IgG conjugated to horseradish peroxidase and diluted 1:300 in PBS) for 2 h at room temperature. The cells were then washed with PBS, and incubated with a diaminobenzidine tetrahydrochloride (DAB) (Sigma-Aldrich) working solution (which produces a brown precipitate in caspase-3 positive cells) for 10 min at room temperature, counterstained with diluted Harris hematoxylin (Sigma-Aldrich) for 2 min., and rinsed in distilled-deionized water. Finally slides were mounted with permount and caspase-3 staining cells visualized by a Zeis Axiovert 200 inverted microscope before being digitally photographed.

2.6. Western blotting

Growth medium was removed from prostate cancer cells 72 h after different sterol treatments, and washed with phosphate-buffered saline (PBS). The cells were then lysed with CellLytic MT mammalian tissue lysis/extraction reagent (125 μ l/10⁷ cells) (Sigma, St Louis, MO), containing a protease inhibitor cocktail. The cells were incubated for 15 min. at 4 °C in a shaker and the cell lysates collected by scraping. The supernatants were recovered by centrifugation at 12,000 g for 10 min. at 4 °C. Final protein concentrations were determined with Bradford solution, using Bio Rad protein assay method and samples stored at -80 °C for Western blot analysis. The cell extracts were boiled for 3 min. in Laemmli sample buffer (Bio-Rad Labs, Richmond, CA). Electrophoresis was performed with 12 % SDS-polyacrylamide gels in SDS-PAGE running buffer (24 mM Tris, 192 mM glycine and 0.1 % (W/V) SDS) and the proteins were transferred to nitrocellulose transfer membranes (Invitrogen, Carlsbad, CA) in transfer buffer (25 mM Tris, 192 mM glycine and 20 % methanol). The membranes were incubated in blocking buffer {3 % powdered blocker (Bio-Rad) diluted in Tris-buffered saline containing 0.1 % Tween-20} for 1 h. The membranes were then probed with polyclonal antibodies for Ndr1 (NDRG1 H-60, Santa Cruz Biotechnologies Inc, CA) at a dilution of 1: 1000 in blocking buffer for 16 h at 4 °C. A secondary antibody conjugated to horseradish peroxidase (Bio-Rad Labs, Richmond, CA) was applied for 1 h at room temperature and after washing, chemiluminescence detection was performed by capturing the image on Fuji Film Luminescent Image Analyzer LAS-3000 mini/LAS 3000 UV mini imager after scanning the membranes, which were treated with LumiGlo chemiluminescent substrate (Cell Signaling) and the integrated intensities were analyzed with the Fuji Film Multi Gauge software (Fuji Film Systems USA, Inc).

2.7. Statistical analysis

Data are given as mean values ($\pm$ SD). In each experiment, the individual data were calculated as the means of triplicates. Differences between treatments groups were determined by two-way ANOVA [35]. In all analyses, a P value less than 0.05 was considered statistically significant.

3. Results

3. 1. Cell viability and growth assays

Since assays for calculating cell viability are necessary for evaluating cell growth, the percentage of PC-3 and DU145 that survived cholesterol and phytosterol treatments were measured by the viability assay. Exposure of each cell line to different sterol treatment options did not result in any significant decrease in viability when compared with control cells as measured by the trypan blue dye-exclusion assay. The mean cell viability of PC-3 following vehicle, cholesterol and phytosterol treatments was 89.8 %, 90.1 %, 91.3 % while the values for DU154 were 93.6 %, 94.2 % and 92.8% respectively (Fig. 1).

After the different sterol treatment options, the high-level of viable cells observed with the trypan blue dye-exclusion assay encouraged the quantitation of cell growth with the versatile Coulter counter, since necrosis from the sterols dosage used was negligible. Fig. 2 shows the effect of sterol supplementation on the proliferation of PC-3 and DU145 cells. Although phytosterol treatment induced a highly significant ($P<0.01$) reduction in the growth of DU145 cells, compared with vehicle-treated cells, the reduction in growth of phytosterol-treated PC-3 cells was not significantly ($P>0.05$) different from control PC-3 cells. In the same cell lines, cholesterol treatment promoted cell growth which was significantly ($p<0.05$) different from the growth observed in vehicle treated cells.

We assessed the impact of various sterol treatments on cell necrosis and thus viability, by measuring the metabolic activity of viable cells using the tetrazolium salt MTT. The manifestation of active metabolism was measured by cell proliferation, based on the spectrophotometric absorbance of MTT, following its reduction to a colored formazan only by metabolically active and growing cells. Fig. 2b represents 72 h sterol supplementation on the proliferation of PC-3 and DU 145 cells. In both cell lines, cholesterol treatment significantly enhanced ($P<0.05$) cell proliferation as measured by MTT absorbance at 570 nm. To the contrary, phytosterol treatment arrested cell growth, which is observed by significant reduction of growth rate ($P<0.05$) from that of vehicle-treated cells.

3.2. Sterol-induced transcriptional changes in growth-suppressors

To evaluate the role of the sterol treatment options on the transcription of an up-stream growth-suppressor and on a downstream androgen-regulated growth-suppressor in cultured PC-3 and DU145 cells, we amplified the genes for *cav-1*, and *NDRG1*. Control gene expression was performed with specific primers for GAPDH. The respective amplification fragments shown in agarose gels for *cav-1*, *NDRG1* and GAPDH were 276, 300 and 598 base pairs as predicted (Fig. 3). Image analysis shows that the intensity of the transcripts for GAPDH was the same for all the samples. The intensity of the phytosterol bands were considered as 100 %. In PC-3 and DU145 cells, the intensity of *NDRG1* transcripts was higher in lanes with phytosterol treated samples (100 %) than those with cholesterol-treated samples (30 % for PC-3 and 60 % for DU145). Remarkably vehicle-treated PC-3 or DU145 cells respectively showed either no bands or the least band intensity (5 % and 28 %). Generally, the same gene expression pattern was observed in both cell lines following the sterol treatment options. The trend in the gene expression pattern was also similar between *NDRG1* and *cav-1*. Thus the intensity of *cav-1* bands was higher (100 % for both cell lines) in phytosterol treated cells (Fig. 3. *cav-1* lanes 3 and 6) than in cholesterol treatment (26 % and 48 % respectively for PC-3 and DU145) (Fig. 3 *cav-1* lanes 2 and 5).

3.3. Cell cycle analysis by fluorescent activated cell sorting

The cell populations were categorized in the FL2 –Area histogram generally as G_0/G_1 phase, or quiescent cells (M1), as S phase or cells in the process of replicating their DNA (M2), and as G_2/M phase, being cells with two full complements of DNA or at mitotic phase (M3) (Fig. 4). On this basis, we observed a significant ($P<0.01$) enrichment of submitotic cell populations (M3) in cholesterol treated DU145 cells (10.3 %) than in vehicle-treated cells (7.6 %). However, the percentage of submitotic cell population of cholesterol-treated PC-3 cells (7.9%) did not differ from vehicle treated PC-3 cells (8.0 %). Interestingly, the least mitotic cell subpopulations were observed following phytosterol treatment (2.9 % and 0 % for PC-3 and DU 145 respectively). Analysis of cells in the sub- G_0/G_1 apoptotic population reveal that unlike vehicle and cholesterol, phytosterols induced the highest apoptosis in PC-3 (71 %) and DU145 (18.7 %) cells respectively. Thus the highest subpopulations of cells during phytosterol-enrichment of PC-3 were mostly in the sub- G_0/G_1 apoptotic population, while the DU145 cells were in the G_0/G_1 or quiescent phase.

3.4. Sterol-induced changes in apoptotic process

Since unregulated cell growth is a feature of the cells inability to undergo apoptosis, we investigated not only the role of the different sterols in cell growth but also in apoptotic process. Sterols generally upregulated the expression of anti-apoptotic gene *Bcl-XL* in PC-3 and DU145 cells, however, the effect is more pronounced in cholesterol treated cells (Fig. 5A and 5C lanes 2 and 5). Whereas cholesterol treatment significantly ($p > 0.05$) upregulated the expression of anti-apoptotic gene *Bcl-2* in both cell lines, downregulation in expression of this gene by phytosterol was most marked in DU145 (Fig. 5 B and 5C lane 6).

Alternatively, RT-PCR results show that only phytosterol-treated cells showed detectable bands for the pro-apoptotic *Bcl-Xs* transcripts (Fig. 5C lanes 3 and 6). Again, results for quantitative PCR showed clearly that in both cell lines, only phytosterol treated cells overexpressed *Bcl-Xs* gene (Fig. 5 A and 5B). To explore the possibility that *cav-1* may regulate the downstream tumor suppressor *NDRG1* by activating a *p53*-dependent pathway, we assessed the transcription of *p53* in the different sterol treatment options. Our results show that in both cell lines, phytosterol treatment caused a highly significant ($P < 0.05$) upregulation of *p53* gene expression as compared to its downregulation in cholesterol treated cells (Fig. 5C and 5D).

From an immunocytochemical perspective, the assumption of “typical” apoptotic morphology in both prostate cancer cells (Fig. 6C III and VI) during phytosterol treatment is consistent with apoptotic signaling of phytosterols. These cells took on a smoother, more spherical shape that gave them a foamy appearance. Cell shrinkage was another common apoptotic morphology observed during phytosterol treatment of both cell lines. Phytosterol-induced pro-apoptotic biochemical changes were also observed in the detectable reaction between polyclonal affinity-purified rabbit antibody and caspase-3. These cells showed various amounts of reactivity with these antibodies demonstrated in the darkly-stained and diffused or scattered cytoplasmic patterns. On the other hand, normal (Fig. 6C I and IV) and cholesterol-treated (Fig. 6C II and V) cells were rather more spindle-shaped, and having less darkly stained patterns indicating little or no detectable reaction with this antibody.

3.5. Western blot analysis of expressed NDRG1 protein

To determine the amount of NdrG1 expressed in the extracted protein preparation, a Western blot analysis with NdrG1 anti-polyclonal antibody was performed. Unlike in transcription, protein immunoblotting analysis did not show any significant difference in the expression of NdrG1 in vehicle- and cholesterol-treated PC-3 and DU145 cell lines (Fig. 7, lanes 1 and 2; 4 and 5). However, especially in PC-3 cell lines, NdrG1 was more expressed during phytosterol treatment (lane 3), while its increased expression in DU145 cells (lane 6) was not significant ($P > 0.05$). Better still, quantification of band intensities show that NdrG1 expression seemed insensitive to cholesterol enrichment while the protein expression was significantly ($P < 0.01$) altered during phytosterol treatment.

4. Discussion

This study describes the importance of sterols in the transcription of upstream and downstream prostate cell growth-suppressors and their relationship of the sterols to cell growth and or apoptosis. To start with, necrosis by the treatment doses and the corresponding cell growth response were analyzed by viability assay. The calculated percentage viability showed that these sterol doses had no adverse effects on cell viability, and caused no significant necrosis. This treatment regimen enabled sterol supplementation encapsulated in an amount of delivery-vehicle (β -CD) corresponding with the determined optimum amount for a previous mycoplasma cells study [31]. The few cases (less than ten

percentage) of necrotic cells found in most treatment regimens strongly favor cell growth measurement with the Coulter counter - a versatile cell counting analyzer - which does not differentiate cell morphology, necrotic state or composition. The limitations of these viability assays were complemented with the CellTiter 96 non-radioactive cell proliferation assay (MTT), which determines viable cell number from proliferating and cytotoxic cells [36,37]. As different cell types and cell status show different levels of metabolic activity, factors that affect cytoplasmic volume or physiology, such as necrosis would influence the metabolic activity of cells, therefore dictating the relationship between cell number and absorbance. Thus MTT assay, which specifically measure states of metabolic activity, especially in proliferating cells, which are metabolically more active than nonproliferating or resting cells [38], serves the dual purpose of determining cell viability and cell proliferation.

It is predictable that in both cell lines, cholesterol treatment would support and maintain prostate cancer cell growth process. It is equally not surprising that cholesterol-rich cells grew significantly more than vehicle-treated cells. When quantified as percentage cell growth, the magnitude of growth agreed with previously observed results [39]. In contrast, phytosterols triggered a decrease in the growth of both prostate cancer cell lines relative to cholesterol and control groups. The cholesterol-induced proliferation, which was demonstrated by MTT assay that measured metabolic activity, may be linked with established role of cholesterol in the regulation of membrane-bound proteins, enzymes and several signal transducing agents [30]. To the contrary, MTT assay revealed a major reduction in cell proliferation during phytosterol treatment as compared to vehicle treatment, thus confirming the inhibitory effects of phytosterols on prostate cell growth. An elaborate significance of cholesterol-induced cell growth may be explained by its role in the intrinsically spontaneous formation of liquid-ordered aggregates known as lipid rafts in cell membranes [40]. The typically high concentration of cholesterol in rafts is widely accepted to be important in cell signaling since it can sequester signaling proteins in close proximity with each other, with a resultant influence on cancer cell growth [40]. Conversely, the reduced cell growth associated with phytosterol enrichment has been attributed to its inhibition of cholesterol absorption and its replacement of the membrane cholesterol [19]. Also, phytosterols are known to decrease cholesterol synthesis at the level of HMG-CoA reductase gene expression, without causing the commonly observed high cholesterol influx from the plasma membrane to the endoplasmic reticulum [41]. Since cholesterol depletion disrupts the signaling processes of rafts [20], membrane cholesterol replacement by phytosterols may potentially disorder raft associated signal transduction pathways that favor cell growth. Fresh evidence shows that changes to sterol structure diminished the capacity to mimic cholesterol functions in the membrane [21]. To confirm this, we investigated the effect of two structurally dissimilar sterols on the expression of two genes. These are cav-1, a membrane protein that regulates a variety of signaling pathways [24], and *NDRG1*. Both gene products are respectively associated with upstream and downstream signaling of prostate cell growth. Here, we showed for the first time that cholesterol enrichment attenuated the co-expression of *NDRG1* (although it unperturbed NdrG1 protein levels), a downstream androgen-regulated cell growth- and metastasis-suppressor gene, alongside cav-1, an upstream signaling lipid raft-rich protein [42]. We also observed the altered expression of NdrG1 proteins especially in phytosterol-rich cellular protein immunoblots of PC-3, which agreed with our RT-PCR data. The apparent inconsistency between attenuated *NDRG1* transcription and unperturbed NdrG1 translation in cholesterol-rich cells may be explained by known posttranscriptional regulatory mechanisms that determine protein lifetimes. Conceptually, constitutively produced mRNA may either have been translated continuously, with the level of protein controlled by its degradation rate, or that a short-lived mRNA encodes the protein, which may be highly stable, so as to persists for very long periods in the cells. This view is consistent with recent studies which report that unlike

NDRG1 gene, NdrG1 protein outlasts the withdrawal of its inducing stress factors, like hypoxia because of the high protein stability in every studied cell [43,44]. Altogether, *NDRG1* gene was subjected to independent confirmation of its expression pattern and validated by either RT-PCR or immunoblotting. Various studies show that poorly progressed and more aggressively invasive tumors show a low expression of *NDRG1* [5,42,45]. So, the reduced transcription of this growth-suppressor gene by cholesterol supports cholesterol's role in promoting cell growth or poor prognosis of disease. The influence of cholesterol-enrichment on cell growth, and the concomitant attenuation of *cav-1* expression, has been reported for most oncogenically transformed and human cancer cells [46]. Our results show a very modest or diminished expression of *cav-1* gene in vehicle (β -CD)-treated metastatic prostate cancer cells PC-3 and DU145. This contrasts greatly with the current paradigm that metastatic prostate cancer cells have a tendency to exhibit increased expression of *cav-1* [47,48]. However, our result does not seem to challenge this paradigm, but rather suggests that the lowly expressed *cav-1* gene, may be hampered by the vehicle or sterol carrier (2-hydroxypropyl)- β -cyclodextrin (β -CD), which is a membrane cholesterol chelating molecule [49]. Analogs of this vehicle or sterol carriers, such as methyl- β -cyclodextrin (M β CD) are reportedly potent cholesterol chelating agents that disrupt membrane domains, resulting in attenuation of *cav-1*-mediated signaling [49,50]. Thus, the low *cav-1* expression attributed to cholesterol chelation may truly result from M β CD inactivation of *cav-1* or its transcriptional inactivation [22]. Surprisingly, our results showed that, cholesterol and phytosterol treatments differentially enhanced the expression of *cav-1*. The restoration by sterols of the lost *cav-1* level in vehicle-treated cells corroborates recently observed reinstatement *cav-1*- regulated signaling by restoring cholesterol to cyclodextrin-treated cells [49]. In our cells, treatment with anti-tumorigenic phytosterols induced a much higher expression of *cav-1* than during cholesterol treatment. Typically, a low level of *cav-1* is generally associated with anti-mitotic or tumor suppressive properties in various human cancers [51,52], and thus consistent with the exhibition of increased expression of *cav-1* or its oncogenic effects in metastatic prostate cancer cells [47,48]. This scenario suggests that the two putative oncogenic and tumor suppressive drugs, which are namely cholesterol and phytosterols, may respectively induce the upregulation or down-regulation of *cav-1* expression in PC-3 and DU145 cells. On the contrary, we observed that the pro-apoptotic phytosterols induced a higher expression of *cav-1* especially in PC-3 than the anti-apoptotic cholesterol, wrongly suggesting that the improved expression of *cav-1* in sterol-treated prostate cancer cells is consistent with apoptosis. The apparent incongruity of the pro- and anti-apoptotic expression of *cav-1* has been explained variously as dependent on cell type-specific effects, methylation status, and the employed apoptosis inducers [53]. Accordingly, recent reports show that elevated cholesterol levels increases genomic methylation, which underlies transcriptional suppression of various genes [54,55]. Thus, this phenomenon may partly explain the observed comparative suppression of *cav-1* expression in cholesterol-rich cells as against phytosterol treated cells. Regardless of this, the concurrent cholesterol-induced cell proliferation may be attributed to its known effect on a host of cellular oncogenes like *N-Myc* (an antagonist and repressor of *NDRG1*) [56], whose tumorigenicity is revealed by downregulated expression of tumor suppressors like *cav-1* [53]. Our demonstration of low *NDRG1* transcription in cholesterol-treated cells is consistent with the milieu that is conducive for the recognized synergism between *cav-1* and other genes that perpetuate cell immortalization [53]. By extension, the pro-apoptotic and tumor suppressive character of *cav-1* in phytosterol-treated prostate cancer cells may be gleaned from the concomitant overexpression of the tumor suppressor, *NDRG1*, which substantiates the view that *cav-1* does not have a direct role in growth regulation, aside from synergism with other genes to exert the required effect [53]. Thus its apparent incongruity as an oncogene or tumor suppressor may be attributed to its possession of numerous recognized peptide domains that are attributed with opposing functions [53]; thus the modifying factors that determine *cav-1* effects could be determined by the synergistic

relationship with the cell or tissue type-associated oncogenes or tumor suppressors. The fact that *cav-1* and *NDRG1* are tumor suppressors [45,46], which concertedly respond to cholesterol treatment by attenuated transcription of their genes, suggests their mutual relationship involves the regulation of either gene by the other, or co-regulation of both genes by cholesterol. Current evidence suggests that *NDRG1* is regulated by *cav-1*, which functions as a scaffolding protein that interacts with, and regulates a variety of signaling pathways [24], among them, androgen-regulated signals [40], such as *NDRG1*. Our current effort to explain the regulatory role of *cav-1* on the expression of *NDRG1* gene exploits *cav-1* gene silencing procedures.

The high phytosterol-induced expression of growth suppressors like *cav-1* and *NDRG1* in PC-3 and DU145 cells suggests that, phytosterols regulate cell activities by repressing growth or by promoting apoptosis. Our hypothesis is supported by abundant evidence that cellular apoptosis in fibroblast, epithelial and rat pituitary adenoma cells is associated with overexpressed *cav-1* [13,57]. The growth-suppressive effects associated with the high expression of these genes were justifiably associated with our observed reduction in cell growth following phytosterol treatment. Besides, recent reports show that *NDRG1* is associated with *p53*-mediated apoptosis [45], revealing that the observed phytosterol-mediated increase in *cav-1* and *NDRG1* expression, and the suppressed cell growth, involve apoptosis. Current evidence shows that *NDRG1* mediates caspase activation and apoptosis, in conformity with its direct transcriptional targeting by *p53* [58]. The transcriptional activation of *NDRG1* by *p53* is supported by recent identification of a *p53* binding site on the *NDRG1* promoter region [58]. The observed suppression of growth and the induced expression of tumor suppressors, *cav-1* and *NDRG1* by phytosterols have impelled investigation of whether growth suppression of prostate cancer cells by phytosterols is an apoptotic process.

Apoptosis in these cells was investigated by the expression of *Bcl-2* gene and its related family member *BclX* in PC-3 and DU145 cells. Results showed that cholesterol-rich cells overexpressed anti-apoptotic gene transcripts *Bcl-2* and *Bcl-XL*, while *Bcl-Xs*, the pro-apoptotic spliced form of *BclX* was only upregulated by phytosterols. Upregulated expression of the pro-apoptotic isoform *Bcl-Xs* during phytosterol treatment is consistent with prevailing evidence that phytosterols induce tumor-suppression and apoptosis [29,59]. Molecular and genetically, we demonstrated phytosterol-induced tumor suppression and apoptosis, through cell growth arrest, overexpressed pro-apoptotic gene *Bcl-Xs*, and the tumor-suppressor genes, *cav-1*, *p53* and *NDRG1*, in prostate cancer cells. Also, caspase-3, a key effector of the apoptotic machinery of cells, and the hallmark of full commitment to cellular disassembly, was highly localized within various cytoplasmic regions of phytosterol-treated cells as compared to vehicle and cholesterol treatment. This is consistent with reports that caspase activation or cleavage to active caspase is a hallmark of almost all apoptotic systems [54], and occurs close to the inside surface of the cellular membrane, and then transferred to the cytoplasm, and a final transfer to the nuclear region [55]. These punctuate cytoplasmic patterns of caspase staining observed in phytosterol-activated pro-apoptosis cells fits the above explanation. Conversely, the indistinct caspase staining found in vehicle or cholesterol-treated cells attests to its pro-caspase form in normal non-apoptotic cells, and its intact cleavage site that lacks the normal antibody binding epitope found in caspases of apoptotic cells [54].

Further, flow cytometric analysis revealed especially in DU145 cells, the presence of a higher mitotic subpopulation in cholesterol-rich cells compared to phytosterol-rich cells. The negligible mitotic cell subpopulation found in phytosterol-treated cells as against the vast number of cells within the sub-G₀/G₁ or G₀/G₁ phases suggests outright sterol induction of apoptosis, or the induction of G₁/S cell cycle phase arrest in readiness for apoptosis.

Accordingly, our demonstration that phytosterols treatment not only caused the reduction in mitotic cell subpopulation, but also elevated the expression of *cav-1* and *p53*, implicates its induction of genes involved in the arrest of cell cycle phases at various points prior to apoptosis. This is corroborated by experimental evidence that overexpressed *cav-1* and *p53* block cells in the G₀/G₁ and G₂ phases of the cell cycle respectively [60,61]. The signaling mechanism by which phytosterols induce apoptosis is not fully established, but may include the induction of cell cycle phase arrest through *p53* mediation of *cav-1*-regulated apoptosis by transcriptional activation of *NDRG1*.

In summary, our study revealed that cholesterol treatment supported and maintained prostate cancer cell growth and downregulated the expression of *p53*, and *cav-1*, which are pro-apoptosis tumor-suppressor and scaffolding proteins that regulate a variety of signaling pathways that include androgen-regulated signals. Cholesterol also downregulated the expression *NDRG1*, an androgen-regulated tumor-suppressor, that is associated with apoptosis. Conversely, we demonstrated that phytosterols contradicts cholesterol effects on cell growth, by upregulating the expression of these pro-apoptosis growth-suppressors. Finally, our study confirmed that phytosterols induced growth-suppression and apoptosis by down-regulating anti-apoptosis genes. In conclusion, this study has demonstrated that cholesterol and phytosterols enrichment differentially regulate prostate cell growth, the expression of tumor suppressor *p53*, its upstream pro-apoptosis counterpart *cav-1*, and *NDRG1* a downstream androgen-regulated equivalent. This therefore correlates sterol-enrichment to cell growth or apoptosis, and reveals novel sterol-induced signal transducers that may constitute biomarkers for prostate cancers with cholesterol etiology, or which may respond to therapeutic intervention by phytosterols.

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Figure 1.

Effect of sterol supplementation on viability of PC-3 and DU145 cells. Viability of cells was measured by trypan blue dye-exclusion method after 72 h. The number of cells were then counted with a hemocytometer on a microscope under $10\times$ objective. The treatments were assayed in triplicates.

Figure 2.

Fig. 2a. Effect of sterol supplementation on growth of PC-3 and DU145 cells. Cells were treated with vehicle (VEH or β -CD), cholesterol (CHOL) and phytosterols (CAMPSIT). Cell growth was measured with a Coulter counter after 72 h of sterol treatment. The results are the mean $\pm$ S.D All the assays were duplicated for at least three independent experiments. Fig. 2b. Effect of sterol supplementation on proliferation of PC-3 and DU145 cells. Cells were treated with VEH Vehicle (β -CD); CHOL (Cholesterol) and CAMPSIT (campesterol/ β -sitosterol). Cell proliferation was determined by CellTiter non-radioactive cell

proliferation assay based on formazan dye formation that is detectable in a 96-well plate reader. The results are the mean $\pm$ S.D. All the assays were duplicated for at least three independent experiments.

Figure 3.

A. Results of RT-PCR showing the effect of sterols on *NDRG1* and *Caveolin1*.

Cyclodextrin-complexed cholesterol or phytosterols {campesterol and β -sitosterol mixture (CAMPSIT); 10: 75 % } were supplemented in prostate cancer cell lines for 3 days accompanied by analysis of *NDRG1* & *Caveolin 1* from their cDNAs. Lane 1 – Cyclodextrin (vehicle) supplemented PC 3 cells; lane 2 – Cholesterol supplemented PC3 cells; Lane 3- CAMPSIT supplemented PC 3 cells; Lane 4 – Cyclodextrin (vehicle) supplemented DU145 cells; lane 5 – Cholesterol supplemented DU145 cells; Lane 6- CAMPSIT supplemented DU145 cells. PCR- amplified bands of *NDRG1* (A) and *caveolin 1* (B) were quantified using Quantity One program. The histograms show quantification of the intensity of the bands, calibrated to the intensity of GAPDH bands, and expressed as folds change of gene. Each experiment was repeated at least three times. The figure shows a representative amplification.

Figure 4.

A. Representative histogram from flow cytometry demonstrating cycling cell population stained with propidium iodide. PC-3 (a–c) and DU 145 (d–e) were respectively enriched with vehicle, cholesterol and phytosterol, then sorted into different stages of mitosis by flow cytometry. Gates M1 are cells in G0/G1 phase ; gates M2 are cells in S-phase and gates M3 are cells in G2-M phase. **B.** Summary of percentage cells that maintain two complements of DNA or are at mitotic phase (M3). Graph represents the means $\pm$ S.D. for three independent experiments

Figure 5.

Real time RT-PCR showing the effect of sterols on expression of various genes. Cyclodextrin (β-CD)-complexed cholesterol or phytosterol (10 % campesterol: 75 % β-sitosterol) was added to prostate cancer cells in culture for 3 days. cDNA from cells was amplified by PCR using appropriate primers. **A** and **B**. Real time RT-PCR of mRNA expression for Bcl-XL, Bcl-2 and Bcl-X in PC-3 and DU 145 respectively. **C**. Real time RT-PCR for p53 expression in PC-3 and DU145. **D**. Gel analysis of Real time RT-PCR products: PC-3 treatment: lanes 1–3; Vehicle (β-CD), Cholesterol and Phytosterols. DU 145 treatment: lanes 4–6; Vehicle (β-CD), Cholesterol and Phytosterols.

Figure 6.

Fig. 6a. Colorimetric assay of caspase-3-activity. PC-3 cells were treated with vehicle (control), cholesterol and phytosterols. Apoptosis was assayed by caspase-3 activity in a microtiter plate reader at 405 nm.

Fig. 6b. Colorimetric assay of caspase-3-activity. DU145 PC-3 cells were treated with vehicle (control), cholesterol and phytosterols. Apoptosis was assayed by caspase-3 activity in a microtiter plate reader at 405 nm.

Figure 6c. Immunocytochemical detection of caspase-3 in sterol-treated and control PC-3 and DU 145 cells (A and B respectively). Representative photomicrographs of cells treated with vehicle (I and III), cholesterol (II and IV) and phytosterols.

Figure 7.

(A). Representative Western blot analysis of NDRG1 in PC-3 (Lanes 1–3) and DU145 (Lanes 4–6) cell lines. Vehicle treated cells (lanes 1 and 4), cholesterol treatment (lanes 2 and 5) and phytosterol –treated cells (lanes 3 and 6). (B) The intensities of NDRG1 bands corresponding to different sterol treatment options were quantified and the values shown on the histogram. The values represent means $\pm$ SD of experiments performed in triplicates.