Withania somnifera root extract inhibits fatty acid synthesis in prostate cancer cells

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1. Introduction

Metastatic prostate adenocarcinoma remains a leading cause of mortality in American men. Diagnosis of >170,000 new cases of prostate cancer and death of >30,000 men with this disease was anticipated in the United States in 2019 alone by the American Cancer Society. Metabolic adaptation is one of the molecular traits anticipated in the United States in 2019 alone by the American Cancer Society. Metabolic adaptation is one of the molecular traits of many malignancies including prostate adenocarcinoma. Oxidative phosphorylation is the primary source of ATP production in normal cells but neoplastic cells exhibit increased reliance on less efficient aerobic glycolysis for meeting cellular energy demand, a phenomenon widely known as the Warburg effect. Increased de novo synthesis of fatty acids is another striking metabolic deregulation especially in prostate cancer. Fatty acid synthesis begins with acetyl-CoA that is eventually converted to palmitic acid [a 16-carbon saturated fatty acid (C16:0)], myristic acid [a 14-carbon saturated fatty acid (C14:0)], and stearic acid [a 18-carbon saturated fatty acid (C18:0)] through a series of catalytic reactions. However, palmitic acid is...
the most abundant de novo synthesized saturated fatty acid. In proliferating cancer cells, the fatty acids and their metabolic intermediaries are utilized for ATP production through mitochondrial β-oxidation, cell signaling such as palmitoylation of oncoproteins (e.g., Wnt and p21Ras) or maintenance of membrane integrity. Both natural products (e.g., green tea-derived epigallocatechin-3-gallate) and synthetic chemicals (e.g., cerulenin and orlistat) have been explored as potential inhibitors of fatty acid synthesis for cancer therapy but their clinical development was limited for a variety of reasons, including stability (cerulenin), poor bioavailability (orlistat), and side effects (appetite suppression and weight loss). Therefore, a safe and effective inhibitor of fatty acid synthesis for targeting this metabolic deregulation for cancer therapy and/or cancer chemoprevention is still desirable.

Plants used in the traditional and complimentary medicine (e.g. Ayurvedic medicine) continue to be explored for treatment and chemoprevention of cancer. Withania somnifera is one such medicinal plant native to Asia that is used heavily in Ayurvedic medicine. The root or leaf of Withania somnifera is a key component of many Ayurvedic formulations. Dietary supplements of Withania somnifera are available over the counter in the United States. Several pharmacological effects of Withania somnifera extract have been described such as effect on male reproductive function, neuroprotective effect, and anticancer effects. In vivo anticancer activity of an alcoholic extract of Withania somnifera root was initially documented in a murine sarcoma model. Another interesting preclinical pharmacological effect of Withania somnifera extract includes suppression of cyclophosphamide-induced toxicity in mice. Several investigators have also reported cancer chemopreventive effects of Withania somnifera root in mice, including 20-methylcholanthrene-induced fibrosarcoma, 1,2-dimethylbenz[a]anthracene-induced skin cancer, and benzo(a)pyrene-induced forestomach papillomagenesis without any side effects. In a human prostate cancer cell line (PC-3), Withania somnifera extract inhibited cell viability in association with suppression of proinflammatory interleukin-8 and cyclooxygenase-2 expression and irreversible arrest in G2/M phase of the cell cycle. Anticancer and chemopreventative effects of Withania somnifera are primarily attributed to steroidal lactones (e.g., withaferin A) collectively known as withanolides.

In this study, we investigated the effect of ethanol extract of Withania somnifera root (WRE) on fatty acid synthesis using an androgen-sensitive (LNCaP) and a castration-resistant (22Rv1) human prostate cancer cell line as a potential mechanism for its antiproliferative activity. We demonstrate that WRE is a potent inhibitor of fatty acid synthesis in both cell lines.

2. Materials and methods

2.1. Determination of withaferin A content in Withania somnifera root extract

The root of Withania somnifera contains many phytochemicals, including withaferin A, withanolide A, and withanone to name a few. In this study, WRE standardized for one of its bioactive phytochemicals (withaferin A) was used, and hence the concentrations for cellular studies are indicated in μM corresponding to the withaferin A content. The ethanol extract of WRE was prepared as described by us previously. ChromaDex (Los Angeles, CA) was outsourced for determination of withaferin A content and that of other withanolides in the WRE preparation used in this study. A 250 mg/mL alcoholic extract was diluted (1:100), sonicated for 30 min, and then filtered through a 0.45 μm filter. Content of withaferin A and other withanolides in the WRE preparation was determined by High-Performance Liquid Chromatography using a Luna 2.5 μm C18(2)-HST 100A column. The flow rate was 0.5 mL/min. The mobile phase consisted of water (solution A) and acetonitrile (solution B). Chromatographic conditions were as follow: 65% A and 35% B for 0–9 min; 45% A and 55% B for 9–10 min; 5% A and 95% B for 10–14 min; and 65% A and 35% B for 14–18 min. Withanone and withanolide A were not detectable but the level of withaferin A (5.76 mg/mL) was higher by about 7.5-fold when compared with that of withanoside V or 1,2-deoxywithastramonolide.

2.2. Reagents and cell lines

Reagents for cell culture were purchased from Invitrogen-Life Technologies (Carlsbad, CA). Antibodies against fatty acid synthase (FASN), acetyl-CoA carboxylase 1 (ACC1), p-Akt (S473), and c-Myc were from Cell Signaling Technology (Danvers, MA); antibodies against ATP citrate lyase (ACLY) and carnitine palmitoyltransferase 1A (CPT1A) were from Abcam (Cambridge, MA); and anti-β-Actin antibody was from Sigma-Aldrich (St. Louis, MO). Human prostate cancer cell lines (22Rv1 and LNCaP) were purchased from American Type Culture Collection (Manassas, VA), and maintained as recommended by the provider. Both cell lines were last authenticated by us in March of 2017. Details of stable transfection of 22Rv1 cells with pcDNA3 empty vector (Vec) and the same vector encoding c-Myc and their culture conditions have been described by us previously.

2.3. Western blotting

Details of preparation of whole cell lysates and western blotting have been described by us previously. Densitometric quantitation of the immunoreactive band of interest was done using UN-SCAN-IT software (Silk Scientific, Orem, UT). Blots were stripped and re-probed with anti-β-Actin antibody for protein normalization.

2.4. Confocal microscopy

Cells were cultured on glass coverslips and treated with WRE for 24 h. Cells were then processed for immunofluorescence microscopy as described by us previously. The primary antibodies used were anti-ACLY (1:2000 dilution), anti-ACC1 (1:1000 dilution) and anti-FASN (1:1000 dilution). DRAQ5 was used to stain nuclei. For CPT1A, cells were also treated with 100 nM Mitotracker Red at 37 °C for 30 min to stain mitochondria followed by incubation with anti-CPT1A antibody (1:3000 dilution). Cells were observed under a confocal microscope. Corrected total cell fluorescence (CTCF) was quantitated using ImageJ software.

2.5. Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Real-time qRT-PCR was performed essentially as described by us previously. The primers used were: ACYI Forward: 5'-GGTGTCGCTGTTGAAGTCAGAG-3’; ACYI Reverse: 5’-ACATGTCGTCGAGACCT-3’; ACC1 Forward: 5’-ATGGGTCAACCCATGGTCT-3’; ACC1 Reverse: 5’-TCTTAAAGAGTGGTTCAGCC-3’; FASN Forward: 5’-AGTACAACACCCAGGGCCAAAGT-3’; FASN Reverse: 5’-TCGATGACGGTGAGGTATCT-3’; CPT1A Forward: 5’-TCCAGTGGCTTATCCTGTTG-3’; CPT1A Reverse: 5’-CTAACCCAGGTTGCTTCTGG-3’; 18S rRNA Forward: 5’-GCATCATTCGCACTTTCTCAGA-3’; GAPDH Reverse: 5’-GGACTCTAGCCGTCCTCAGA-3’. GAPDH was used as a normalization control. The amplification conditions were as follows: 95 °C for 15 s, 60 °C for 1 min, and 72 °C for 30 s for 40 cycles.
Fig. 1. Western blots showing effects of WRE treatment on fatty acid metabolism-related protein levels in LNCaP and 22Rv1 cells. (A) Immunoblotting for ACLY, ACC1, FASN, CPT1A and β-Actin proteins using whole cell lysates from LNCaP and 22Rv1 cells after treatment with ethanol or the indicated doses of WRE. (B) The graphs show densitometric quantitation of each protein from 3 independent western blotting experiments (mean ± SD, n = 3). *Significantly different (P < 0.05) compared with ethanol-treated control by one-way ANOVA with Dunnett’s adjustment.
Fig. 2. WRE treatment decreased the expression levels of fatty acid metabolism proteins in LNCaP and 22Rv1 cells. (A) Representative confocal images (60× objective magnification) for ACLY, ACC1, FASN and CPT1A proteins (green fluorescence) in ethanol- or WRE-treated (24-h treatment) LNCaP and 22Rv1 cells. DRAQ5 (blue fluorescence) and Mitotracker (red fluorescence) were used to stain nuclei and mitochondria, respectively. (B) Quantitation of corrected total cell fluorescence (CTCF) for ACLY, ACC1, FASN, and CPT1A protein expression in WRE-treated LNCaP and 22Rv1 cells. Results shown are mean ± SD (n = 6–14) from two independent experiments. *Significantly different (P < 0.05) compared with ethanol-treated control by one-way ANOVA with Dunnett’s adjustment. Results were consistent in replicate experiments.
2.6. Determination of acetyl-CoA, total free fatty acids, and neutral lipid droplets

Intracellular levels of acetyl-CoA and total free fatty acids were determined using kits from BioVision (Milpitas, CA) and Sigma-Aldrich (St. Louis, MO), respectively, as recommended by the supplier of the kit. For neutral lipid droplets, BODIPY staining was performed. Cells were plated on coverslips, treated with ethanol or WRE for 24 h followed by incubation with 1 μg/mL of BODIPY for 1 h at room temperature prior to microscopic visualization. At least four non-overlapping images were captured from each section and number of neutral lipid droplet positive cells was quantified using ImageJ software.

2.7. Colony formation, cell proliferation, and apoptosis assays

For clonogenic assay, cells (500 cells) were plated in 6-well plates in triplicate. After overnight incubation, cells were treated with ethanol or specified concentrations of WRE. The medium
containing ethanol or WRE was changed every third day. After 12 days, cells were fixed with methanol and stained with crystal violet for 30 min. Colonies were counted using GelCount (Oxford Optonix, Abingdon, UK). Cell proliferation was determined by the MTS assay using a kit from Promega (Madison, WI) as suggested by the manufacture. Apoptosis was measured by flow cytometry using Annexin V kit from BD Biosciences (San Jose, CA) according to the protocol of the manufacturer.

2.8. Statistical analysis

Statistical tests were performed using GraphPad Prism (version 7.02). Statistical tests were performed using one-way analysis of variance (ANOVA) followed by Dunnett’s test (dose-response) or Bonferroni’s test (multiple test).

3. Results

3.1. WRE downregulated expression of fatty acid metabolism proteins in LNCaP and 22Rv1 cells

Initially, we performed western blotting to determine the effect of WRE treatment on protein levels of key fatty acid metabolism proteins, including ACLY (an enzyme responsible for conversion of citrate to acetyl-CoA), ACC1 (an enzyme responsible for conversion of acetyl-CoA to malonyl-CoA), FASN (an enzyme complex responsible for synthesis of saturated fatty acids like palmitic acid), and CPT1A (a mitochondrial enzyme responsible for the formation of acyl carnitines for mitochondrial transport of fatty acids for \( \beta \)-oxidation). As can be seen in Fig. 1A, protein levels of ACLY, ACC1, FASN, and CPT1A were decreased upon WRE treatment in both
LNCaP and 22Rv1 cells. Statistically significant downregulation of ACC1 and FASN proteins in WRE-treated LNCaP and 22Rv1 cells was noticeable even after 8 h of treatment (Fig. 1B).

Fig. 2A depicts confocal microscopic images for ACLY, ACC1, FASN, and CPT1A proteins in ethanol-treated and WRE-treated LNCaP and 22Rv1 cells. Quantitation of the immunofluorescence (CTCF) confirmed statistically significant downregulation of these proteins in WRE-treated cells in comparison with corresponding solvent-treated control cells (Fig. 2B). The mRNA levels of ACLY, ACC1, FASN, and CPT1A proteins were also significantly reduced upon 24-h WRE treatment in both cell lines (Fig. 3). Collectively, these results indicated suppression of ACLY, ACC1, FASN, and CPT1A proteins and mRNA levels by WRE treatment in LNCaP and 22Rv1 cells.

3.2. WRE treatment decreased intracellular levels of acetyl-CoA and total free fatty acids

The intracellular level of acetyl-CoA was lower by 45%–53% in WRE-treated LNCaP cells when compared to ethanol-treated control (Fig. 4A). Likewise, WRE treatment resulted in a statistically significant decrease in intracellular levels of acetyl-CoA in 22Rv1 cells (Fig. 4A). WRE-treated cells also exhibited a significant decrease in intracellular levels of total free fatty acids when compared to ethanol-treated control cells (Fig. 4A). Neutral lipid droplets following staining with BODIPY in ethanol-treated control cells and WRE-treated LNCaP and 22Rv1 cells can be visualized in Fig. 4B. The number of neutral lipid droplet positive cells was lower by 64%–82% in WRE-treated LNCaP cells and by 66%–87% in 22Rv1 cells following exposure to WRE (Fig. 4C). Collectively, these results indicated that WRE is a potent inhibitor of fatty acid synthesis in both LNCaP and 22Rv1 cells.

3.3. Comparison of WRE with Cer or Eto for inhibition of cell proliferation, apoptosis induction, and suppression of total free fatty acids level

Next, we compared the effects of WRE with those of cerulenin (Cer) and etomoxir (Eto) on cell proliferation, apoptosis induction, and total free fatty acids levels. Cer is an antifungal agent that is known to inhibit fatty acid synthesis by binding to one of the subunits of the FASN complex. Eto irreversibly inhibits CPT1 that is localized on the outer side of the mitochondrial inner membrane. Cer and Eto were not used as positive controls, but instead used to demonstrate that, at equimolar concentration, WRE is a better suppressor of fatty acid synthesis than the other two agents. For direct comparison, it was only logical to use the same dose and treatment conditions for WRE, Cer, and Eto. As shown in Fig. 5A, proliferation of LNCaP and 22Rv1 cells was inhibited significantly upon treatment with WRE. Proliferation of these cells was not affected by Cer or Eto. We also determined the effects of WRE, Cer, and Eto on apoptosis induction because fatty acid synthesis inhibitors are known to promote apoptosis. Consistent with inhibition of cell proliferation data (Fig. 5A), WRE treatment resulted in a dose-dependent and statistically significant apoptosis induction in both LNCaP and 22Rv1 cells, but this effect was not seen in Cer or Eto-treated cells (Fig. 5B). Finally, WRE treatment, but not Cer or Eto treatment, caused suppression of intracellular levels of total free fatty acids (Fig. 5C). These results further established potency of WRE for inhibition of fatty acid synthesis.

3.4. Role of c-Myc and caAkt in WRE-mediated suppression of fatty acid synthesis

To gain insights into the mechanism underlying WRE-mediated suppression of fatty acid synthesis, we determined its effect on protein levels of c-Myc and Akt, which have been implicated in regulation of fatty acid synthesis. The level of p-Akt (S473), which is indicative of Akt activation, was higher in cells transfected with caAkt plasmid when compared to empty vector transfected Vec cells (Fig. 6A). Level of p-Akt (S473) was decreased following WRE treatment in Vec and caAkt overexpressing 22Rv1 cells (Fig. 6A). However, overexpression of caAkt did not have a meaningful impact on suppression of ACLY, ACC1, and FASN proteins (Fig. 6A). In addition, colony formation inhibition by WRE exposure was not affected by caAkt overexpression (Fig. 6B). One puzzling observation from this experiment was inhibition of colony formation by caAkt overexpression, but reasons for this unexpected observation are not clear. Nevertheless, these results ruled out a role for Akt in fatty acid synthesis inhibition by WRE.

Western blotting confirmed overexpression of c-Myc in stably transfected 22Rv1 cells in comparison with empty vector transfected Vec cells (Fig. 6C). Downregulation of ACC1 and FASN proteins by WRE treatment was partially abrogated by overexpression of c-Myc (Fig. 6C). Furthermore, overexpression of c-Myc conferred partial protection against WRE-mediated inhibition of colony formation especially at 20 and 40 nM concentrations (Fig. 6D). A similar effect of c-Myc overexpression was seen on WRE-mediated inhibition of total free fatty acids levels (Fig. 6E). These results partially implicated c-Myc in suppression of fatty acid synthesis by WRE treatment.

4. Discussion

The present study identifies a novel pharmacological effect of fatty acid synthesis inhibition by WRE treatment in androgen-sensitive and castration-resistant human prostate cancer cells. Inhibition of fatty acid synthesis by WRE treatment is accompanied by downregulation of mRNA and protein levels of ACLY, ACC1, FASN, and CPT1A. While ACLY, ACC1, and FASN enzyme proteins play an important role in the synthesis of fatty acids, CPT1A is critical for mitochondrial uptake of fatty acids for their eventual β-oxidation for energy production. The fatty acid synthesis enzyme proteins are implicated in the pathogenesis of prostate cancer. For example, studies have identified a novel feedback loop involving ACLY, 5′-adenosine monophosphate-activated protein kinase, and androgen receptor in prostate cancer cells. These investigators showed that inhibition of ACLY in castration-resistant prostate cancer cells.
promoted activation of 5′ adenosine monophosphate-activated protein kinase leading to suppression of androgen receptor levels, and inhibition of cell proliferation. Overexpression of ACC1 and FASN has been reported in early-stage prostatic intraepithelial neoplasia and/or in advanced-stage prostate adenocarcinoma. It was also shown very recently that CPT1A supports castration-resistant prostate cancer by supplying acetyl groups for histone acetylation and thereby promoting growth and antiandrogen resistance. Thus, it is reasonable to conclude that key enzymes of the fatty acid synthesis pathway are novel targets of anticancer activity of WRE in prostate cancer cells. The WRE preparation used in this study was standardized for withaferin A, one of the withanolide constituents of this herbal preparation. Even though withaferin A was the most abundant withanolide in the WRE preparation, it is possible that minor constituents may also contribute to the fatty acid synthesis inhibition by WRE. Additional work is necessary to explore this possibility but we can conclude that fatty acid synthesis inhibition by WRE may contribute to its anticancer activity in prostate cancer cells. In this context, however, it is important to point out that withaferin A has been shown to inhibit growth of xenografted human prostate cancer cells in immune compromised mice as well as prevent prostate cancer development in transgenic mouse models.

We have shown previously that certain other naturally-occurring cancer chemopreventive agents like phenethyl isothiocyanate from watercress and sulforaphane from broccoli are able to inhibit fatty acid synthesis in prostate cancer cells. Once common structural similarity in withaferin A from WRE and above listed isothiocyanates is that they all are Michael acceptors (electrophile) capable of reacting with nucleophiles. Studies have shown interaction of withaferin A with cysteine residue in several proteins including vimentin and β-tubulin. One interesting question requiring further interrogation in future studies is whether withaferin A from WRE can interact with proteins of fatty acid metabolism pathway.

In conclusion, the present study reveals that WRE is a novel inhibitor of fatty acid synthesis in prostate cancer cells. We also found that suppression of fatty acid synthesis inhibition by WRE is mediated by c-Myc downregulation.

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Declaration of competing interest

None of the authors has any conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtcmce.2020.02.002.

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