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Analysis of autophagic flux in response to sulforaphane in metastatic prostate cancer cells

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Abstract

Scope—The phytochemical sulforaphane has been shown to decrease prostate cancer metastases in a genetic mouse model of prostate carcinogenesis, though the mechanism of action is not fully known. Sulforaphane has been reported to stimulate autophagy, and modulation of autophagy has been proposed to influence sulforaphane cytotoxicity; however, no conclusions about autophagy can be drawn without assessing autophagic flux, which has not been characterized in prostate cancer cells following sulforaphane treatment.

Methods and Results—We conducted an investigation to assess the impact of sulforaphane on autophagic flux in two metastatic prostate cancer cell lines at a concentration shown to decrease

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Designed the research: GWW, VIP, EH. Conducted the research: GWW, SW, YF, ZPS. Wrote the manuscript: GWW. Evaluated the data and edited the manuscript: GWW, SW, YF, ZPS, CSM, DEW, RHD, VIP, EH.

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metastasis *in vivo*. Autophagic flux was assessed by multiple autophagy related proteins and substrates. We found that sulforaphane can stimulate autophagic flux and cell death only at high concentrations, above what has been observed *in vivo*.

Conclusion—These results suggest that sulforaphane does not directly stimulate autophagy or cell death in metastatic prostate cancer cells under physiologically relevant conditions, but instead supports the involvement of *in vivo* factors as important effectors of sulforaphane-mediated prostate cancer suppression.

Keywords

autophagy; cancer; flux; prostate; sulforaphane

1 Introduction

Sulforaphane (1-isothiocyanato-4-methylsulfinylbutane) is a plant-derived isothiocyanate that has been the subject of scientific investigation for several decades. The compound is bioactive and induces an array of cellular responses that are associated with health benefits [1]. In the cancer field, sulforaphane has long been known as an effective “blocking agent” [2]. Sulforaphane is a well-characterized inducer of cytoprotective enzymes, particularly the Phase I and Phase II detoxifying enzymes controlled by nuclear factor erythroid-derived 2 factor 2 (nrf2) transcription factor [3]. This effect has been shown to have an important role in genome protection by increasing a cell’s ability to neutralize and remove electrophiles and reactive intermediates and effectively limit cancer initiating events.

More recent work has suggested that sulforaphane may also possess cancer-suppressive properties in addition to its blocking activity. A number of investigations have demonstrated a cytotoxic response in transformed cells relative to normal cells at equal pharmacological concentrations, though the sensitivity to sulforaphane between cell lines is highly variable [4, 5]. High concentrations of sulforaphane (i.e. pharmacological concentrations exceeding a typical dietary exposure) have been reported to stimulate stress-response pathways in addition to the antioxidant response mediated by nrf2, including increased heat shock protein and chaperone expression [6], increased proteasomal degradation capacity [6], cell-cycle arrest [1] and induction of autophagy (specifically macroautophagy) [7, 8]. Modulation of autophagy is a particularly interesting finding because autophagy is known to be dysregulated in cancer cells and has been proposed as a direct therapeutic target or as a chemo-sensitizer to enhance the efficacy of other agents [9]. Over 40 clinical trials are currently registered with the National Institutes of Health investigating modulation of autophagy as a therapeutic strategy (clinicaltrials.gov).

Autophagy is the process by which cellular substrates are engulfed by a double membrane vacuole and delivered to the lysosome for degradation [10]. The double membrane is marked by lipidated LC3 (LC3-II, LC3-PE) and sequesters substrates either non-specifically or specifically through autophagy adaptor proteins (e.g. p62/SQSTM1). Autophagic activity (i.e. autophagic flux) can thus be assessed by monitoring the levels of these proteins [11, 12]. Although autophagy has been implicated in the cellular response to sulforaphane both *in vitro* in prostate cancer cell lines [7] and *in vivo* in a mouse model of prostate cancer [13],

no study has characterized the effect on flux in prostate cancer cells. Because autophagy is a dynamic process, movement of substrates through the degradation pathway must be addressed to assess whether sulforaphane enhances or impairs autophagy [11]. We also now know that autophagy and apoptosis often co-occur and share a subset of molecular components [14], suggesting induction of autophagy may be a concurrent but inconsequential result of apoptotic stimulation in response to sulforaphane. In addition, nearly all previous *in vitro* studies characterizing autophagy and cell death in prostate cancer cells utilize sulforaphane at concentrations above what is relevant *in vivo* or for treatment periods that do not match *in vivo* sulforaphane pharmacokinetics [4, 7, 15–28]. We therefore undertook an investigation to clarify the relationship between autophagy, apoptosis and sulforaphane in metastatic prostate cancer cells under more physiologically relevant conditions.

2 Materials and methods

2.1 Chemicals and Reagents

R,S-Sulforaphane was purchased from LKT Laboratories (St. Paul, MN, USA) and resuspended in dimethyl sulfoxide (DMSO) (EMD Millipore, Darmstadt, Germany). Chaetocin was purchased from Sigma-Aldrich (St. Louis, MO, USA) and resuspended in DMSO. Ammonium chloride (NH₄Cl) (Sigma-Aldrich) was prepared in sterile water. Mitochondrial dye MitoTracker Deep Red FM was purchased from Invitrogen (Carlsbad, CA, USA) and resuspended in DMSO. Primary antibodies for p62 (Santa Cruz Biotechnology), LC3A/B (Cell Signaling Technology, Danvers, MA, USA), cleaved poly-ADP ribose polymerase (cPARP) (Cell Signaling Technology), cytochrome c (CYCS) (Santa Cruz Biotechnology) and β -actin (Sigma-Aldrich) were used in accordance with the manufacturer's protocol. HRP-conjugated secondary antibodies (Santa Cruz Biotechnology) for Western Blotting detection and AlexaFluor-conjugated secondary antibodies for immunofluorescence/confocal imaging (Invitrogen) were used in accordance with the manufacturer's protocol.

2.2 Cell Lines and Culture Conditions

PC3 [androgen receptor (AR) negative] and LNCaP (AR positive) metastatic prostate cancer cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in RPMI-1640 media with L-glutamine supplemented with fetal bovine serum (FBS, 50 ml FBS/500 ml media) at 37°C 5% CO₂. Cell lines were validated by Idexx Radil (Columbia, MO, USA) on December 24, 2012. Subconfluent cells were treated under the indicated conditions prior to harvest. Treatment reagents were used at the following concentrations: sulforaphane as indicated, 30 mM NH₄Cl, 500 nM chaetocin (CHAE). DMSO, or other appropriate carrier control, was used as needed for control treatments. For serum deprivation/starvation (SS), cells were rinsed thoroughly in PBS three times then cultured in RPMI-1640 with L-glutamine for the treatment period.

2.3 Protein Preparation and Western Blot Analysis

Protein lysates were prepared in RIPA cell lysis buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 20 mM TRIS pH 8.0) supplemented with protease

inhibitor cocktail (Thermo, Waltham, MA, USA). Cell lysate was cleared of insoluble material by centrifugation at 4°C (10 minutes, 13,000 RPM) and quantitated by the DCA Protein Assay (BioRad, Hercules, CA, USA). Equal amounts of protein were separated by SDS-PAGE and blotted to a PVDF membrane (BioRad) using NuPAGE Reagents and equipment in accordance with the manufacturer's protocol (Invitrogen). Membranes were blocked and probed for the indicated proteins following standard protocols. For protein detection membranes were incubated in SuperSignal West Femto Reagent (Thermo) and developed on the AlphaInnotech FluorChem 8900 system (ProteinSimple, San Jose, CA, USA). Densitometric analyses were performed on the native membrane image using AlphaInnotech FluorChem 8900 software (ProteinSimple). For each membrane, the relative densitometric value of each replicate for a given probe was normalized to the corresponding relative level of the normalizing protein (β -actin). For graphing, treatments are expressed relative to Control (set to the value 1).

2.4 Mitochondrial Staining and Confocal Imaging

Cells were grown and treated on glass coverslips. Mitochondria were stained using MitoTracker Deep Red FM probe following the manufacturer's protocol. The MitoTracker probe was added at 200 nM during the final 30 minutes of cell treatment and then cells were prepared for immunostaining following standard protocols. Briefly, cells were rinsed in PBS and fixed/permeabilized in ice-cold 100% methanol for 10 minutes at -20°C. Cells were blocked in 1X PBS, 5% BSA, 0.3% Triton X-100. Cells were probed with primary antibodies in 1X PBS, 1% BSA, 0.3% Triton X-100 overnight at 4°C. After incubation with secondary antibodies cells were rinsed three times in PBS, with the final rinse containing DAPI nuclear stain. Coverslips were mounted on glass slides using ProLong Gold Antifade Reagent (Invitrogen) and allowed to set. Slides were visualized on a Zeiss LSM 510 Meta Confocal Microscope (Oberkochen, Germany) at the Center for Genome Research and Biocomputing (CGRB) at Oregon State University.

2.5 Quantitative Real-Time PCR

Total RNA was harvested by TRIzol reagent in accordance with the manufacturer's protocol (Invitrogen). cDNA was prepared from 1 μ g RNA using the SuperScript III kit from Invitrogen. cDNA was amplified by Fast SYBR Green Reagent in accordance with the manufacturer's protocol (Invitrogen) on a 7900HT Real Time PCR Machine (Applied Biosciences).

Primers: GAPDH (sense 5'-CGAGATCCCTCCAAAATCAA-3', antisense 5'-TTCACACCCATGACGAACAT-3'); HMOX-1 (sense 5'-CTTCTTCACCTTCCCCAAC-3', antisense 5'-GCTCTGGTCCTTGGTGTGTCATA-3'); p62 (sense 5'-CATCGGAGGATCCGAGTGTG-3', antisense 5'-TTCTTTTCCCTCCGTGCTCC-3').

2.6 Statistical Analysis

Graphing and statistical analyses were performed using GraphPad Prism Software (La Jolla, CA, USA). Figures depict one representative experiment. Graphs depict mean + SEM for at least two independent experiments. Statistical significance was determined by Student's *t*-

Test or one-way analysis of variance (ANOVA) with Tukey's post-test where appropriate. Significance is indicated by asterisk, with * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

3 Results

3.1 Sulforaphane does not directly influence autophagic activity in metastatic prostate cancer cells

To test whether sulforaphane has any direct influence on autophagic activity in metastatic prostate cancer cells, PC3 and LNCaP cells were treated with sulforaphane under conditions relevant to an *in vivo* therapeutic exposure (15 μ M for 4 hours) [29]. Cells were co-treated in the presence of the lysosomotropic degradation inhibitor ammonium chloride (AC; NH_4Cl) to assess delivery of substrates to the lysosome [11, 12]. Protein levels of the autophagy adaptor p62 (SQSTM1) and LC3 [microtubule associated protein 1 light chain 3 (MAP1LC3)] were monitored to determine autophagic activity. Culturing in AC for 4 hours indicated both cells types are undergoing constant autophagic flux as assessed by an accumulation of LC3-II (Fig. 1, Lane 1 and 2). In PC3 cells, serum starvation (SS) (positive control for flux) stimulated autophagic activity as indicated by a significant increase in p62 protein level and accumulation of LC3-II (Fig. 1A). No significant change in p62 protein or LC3-II was noted in sulforaphane treated cells (Fig. 1A), indicating no influence on autophagic flux. Analyses in LNCaP cells also showed that sulforaphane did not influence autophagic activity (Fig. 1B).

3.2 Sulforaphane does not influence autophagic flux following prolonged exposure

Sulforaphane leads to a concentration-dependent increase in reactive oxygen species (ROS) and activation of cytoprotective programs [1, 16, 30], suggesting prolonged exposure to sulforaphane may indirectly influence autophagic flux. To address this issue, PC3 cells were treated with sulforaphane and/or various flux-modifiers for 24 hours to determine whether sulforaphane inhibits or stimulates autophagic flux (Fig. 2).

We did not find sulforaphane to influence autophagic flux following an extended 24 hour treatment period. Sulforaphane did not decrease flux: we observed no increase in p62 or LC3-II in sulforaphane-treated cells as is seen in AC-treated cells (Fig. 2A); we also noted no increase in p62 or LC3-II under conditions of high flux.

Sulforaphane also did not increase flux under these conditions: we observed no increase in p62 in sulforaphane-treated cells as is seen in SS-treated cells (positive control for flux) in the presence of AC (Fig. 2A). In agreement with no increase in p62 protein in sulforaphane-treated cells, sulforaphane did not significantly increase p62 gene expression at this concentration (Fig. S1).

In addition to p62 and LC3 as indicators of autophagic flux, we also assessed potential mitochondrial involvement since mitochondria are reportedly required for sulforaphane-stimulated cell death [19] and damaged mitochondria are both a ROS source and an autophagy substrate [31]. Flux analysis indicated that mitochondria are not significantly turned over through the lysosome during our treatment period and that sulforaphane does not stimulate mitochondrial turnover as assessed by cytochrome c (CYCS) levels (Fig. 2A). We

also observed no colocalization of mitochondria and LC3 puncta in sulforaphane-treated cells (Fig. 2B), confirming that mitochondria are not directed toward sites of autophagy following sulforaphane treatment. Analysis of neutral lipid as an autophagy indicator by counting neutral lipid-positive cells also suggested sulforaphane does not influence autophagic flux (data not shown) [32–34].

Visualization of LC3 puncta also did not suggest an increase in flux (Fig. 2B). We noted LC3 puncta in both control and sulforaphane-treated cells, which is in agreement with constitutive flux we observed in the flux assay (Fig. 1, Fig. 2A). Although sulforaphane did lead to an increase in LC3-II at 24 hours, we also observed an increase in LC3-I (Fig. 2A, Fig. S1B). This resulted in no observed increase in LC3-II/LC3-I ratio, indicating no increase in autophagic flux. This may be a result of increased LC3 gene expression in response to sulforaphane, which has been observed in breast cancer cells [35].

Previous work has suggested that altering autophagic activity can influence apoptotic signaling in response to cytotoxic agents in cancer cells [9] and sulforaphane has been reported to induce apoptosis in prostate cancer cells [4, 7, 13]. We therefore tested whether inhibiting lysosomal degradation (AC treatment) or enhancing autophagy through SS sensitizes prostate cancer cells to 15 μ M sulforaphane using cleaved poly-ADP ribose polymerase (cPARP) levels as a readout for apoptotic signaling (Fig. 2A). Blocking lysosomal degradation or enhancing flux did not induce apoptosis in PC3 cells. Blocking degradation or enhancing flux also did not sensitize cells to sulforaphane. Sulforaphane alone was unable to directly stimulate apoptosis under these treatment conditions. Simultaneously driving autophagic flux while inhibiting lysosomal degradation did not trigger apoptosis or sensitize cells to sulforaphane. In contrast, exposing cells to chaetocin (CHAE), a potent ROS-inducer and positive control for cytotoxicity [36], strongly triggered cell death.

3.3 High-concentration sulforaphane stimulates autophagic flux and cell death

To test whether sulforaphane influences autophagic flux at higher concentrations we treated LNCaP cells with increasing concentrations of sulforaphane (Fig. 3). Cells were transiently exposed to sulforaphane for 4 hours to mimic an *in vivo* exposure [29]. Cells were treated with chaetocin (CHAE) in parallel as a positive control. Sulforaphane did stimulate autophagic flux at high concentrations (Fig. 3A and 3B). At 150 and 300 μ M we observed a decrease in p62 protein and an increase in LC3-II. Sulforaphane treatment in the presence of ammonium chloride (AC) partially rescued the decrease in p62 protein and showed an increase in LC3-II protein (Fig. 3B), confirming protein degradation through the lysosomal pathway. Sulforaphane treatment at 150 and 300 μ M also lead to a phenotypic response (Fig. S2) but was not sufficient to directly stimulate cell death in LNCaP cells within 4 hours, whereas chaetocin potently stimulated cell death as evidenced by the appearance of cleaved PARP (cPARP) (Fig. 3A).

Although we did not observe induction of apoptosis at 4 hours, high-concentration sulforaphane could lead to cell death at a later time point if the cells sustain an irrecoverable amount of stress. To test this, LNCaP cells were treated for 4 hours as in Fig. 3A and then the medium was replaced with normal growth medium for 40 hours and the cells were

monitored for recovery as evidenced by continued growth. As seen in Fig. 3C, control (DMSO) and 15 μ M sulforaphane-treated cells continued to grow to confluence, as was expected given no indication of cell stress (Fig. 1A, Fig 3A, Fig. S1). All chaetocin-treated cells were dead 40 hours after media removal, consistent with the apoptotic signaling we observed at 4 hours (Fig. 3A). Cells transiently exposed to sulforaphane at 150 μ M did recover from the treatment, suggesting that this concentration is not sufficient to overcome cytoprotective responses stimulated by sulforaphane. Sulforaphane at 300 μ M was sufficient to cause an irrecoverable amount of stress as evidenced by cellular debris and minimal outgrowth of LNCaP cells 40 hours after media removal (Fig. 3C, Fig. S3).

4 Discussion

Previous investigations have suggested the involvement of autophagy in sulforaphane-mediated cytotoxicity in prostate cancer cells based on the presence of autophagosomes and LC3-II production [7, 13]; however, the presence of these markers cannot differentiate between induction or inhibition of autophagic flux. These same markers would be expected to accumulate under conditions where autophagosome-lysosome fusion is impaired. To clarify the relationship between sulforaphane, autophagy and cell death we tested whether sulforaphane has any influence specifically on autophagic flux in metastatic prostate cancer cells at a concentration associated with decreased metastasis *in vivo* (15 μ M) [29]. Our results show that sulforaphane cannot directly stimulate autophagic flux and cell death in metastatic prostate cancer cells at this concentration *in vitro*, but can directly stimulate flux and lead to cell death at higher concentrations (Fig. 1, Fig. 2, Fig. 3, Fig. S1B). This is consistent with previous investigations showing that high levels of sulforaphane and/or extended treatment periods are required to trigger cell death in metastatic prostate cancer cells *in vitro* [4, 7, 15–22].

In our analysis of sulforaphane as a flux-modifier under conditions where flux is already impaired or up-regulated (AC and SS, respectively) (Fig. 2), we found that sulforaphane does not further influence flux, suggesting no context specificity for a sulforaphane effect on flux. We also noted that modulation of flux did not sensitize cells to sulforaphane at the *in vivo* therapeutic concentration of 15 μ M even at an extended 24 hour treatment period (Fig. 2). Recent work in pancreatic carcinoma cells using different autophagy modifiers (chloroquine and rapamycin) has also demonstrated that these agents do not alter sulforaphane cytotoxicity [37]. Autophagy therefore may, under some conditions, influence sulforaphane cytotoxicity [35, 38], but the effect is cell-type and concentration dependent.

In this investigation we chose a 4 hour sulforaphane exposure because *in vivo* data characterizing decreased prostate cancer metastasis following sulforaphane treatment showed that sulforaphane quickly reaches a plasma concentration peak of ~16 μ M and is then rapidly eliminated from circulation in 4 hours (Fig. 1, Fig. 3) [29]. The *in vivo* pharmacokinetics observed in this model of prostate cancer are in agreement with other *in vivo* analysis in mice and rats showing a spike in plasma concentration shortly after exposure followed by rapid elimination from the plasma and tissues [23–28]. Under these shorter transient treatment periods intended to simulate an *in vivo* exposure, we found that only very high concentrations of sulforaphane are able to directly lead to an increase in

autophagic flux and cell death in LNCaP cells (Fig. 3). Recovery and continued growth after transient sulforaphane treatment has also been noted in two colon cancer cell lines [39, 40]. Based on these findings, we conclude that modulation of autophagic flux is not a primary response to sulforaphane in metastatic prostate cancer cells at physiologically relevant concentrations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

SF	sulforaphane
AC	ammonium chloride
SS	serum starvation
CHAE	chaetocin
ROS	reactive oxygen species
cPARP	cleaved poly-ADP ribose polymerase

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Fig. 1.

Sulforaphane does not directly stimulate autophagic flux in metastatic prostate cancer cells. (A) PC3 or (B) LNCaP cells were grown for 4 hours under the appropriate conditions to assess autophagic flux. Lane 1: no treatment (DMSO control). Lane 2: lysosomal degradation inhibitor ammonium chloride (AC). Lane 3: sulforaphane (SF) 15 μ M and AC. Lane 4: serum starvation (SS) and AC. One representative experiment of multiple independent experiments pictured (upper). Relative levels of p62 and LC3-II from two independent experiments were graphed and analyzed (lower). Proteins are expressed relative to β -actin.

Fig. 2.

Sulforaphane does not influence autophagic flux following prolonged exposure. (A) PC3 cells were treated for 24 hours under varying conditions to assess flux. Lane 1: DMSO (control). Lane 2: sulforaphane (SF) 15 μ M. Lane 3: ammonium chloride (AC). Lane 4: SF and AC. Lane 5: serum starvation (SS). Lane 6: SF and SS. Lane 7: AC and SS. Lane 8: SF, AC and SS. Lane 9: chaetocin (CHAE). Cleaved poly-ADP ribose polymerase (cPARP) was assayed to assess apoptotic signaling. The autophagy adaptor/substrate p62, autophagosome marker/substrate LC3, and mitochondrial marker cytochrome c (CYCS) were assayed to assess autophagic flux. β -actin was probed as a loading control. LC3 (LE) indicates a Longer Exposure during membrane visualization. (B) PC3 cells treated with DMSO (control) (upper) or SF (lower) for 24 hours then processed for visualization of mitochondria (Green) and LC3 (Red). Scale bar is 10 μ m.

Fig. 3.

High-concentration sulforaphane stimulates autophagic flux and cell death. (A) LNCaP cells were treated with increasing amounts of sulforaphane (SF) for 4 hours. Cells were also treated with chaetocin (CHAE) as a positive control for cytotoxicity. Cleaved poly-ADP ribose polymerase (cPARP) was assayed to assess apoptotic signaling. The autophagy adaptor/substrate p62 and autophagosome marker/substrate LC3 were assayed to assess autophagic flux. β -actin was probed as a loading control. Representative blot from two independent experiments shown. (B) LNCaP cells were treated for 4 hours with high concentrations of sulforaphane in the absence or presence of ammonium chloride (AC) to impair lysosomal degradation. p62 and LC3 were assayed to assess autophagic flux. β -actin was probed as a loading control. (C) LNCaP cells were treated with SF at the indicated concentration or with CHAE for 4 hours. Media was then replaced with normal growth media and the cells allowed to recover for 40 hours. Representative bright-field image shown at 4 $\times$ magnification. Scale bar is 500 μ m. Higher magnification can be seen in Fig. S3.