Brief Report

Sulforaphane pre-treatment improves macrophage killing

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Abstract: Binge drinking is associated with increased mortality and morbidity. *Burkholderia pseudomallei*, the causative agent of pneumonic melioidosis can occur in healthy humans; however, binge alcohol intoxication is a major risk factor. Previous findings indicate that a single binge alcohol episode increases *Burkholderia* spp. infection by reducing alveolar macrophage function. The aim of this study was to test the ability of the phytonutrient sulforaphane (SFN) to rescue the phagocytic function of alveolar macrophages when infected with *Burkholderia* spp. in vitro. *B. thailandensis* E264 was used as a useful BSL-1 model to determine the effects of SFN pre-treatment. The primary outcome was macrophage phagocytic killing, while the secondary outcome was the nuclear factor (erythroid-derived 2)-like (Nrf2) signaling response measured by western blot analysis. Results indicate that alveolar macrophages pre-treated with SFN (5 µM) and challenged with 0.2% (v/v) alcohol for 3 or 8 h prior to live *B. thailandensis* infection improved intracellular killing of *B. thailandensis* ~2-fold compared to MHS cells treated with alcohol alone. These data demonstrate that SFN may be an effective pre-treatment option to prevent alcohol mediated immune dysfunction and restore macrophage phagocytic killing of *Burkholderia* spp. and other similar opportunistic pathogens.

Keywords: Sulforaphane; Alcohol; *Burkholderia*; Binge drinking; MHS cells; Melioidosis

1. Introduction

Alcohol abuse in the form of binge alcohol consumption, is commonly associated with liver, brain, and gastrointestinal tract disorders, yet alcohol-induced dysfunction of the lung microenvironment is becoming increasingly important to understand. Binge alcohol intoxication can be a serious risk factor for pulmonary bacterial infections [1]. Melioidosis is an emerging tropical disease characterized by pneumonia, with mortality rate up to 53% globally; pneumonia being the clinical presentation in half of all reported cases [2]. Nearly 40% of melioidosis cases report binge alcohol use as a major risk factor. Endemic to Northern Australia and South East Asia, *Burkholderia pseudomallei* is the causative agent of melioidosis; however, the genus includes opportunistic *B. thailandensis* that coexists with *B. pseudomallei* in the soil and has been identified sporadically in the midwestern United States [3-5]. Additionally, *in vitro* and *in vivo* models of alcohol intoxication have demonstrated a dose- and time-dependent effect that can alter initial host-pathogen interactions and permit less-virulent pathogens to persist and cause disease. Opportunistic pathogens of considerable interest include *Escherichia coli*, *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *B. thailandensis* [6-9].

The exact mechanisms by which alcohol can increase the virulence of opportunistic bacteria is less understood; however, a major focal point appears to be innate immune dysfunction [10]. Alveolar macrophages (AMs) are the first line of defense during pulmonary infections, generally located in the distal respiratory tract, and are critical for detecting, capturing, and eliminating pathogens while initiating the early host immune response [11]. Moreover, *Burkholderia* spp. are predominately opportunistic pathogens with a high tolerance to antibiotics and expressing virulence mechanisms enable them to
survive inside of phagocytic cells [12]. We have shown previously that a single alcohol insult in a binge-like manner alters phagocytosis in the murine AM cell line MH-S, resulting in the survival and replication of *B. thailandensis* in vitro [11]. Furthermore, *B. thailandensis* infectivity was increased 24 h post infection, with bacteria reaching the blood and colonizing the lungs *in vivo* [9]. However, a treatment option that can mitigate the toxic effects of alcohol and provide protection from *Burkholderia* spp. infection remains to be elucidated.

Sulforaphane (SFN) is a promising phytonutrient that can affect cytoprotective cellular processes. [13]. SFN can stimulate phase II detoxification response in host cells and induce cytoprotective measures via activation of nuclear factor (erythroid-derived 2)-like (Nrf2). Nrf2 is a redox-sensitive transcription factor that regulates the activation of the antioxidant response element (ARE) and has a significant role in cellular protection from cigarette smoke and chronic alcoholism [14-17]. Moreover, phagocytic activity was improved in RAW 264.7 macrophages after SFN pretreatment in the absence of alcohol [17]; however, utilizing SFN to prevent lung macrophage dysfunction from the toxic effects of alcohol is less well understood.

This study tested the ability of SFN to protect AMs from the toxic effects of alcohol *in vitro*. Our working hypothesis was that SFN pre-treatment of MH-S cells prior to a binge alcohol insult, would prevent phagocytic dysfunction and neutralize intracellular bacteria. The current study extends ongoing characterization of SFN to cytoprotection effects of another tissue, lungs (i.e., MH-S cell line), utilizes a biologically relevant dose of SFN to prevent the toxic effects of binge alcohol exposure, and extends pulmonary infection research to another opportunistic pathogen, *B. thailandensis*.

2. Materials and Methods

2.1. Dose-response curves: sulforaphane and alcohol

Murine AM cell line (MH-S ATCC, CRL-2019) was used in these studies at passages ≤ 6 and ≥ 90% confluency. Cells were regularly grown in T-75 cell culture flasks in phenol red-free RPMI-1640 medium (Gibco, Life Technologies) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 10 mM HEPES, 0.1 mM non-essential amino acids, 1.5 g/L sodium bicarbonate, 50 U/mL penicillin, and 50 mg/mL streptomycin. Cells were incubated at 37°C and 5.5% CO₂. Cells were seeded in 24-well cell culture plates at 1 × 10⁶ cells/well and incubated in RPMI-1640 media. Low evaporative cell culture plates, a compensating system previously described by Jimenez et al. [11] were used for all experiments to avoid evaporation of alcohol during treatments. RPMI-1640 media supplemented with 0.2 or 0% v/v alcohol (ethanol absolute, 200 proof, Fisher BioReagents) were used based on biological relevancy and MH-S cell viability > 85%.

To select a dosage of SFN for our study we generated a dose-response curve. Cells were cultured with 0, 2.5, 5, 10, 20, 50 µM SFN for 2 or 24 h (Fig. 1). Five µM SFN concentrations were used for all further experiments based on ≥ 95% cell viability utilizing the Trypan blue cell viability test (Fig. 1A). The effects of alcohol on MH-S cell viability were tested in a similar procedure (Fig. 1B). For all binge alcohol treatments, alcoholic media changes were used to ensure consistent alcohol concentration was maintained. Viability assays were run in at least 4 experimental replicates and at least two independent experiments were performed with similar results. Cells were either infected with live *B. thailandensis* E264 for CFU determination, NO assay, or further processed for Western blot analysis.

2.2. Western blot analysis

Western blots were processed using methods previously described in Done et al. [18] with the following modifications: whole cell lysates were harvested from culture using lysis buffer (1% Triton-X-100, 3% Protease-Phosphatase Inhibitor cocktail in 1X PBS).
Sample buffer was added to samples (250 mM Tris-HCl pH 6.8, 8% SDS, 40% glycerol, 10% β-Mercaptoethanol, and 0.02% Bromophenol Blue) and sonicated 3X for 10 sec each with 2 min rest on ice. Samples were stored at -80°C until western blot assays were run.

Protein concentrations were determined using the Bradford method. Equal protein concentrations (20 µg) were loaded and electrophoresed at 180 mV for 65 min then transferred onto nitrocellulose membranes for an additional hour with voltage set at 20 mV. Membranes were blocked for one hour at room temperature in blocking buffer (TBS + 5% BSA) followed by multiplex antibody incubation. Primary antibody incubation occurred with Nrf2 rabbit monoclonal antibody 1:500 dilution (Cell Signaling Technologies – 12721). Antibody was diluted with TBST plus 5% BSA for 48 h at 4 ºC with gentle shaking. β-Actin mouse monoclonal was used as an internal control at a 1:2000 dilution (Santa Cruz Biotechnology – sc47778) and gentle shaking overnight. Secondary antibodies from Licor Biosciences (Lincoln, NE) anti-rabbit and anti-mouse were co-incubated with a dilution of 1:20,000 in TBS + 1% BSA with gentle shaking at room temperature for one hour. All blots are normalized to β-Actin.

Membranes were imaged using an Odyssey Fc Infrared Imager (Licor Biosciences) and densitometry analysis was conducted using Image Studio Lite Software (Licor Biosciences). Each treatment was normalized to its own internal control (β-Actin) before making comparisons across treatments. Each western blot assay was performed in duplicate and repeated experimentally at least twice with similar results.

2.3. Intracellular killing and nitric oxide (NO) assay

Confluent MH-S cells were grown in media supplemented with 0 or 5 µM SFN for 24 h or media supplemented with 0 or 0.2% (v/v) alcohol for 3 or 8 h. Additionally, MH-S cells were pre-treated with 0 or 5 µM SFN for 24 h, and immediately challenged with 0 or 0.2 % (v/v) alcohol for 3 or 8 h. Cell monolayers were then co-cultured with viable B. thailandensis at a multiplicity of infection (MOI) of 1:1 for 3 h at 37 ºC, and with 5.5% CO₂ to allow phagocytosis to occur. At a MOI of 1:1 cell viability was ≥ 90%. After 3 h, extracellular bacteria were removed by washing the cells with DPBS and replacing culture media supplemented with 250 µg/mL of kanamycin for 1 h. Thereafter, the cell monolayers were incubated at 37 ºC in media with 50 µg/mL kanamycin for 2 h to completely remove any residual extracellular and attached bacteria. Intracellular killing was allowed to remain for 3 h post infection (PI). The infected cell monolayers were additionally washed, and cell culture media was removed. To determine the ability of MH-S cells to suppress and kill intracellular bacteria, cell monolayers were lysed with DPBS containing 0.1% Triton X-100, homogenized, centrifuged, resuspended in media and serial diluted. Viable intracellular bacteria were quantified by plating serial dilutions of the lysate, and average CFUs were determined utilizing the following equation:

\[
\text{CFU's} = \frac{\text{DF} \times \text{volume spread over surface of LB plate}}{\text{DF}}
\]

\[
\text{DF} = \text{dilution factor utilized to grow bacteria}
\]

\[
\text{CFU's} = \text{number of total colonies counted on a single plate}
\]

In order to determine the effects of binge alcohol exposure on NO produced by infected MH-S cells, cell culture supernatant was collected prior to macrophage lysing from each group as described earlier. Levels of nitrates in the media were assayed immediately upon collection. Nitrite concentration was determined using the Griess reagent (Promega, Madison, WI, USA). Dilutions of sodium nitrite in RPMI culture media supplemented with 0 or 5 µM SFN were used as standards. Absorbance at 550 nm was read for each treatment sample in triplicate and replicated in at least three independent experiments with similar results.

2.4. Statistical analysis
The data analysis was completed using Prism 5.0 software (Graph Pad, 5.04, San Diego, CA). Assay replicate independence and significance was determined by two-way ANOVA with Bonferroni multiple comparisons, and Student’s t-test. Each N represents at least 4 experimental replicates (i.e. identical experimental wells) and at least 2 assay replicates from each experimental replicate. Each cell culture experiment was conducted independently at least twice on different days. Additional statistics were performed using R, and non-parametric, unequal variances. A P value ≤ 0.05 was considered significant.

3. Results

3.1. SFN and alcohol effects on MH-S cell viability

SFN dose-response curves were generated to determine the effects on MH-S cell viability (Fig 1). In media supplemented with 2.5 or 5 μM SFN, viability was not different compared to control at 2 h incubation; viability was not different compared to 24 h post incubation (Fig 1A). Cells treated with 10 μM SFN reduced viability to 95% and 90% at 2 and 24 h incubation respectively. Viability was reduced to approximately 85% in cells treated with 20μM and approximately 80% with 50 μM after a 24 h incubation (Fig 1A). SFN treatment at 2 and 24 h did not significantly affect MH-S cell viability when concentrations were 10 μM or less; however, 20 and 50 μM were significantly different (P ≤ 0.05 by Student’s t-test).
Figure 1. Effects of sulforaphane and alcohol on alveolar macrophages viability. Cells were grown in RPMI-1640 media. Zero, 2.5, 5, 10, 20, or 50 µM sulforaphane was used for SFN dose response experiments (A), for alcohol dose response experiments 0, 0.08, 0.2, or 0.4% alcohol v/v (biological grade) was used (B). Cell viability was determined by Trypan blue exclusion at 2, 3, or 24 h relative to 1 x 10^6 cells per well. Symbols represent the average viability ± 1 SE of at least two independent experiments. Asterisks represent statistical significance between time points within the same group by Students t-test. *, P ≤ 0.05. N = 4 represents at least 4 experimental replicates with 2 assay replicates per well. Each cell culture experiment was conducted independently at least twice on different days.

The effect of alcohol on MH-S cell viability was determined in a similar manner using alcohol concentrations ranging 0.08% - 0.4% for 3 and 24 h exposures. Cell viability was approximately 95% after 0.08% alcohol treatment; no significant difference was measured after 3 or 24 h alcohol incubation. Cell viability was decreased to 90% after 0.2% alcohol treatment; there was no difference between 3 or 24 h alcohol incubation. After 0.4% alcohol exposure MH-S cell viability was reduced to approximately 80% and 58% after 3 and 24 h incubation respectively and were significantly different (P ≤ 0.05 by Students t-test). For all subsequent experiments SFN (5µM) and alcohol (0.2%) were used. These doses represent biologically relevant conditions for SFN treatment and a single binge-like alcohol episode that maintained cell viability ≥ 90%.

3.2. SFN pre-treatment increases Nrf2 protein expression.

Western blot analysis was utilized to determine Nrf2 expression after 5 µM SFN treatment and to test the ability of SFN to protect MH-S cells from alcohol-induced attenuation of Nrf2 expression (Fig 2). MH-S cells pre-treated with 5 µM SFN for 24 h followed by an alcohol challenge with 0.2% (v/v) for 3 or 8 h, increased Nrf2 expression approximately 3 or 3.5-fold respectively, compared to alcohol treated cells in the absence of SFN. Cells treated with SFN and challenged with alcohol were significantly different compared to control (* P ≤ 0.05, ** P ≤ 0.01 by two-way ANOVA). Treatment with 5 µM SFN alone increased Nrf2 protein content approximately 2-fold compared to control. Treatment with 0.2% alcohol (v/v) for 3 or 8 h expressed Nrf2 similarly to untreated control (Fig. 2 A and B). To rule out any confounding effect of varying culture times on protein expression, two groups of control cells were generated, one harvested at the same time as SFN pre-treatment, and one harvested 8 h after the first harvest. There was no difference between untreated and 8-h untreated cells. The comparisons for treatment versus control were made relative to untreated control (data not shown).
Protein expression of Nrf2 in alveolar macrophage (AMs). Cells pre-treated with SFN, Alcohol (ALC), or SFN then ALC. AM cells were treated for 24 h with SFN (5 μM) before challenging cells with 0 or 0.2 % (v/v) alcohol for 3 or 8 h. To establish a baseline, cells were treated with alcohol (0.2%) for 3 or 8 h without SFN pre-treatment. Equal concentrations (20 μg) of cellular lysate were resolved by 10% SDS-PAGE, transferred to the nitrocellulose membranes, and immunoblotted with primary rabbit monoclonal antibody for Nrf2. Secondary antibodies, anti-rabbit and anti-mouse were co-incubated and imaged with Odyssey infrared imaging. β-Actin mouse monoclonal was used as an internal control. (B) Nrf2 fold change. Fluorescent densitometry analysis of Nrf2 expression was conducted using Image Studio Lite and each treatment was normalized to its own internal control (relative to (β-Actin) before making comparisons across treatments. Each bar represents the average of two independent experiments with no statistical difference ± 1 SEM. * P ≤ 0.05, ** P ≤ 0.01 by two-way ANOVA, N = 3. Each N represents at least 3 experimental replicates (i.e. identical experimental wells).

3.3. SFN treatment prior to binge alcohol exposure increases intracellular killing and nitric oxide (NO) production

Building upon our Western blot findings, we tested the ability of 5 μM SFN to protect MH-S cells from alcohol-induced phagocytic dysfunction during an infection with live B. thailandensis (Fig 3). An increase in bacterial survival was observed in MH-S cells treated with 0.2% v/v alcohol for 3 or 8 h compared to untreated or SFN treatment alone; there was no difference between 3 or 8 h alcohol incubation (Fig. 3A). MH-S cells pre-treated with SFN decreased intracellular survival of B. thailandensis approximately 2-fold compared to cells incubated in alcohol in the absence of SFN (Fig. 3A). The number of viable
B. *thailandensis* collected from SFN pre-treated cells challenged with alcohol for 3 or 8 h was similar to untreated cells and cells treated with SFN alone (Fig. 3A).

Nitric oxide (NO) was measured as a readout for phagocytic activity during live *B. thailandensis* infection (Fig 3B). MH-S cells pre-treated with SFN and challenged with alcohol for 3 or 8 h, increased NO production compared to alcohol treated cells in the absence of SFN. MH-S cells challenged with 0.2% v/v alcohol for 3 h significantly decreased NO production compared to untreated or SFN treated cells (**, P ≤ 0.01; ***, P ≤ 0.001 by two-way ANOVA). There was no difference between untreated and SFN treated cells; MH-S cells treated with SFN and challenged with alcohol produced NO similar to untreated and SFN treated cells (Fig 3B).

4. Discussion

Binge alcohol intoxication is defined as elevating blood alcohol concentration to 0.08% or greater; a high alcohol concentration for a short period of time can have a profound effect on innate immunity. In our current study, binge alcohol is defined by the dose (0.2% v/v) and conditions of exposure (i.e., temporal effects at 3 and 8 h) [9, 19]. Melioidosis is an emerging pneumonic disease that is linked to binge alcohol use; however, the extent of mitigating the deleterious effects alcohol on lung dysfunction is less well understood. In addition, the ability of phytoneutrient SFN to protect from alcohol-induced phagocytic dysfunction during a *Burkholderia* spp. infection is unknown. In the current study we have shown that pre-treatment of SFN significantly improved intracellular killing of *B. thailandensis in vitro*.
During alcohol-induced lung immune dysfunction, the upper airway is the first barrier to fail in the clearance of airborne pathogens [21]. High levels of alcohol for a short period of time (e.g., binge alcohol) trigger oxidative stress pathways in AMs, thereby impairing AM phagocytic function and pathogen clearance [22]. Although macrophages are derived from monocytes, the ability of AMs to be recruited and differentiated into the alveolar space suggests alcohol may affect resident macrophages of other tissues differently than the AMs present in the lung microenvironment [23]. During a binge alcohol episode, the diminished response of local lung AMs render the host susceptible to prolonged infections. We have shown previously that binge alcohol intoxication increases lung Burkholderia spp. load for 72 h P I in vivo [9]. There is some evidence to suggest binge alcohol produces an over-exuberant pro-inflammatory response during infection. However, AMs tend to exhibit an immunosuppressed phenotype [24, 25].

In the present study we observed a significant 2-fold decrease in B. thailandensis intracellular survival when MH-S cells were pretreated with 5 μM SFN for 24 h prior to binge alcohol exposure. MH-S cells pretreated with SFN and challenged with a single bout of alcohol for 3 or 8 h significantly improved NO production in the context of infection compared to alcohol treated cells in the absence of SFN (Fig. 3B). MH-S cells treated with SFN alone did not express significantly more NO; however, MH-S cells treated with SFN alone enhanced phagocytic activity compared to untreated controls (compare Fig 3 A and B), indicating possible alternative mechanisms of intracellular killing independent of NO production. SFN (5 μM) was selected based on biological relevance and non-toxic effects; alcohol (0.2% v/v) is non-toxic in vitro, increases Burkholderia spp. infection in vivo and represents the higher end of the blood alcohol range observed in humans [20]. Duration of alcohol exposure up to 8 h did not decrease the efficacy of the SFN pre-treatment.

To better understand the modulatory effect of SFN pre-treatment of MH-S cells, Western blot analysis was utilized to quantify Nrf2 protein activation as an indication of induced cytoprotection from the effects of binge alcohol intoxication. An increase in the Nrf2 regulated AM antioxidant response element (ARE) pathway may explain, in part, the recovery of NO expression and increased intracellular killing when cells were treated with SFN prior to binge alcohol intoxication and infection [26]. We observed an approximate 2-fold increase in Nrf2 protein expression when MH-S cells were treated with SFN prior to 3 or 8 h binge alcohol exposure. Nrf2 is a key transcription factor which activates ARE regulated genes once it translocates to the nucleus [27]. Our current data confirms Nrf2 activation from SFN treatment in murine MH-S cells. Although outside the scope of this study, benzyl isothiocyanate (BITC) a compound that stimulates the Nrf2-dependent pathway similar to SFN, was shown to enhance alcohol and aldehyde dehydrogenase (ADH and ALDH) enzymes in murine hepatoma cells in an additive manner [28]. Our current data are consistent with previous studies showing that cell alcohol detoxification is controlled, at least in part, by the Nrf2-dependent pathway [29]. Taken together, SFN pre-treatment of MH-S cells is sufficient to reduce binge alcohol toxic effects and protect macrophage phagocytic function in the described cell line. Perhaps a limitation of this study was the use of a single immortalized cell line. Furthermore, whether alcohol metabolism is elevated to clear the alcohol quicker in response to Nrf2 activation, or if Nrf2 indirectly improves cellular function by increasing general protection against toxic compounds prior to bacterial infection, cannot be determined and should be fully elucidated in future in vitro and in vivo studies. Nevertheless, we provide evidence for SFN, a potential phytonutrient treatment that can provide protection from alcohol-induced phagocytic dysfunction. Similar studies utilizing RAW 264.7 macrophage-like cells have shown inactive properties of SFN to reduce macrophage migration inhibitory factor (MIF) and improve phagocytosis [17].

The SFN-induced increase in proinflammatory NO, is in stark contrast with Lin et al. [30] and Weiss et al. [31], who reported SFN displayed more significant downregulation of iNOS expression via Nrf2 activation. This contradiction can be explained by significant differences in the experimental designs. In the present study, lung-MH-S cells were treated with 5 μM for 24 h, rather than peritoneal macrophages treated with SFN for 6 h
as reported by Lin et al [30]. Furthermore, a significant reduction in pro-inflammatory markers were not observed at 5 μM, but rather at 20 or 50 μM concentrations as reported by Western blot analysis [30, 32]. These results could be explained by a hormetic effect of SFN, whereby low doses of a compound confer a beneficial stimulus for the cell but increasing dosage can itself induce negative effects [33]. Our cell viability data are in agreement with the hormesis model and previous findings. Taking the hormesis model and mechanisms of SFN into account, together the findings of Lin et al. [30] and our current results would suggest that lower, biological levels of SFN provide beneficial effects whereas higher levels suppress immune function. Collectively, our data suggest a dose dependent and temporal effect of SFN that may have different phenotypic effects that are tissue specific.

Current therapeutic strategies to mitigate alcohol induced AM oxidative stress may include treatments with over-the-counter antioxidant N-acetylcysteine (NAC) or glutathione (GSH) that are pharmacologic treatment options for many substance-use disorders [34]. Such treatments could potentially restore pathogen clearance by AMs [35, 36]. However, no current data is available to investigate the therapeutic potential of NAC or GSH to alleviate the effects of alcohol intoxication in the context of infection. Furthermore, Nrf2 as a therapeutic target is theoretically more efficacious because it regulates many different antioxidant enzymes and cytoprotective proteins [35]. Modulating the whole system by activating Nrf2, instead of supplementing with one or two small molecular antioxidants, would likely be a much more effective approach.

5. Conclusion

In this study, we showed for the first time that in murine MH-S cells (1) SFN pretreatment upregulates Nrf2 protein expression; (2) SFN pretreatment improves intracellular *Burkholderia* killing and stabilizes NO production after binge alcohol exposure. Understanding the mechanisms of SFN action can be useful to identify an effective supplement dosage for at risk melioidosis patients and other pneumonic infections with a disposition for alcohol abuse or other abused drugs. Further exploration of these direct and indirect avenues of therapeutic manipulation will be of benefit to the overall health of persons who suffer from alcohol use disorders.

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**References**


27. Kessler, T.W.; Wakabayashi, N.; Biswal, S. Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. Annu Rev Pharmacol Toxicol. 2007, 47, 89-116. DOI: 10.1146/annurev.pharmaco.46.120604.141046


