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Identification and quantitative analysis of cellular proteins affected by treatment with withaferin a using a SILAC-based proteomics approach

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ABSTRACT

Ethnopharmacological relevance: Withaferin A (WA) is a major bioactive compound isolated from the medicinal plant *Withania somnifera* Dunal, also known as "Ashwagandha". A number of published reports suggest various uses for WA including its function as an anti-inflammatory and anti-angiogenic drug molecule. The effects of WA at the molecular level in a cellular environment are not well understood. Knowledge of the molecular mechanism of action of WA could enhance its therapeutic value and may reveal novel pathways it may modulate.

Materials and methods: In order to identify and characterize proteins affected by treatment with WA, we used SILAC- based proteomics analysis on a mouse microglial cell line (N9), which replicates phenotypic characteristics of primary microglial cells.

Results: Using stable isotope labeling of amino acids in cell culture (SILAC) and mass spectrometry (MS), a total of 2300 unique protein groups were identified from three biological replicates, with significant expression changes in 32 non-redundant proteins. The top biological functions associated with these differentially expressed proteins include cell death and survival, free radical scavenging, and carbohydrate metabolism. Specifically, several heat shock proteins (Hsps) were found to be upregulated, which suggests that the chaperonic machinery might be regulated by WA. Furthermore, our study revealed several novel protein molecules that were not previously reported to be affected by WA. Among them, annexin A1, a key anti-inflammatory molecule in microglial cells was found to be downregulated. Hsc70, Hsp90α and Hsp105 were found to be upregulated. We also found sequestosome1/p62 (p62) to be upregulated. We performed Ingenuity Pathway Analysis (IPA) and found a number of pathways that were affected by WA treatment.

Conclusions: SILAC-based proteomics analysis of a microglial cell model revealed several novel proteins whose expression is regulated by WA and probable pathways regulated by WA.

1. Introduction

Roots, leaves and berries of *Withania somnifera* have been used in traditional medicine to treat a wide variety of conditions including chronic fatigue, dehydration and rheumatism (Baitharu et al., 2013; Mishra et al., 2000; RajaSankar et al., 2009). Withaferin A (WA) is one of the most active and highly studied bioactive compounds extracted from *W. somnifera* (Bhattacharya et al., 1997). It has been studied for its anti-inflammatory (Maitra et al., 2009; Sabina et al., 2008), anti-tumor (Li et al., 2015), anti-angiogenic (Mohan et al., 2004) and pro-apoptotic (Oh et al., 2008a) properties. These studies suggest that WA could be modulating a number of pathways based on the context of cell type. Identification of these pathways can further help in elucidating the molecular mechanism of action of WA, and the feasibility of its application as a therapeutic agent.

Based on the anti-inflammatory activity of WA, we chose to study its effect on microglial cells, which are the resident phagocytes and immune cells of the brain. They constitute 5–20% of all the glial cells in the central nervous system (CNS) (Lawson et al., 1990). They are one of the major mediators of inflammatory responses in the CNS. The response of microglial cells to neuronal injury is termed 'reactive gliosis' or 'microgliosis' (Streit et al., 1999). Neurodegenerative diseases often show evidence of reactive gliosis with the persistent production of pro-inflammatory cytokines. The hallmarks of pathology in Alzheimer's disease

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include neuritic plaques, neurofibrillary tangles and neuroinflammation induced by microgliosis (Cotter et al., 1999). There is evidence for activation of microglia in multiple sclerosis subsequent to infiltration of macrophages and dendritic cells (Carson, 2002). Activated microglia are also suspected to be the source of elevated levels of inflammatory cytokines in the substantia nigra of patients with Parkinson's disease (Kim and Joh, 2006).

In the current study, we chose to use the N9 mouse microglial cell line, which closely replicates characteristics of primary microglia, in order to first understand the mechanism of action of WA on resting microglial cells. We used stable isotope labeling by amino acids in cell culture (SILAC) as a quantitative approach to study global changes in the proteome of N9 cells upon treatment with WA. We find that treatment with WA for 24 h differentially regulates the expression of several proteins involved in diverse pathways including anti-oxidant response and protein folding.

2. Methods

2.1. Reagents

WA was purchased from Sigma (St. Louis, MO). The SILAC labeling kit was purchased from Thermo Scientific. Antibodies were purchased from the following sources: anti-Hsp90 and anti-Hsc70 from Enzo Life Sciences (Farmingdale, NY), anti-Hsp105 from Abcam (Cambridge, MA), anti-p62 from Proteintech (Chicago, IL), anti-annexinA1 from Cell Signaling (Danvers, MA), anti-iNOS from Millipore (Billerica, MA), and anti-GAPDH from Meridian Life Science (Memphis, TN).

2.2. Cell culture and SILAC labeling

N9 mouse microglial cells were initially cultured in DMEM with 10% FBS, L-glutamine, and penicillin and streptomycin at 37 °C and 5% CO_2. Cells were grown in T25 cm^2 flasks for stable isotope labeling of amino acids in cell culture (SILAC) with heavy and light amino acids (Ong et al., 2002). N9 were labeled using the Pierce SILAC Protein Quantitation Kit – DMEM (# 89983; Thermo Scientific) as per manufacturer's instructions. Briefly, cells were grown in DMEM containing 10% dialyzed fetal bovine serum (FBS), penicillin/streptomycin, $^{12}C_6$ L-lysine and $^{12}C_6{}^{14}N_4$ L-arginine for light labeling or $^{13}C_6$ L-lysine and $^{12}C_6{}^{14}N_4$ L-arginine for heavy labeling. Both cell populations were passaged for at least five cell doublings for efficient incorporation of the label. Efficiency of labeling was determined to be $>98\%$ by mass spectrometry analysis.

Light labeled and heavy labeled cells were treated with DMSO and WA (2 μM) for 24 h, respectively. Cells were collected by trypsinization. Cell pellets were lysed in buffer containing a final concentration of 100 mM Tris–HCl, 4% SDS and 100 mM DTT at 100 °C for 5 min. Lysates were then sonicated in six pulses of 20% AMPL, followed by centrifugation at 15,000*g* for 20 min at 4 °C. Protein concentrations were determined using Pierce 660 nm protein assay with the ionic detergent compatibility reagent (Thermo Scientific). These experiments were done in triplicate.

2.3. Sample preparation for SILAC analysis

Whole cell lysates were digested using the filter-aided sample preparation kit (Protein Discovery), as developed by Wisniewski and Mann (Wisniewski et al., 2009). Thirty microliters containing 400 μg protein sample were mixed with 200 μL 8 M urea and added to the 30 kDa filter-aided sample preparation spin filter for buffer exchange. Samples were alkylated according to manufacturer's instructions with iodoacetamide for 30 min in the dark. Following alkylation, samples underwent further buffer exchange with 3×100 μL additions of 50 mM ammonium bicarbonate, followed by centrifugation at 14,000*g* for 10 min. Samples were incubated with trypsin at 1:100 (w:w, trypsin:protein) for proteolytic digestion of proteins and incubated overnight at 37 °C. Peptides were collected by centrifugation with the addition of 2×40 μL 50 mM ammonium bicarbonate and 40 μL NaCl and acidified by addition of formic acid to pH 3. Peptides were desalted using Supelco Discovery DSC-18 SPE columns in combination with a Supelco vacuum manifold. Samples were dried using a vacuum concentrator (Thermo) and resuspended in 20 μL of 0.1% formic acid in H_2O. Peptides were fractionated on a Thermo Surveyor HPLC system with a 15 cm × 2.1 mm id strong cation-exchange (SCX) column (PolyLC Inc.) packed with 5 μm 300 Å poly-SULFOETHYL A-SCX material. Three-min fractions were collected using a 30-min gradient, where ammonium formate increased from 5–200 mM in 25% ACN at a flow rate of 250 μL/min. Nine peptide-containing fractions were selected for LC-MS/MS analysis from each biological replicate ($n=3$ total). Peptides were again dried in a vacuum concentrator and resuspended in 10 μL of 0.1% formic acid in H2O.

2.4. Liquid chromatography–tandem mass spectrometry (LC–MS/MS)

SCX peptide fractions were separated on a 10 cm × 75 μm id RP column (New Objective) packed with 5 μm 300 Å C18 material (ProteoPep II). MS/MS analysis was carried out using a hybrid linear ion trap-Orbitrap instrument (LTQ Orbitrap XL, Thermo). A 180 min linear gradient was used, where 0.1% formic acid in ACN increased from 2% to 40%, increasing to 80% at 185 min through 190 min. Orbitrap full MS scans were collected at a mass resolving power of 60,000, with positive polarity in profile mode, and a scan range of m/z 350–1650. The top ten most abundant ions were selected for further fragmentation in the ion trap. Global settings include dynamic exclusion of 180 s, with an exclusion list size of 500, and a repeat count of 1.

2.5. Database searching

Raw files were processed in MaxQuant version 1.3.0.5, a quantitative proteomics software package for the analysis of large, high-resolution MS data sets. The raw files were processed and searched against the current UniprotKB database containing *Mus musculus* (mouse) protein sequences as well as a second MaxQuant database of known contaminants. The search parameters included a constant modification of cysteine by carbamidomethylation and variable modification of methionine oxidation. Additional parameters include multiplicity set to 2, with a heavy set of lysine-6 and arginine-10. The search tolerance was set to 6 ppm and the fragment ion mass tolerance was set to 0.5 Da with a false discovery rate of less than 1%. Statistical analysis was carried out using Perseus software, which assesses the statistical significance of protein expression based on the approach developed by Benjamini and Hochberg (1995). A threshold q-value of 0.05 for the Benjamini–Hochberg false discovery rate was used.

2.6. Data validation by western blotting

N9 cells were treated with either DMSO vehicle control or a range of concentrations of WA (0.3, 1, 2, 3 μM) for 24 h and harvested as previously described (Jinwal et al., 2012). Briefly, cells were lysed in Mammalian-Protein Extraction Reagent (M-PER; Pierce, Rockford, IL) containing 1X protease inhibitor mixture (Calbiochem, Billerica, MA), 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1X phosphatase inhibitor I and II cocktails (Sigma). Protein concentration was determined using the BCA Protein Assay