Fish & Shellfish Immunology 34 (2013) 1383–1389

Contents lists available at SciVerse ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Short communication

Proteomic characterization of the acute-phase response of yellow stingrays *Urobatis jamaicensis* after injection with a *Vibrio anguillarum-ordalii* bacterin

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ARTICLE INFO

Article history:
Received 21 November 2012
Received in revised form
15 February 2013
Accepted 22 February 2013
Available online 5 March 2013

Keywords:
Elasmobranch
Proteomics
Acute-phase response
Yellow stingray
Urobatis jamaicensis
Vibrio

ABSTRACT

Systemic inflammatory responses of mammals and bony fish are primarily driven by coordinated up-regulation and down-regulation of plasma acute-phase proteins. Although this general principle is believed to be universal among vertebrates, it remains relatively unexplored in elasmobranchs. The objective of this study was to characterize acute changes in the plasma proteome of three yellow stingrays *Urobatis jamaicensis* following intraperitoneal injection with a commercial *Vibrio* bacterin. Changes in plasma protein levels were analyzed immediately prior to vaccination (time 0) and at 24 and 72 h post-injection by isobaric tags for relative and absolute quantitation (iTRAQ 4-plex) using shotgun-based nano liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis and *de novo* peptide sequencing. Pooled 2D-LC-MS/MS and *de novo* sequencing data revealed differential expression of 156 distinct plasma proteins between time 0 and at least one post-vaccination time point. Using 1.5-fold change in expression as physiologically significant, 14/156 (9.0%) proteins were upregulated in at least one stingray through at least one experimental timepoint. Upregulated proteins included complement factors, Mx-protein, hemopexin, factor X and prothrombin. Seventy-six of 156 (48.7%) proteins were downregulated in the acute-phase response, including transferrin, apolipoprotein B, heparin cofactor 2, alpha2-macroglobulin, and various growth factors. Other differentially upregulated or downregulated proteins included intracellular, cell binding and structural proteins, proteins involved in physiologic processes, and unknown/hypothetical proteins. Selected bioactive factors are discussed for their putative roles in the elasmobranchs acute-phase response. These findings contribute to our understanding of disease processes in elasmobranchs, immunologic phylogeny in vertebrates, and begin the search for potential biomarkers of disease in these ecologically important fish.

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1. Introduction

Systemic inflammatory responses in mammals and bony fish are primarily driven by coordinated up-regulation and down-regulation of plasma acute-phase proteins [1]. In response to infection or injury, acute-phase proteins, including C-reactive protein, ferritin, haptoglobin, and serum amyloid A, are released into the blood stream from hepatic and extra-hepatic sites. Most proteins are believed to isolate and destroy infectious organisms, remove harmful molecules, and further activate the inflammatory process [2]. Other acute-phase proteins are thought to have anti-inflammatory and repair properties that restore homeostasis and counteract collateral damage from inflammation [3]. This intricate balance of agonists, antagonists and feedback loops plays a vital role in successful host defenses against pathogens, toxins, and injury.

Protein expression patterns are an area of interest to identify clinically relevant bioindicators of disease in human and veterinary medicine [4–6]. Biomarker profiles that quantify changes in acute phase protein levels have been found to reflect physiologic and pathologic states in mammals including shock, stress, malnutrition, and infection [7]. Although not widely utilized in non-traditional veterinary species, similar methods may be of benefit as diagnostic and prognostic indicators of disease in fish. Previous studies in rainbow trout *Oncorhynchus mykiss* injected with viral, bacterial, and fungal antigens have illustrated unique inflammatory protein profiles specific to each antigen, suggesting etiologically unique patterns of the acute-phase response in fish [8].

Similar acute-phase proteins may drive the immune response in elasmobranchs and provide opportunities to identify clinically relevant biomarkers of disease. While determination of these

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1050-4648/$ – see front matter Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.fsi.2013.02.024

inflammatory processes represents a critical component in understanding how elasmobranchs respond to disease, evidence of a functional acute-phase response is limited in these species. Genomic and molecular studies indicate the presence of C-reactive protein [9], hemopexin [10] and amyloid-P [11] in elasmobranchs. It is anticipated that more acute phase proteins are likely present, but still unknown, including the possibility elasmobranchs possess novel bioactive proteins.

The objective of this study was to identify changes in the plasma proteome of yellow stingrays *Urobatis jamaicensis* following immunostimulation with a commercial *Vibrio* bacterin. Changes in plasma protein levels were analyzed by isobaric tags for relative and absolute quantitation (iTRAQ 4-plex) using shotgun-based nano liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis and *de novo* peptide sequencing. Proteins identified against the NCBI database with putative immunologic and physiologic functions are discussed for their role in inflammation and potential use as biomarkers of disease.

2. Materials and methods

2.1. Experimental design

Three, adult yellow stingrays (2 females/1 male, 380–500 gms) that were wild caught off the southern coast of Florida were transported to the University of Georgia, College of Veterinary Medicine Aquatic Research Laboratory. Rays were housed in individually partitioned sections of a 3000 L aquarium with sand substrate and recirculating artificial sea water (Instant Ocean® Sea Salt, Aquarium Systems, Inc., Mentor, OH) at 21 °C, 30 parts per thousand salinity. Rays were fed a daily, rotating diet of shrimp, fish, and clam *ad libitum* and allowed 2 mos acclimation. Prior to the experiment, rays were fasted for 48 h and offered food once daily beginning on experimental day 1.

Five hundred microliters (μL) of blood was collected in lithium heparin plasma separator tubes (BD Microtainer® Green tubes, Franklin Lakes, NJ) from each ray at time 0 to establish resting plasma protein levels. Blood collection was performed via intracardiac puncture using a 22 G × 1″ needle and 3-ml syringe (Kendall Monoject, Tyco Healthcare Group LP, Mansfield, MA) under general anesthesia by immersion in 75 mg/L MS-222 (Finquel, Argent Chemical Laboratories Inc., Redmond, WA) buffered 1:1 by weight with sodium bicarbonate. Plasma separator tubes were centrifuged for 10 min at 3000 × g and approximately 150–200 μL of plasma was pipetted off and frozen at −80 °F until the conclusion of the study.

Immediately following blood collection, stingrays were injected intraperitoneal with 0.15 mL of Vibrogen 2® (Intervet/Schering-Plough Animal Health, Summit, NJ), a commercial fish vaccine containing three strains of *Vibrio anguillarium,* which is known to produce disease in elasmobranchs [12,8]. An additional 500 μl of blood was collected at 24 and 72 h post-injection to serve as experimental samples representative of the acute phase response [13]. Plasma was sent to Cornell University Proteomics and Mass Spectrometry Core Facility (Ithaca, NY) for protein level quantification and proteomic analysis.

2.2. Analysis of plasma

Plasma protein concentrations were estimated by the Bradford method [14] and qualitatively visualized through standard 1-dimensional gel separation techniques. Although immunodepletion of albumin is performed with mammalian plasma to increase iTRAQ assay sensitivity to lower abundance proteins, this was not necessary for yellow stingray plasma and is consistent with studies indicating low to nonexistent plasma albumin levels in elasmobranchs [15].

Change in protein expression between time 0 and post-injection time points was quantified for each ray by isobaric tags for relative and absolute quantitation (iTRAQ 4-plex) profiling. One-hundred μl aliquots of each of the nine samples were labeled with iTRAQ 4-plex tags. The time 0 samples were labeled with two tags to serve as an internal technical replicate. Resting plasma samples were labeled 114 and 115. The 24-h samples were labeled 116 and the 72-h samples were labeled 117. The mix tags labeled samples were constructed by first dimensional high pH RP separation of tryptic peptide mixtures by Ultimate3000 MDLC platform with built-in fraction collection option, autosampler, and UV detection (Dionex, Sunnyvale, CA). The tandem mass tagged tryptic peptides were reconstituted in 20 mM ammonium formate (NH_4FA) pH 9.5 in water (buffer A), and loaded onto an XTerra® MS C18 column (3.5 μm, 2.1 × 150 mm, in water) (Waters Corp, Milford, MA) with buffer A and 80% acetonitrile (ACN)/20% 20 mM NH_4FA (buffer B).

Liquid chromatography was performed using a gradient from 10% to 45% of buffer B for 30 min at a flow rate 200 μL/min. Forty-eight fractions were collected at 1 min intervals in a 96-well plate and pooled into a total of ten fractions based on UV absorbance at 214 nm. Fractions were pooled into the final ten fractions by disparate first dimension fractions (retention time multiplexing) using concatenation strategy. All ten pooled peptide fractions were dried and reconstituted in 2% ACN/0.5% formic acid for Nano LC-MS/MS analysis.

2D-LC-MS/MS analysis was performed on equal mixtures of tag-labeled digests. Nano LC-MS/MS analysis was carried out using an LTQ-Orbitrap Velos mass spectrometer (Thermo-Fisher Scientific, San Jose, CA) equipped with nano ion source via high energy collision dissociation (HCD) and interfaced with an UltiMate3000 RSLC nano system (Dionex, Sunnyvale, CA). Ten μL aliquots of each pH RP peptide fraction was injected onto a PepMap C18 trap column (5 μm, 300 μm × 5 mm) for desalting at 20 μL/min flow rate. Fractions were then separated on a PepMap C-18 RP nano column (3 μm, 75 μm × 15 cm) and eluted for 90 min in a gradient of 5%–38% ACN in 0.1% formic acid at 300 nL/min followed by a 3-min ramping to 95% ACN-0.1% FA and a 5-min holding at 95% ACN-0.1% FA. The column was re-equilibrated with 2% ACN-0.1% FA for 20 min prior to the next run.

The eluted peptides were detected by Orbitrap through nano ion source containing a 10-μm analyte emitter (New Objective, Woburn, MA). The Orbitrap Velos was operated in positive ion mode with nano spray voltage set at 1.5 kV and source temperature at 275 °C with nitrogen as the collision gas. Calibration was performed internally using the background ion signal at m/z 445.120025 as a lock mass or externally using a Fourier transform (FT) mass analyzer. The instrument was run on data-dependent acquisition (DDA) mode using FT mass analyzer for survey MS scans of precursor ions followed by ten data-dependent HCD-MS/MS scans for precursor peptides with multiple charged ions above a threshold ion count of 7500 with normalized collision energy of 45%. MS survey scans were conducted at a resolution of 30,000 FWHM at m/z 400 for the mass range of m/z 400–1400 and MS/MS scans were conducted at 7500 resolution for the mass range of m/z 100–2000. All data was acquired under Xcalibur 2.1 operation software (Thermo-Fisher Scientific, San Jose, CA). All MS and MS/MS raw spectra data from iTRAQ experiments were processed using Proteome Discoverer 1.2 (PD1.2, Thermo Scientific, San Jose, CA).

The spectra from each DDA file was output as a Mascot generic format (MGF) file for a database search against the NCBInr database with taxonomy: Chordata (vertebrates and relatives),using in-house license Mascot Deamon (version 2.3.02, Matrix Science, Boston, MA). An automatic decoy database search was performed in