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Fish skeletal muscle tissue is an important focus of immune reactions during pathogen infection

Cristián A. Valenzuela [a, b], Rodrigo Zuloaga [a, b], Matías Poblete-Morales [b, c], Tamara Vera-Tobar [a], Luis Mercado [d], Ruben Avendaño-Herrera [b, c, e], Juan Antonio Valdés [a, b, e, **], Alfredo Molina [a, b, e, *]

[a] Universidad Andres Bello, Laboratorio de Biotecnología Molecular, Departamento de Ciencias Biológicas, Facultad Ciencias Biológicas, 8370146 Santiago, Chile
[b] Interdisciplinary Center for Aquaculture Research (INCAR), 4030000 Concepción, Chile
[c] Universidad Andres Bello, Laboratorio de Patología de Organismos Acuáticos y Biotecnología Acuícola, 2520000 Viña del Mar, Chile
[d] Laboratorio de Genética e Inmunología Molecular, Instituto de Biología, Pontificia Universidad Católica de Valparaíso, 2340000 Valparaíso, Chile
[e] Universidad Andres Bello, Centro de Investigación Marina Quintay (CIMARQ), Facultad de Ecología y Recursos Naturales, 2340000 Valparaíso, Chile

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ABSTRACT

Skeletal muscle in mammals can express and secrete immune-related molecules during pathogen infection. Despite in fish is known that classical immune tissues participate in innate immunity, the role of skeletal muscle in this function is poorly understood. To determine the immunocompetence of fish skeletal muscle, juvenile fine flounder (*Paralichthys adpersus*) were challenged with *Vibrio ordalii*. Different Toll-like receptors, pro-inflammatory cytokines (TNFα, Il-1β, and IL-8), and immune-effector molecules (NKEF and the antimicrobial peptides hepcidin and LEAP-2) were analyzed. Infection initially triggered IL-1β upregulation and P38-MAPK/AP-1 pathway activation. Next, the NFκB pathway was activated, together with an upregulation of intracellular Toll-like receptor expressions (*tlr3, tlr8a tlr9,* and *tlr21*), TNFα production, and *leap-2* expression. Finally, transcriptions of *il-1β, il-8, tnfα, nkef-a*, and *hepcidin* were also upregulated. These results suggest that fish skeletal muscle is an immunologically active organ that could play an important role against pathogens.

1. Introduction

Innate immunity in fish is divided into physical, cellular, and humoral components. The skin, which is the first line of defense against pathogens, is covered by a mucus that contains transferrin, lysozyme, antimicrobial compounds, protease inhibitors, complement factors, lectins, and interferons, among other elements (Manning, 1998). Regarding cellular components, different cell types can identify different pathogen-associated molecular patterns (PAMPS), such as lipopolysaccharides, peptidoglycan, and viral nucleotides (Rauta et al., 2014). In turn, these cellular immune components are triggered by a wide range of pattern recognition receptors, including Toll-like receptors (TLRs), NOD-like receptors, and RIG-like receptors (Li et al., 2016). Among the humoral components fish can express a wide variety of cytokines similar to those found in mammals (Plouffe et al., 2005). These include the tumor necrosis factor (TNF α and β), interferon, chemokines, and interleukins (IL-1β, TNFα, or IL-8) (Rajendran et al., 2012a, 2012b; Rebl et al., 2010; Savan, 2004; Zhang et al., 2014; Zou et al., 1999). Furthermore, several studies support that costimulatory molecules, antigen-presenting molecules (Katzenback, 2015; Li et al., 2013; Schmitt et al., 2015; Sever et al., 2014; Zhang et al., 2014), and effector molecules, including antimicrobial peptides (e.g. pardaxin, dicentracin, piscidin, pleurocidin, hepcidin, chrysophsin,

Abbreviations: TBS, tris-buffered saline; TLRs, Toll-like receptors; TNF, tumor necrosis factor.

* Corresponding author. FONDAP, Interdisciplinary Center for Aquaculture Research (INCAR), Departamento de Ciencias Biologicas, Facultad Ciencias Biológicas, Universidad Andres Bello. Chile.
** Corresponding author. FONDAP, Interdisciplinary Center for Aquaculture Research (INCAR), Departamento de Ciencias Biologicas, Facultad Ciencias Biológicas, Universidad Andres Bello. Chile.
E-mail addresses: valenzuela.i.cristian@gmail.com (C.A. Valenzuela), r.zuloaga@uandresbello.edu (R. Zuloaga), matias.poblete.m@gmail.com (M. Poblete-Morales), ta.vera@uandresbello.edu (T. Vera-Tobar), luis.mercado@pucv.cl (L. Mercado), ravendano@unab.cl (R. Avendaño-Herrera), jvaldes@unab.cl (J.A. Valdés), amolina@unab.cl (A. Molina).

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cathelicidin, liver-expressed antimicrobial peptide 2 (LEAP-2), and defensin), play relevant roles in immunity (Bae et al., 2016; Broekman et al., 2013; Meloni et al., 2015; Santana et al., 2016).

Skeletal muscle is the largest cellular compartment of the body and is important for immunological reactions (Wiendl et al., 2005). Several studies indicate that skeletal muscle in higher vertebrates is an immunologically active organ that plays an essential role during infectious events. This organ expresses a wide variety of immune molecules, such as innate immunity receptors (e.g. TLRs, RIG-like receptors, and NOD-like receptors), pro-inflammatory cytokines (e.g. IL-1β, TNFα, IL-6, INFγ, and IL-8), and major histocompatibility complexes, among others (Figarella-Branger et al., 2003; Frost and Lang, 2007; Hohlfeld and Engel, 1994; Nagaraju, 2001). Considerable evidence indicates that mammalian muscle cells play an active, rather than a passive, role in the immune response (Frost and Lang, 2007; Marino et al., 2011; Wiendl et al., 2005).

Immunocompetence in fish is associated with the head kidney (Rauta et al., 2012), the spleen the thymus, and the gut-associated lymphoid tissue (Zapata and Amemiya, 2000). The gills also play a role in the fish immune response (Lovy et al., 2006). More recently, it has been reported that fish skeletal muscle can also express immune-related genes in response to different stimuli, including exercise (Magnoni et al., 2015), food deprivation (Valenzuela et al., 2015), and pathogen infection (Chatterjee et al., 2016). This immune response has been verified in cultured myoblasts after stimulation with pro-inflammatory cytokines (Pooley et al., 2013) or lipopolysaccharides (Aedo et al., 2015).

Therefore, it is particularly relevant to study the potential immunocompetence of fish skeletal muscle during infection. This study examined the capacity of skeletal muscle to deploy a pathogen-induced response in fine flounder (*Paralichthys adpersus*), a flatfish species with great farming potential for the aquaculture industry in the southeast Pacific (Fuentes et al., 2008). Juvenile fine flounders were challenged with *Vibrio ordalii*, and transcript levels of TLRs, pro-inflammatory cytokines, and effector molecules were quantified. Additionally, the protein levels of TLR9, pro-inflammatory cytokines (TNFα and IL-1β), and key molecules involved in TLR signaling pathways (myeloid differentiation primary response gene 88 [MyD88], activator protein 1 [AP-1], P38 mitogen-activated protein kinase [P38], and the nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha [IkBα]) were evaluated.

2. Materials and methods

2.1. Ethics statement

This study adhered to animal welfare procedures and was approved by the bioethical committees of the Universidad Andres Bello and the National Commission for Scientific and Technological Research of the Chilean government.

2.2. Bacterial culture

The *V. ordalii* Vo-LM-18 strain was used. This strain was initially identified as *V. ordalii* with standard phenotyping following the procedures described by Silva-Rubio et al. (2008). This result was confirmed using PCR analysis based on the vohB (haemolysin) gene (Avendaño-Herrera et al., 2014). Vo-LM-18 was routinely grown on trypticase soy broth or agar supplemented with 1% (w/v) NaCl (TSB-1 and TSA-1, respectively) and incubated at 18 ± 1 °C for 48–72 h for all tests. Following collection in 2008, stock cultures were frozen and stored at −80 °C in Criobilles tubes (AES Laboratory) or in TSB-1 containing 15% (v/v) glycerol.

2.3. Fish husbandry, bacterial challenge, and sampling

Sexually immature, one-year-old juvenile fine flounder (10 ± 4 g average weight) were obtained from the Centro de Investigación Marina de Quintay (Valparaíso, Chile). Fish (20 per tank) were maintained in four circular fiberglass tanks (1.85 m diameter) with a 30 cm water column and water turnover of 13.4 L min^{-1} (i.e. one water clearance per hour). All tanks were maintained at 14 ± 1 °C and under a 12:12 light:dark photoperiod. Fish were fed once daily with 3 mm of commercial pellet containing 45% protein, 22% lipids, 16% carbohydrates, 1% crude fiber, 7% ashes, and 10% humidity (Skretting, Puerto Montt, Chile).

For infection, fish were bath-challenged with *Vibrio ordalii* Vo-LM-18 at a concentration of 2.59×10^7 CFU/mL of seawater. After 2 h of exposure, the seawater was changed, and fish were kept in the same tanks. Control fish were bath-challenged with just TSB-1 (i.e. without bacteria).

Fish samples were obtained from both groups at 2, 4, and 10 days post-infection (dpi). At the 2 and 4 dpi sampling points, six individuals were sampled from each group (n = 6), whereas three individuals from each group were sampled at 10 dpi (n = 3). All sampled fish were first anesthetized for collection using 3-aminobenzoic acid ethyl ester (100 mg/L) and then euthanized using an anesthetic overdose (300 mg/L). A cross section of fast-white myotomal muscle was collected at a 0.50 fork length. Muscle samples were immediately frozen in liquid nitrogen and stored at −80 °C until further processing for protein and RNA extractions.

2.4. Genomic DNA extraction and electrophoresis on agarose gel

Genomic DNA was extracted from skeletal muscle using the Quick-gDNA MiniPrep Kit (Zymo Research, Irvine, CA, USA) following the manufacturer's recommendations. DNA was quantified by spectrophotometry using NanoDrop technology with the Epoch Multi-Volume Spectrophotometer System (BioTek, Winooski, VT, USA). Only DNA with an approximate A260/280 ratio of 1.8 was used. Assessments of DNA quality and visualization were performed by electrophoresis on 2% agarose gel in 1X Tris-acetate-EDTA containing ethidium bromide.

2.5. Western blotting

Total protein was extracted from skeletal muscle (0.1 g) in 1 mL of lysis buffer containing 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 5 mM Na3VO4, 20 mM NaF, 10 mM sodium pyrophosphate, and a protease inhibitor cocktail (Calbiochem, San Diego, CA, USA), centrifuged at 12,000x*g*, and solubilized at 4 °C. Protein concentration was determined using a Pierce BCA Protein Assay Kit (Thermo Scientific, Hanover Park, IL, USA). Total protein (50 μg) was loaded into each lane, separated by 10–15% SDS-PAGE, transferred to polyvinylidenediflouride membranes (Millipore, Bedford, MA, USA), and blocked for 1 h at room temperature in 2% ECL Advance Blocking Agent (GE Healthcare, Buckinghamshire, UK) dissolved in tris-buffered saline (1X TBS). Primary antibody incubations (phosphorylated or total protein) were performed overnight at 4 °C. Antibodies were purchased from Cell Signalling (Beverly, MA, USA) or Abcam (Cambridge, UK), or were locally produced by the Immunological Markers in Aquatic Organisms Group (Pontificia Universidad Católica de Valparaiso, Valparaiso, Chile). Antibody details and validations are available in Supplementary Figure 1 and Table 1.

Membranes were washed with 1X TBS and incubated for 1 h at room temperature with the appropriate secondary antibody. After washing, the membranes were visualized using a high-sensitivity enhanced chemiluminescence kit (ECL Prime western Blotting