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Immune modulatory properties of 6-gingerol and resveratrol in Atlantic salmon macrophages

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ABSTRACT

The innate immune system provides the first barrier to infection and disease through various processes including activation of inflammation. Inflammation is a biological process whereby immune cells, including macrophages, respond to, and work together to eliminate, damage from injury and disease. Chronic or sustained inflammation, however, can be detrimental to the health and growth of animals, including fish. Immune modulating functional plant compounds, supplemented as feed additives, may be safe and natural approaches to controlling inflammation and disease. This project employed an *in vitro* cell model to assess the effect of two plant phytoextracts, 6-gingerol and resveratrol, on Atlantic salmon macrophages. To determine if these plant compounds can modify Atlantic salmon macrophage function, primary macrophages were isolated from the head kidney, cultured and pre-treated with the compound for 24 hours. The cells were then challenged with a bacterial pathogen-associated molecular pattern (PAMP), lipopolysaccharide (LPS). To determine if the plant compound pre-treatment modifies macrophage response to LPS stimulation, the mRNA expression of anti-bacterial and cytokine genes (COX2, TNFα, IL-1β, IL-8, Lect-2 and sTLR5) was analyzed by real-time quantitative polymerase chain reaction (qPCR). In addition, the effects of compound pre-treatment on macrophage ROS production and phagocytic responses were analyzed via flow cytometry. We found that both 6-gingerol and resveratrol modified macrophage function, with 6-gingerol having an overall greater effect on expression of the genes analyzed, suggesting that these functional plant compounds may be good candidates as feed additives and should be investigated further.

1. Introduction

Aquaculture production of finfish, such as the Atlantic salmon (*Salmo salar*), has increased substantially over the past decade and is the fastest growing food-producing industry in the world (Pettersen et al., 2015). Intensive farming, however, can expose salmon to environmental stressors such as temperature shifts, as well as high stocking density, pathogens (e.g., viral such as salmon alpha virus, and bacteria such as *Aeromonas salmonicida*) and parasites (e.g., sea lice); this can lead to the development of infectious disease, which can cause acute and chronic inflammation (Kent, 2000; McLoughlin and Graham, 2007; Palacios et al., 2010; Pettersen et al., 2015). Acute inflammation, which occurs immediately, is a biological process whereby immune cells such as macrophages respond to, and eliminate, damage from injury and disease. While the acute response is critical to fight infectious disease, chronic or sustained inflammation decreases salmonid growth rate and has a negative impact on production and fish health (Johansen and Overturf, 2006). Fish meal and fish oil are the most common ingredients in aquafeed (Caruso, 2015). However, both are limited resources and are being replaced with plant-based ingredients, such as soybean meal, which can induce inflammation in several fish species (Baeverfjord and Krogdahl, 1996; Van den Ingh et al., 1991; Urán et al., 2008; Montero et al., 2010). A safe and natural approach to controlling inflammation in aquaculture is required to promote overall fish health.

It is possible that immune modifying functional plant compounds could be used to enhance the fish immune system, which could aid in controlling disease and inflammation. Functional plant compounds have been used as additives in livestock feed in recent years and have had many benefits such as immunomodulatory properties, reduced incidence of disease, increased feed intake, growth promotion and improved reproductive parameters (reviewed by Yitbarek, 2015; Kumar et al., 2014). Several plants have immune modifying properties such as antioxidant, anti-inflammatory, antimicrobial, antifungal, and antiviral

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functions, due to the presence of functional compounds, such as vitamins, phenols, thiols, flavonoids, and essential oils (Watzel, 2008; Citarasu, 2010). When supplemented into the diet, several plants and plant compounds such as ginger (Shakya, 2015; Nya and Austin, 2009), garlic (Militz et al., 2013), cumin (Yilmaz et al., 2012), curcumin (Manju et al., 2011) and vitamin E (Liu et al., 2014), among others, have been shown to positively modify health, growth and resistance to stress and disease in some fish. 6-gingerol (a main component of the ginger plant) and resveratrol (found in red grapes, blueberries and raspberries), are natural phenols known to have anti-inflammatory and anti-oxidative properties in mammals (reviewed in Mashhadi et al., 2013; Watzel, 2008). The effects of 6-gingerol and resveratrol on the immune function of Atlantic salmon macrophages are unknown.

Macrophages, and their precursor cells (monocytes), are among several important cells of the immune system that regulate many different properties of the immune response to infection and disease, including both acute and chronic inflammation. To eliminate infection and disease, macrophages initiate a cytokine cascade, releasing several cytokines, such as interleukins, that orchestrate a variety of processes including the control of inflammation, cellular proliferation, chemotaxis and tissue repair (Dinarello, 2007). In addition, they engulf foreign particles, or pathogens, through a process called phagocytosis and subsequently destroy the particles by producing reactive oxygen and reactive nitrogen species (reviewed in Rees, 2010). Several mammalian *in vitro* macrophage studies have determined that pre-treatment with different plant derivative compounds, such as gingerol (Lee et al., 2009; Tripathi et al., 2007), curcumin (Guimaraes et al., 2013), resveratrol (Qureshi et al., 2012; Zong et al., 2012) and quercetin (Angeloni and Hrelia, 2012; Mu et al., 2001), can modify the macrophage inflammatory and oxidative response to lipopolysaccharide (LPS), a pathogen associated molecular pattern (PAMP) that stimulates an antibacterial immune response. There is a paucity of information available on the effects of functional plant compounds on the immune function of fish macrophages. In fish, a heterogeneous population of adherent immune cells, consisting mostly of macrophages, but also monocytes, can be isolated from the head kidney, the main hematopoietic organ of the fish (Joerink et al., 2006; MacKenzie et al., 2003; Neumann et al., 1998). These adherent head kidney leukocytes will henceforth be referred to as macrophages. This study employed an *in vitro* cell model to assess the effects of the plant phytoextracts, 6-gingerol and resveratrol, on the Atlantic salmon macrophage ability to phagocytize and produce ROS, as well as their effect on macrophage antibacterial and inflammation-relevant transcript expression following LPS challenge. The aim of this study was to determine if 6-gingerol and resveratrol may be good candidates as immune modifying food additives by examining their effects on Atlantic salmon head kidney macrophage immune function.

2. Materials and methods

2.1. Animals

The Atlantic salmon (0.5 +/− 0.1 kg) that were used for this experiment were reared in the Dr. Joe Brown Aquatic Research Building (JBARB) of the Ocean Sciences Centre in 3800 L tanks, and kept at 12° C with 95–110% oxygen saturation, using a flow-through seawater system. All procedures in this experiment were approved by Memorial University of Newfoundland's Institutional Animal Care Committee (protocol 15-72-MR), based on the guidelines of the Canadian Council of Animal Care.

2.2. Macrophage isolation

Head kidney macrophages were isolated as previously described for other fish species (Joerink et al., 2006; MacKenzie et al., 2003; Neumann et al., 1998) with some modifications. The fish were netted and euthanized with an overdose of MS222 (0.4 g/L, Syndel Laboratories, Vancouver, BC, Canada). The anterior portion of the kidney, also referred to as the head kidney, was removed and placed in isolation media: 500 ml of Leibovitz-15 medium (L-15 Gibco, Carlsbad, CA, USA) supplemented with 2.5% fetal bovine serum (FBS, Gibco), 1% penicillin/streptomycin (Gibco) and 27.5 mg of heparin (Sigma-Aldrich, St. Louis, MO, USA). The head kidney was minced through a 100 µM nylon cell strainer (Thermo-Fisher Scientific, Waltham, MA, USA), placed on a 34/51% Percoll gradient (GE Healthcare, Uppsala, Sweden) prepared with 5% HBSS (Gibco) to ensure an isotonic solution, and centrifuged at 500 x g for 30 min at 4° C. Following centrifugation, the interface between the 34% and 51% gradient, which contains mostly macrophages, was collected and washed twice in isolation media at 500 x g for 5 min at 4° C. The cells were re-suspended in culture media (L-15 supplemented with 5% FBS and 1% penicillin/streptomycin), and viable cells were counted on a haemocytometer using Trypan Blue (Sigma-Aldrich) exclusion. The cells were then seeded in 6-well culture plates (Corning, Corning, NY, USA) at 1×10^7 cells in 2 ml of culture media (for respiratory burst and phagocytosis assays) or 3×10^7 cells in 2 ml of culture media (for gene expression studies) per well and incubated at 15° C for 24 h to allow cell adherence. Following the 24 h incubation, cells were washed twice in culture media to remove non-adherent cells, and the media was replaced with fresh culture media.

2.3. Respiratory burst assay

Twenty-four hours after seeding macrophages, the culture media was removed and cells were washed once in culture media. The media was replaced with new culture media containing either 10 µM or 100 µM of 6-gingerol, diluted in ethanol, 10 µM, 30 µM or 50 µM of resveratrol, diluted in ethanol, or a vehicle control (1 µl of ethanol, equivalent to the maximum volume of ethanol used in experimental conditions), for 24 h. These concentrations were based on previous mammalian literature (Ippoushi et al., 2003; Lee et al., 2009, Leiro et al., 2002). We found that 100 µM of resveratrol appeared to kill the cells (as noted by floating cells and a lower concentration of RNA); therefore, 50 µM of resveratrol was the highest concentration tested. Following 24 h, the culture media was replaced with respiratory burst assay buffer (L-15 media + 1% BSA + 1 mM $CaCl_2$). One microlitre of dihydrorhodamine 123 (DHR) (5 mg/ml) was diluted in 1 ml of PBS and 50 µl of the dilution was added to the cells for 15 min. Following DHR addition, 200 µM of phorbol myristate acetate (PMA), or sterile phosphate buffered saline (PBS; Sigma-Aldrich) for a negative control, was added to the cells for 45 min to stimulate reactive oxygen species (ROS) production (Kalgraff et al., 2011). PMA is a small chemical compound that mimics diacylglycerol, an activator of protein kinase C (PKC), which activates NAPDH oxidase, resulting in ROS production. It has been used in measurements of the respiratory burst response in both vertebrates and invertebrates (Kalgraff et al., 2011) and has also been used in mammalian *in vitro* functional plant compound studies (Pečivová et al., 2015). Cells were removed from the plate using trypsin-EDTA (0.25%) (Thermo Fisher Scientific), washed in culture media, centrifuged for 5 min, 500 x g, at 4 °C and re-suspended in fluorescence-activated cell sorting (FACS) buffer (PBS + 1% FBS). Before flow cytometry analysis, cells were stained with 1 µg/ml of propidium iodide (PI) to stain and exclude dead cells from the analysis. Fluorescence was detected from 10,000 cells using a BD FACS Aria II flow cytometer and analyzed using BD FACS Diva v7.0 software (BD Biosciences, San Jose, CA, USA). The non-PMA stimulated vehicle control cells were used to define the region of ROS negative cells and based on this gating the FITC positive cells were identified. The mean fluorescence intensity and percentage of FITC-positive cells were determined for each condition.