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Effects of resveratrol on growth and skeletal muscle physiology of juvenile southern flounder

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ABSTRACT

Resveratrol is a naturally occurring antioxidant that has been widely studied in mammals due to its potential to extend lifespan. However, antioxidants may also limit protein damage and therefore reduce rates of protein degradation, providing a potential avenue for enhancing growth in an aquaculture setting. The present study tested the hypotheses that in Southern flounder, *Paralichthys lethostigma*, resveratrol would decrease protein carbonylation and 4-HNE (indicators of protein and lipid oxidative damage, respectively), levels of ubiquitinylation and LC3 (indicators of non-lysosomal and lysosomal protein degradation, respectively), while having no effect on S6K activation (indicator of protein synthesis). These effects were predicted to increase growth rate. Mitochondrial volume density was also examined since resveratrol may lead to the proliferation of mitochondria, which are the principal source of reactive oxygen species (ROS) that cause oxidative damage. Juvenile fish (n = 142) were fed a control diet or a diet supplemented with 600 μg resveratrol per g of food for 16 weeks. Fish treated with resveratrol had a 9% greater length and 33% greater body mass than control fish after 16 weeks. Additionally, there was lower protein carbonylation and lipid 4-HNE within the muscle tissues of treated fish, indicating decreased oxidative damage, and reduced protein ubiquitinylation in the resveratrol fed flounder, indicating less protein degradation. However, there was not a significant difference in LC3, S6K activation, or mitochondrial volume density. These results suggest that resveratrol has positive effects on growth due to its antioxidant properties that reduce non-lysosomal protein degradation.

1. Introduction

Muscle growth and atrophy are dependent on the net balance between protein synthesis and degradation (Millward et al., 1975; Goldspink, 1991; Houlihan, 1991; McCarthy et al., 1994; Mente et al., 2011). In aquaculture, there is considerable emphasis on maximizing net protein synthesis and growth, particularly in muscle, and this can be achieved by increasing rates of protein synthesis and/or decreasing rates of protein degradation. Protein synthesis in muscle is primarily regulated by the mechanistic target of rapamycin (mTOR) (Hemmings and Restuccia, 2012), and an increase in the activation of mTOR and its downstream targets is typically used as an indicator of enhanced rates of protein synthesis (Hemmings and Restuccia, 2012). Protein degradation in muscle occurs primarily through the non-lysosomal ubiquitin–proteasome and the lysosomal autophagy pathways (Bonaldo and Sandri, 2013). Non-lysosomal protein degradation begins when damaged or unnecessary proteins are tagged by multiple ubiquitin molecules. These marked proteins are then recognized and broken down by the proteasome complex (Bonaldo and Sandri, 2013). Lysosomes are acidic organelles that receive material to be degraded via endocytosis, phagocytosis or, in the case of muscle protein turnover, autophagy (Dell'Angelica et al., 2000; Saftig and Klumperman, 2009; Bao et al., 2010). In autophagy, lysosomes fuse with autophagosomes to form autolysosomes, where degradation ensues (Saftig and Klumperman, 2009), and various lysosomal hydrolyses and lysosomal membrane proteins target specific substrates for degradation (Conus and Simon, 2008; Saftig and Klumperman, 2009).

Reactive oxygen species (ROS) are produced naturally during electron transport, phagocytosis and by specific enzymes such as oxidases (Rice-Evans and Burdon, 1993). ROS can cause oxidation of proteins, peroxidation of lipids, and breaks in DNA (Latruffe and Rifler, 2013). In most cells, ROS are primarily generated within the mitochondria where oxygen is converted into the superoxide anion, which occurs

Abbreviations: LC3, microtubule-associated protein 1A/1B-light chain 3; LDL, low-density lipoprotein; mTOR, mechanistic target of rapamycin; PBS, phosphate buffered saline; P-S6K, phosphorylated S6K; ROS, reactive oxygen species; S6K, p70S6 kinase; TEM, transmission electron microscopy; TTBS, tris buffered saline with 0.1% Tween; 4-HNE, 4-hydroxynonenal

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when dioxygen is reduced by one electron (Latruffe and Rifler, 2013). Cells have mechanisms to manage ROS levels, such as antioxidant enzymes (Nyström, 2005), and dietary antioxidant molecules such as vitamins E and C (Hamre et al., 2004). However, an oxidative challenge can occur if the formation of ROS exceeds the organism's antioxidant defenses, and at least some oxidative damage is an unavoidable consequence of respiration (Rice-Evans and Burdon, 1993; Pandey and Rizvi, 2011; Latruffe and Rifler, 2013). For example, protein carbonylation is an irreversible modification that occurs fairly rapidly during oxidative stress and is a commonly measured indicator of ROS induced protein damage (Dalle-Donne et al., 2003; Nyström, 2005). Oxidation of proteins can lead to unfolding, which may increase the exposure of hydrophobic amino acid residues to the aqueous environment (Grune et al., 2004; Nyström, 2005). This conformational change makes the protein more vulnerable to ubiquitin tagging, and thus proteosomal degradation (Dukan et al., 2000; Bota et al., 2002; Grune et al., 2003, 2004; Nyström, 2005), suggesting that excessive protein carbonylation may reduce net protein synthesis and limit growth.

Resveratrol is an antioxidant compound in the stilbene group of polyphenols that can be found in the skins of red grapes as well as other plants such as peanuts (Latruffe and Rifler, 2013). Resveratrol has been implicated in protection against aging, obesity, and development of insulin resistance in rodents (Baur et al., 2006; Lagouge et al., 2006; Park et al., 2012). Resveratrol scavenges ROS directly, and also increases the level of mitochondrial antioxidant enzymes and modifies cell signaling pathways and kinase activities (Kairisalo et al., 2011; Xia et al., 2011; Giovannini and Masella, 2012). Thus, resveratrol's antioxidant properties might be expected to reduce protein degradation and potentially enhance growth rates.

As in mammals, fish tissues generate small quantities of ROS during normal metabolism (Rice-Evans and Burdon, 1993; Hamre et al., 2004) and dietary supplementation with antioxidants has been shown to enhance growth in fishes (Tocher et al., 2002; Wang et al., 2003; Lee and Dabrowski, 2004; Gao et al., 2012). However, while resveratrol has been found to increase the longevity of a short-lived seasonal fish *Nothobranchius furzeri* (Valenzano et al., 2006), the effect of resveratrol on growth in fishes has not been investigated. The Southern flounder *Paralichthys lethostigma* is a marine flatfish inhabiting estuarine and shelf waters of the south Atlantic and Gulf coasts of the U.S. High commercial value, declining natural populations, and wide temperature and salinity tolerances make the southern flounder a versatile species for aquaculture (Daniels et al., 2010). Additionally, spawning and larval rearing techniques for this species have been documented (Watanabe et al., 2006; Daniels et al., 2010). The present study tested the hypothesis that resveratrol supplementation in juvenile Southern flounder would decrease markers of muscle oxidative damage and protein degradation, and therefore lead to an increase in growth.

2. Materials and methods

2.1. Animal maintenance

Adult southern flounder broodstock held in photothermally controlled tanks were induced to spawn by using luteinizing hormone-releasing hormone analog (Watanabe et al., 2006) at the University of North Carolina Wilmington Aquaculture Facility (Wrightsville Beach, NC). Embryos were hatched and larvae reared according to published protocols (Daniels and Watanabe, 2003). Early juveniles were raised in 1000-L recirculating tanks and fed a commercially prepared diet (50% protein and 15% lipid) (Skretting, Vancouver, British Columbia) until the experiment was started.

The experimental system consisted of 6 circular, 680-L tanks at the University of North Carolina Wilmington. Temperature was maintained at 21 ± 1 °C and salinity was 33 ± 1 ppt. Levels of ammonia (0 to 0.1 ppm), nitrate (10 to 25 ppm), nitrite (0 to 0.1 ppm), dissolved oxygen (6.5 to 7.8 mg/L), as well as pH (7.7 to 8.0) were measured throughout the experiment, and these parameters were maintained within the optimum levels of southern flounder culture as described by Daniels and Watanabe (2003). Uniform water quality and temperature were maintained in all the tanks, as they were supported by a recirculating aquaculture system, including mechanical and biological filters and a UV sterilizer. Total system volume, including the six experimental tanks was 3800 L. A single cohort of 144 juvenile southern flounder were used in the experiments (mean initial weight: 28.55 ± 1.59 g; mean initial total body length: 13.62 ± 0.28 cm). Light conditions were set to 12 h of light and 12 h of darkness. Fish were held for two weeks to adjust to new tank conditions before trials began. During this holding period, fish were fed a standard aquaculture diet (47% protein, 12% lipid, Table 1: control diet). After two weeks, the control diet and resveratrol diets were added to appropriate tanks and food consumption, as well as length measurements were recorded (body mass was measured at three sub-sampling periods throughout the experiment, further detailed below). Fish were maintained and processed according to the UNCW Institutional Animal Care and Use Committee standards.

2.2. Diets and growth

Two isonitrogenous (47%) and isolipidic (12%) control and resveratrol-based (0.06%) diets were prepared (Table 1). The formulation of the basal diet was according to recent published information on the nutrient requirements of southern flounder (Alam et al., 2009, 2011). All ingredients were the same in both diets except α-cellulose was added in the control diet as a filler instead of resveratrol. Resveratrol was supplemented in the test diet at a dose of 600 μg/g of diet. This concentration was chosen because it had been used previously by Valenzano et al. (2006) to demonstrate resveratrol's impacts on longevity in turquoise killifish. Control and resveratrol diets were made at the UNC Wilmington aquaculture facility (Table 1) as described by Alam et al. (2011). Three replicate tanks were assigned to both the treatment and control groups, with 24 fish per tank. Flounder were fed twice a day at 10:00 am and 5:00 pm until satiation and food consumption was recorded for each tank. Fish were considered satiated once aggressive pursuit of food slowed and food remained untouched for several minutes.

Table 1
Composition (g/100 g diets) of diets for control and resveratrol treated fish.

Ingredients	Control diet	Resveratrol diet
	% in diet	% in diet
Solvent-extracted soybean meal [a]	20	20
Menhaden fish meal [b]	37	37
Poultry meal [c]	20	20
Wheat starch	6.94	6.94
Wheat gluten (binder) [d]	4	4
Menhaden fish oil [e]	5	5
Soybean lecithin [f]	1	1
Vitamin premix [g]	3	3
Mineral premix [g]	3	3
Resveratrol [h]	0	0.06
Cellulose	0.06	0
Total	100	100
Proximate composition		
Protein (%)	47	47
Lipid (%)	12	12
Energy (kj/g diet) [i]	16.9	16.9

[a] Southern States, Wallace, NC, USA (solvent extracted, crude protein 47.5%).
[b] Omega protein, Houston, TX, USA (crude protein 60%, lipid 10%).
[c] Mallick Aquiafeed, Wallace, NC, USA (crude protein 60%, lipid 10%).
[d] Sigma-Aldrich, St. Louis, MO, USA (crude protein 80%).
[e] Virginia Prime Silver, Omega Protein, Hammond, LA, USA.
[f] DM, IL,USA.
[g] Tomita Pharmaceutical Company, Kagoshima, Japan as Alam et al. (2011).
[h] Mega Resveratrol, Danbury, CT, USA (certified 99% pure trans-resveratrol).
[i] Calculated based on carbohydrates, proteins and lipids are 17.2, 23.6, and 39.5 kJ g^{-1}, respectively (Blaxter, 1989).