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IGF-I and amino acids effects through TOR signaling on proliferation and differentiation of gilthead sea bream cultured myocytes

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ABSTRACT

Skeletal muscle growth and development is controlled by nutritional (amino acids, AA) as well as hormonal factors (insulin-like growth factor, IGF-I); however, how its interaction modulates muscle mass in fish is not clearly elucidated. The purpose of this study was to analyze the development of gilthead sea bream cultured myocytes to describe the effects of AA and IGF-I on proliferating cell nuclear antigen (PCNA) and myogenic regulatory factors (MRFs) expression, as well as on the transduction pathways involved in its signaling (TOR/AKT). Our results showed that AA and IGF-I separately increased the number of PCNA-positive cells and, together produced a synergistic effect. Furthermore, AA and IGF-I, combined or separately, increased significantly Myogenin protein expression, whereas MyoD was not affected. These results indicate a role for these factors in myocyte proliferation and differentiation. At the mRNA level, AA significantly enhanced PCNA expression, but no effects were observed on the expression of the MRFs or AKT2 and FOXO3 upon treatment. Nonetheless, we demonstrated for the first time in gilthead sea bream that AA significantly increased the gene expression of TOR and its downstream effectors 4EBP1 and 70S6K, with IGF-I having a supporting role on 4EBP1 up-regulation. Moreover, AA and IGF-I also activated TOR and AKT by phosphorylation, respectively, being this activation decreased by specific inhibitors. In summary, the present study demonstrates the importance of TOR signaling on the stimulatory role of AA and IGF-I in gilthead sea bream myogenesis and contributes to better understand the potential regulation of muscle growth and development in fish.

1. Introduction

The gilthead sea bream (*Sparus aurata* L.) has become one of the most important species for Mediterranean aquaculture over the last 30 years, overcoming the capture fisheries production (FAO, 2012). However, despite its commercial interest, more research is needed to better understand its growth and development.

The growth pattern in fish differs from other vertebrates, since most fish can grow in length and weight until they die (Johnston et al., 2011; Talbot, 1993). The majority of this growth is due to accretion of muscle tissue, mostly the white skeletal muscle (Johnston, 2006; Mommsen, 2001). Contrary to most vertebrates, this muscle growth implies not only muscle hypertrophy (increase in fiber size), but also hyperplasia (new muscle fibers formation), which is mediated by muscle satellite cells (Stoiber and Sänger, 1996). During skeletal muscle development, precursor cells become myoblasts, which undergo proliferation, cell cycle exit, differentiation and then, fusion to form multinucleated myofibers (Chargé and Rudnicki, 2004; Johnston, 2006).

Myogenesis in fish is regulated by several growth and transcription factors expressed in a sequential manner (García de la serrana et al., 2014; Johnston, 2006). Some of these myogenic regulatory factors (MRFs) are essential for muscle lineage determination and cell proliferation (Myf5 and MyoD) while others contribute to the initiation and maintenance of the differentiation program, which turns myoblasts into myotubes (MRF4 and Myogenin). In addition to MRFs, growth is hormonally regulated mainly by the hypothalamic-pituitary axis through the growth hormone (GH) and the insulin-like growth factors (IGFs) system (Fuentes et al., 2013; Le Roith et al., 2001; Montserrat et al., 2007a; Reindl and Sheridan, 2012; Reinecke et al., 2005; Wood et al., 2005). Interestingly, it has been demonstrated that IGFs (IGF-I and IGF-II) stimulate *in vitro* nutrients uptake and protein synthesis (Castillo et al., 2004; Codina et al., 2008; Montserrat et al., 2012), as well as

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myoblast proliferation, which has been observed in gilthead sea bream myocytes using a proliferating cell nuclear antigen (PCNA) immunocytochemical technique (Rius-Francino et al., 2011) and in rainbow trout myocytes by BrdU labeling (Gabillard et al., 2010).

In addition to the endocrine factors, fish growth is a multifactorial process influenced by nutritional, genetic and environmental factors (Garcia de la serrana et al., 2012). Amino acids (AA) are important precursors that stimulate protein synthesis mainly activating the target of rapamycin (TOR) nutrient-sensitive signaling pathway (Kim, 2009; Meijer, 2003). Insulin and growth factors (e.g. IGFs) also activate the TOR signaling pathway through its ability to induce the phosphoinositide 3-kinase (PI3K)/AKT signaling pathway (Glass, 2010), resulting in an increase in protein synthesis via indirect activation of TOR (Vander Haar et al., 2007). In vertebrates, it is commonly known that TOR integrates signals from nutrients, energy status and growth factors, and that is an essential regulator of cell growth by controlling cell cycle, gene transcription, cytoskeleton organization and protein synthesis among other functions (Destefano and Jacinto, 2013; Dowling et al., 2010; Edinger and Thompson, 2002; Gough, 2012; van Dam et al., 2011; Yang et al., 2008). TOR is present in two distinct multi-protein complexes: TORC1 (Raptor) that is rapamycin and nutrient-sensitive, and TORC2 (Rictor), which is rapamycin and nutrient-insensitive. TORC1 promotes cellular growth, proliferation and metabolism by stimulating protein synthesis through 4EBP1 and 70S6K either in fish as in mammals (Sarbassov et al., 2005; Seiliez et al., 2008). Moreover, TORC2 controls various metabolic processes and promotes cell proliferation and survival by facilitating the phosphorylation of the active loop of AKT by the phosphoinositide-dependent kinase-1 (PDK1), which is necessary for AKT activation (Destefano and Jacinto, 2013; Foster and Fingar, 2010; Liao et al., 2008; Manning and Cantley, 2007; Sarbassov et al., 2005). Hence, AKT also regulates cell metabolism, growth and survival by inhibiting the forkhead family of transcription factors, FOXO (Calnan and Brunet, 2008; Héron-Milhavet et al., 2006; Manning and Cantley, 2007; Vadlakonda et al., 2013). Nevertheless, studies exploring the role of AA on fish myogenesis as well as the signaling pathways involved are very scarce. Seiliez et al. (2008) showed in rainbow trout *in vivo* and *in vitro* the importance of nutritional factors on the activation of TOR and its downstream effectors. Moreover, a study in Atlantic salmon using cultured myocytes has demonstrated the effects of AA alone or combined with IGF-I up-regulating the expression of different members of the IGF system (Bower and Johnston, 2010); thus supporting a nutritional stimulatory role on muscle growth.

In this framework, the main aim of the present study was to investigate the role of AA and growth factors in muscle growth and development in gilthead sea bream *in vitro* using a primary cell culture system. Thus, we analyzed the effects of IGF-I and AA on protein expression of PCNA and the myogenic factors, MyoD and Myogenin, as well as the gene expression of PCNA and MRFs (MyoD1, MyoD2, Myf5, Myogenin). Furthermore, we investigated the action of these molecules on the mRNA expression of TOR, AKT2 and its downstream effectors (4EBP1, 70S6K and FOXO3, respectively), as well as the activation by phosphorylation of these signaling pathways.

2. Materials and methods

2.1. Animals

Fish were obtained from a commercial fishery located in the north of Spain and maintained at the facilities of the School of Biology at the University of Barcelona (Spain) in 0.4 m^3 tanks with a temperature-controlled seawater recirculation system (21 ± 1 °C) and 12L:12D photoperiod. Fish were fed *ad libitum* twice daily with a commercial diet (Skretting, Burgos, Spain) and fasted for 24 h previously to the isolation of muscle cells. All animal handling procedures were conducted with the Ethics and Animal Care Committee of the University of Barcelona approval, following the EU, Spanish and Catalan Government-established norms and procedures.

2.2. Myocyte cell culture

For the different studies a total of twenty independent primary cultures of muscle satellite cells were performed as described by Montserrat et al. (2007b). We used approximately 40 gilthead sea bream (*S. aurata* L.) juveniles from 4 to 24 g body mass per culture. Cells were cultured in 6 well-plates (9.6 cm^2/well) for Western blot and quantitative real-time PCR (qPCR) studies and in 12 well-plates containing glass cover slips (2.55 cm^2) for immunofluorescence and immunocytochemistry analyses. Cells were counted, diluted and plated to a final density of 0.2–0.25 10^6 cells/cm^2 for both types of plates. The cultures were maintained at 23 °C in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 0.11% NaCl, 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic solution (A/A). The development of the cells was first analyzed throughout the culture (days 2, 4, 8 and 12) in order to describe the proliferation and differentiation patterns using immunocytochemical and immunofluorescence assays as described below.

2.3. Experimental treatments

For the different studies, myocytes at day 4 were used. This day of culture development was chosen because at this point, the cells retain the ability to proliferate and have also the capacity to start fusing and differentiating (Montserrat et al., 2007b). The cells were held for 12 h with DMEM containing 0.02% FBS and 1% A/A, then starved for 5 h with medium B (without amino acids, AA) containing 10% Earle's Balanced Salt Solution (EBSS, E7510) + 1% MEM vitamins (M6895) + 0.9% NaCl + 0.13% BSA. Subsequently, the cells were maintained in medium B alone (Control) or were treated with human IGF-I at 100 nM and/or an AA cocktail, which contained the concentration of AA of a standard cell culture medium, previously reported by Lansard et al. (2010) (1% MEM Amino Acids Solution (M5550) + 1% MEM Non-essentials Amino Acids Solution (M7145)). The incubations lasted for 3 h in the case of Western blot experiments and for 6 h for the rest of analyses. When using for Western blot studies the specific inhibitors of TOR, rapamycin (R8781) at 100 nM, and AKT, wortmannin (W3144) at 1 μM, these were added for the last 30 min of starvation and during subsequent incubation with the different treatments. Once the incubation time passed, wells were washed twice with cold phosphate buffered saline (PBS) and the samples were recovered accordingly depending on the assay type to be performed. All reagents were obtained from Sigma–Aldrich (Tres Cantos, Spain) except human IGF-I that was from Bachem (Weil am Rhein, Germany) and all plastic ware and glass cover slips were from Nunc (LabClinics, Barcelona, Spain).

2.4. Immunocytochemistry

Cell proliferation was analyzed by immunostaining using a commercial PCNA staining kit (Cat. No. 93-1143. Life Technologies, Alcobendas, Spain). After washing, cells were fixed at room temperature in 4% paraformaldehyde (PFA, Sigma–Aldrich, Spain) for 15 min, washed, and postfixed for 5 min in 50% and 70% ethanol. Briefly, coverslips were incubated in PCNA staining reagents following the suggested manufacturer's protocol. Coverslips were incubated in a blocking solution to prevent non-specific binding

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