



Differential effects on proliferation of GH and IGFs in sea bream (*Sparus aurata*) cultured myocytes

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ABSTRACT

Primary culture of gilthead sea bream skeletal muscle cells was used to examine the effects of growth hormone (GH) and insulin-like growth factors (IGFs) in fish muscle proliferation and growth. Proliferation was measured as the percentage of positive cells expressing the proliferating cell nuclear antigen (PCNA) analyzed by immunocytochemistry. First, the effects of GH from two different origins (mammals and fish) were tested. GH from human (hGH) did not stimulate proliferation except at 3 h at the dose of 1 nM. On the other hand, sea bream GH (sbGH) significantly stimulated proliferation, without differences between the three incubation times studied (3, 6, and 18 h), at the dose of 10 nM, demonstrating that the homologous hormone has a more potent effect. In addition, the results with the IGFs indicated that both peptides, IGF-I and IGF-II significantly stimulated proliferation of sea bream myocytes, but IGF-II showed higher effects than IGF-I, and even than those of sbGH. Finally, the combinations of peptide treatments (GHs with IGFs) indicated that IGF-I has higher effects on proliferation when it is combined with GHs compared with IGF-I alone, while IGF-II has similar effects alone or combined with either GH. These results indicate that IGF-II may have an important role on muscle proliferation that appears to be independent of GH. On the contrary, IGF-I seems to play a synergistic action with GH stimulating myocyte proliferation.

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1. Introduction

Gilthead sea bream, *Sparus aurata*, is a widely cultured fish in the southern Mediterranean area and represents an important source of protein in the European diet. The optimization of sea bream growth has been improved in the last years, but it is still far from achieving a high level of competence and flesh quality. Mechanisms of muscle growth have been studied mainly in salmonids and fish model species [20,32]. Nevertheless, basic research is still needed to understand muscle growth and the main regulatory molecules in marine fish species, sea bream for instance. Skeletal muscle growth in fish, as in other vertebrates, is largely regulated by the activity of satellite cells, which in turn depend on numerous extracellular signals and intracellular factors [20,23]. Among these, growth hormone (GH) and the insulin-like growth factors (IGFs) exert an important role in the regulation of all major physiological processes, including somatic growth, metabolism and reproduction [13,21,22]. These effects are generally well known on vertebrates, although information in fish muscle is less available [7,10,28,30].

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The IGF system includes the growth factors IGF-I and IGF-II, their corresponding receptors and the IGF binding proteins (IGFBPs). This system has been extensively studied in mammals [28], but there are also several studies in fish [4,8]. The IGF-I is synthesized predominantly in the liver and it is an important mediator of the action of GH during postnatal life. On the other hand, IGF-II shows little dependence on GH and it is more related to fetal growth [6]. In teleosts, IGFs are also mainly produced by the liver, but a wide variety of extrahepatic tissues, such as skeletal muscle, produce IGFs locally too, where they may act in a paracrine or autocrine manner [11]. In different studies in fish *in vivo*, IGF-I expression and fish growth have been positively related [12]. For example, in different conditions, as with vegetable replacement in fish diets [15], starvation [16] and compensatory growth [24]. Furthermore, several *in vitro* studies on fish myoblasts have demonstrated the stimulatory effects of IGF-I and IGF-II on the activation of MAPK and PI3K/Akt pathways [7,10,25]. The dose response effects of IGF-I on the proliferation of primary cultures of rainbow trout, *Oncorhynchus mykiss* myocytes have been demonstrated using the thymidine uptake technique [7]. The mitogenic and metabolic effects of IGF-II were also investigated on muscle cells of sea bream showing an increased effect only in the presence of 5% FBS [10]. In addition, Gabillard and coworkers [14] used the BrdU labeling technique to check the effects of IGFs on

trout myocytes proliferation. On the other hand, Bouraoui and coworkers [3] used the PCNA immunocytochemical technique to test the effects of IGF-I on rainbow trout pre-adipocytes proliferation. Furthermore, muscle differentiation in Atlantic salmon, *Salmo salar*, and sterlet, *Acipenser ruthenus* has also been demonstrated to be stimulated by IGF-I [27,37].

As in other vertebrates, GH is produced by the pituitary gland in teleosts. It exerts its action directly, by binding to a transmembrane receptor, the GH receptor (GHR), in its target tissues such as muscle, or indirectly through local GH-induced IGF-I production. In sea bream, Duguay and coworkers [11] demonstrated that GH administration *in vivo* caused an increase in the amount of IGF-I mRNA in the liver, while no changes in IGF-II mRNA levels were detected in any tissue, suggesting that they play distinct roles in growth. Moreover, they showed that muscle IGFs did not respond to GH stimulation at all. Contrary, Vong and coworkers [36] demonstrated that, in common carp, *Cyprinus carpio*, GH administration *in vivo* increased the muscle expression of IGF-II, while IGF-I expression was not affected. Therefore, further studies are needed to investigate the effects of this hormone on fish muscle growth and how they are mediated.

The aim of the present study was to elucidate the role of GH and IGFs on sea bream muscle proliferation. We studied the relationship between these hormones and their mutual effects on cell proliferation in primary cultures of sea bream satellite cells and found IGF-II as the most important regulator of growth in this cellular model.

2. Materials and methods

2.1. Animals

Gilthead sea bream (*S. aurata*) juveniles were obtained from Grupo Tinamenor (Pesués, Cantabria, Spain) and maintained in optimal conditions in the facilities of the Servei d'Estabulari of the Faculty of Biology at the University of Barcelona, in tanks of 200 L with a closed-water flow circuit with water at a temperature of $21 \pm 1^\circ\text{C}$. Fish were fed *ad libitum* once daily with a commercial diet and fasted for 24 h previously to the isolation of muscle cells. All animal handling procedures were approved by the Ethics and Animal Care Committee of the University of Barcelona, following the EU, Spanish and Catalan Government-established norms and procedures.

2.2. Primary cell cultures

To establish the primary cultures of sea bream muscle cells, fish (30–50 for each culture) with an average weight of 22 ± 2.49 g were killed by a blow to the head followed by immersion in 70% ethanol for 30 s to sterilize the external surfaces. White muscle was dissected, and cells were isolated, pooled and cultured following the protocol described previously [25]. Cells were seeded at a density of 2×10^6 cells per well, in 6-well plastic plates (9.6 cm²/well) pretreated with poly-L-lysine and laminin. The cells were maintained at 22°C in DMEM medium (Sigma, Tres Cantos, Spain) supplemented with 1% penicillin–streptomycin and 10% fetal bovine serum (FBS) until further analysis.

2.3. Hormone treatments

To perform the proliferation studies the stage of day 4 in culture was chosen because the cells are still mononucleated, since they start the process of fusion on day 6 [25]. Furthermore at day 4, the culture is well stabilized and the cells are grown enough to provide the necessary amount of material for analysis.

To check the effect of GH, IGF-I and IGF-II on myocytes proliferation, cells on day 4 after seeding were washed and starved for 5 h with DMEM with 0.02% FBS to restrict cell growth. Next, the cells were incubated with DMEM with 2% FBS for 3, 6, and 18 h supplemented with the hormone indicated for each experimental condition. The need of FBS in the media for proper cell proliferation in response to the hormonal treatments was demonstrated after unsuccessful preliminary experiments in media without FBS and bovine serum albumin (BSA) instead (data not shown), as was previously reported for trout myocytes [7]. The doses used in the study were selected according to previously performed dose–response experiments. The treatments used were as follows: (1) human GH, hGH (GenWay Biotech, Inc., CA) at concentrations of 1 and 10 nM; (2) sea bream GH, sbGH (GenWay Biotech, Inc., CA) at a concentration of 10 nM; (3) human IGF-I, hIGF-I (Bachem, Germany) at concentrations of 10 and 100 nM; and (4) human IGF-II, hIGF-II (Bachem, Germany) at concentrations of 10 and 100 nM. Moreover, we checked the combined effect of: (5) hGH (10 nM) and both IGFs (I and II) (100 nM) and (6) sbGH (10 nM) and IGFs (I and II) (100 nM). Control cells were kept in DMEM with 2% FBS. The entire experiment for each hormone treatment was repeated three times independently with wells run in duplicate.

2.4. Cell proliferation assay

Cell proliferation was evaluated by immunocytochemical detection of the proliferating cell nuclear antigen (PCNA) with a commercial kit following the manufacturer's instructions (PCNA Staining Kit, Invitrogen, Barcelona, Spain). Briefly, when the incubation time ended at 3, 6 or 18 h after the addition of the hormones, the media was removed and the cells were washed in PBS and fixed in paraformaldehyde 4% for 20 min. Then, cells were washed in ethanol 50% and 70% for 5 min. Subsequently, the cells were washed three times in PBS, and then incubated with a mouse anti-PCNA monoclonal antibody (clone PC10) for 1 h. The goat anti-mouse biotin-labeled secondary antibody was added for 10 min; and the streptavidin–biotin peroxidase complex was applied for 10 more min. Next, 3,3'-diaminobenzidine (DAB) was used as the chromogen. The cells were counterstained with Mayer's hematoxylin for 2 min and washed in tap water. Finally, the cells were dehydrated in a graded alcohol series and mounted with histomount. The PCNA-labeling index was expressed as the percentage of the number of PCNA-labeled nuclei (positive cells) divided by the total number of nuclei examined. A representative image of the cells stained with PCNA is showed in Fig. 1. Pictures were taken with a CC2 camera coupled to a microscope using the analySIS[®] (Soft Imaging System) software. Five fields with approximately 200–400 cells per field were counted for each slide, two slides per condition. The same researcher counted all the slides by personal observation.

2.5. Statistical analysis

Each experiment was performed three independent times for each condition with wells in duplicate. Data are presented as mean \pm SEM. Statistical differences between conditions were analyzed by one-way analysis of variance (ANOVA), followed by the Tukey's test. Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Effects of hGH and sbGH on myocytes proliferation

After the establishment of the myocytes cell culture, first we checked the effect of GH peptides of different origins (mammals

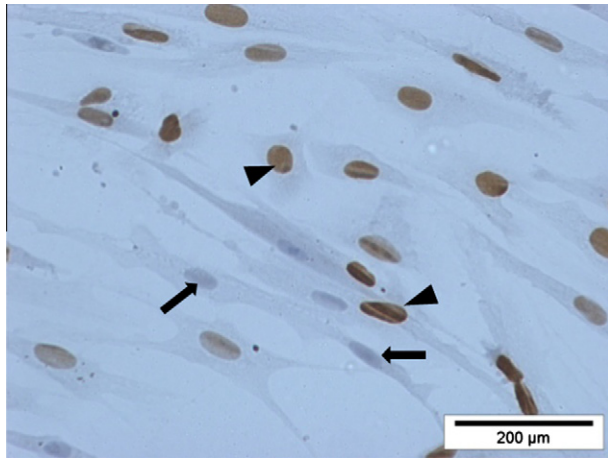


Fig. 1. Light micrograph of sea bream myocytes at day 4 in primary culture. Cell proliferation was assessed by immunocytochemical detection of proliferating cell nuclear antigen (PCNA). PCNA-containing nuclei are stained dark brown (arrow-heads). Counterstaining with Mayer's hematoxylin gave PCNA-negative nuclei a blue color (arrows). Bar = 200 μ m. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and fish) on cell proliferation, as shown in Fig. 2. Cultured muscle cells at day 4 (myocytes) were incubated with human GH (hGH) at two different concentrations (1 and 10 nM) and seabream GH (sbGH) at 10 nM. hGH only caused a significant stimulation on proliferative capacity at 3 h of treatment at the dose of 1 nM. Nevertheless, sbGH caused a significant increase on PCNA positive cells in all conditions tested.

3.2. Effects of hIGF-I and hIGF-II on myocytes proliferation

Following the studies with GH, the effects of human IGFs (hIGF-I and hIGF-II) on myocytes proliferative capacity were analyzed. The results obtained showed that both peptides have a significantly clear stimulatory effect on the percentage of PCNA positive cells at all times (Fig. 3). However, the effects of IGF-II (91–95%) were always much stronger than those of IGF-I (83–85%). In addition,

while no differences were observed among the different concentrations used (10 or 100 nM) within the same peptide, we observed a slight increase on the effects of both hormones throughout the incubation time.

3.3. Combined effects of GH and IGFs on myocytes proliferation

Finally, several treatments based on combinations of the GHs from both origins (mammals and fish) and the human IGFs (I and II) were used to analyze the synergistic effect of these peptides on muscle cell proliferation.

The combination of IGF-I with both GHs increased significantly the percentage of proliferative cells compared to the control, despite no differences were observed at any time using either sbGH or hGH with hIGF-I (Fig. 4). In this case, also a higher number of PCNA positive cells was found with the combinations (89–92%) in relation to the effect observed for IGF-I alone (83–85%) (Fig. 3). Furthermore, a similar increase in proliferation was observed for IGF-II in combination with both GHs, although significant differences were observed between the two different GHs at 6 h. In comparison to the effects caused by IGF-II alone (91–95%) (Fig. 3), significant differences with the IGF-II combined with GH (92–94%) were only observed at 6 h. In addition, as IGF-II alone had higher effects on proliferation than IGF-I, similar results were observed with the combination of IGFs with GH, being the stimulatory effects of GHs + IGF-II significantly higher than those of GHs + IGF-I, specially at 18 h of incubation.

4. Discussion

This is the first time that the *in vitro* effects of GH and IGFs in sea bream muscle cells proliferation in primary culture have been described. The present study aimed to assess the effects of several growth factors and growth hormone and their combination on the proliferative activity of myocytes based on previous studies performed with IGF-I [7] and IGF-II [10] in trout muscle cells. As an indicator of myocytes proliferation, we measured PCNA positive cells. This technique has been previously used to test the proliferative capacity of salmon pre-adipocytes and adipocytes [35] and on rainbow trout pre-adipocytes stimulated with IGF-I [3].

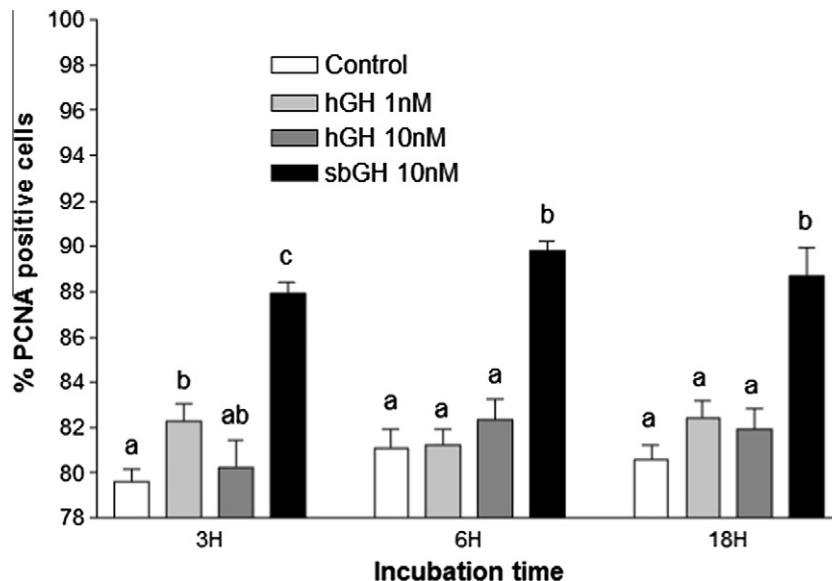


Fig. 2. Stimulation of cell proliferation by human GH (hGH) and sea bream GH (sbGH) in sea bream myocytes. Cells were isolated, seeded and the experiments performed during 3, 6 or 18 h as described in Section 2. Results are expressed as percentage of PCNA positive cells, mean \pm SEM ($n = 3$). Different letters indicate significantly ($P < 0.05$) different values between groups.

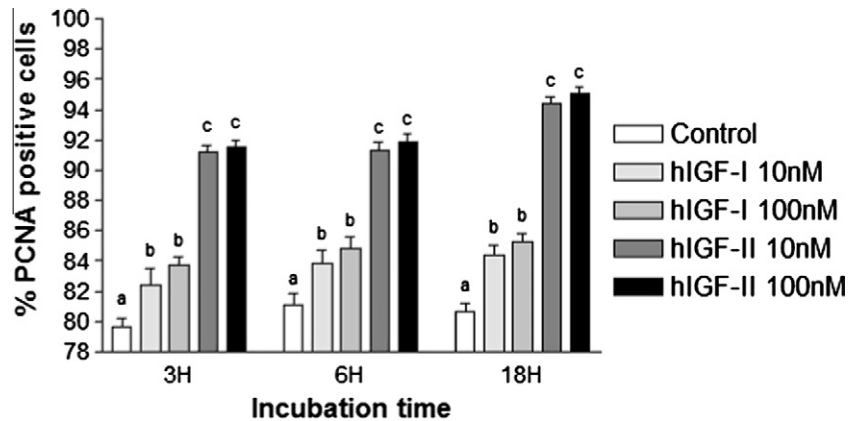


Fig. 3. Stimulation of cell proliferation by human IGF-I (hIGF-I) and human IGF-II (hIGF-II) in sea bream myocytes. Cells were isolated, seeded and the experiments performed during 3, 6 or 18 h as described in Section 2. Results are expressed as percentage of PCNA positive cells, mean \pm SEM ($n = 3$). Different letters indicate significantly ($P < 0.05$) different values between groups.

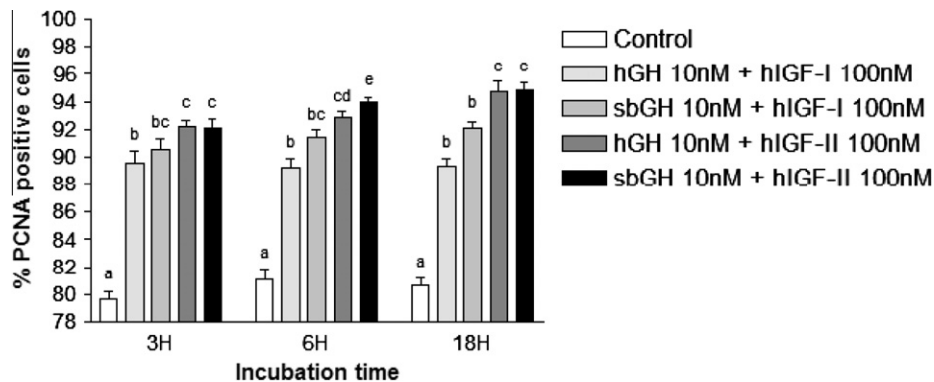


Fig. 4. Combined effects of GH (hGH and sbGH) and IGFs (IGF-I and IGF-II) on cell proliferation in sea bream myocytes. Cells were isolated, seeded and the experiments performed during 3, 6 or 18 h as described in Section 2. Results are expressed as percentage of PCNA positive cells, mean \pm SEM ($n = 3$). Different letters indicate significantly ($P < 0.05$) different values between groups.

First, we checked the effects of GH from two different origins (mammals and fish) on cell proliferation. Myocytes on day 4 were incubated with hGH or sbGH and significant differences on the effects of both peptides were observed, with piscine GH giving significantly higher values in all conditions. Interestingly, GH from teleostean fish is only 30–35% similar to human GH [28], and specifically, sbGH shares 34% identity with hGH. Therefore, such a structural difference can explain our results, where the sbGH showed increased effects due to higher specificity. This is in agreement with the first studies using heterologous hormone in rodents that observed that GH did not affect the proliferation or differentiation of satellite cells [1,2], however more recent literature have demonstrated the proliferative effect of GH in avian skeletal muscle cells using homologous GH [17,19]. This fact reinforces the species specificity of growth hormone.

Furthermore, the present study is the first one to show that proliferation in sea bream satellite cells is significantly stimulated by IGFs. In contrast with GH, IGFs are conserved polypeptides through evolution, and teleostean and human peptides, share up to 80% of identity [6]. Previous studies from Codina and coworkers [10] compared the effects of mammalian and fish peptides on 2-deoxy-D-glucose uptake in trout myocytes, and no differences were found using either one of the peptides. Consequently, we used human IGFs to test their effects on fish satellite cells. Both IGFs (IGF-I and II) showed a rapid and dose independent effect increasing proliferation of sea bream myocytes. This finding is in agreement with previous studies by Castillo and coworkers [7] and Codina and

coworkers [10], who demonstrated that IGF-I and IGF-II, respectively, significantly increase proliferation in rainbow trout (*O. mykiss*) muscle cells. In addition, it has been also shown *in vitro*, that both IGFs stimulate proliferation in embryonic cells from zebrafish (*Danio rerio*) with a similar mitogenic activity between them [29]. Moreover, our results showed significantly higher effects of IGF-II on proliferation than IGF-I. This data is in agreement with the higher expression observed for IGF-II in sea bream muscle in comparison to IGF-I [34], as well as in other fish species [5]. All these data point out for an important role of IGF-II in fish muscle proliferation or growth regulation.

Next, we also studied the combined effects of GHs and IGFs on sea bream myocytes proliferation. First, and according to the percentage values of proliferation obtained with sbGH and both IGFs (IGF-I and II) individually, we may conclude that the highest effect is caused by IGF-II (91–95%), followed by sbGH (87–90%) and lastly by IGF-I (83–85%). These results would suggest that IGF-I has a minor role in proliferation of sea bream myocytes at 4 days of culture than IGF-II or even GH; however, literature in mammals and fish [7,18,26,33] support the importance of IGF-I in muscle growth and this hypothesis would need further demonstration. A possible explanation for this data could be that IGF-I have a more important role at earlier stages of myogenesis (0–3 days). In fact, in cultured salmon myocytes the highest expression of IGF-I is at the beginning of myogenesis, decreasing to a very low levels until the stage of myotubes [5]. On the other hand, if we look at the combined effects of both peptides, GH and IGFs, our results showed that IGF-II

combined with both GHs (from mammals and fish) caused higher effects than the combinations of IGF-I with either GHs. This will support again the important role of IGF-II on satellite cells proliferation. In turn, Bower and coworkers [5] showed in cultured muscle cells of salmon that IGF-II has a higher expression throughout all the culture than IGF-I. The combination of IGF-I with both GHs had similar effects on proliferation in time, the dosage or the origin (human or sea bream) used. In all cases, the combined effects were higher (89–92%) than those of IGF-I alone (83–85%). This finding could indicate that, *in vivo*, IGF-I and GH play a synergistic action stimulating myocyte proliferation; however, there are no previous studies of any kind that have addressed this issue. Despite this, their effects in some other systems like mammalian cardiac tissue and bone were not compatible with an additive or synergistic effect of GH and IGF-I, indicating that perhaps tissue or species specificity exist [9,31].

Lastly, similar results were observed when the growth factor combined with GHs is IGF-II. With the combined treatments of IGF-II and sbGH the synergistic effect observed (92–94%) overcame slightly the effect of IGF-II alone (91–92%), 3 and 6 h after treatment. Moreover, at 18 h of incubation, the IGF-II proliferative effect was the same with or without GH, supporting again a different role for both IGFs on muscle growth and the importance of IGF-II regulating proliferation in sea bream myocytes.

In conclusion, these are the first studies on the effects of GH and IGFs on proliferation of gilthead sea bream myocytes. The results pointed out the important proliferative role of IGFs and GH in separated or combined treatments. Interestingly, IGF-II appears to be the stronger stimulator of proliferation. GH also stimulates muscle cells proliferation, but sea bream GH is more effective than its mammalian counterpart. Furthermore, IGF-I seems to play different roles and to maintain a different synergic relationship with GH than IGF-II. Future experiments must address new questions, like the effects of GH and IGFs on myocyte differentiation throughout the cell culture, analyzing the expression of myogenic factors such as MyoD and myogenin, and the local production of IGFs.

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