INVESTIGACIÓN

Biological activity of a new Growth hormone secretagogue: study in fish and murine cell line
La actividad biológica de un nuevo secretagogo de la hormona de crecimiento: estudio en el pescado y la línea celular murina

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ABSTRACT

Growth hormone (GH) has pleiotropic functions in all vertebrates. In addition to its essential role in the regulation of body growth and development, it can also influence reproduction, immunity, osmoregulation, and behavior. Immune and neuroendocrine systems have bidirectional communications and it is well documented the enhancing action of GH on the teleost immune system. It is desirable controlled GH administration to allow growth and stimulation of the innate immune system of fish. In this study, we have characterized a chemical peptide compound, A228, designed by molecular modeling, which is able to perform the function of a GH peptide secretagogue. In pituitary cell culture, the peptide A228 induces GH secretion. It is able to increase superoxide production in tilapia peripheral blood leukocytes cultures and in a macrophage cell line J774 from mice, therefore using this molecule, innate immune system stimulation is obtained in vitro both in fish and in mammal cell cultures. In this paper is also shown the biological action in vivo of the molecule, to assess growth stimulation in tilapia larvae.

Keywords: Biological activity, Growth hormone secretagogue, murine cell line.

RESUMEN

La hormona del crecimiento (GH) tiene funciones pleotrópicas en todos los vertebrados. Además de su papel esencial en la regulación del crecimiento y el desarrollo del cuerpo, sino que también puede influir en la reproducción, la inmunidad, la osmorregulación, y el comportamiento inmunológico y los sistemas neuroendocrinos tienen comunicaciones bidireccionales y se documenta bien la acción de mejorar la GH sobre el sistema inmune teleósteo. Es es la administración de GH controlada deseable permitir el crecimiento y la estimulación del sistema inmune innato de los peces. En este estudio, hemos caracterizado un compuesto peptídico químico, A228, diseñado por modelado molecular, que es capaz de realizar la función de un péptido secretagogo de GH. En el cultivo de células pituitaria, el A228 péptido induce la secreción de GH. Es capaz de aumentar la producción de superóxido en tilapia periféricos culturas leucocitos de la sangre y en una línea celular de macrófagos J774 de ratones, por lo tanto, el uso de esta molécula, la estimulación del sistema inmune innato se obtiene in vitro tanto en los peces y en cultivos de células de mamíferos. En este documento también se muestra la acción biológica in vivo de la molécula, para evaluar la estimulación del crecimiento en larvas de tilapia.

Palabras clave: actividad biológica, scretagogo, hormona del crecimiento, línea celular murina.

Introduction

Growth hormone (GH) is a pluripotent hormone with essential role in the regulation of body growth and development; it can also influence reproduction, immunity, osmoregulation, and behavior (1). It is produced by the pituitary gland in teleosts as in other vertebrates. GH gene is also expressed in other tissues of fish, especially in lymphoid organs and cells (2). The expression of GH is tightly regulated by several factors.

There are been demonstrated the interactions among elements of the endocrine and immune systems in fish. And it is desirable controlled GH administration to allow growth and stimulation of the innate immune system of fish.

The innate response is the basis of the immune defence of invertebrates and lower vertebrates. In fish, the innate immune response has been considered the essential component in combating pathogen invasions due to the limits placed on their adaptive immune response (3). The macrophage cell lineage represents an important group of cells which play a central role in the initiation and coordination of the immune response.

Among the different peptides involved in the regulation of expression of GH are the GHS. The synthetic GH secretagogues (GHSs) consist of a family of...
The decapeptide A228 (GKFGDLSPHEQ) y A233 (GKFDLSPEHQ) with an internal lactam bond between side chains of underlined amino acids) was manually synthesized on a solid-phase support. The peptide was purified by reverse-phase, high-performance, liquid chromatography up to 95% on a C-18 preparative column with an acetonitrile/water linear gradient. Trifluoracetic acid was used in both solvents for counter-ion pair formation. The correct sequence of the purified peptide was confirmed by electrospray mass spectrometry (Micromass, Manchester, UK). The positive control used was GHRP6 (Liptotec, Barcelona, Spain) lyophilized peptides were reconstituted in PBS.

Primary culture of pituitary cells
The in vitro effects of A233 GHS were examined using cells dispersed from whole pituitary. Mature tilapias of both sexes weighing 300–500 g were used after anesthesia in tricaine methanesulfonate (MS-222, Sigma). Pituitaries were collected aseptically in isotonnic medium (Krebs bicarbonate- Ringer solution, 330 m Osmolal, pH 7.4) supplemented with penicillin (100 IU/ml), streptomycin (0.1 mg/ml), and nystatin (250 IU/ml, all from Sigma). The pituitaries were diced with a sterile razor blade and treated with collagenase for 1 h at room temperature in 2.5 ml trypsin–EDTA solution (0.25% trypsin/C0.02% EDTA in PBS, pH 7.4). Tissues were aspirated repeatedly through a pipette during enzymatic treatment to promote dissociation of cells. The process was terminated by the addition of 0.5 ml (20%) fetal bovine serum (Sigma). Cells were counted on a hemocytometer under a light microscope and viability determined by trypan blue exclusion. Viability of the cells was always >95%. Cells were then plated at a density of 4.0×105 cells/well into a 24-well plate (Falcon, Primaria 24, Becton Dickinson, Franklin Lakes, NJ, USA) at a volume of 300 ml/well of isotonnic medium supplemented with 10% fetal bovine serum. The wells were preincubated for 4 days at 26–28°C under a humidified atmosphere of 95% O2 and 5% CO2, with one change of culture medium at 48 h post-plating. Before each experiment, cells were washed once with serum-free medium. A final 300 ml serum-free medium was added containing A233, GHRP6 (Liptotec), or control medium without hormones. The medium was replaced at 4 h. Incubations were terminated at 8 h, and hormone release was quantified for the 0–4 and 4–8 h intervals. GH release was expressed as secretion per unit volume of medium (ng/ml).

GH secretagoues
Peripherical blood leukocytes were isolated as described by Acosta et al, 2010(12) .Tilapias were anesthetized in tricaine methanesulfonate (MS222), and blood was collected from the caudal vessels using a heparinized syringe. Blood was diluted 1:2 in RPMI-1640 medium, containing 4 μg/mL of gentamicin. The mixture was placed on Ficoll-Paque™ Research Grade (Amersham Pharmacia Biotech, Sweden) and centrifuged at 400 g for 12 min at 4°C. The supernatant was collected, and the leukocytes were washed once with RPMI-1640 medium containing 10% fetal bovine serum. The leukocyte pellet was resuspended in 1 ml of RPMI-1640 medium containing 10% fetal bovine serum and 2 μg/mL of gentamicin. The cell suspension was incubated at 37°C for 30 min to promote the dissociation of the leukocytes. The cell suspension was then washed as described above and resuspended in 1 ml of RPMI-1640 medium containing 10% fetal bovine serum and 2 μg/mL of gentamicin. The cell suspension was incubated at 37°C for 30 min to promote the dissociation of the leukocytes. The cell suspension was then washed as described above and stored at 4°C until the time of the assay. Leukocytes were incubated with 0.2 M Na2HPO4, 0.1 M citric acid (pH 5.0) containing 0.5 mg/ml ortho-phenylenediamine and 5 ml 30% H2O2 was added (100 ml/well). The reaction was stopped 15 min later by adding 50 ml of 2.5 M sulfuric acid per well. The absorbance was measured at 492 nm using the TiterTec Multiskan Plus spectrophotometer. The accepted upper limit of the assay background was 0.094. The lower detection limit of the assay was 0.1 ng/mL. The degree of variation and intra- and interassay coefficients of variation are 3.90 and 13.45% respectively.

Isolation and culture of leukocytes
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Results

The molecules A 228 and A 233 (Fig. 1A) were selected from the virtual libraries described by Rodriguez et al. 2007(10), and, in order to determine in vitro and in vivo whether this novel mimetic molecules function as GHSs, the following assays were conducted on the growth and immune system.

GH in vitro assays using pituitary cell culture

To evaluate the effect of A228 and A233 peptide on GH secretion, we performed an in vitro culture of cells in the pituitary gland of tilapia (Oreochromis sp.). The peptides at a concentration of 10 nM stimulated GH secretion at 8 h by cells in the anterior pituitary gland (Table). The stimulatory effect of GRLN and GHSs on the release of GH in vitro by the pituitary gland has been reported in mammals, birds, and different species of fish (14,15,16, 17, 18).

Determination and characterization of superoxide anion induction in PBLs and macrophage cell line J774

Superoxide anion produced by cultured tilapia (Oreochromis sp.) PBLs were measured by the addition of A 228 as positive control GHRP-6 was included and also A233, which in previous results was capable of enhance superoxide anion production in leukocytes from tilapia head kidney. The peptides induced significant increases in superoxide production. The concentration of peptide used was 10 nM taking in consideration previous results (19), the increase of superoxide anion production was statistically higher in cells stimulated with the peptides, to compare with no treated cells, among the different treatments there were not statistical differences, in both cultures, PBLs (Figure 2) this effect is similar to that reported by Acosta et al. 2010,(12) who stimulated phagocytic peripheral blood cells of tilapia (Oreochromis sp.) with recombinant tiGH (Oreochromis hnororum).

The results obtained in the production of superoxide anion with the macrophage cell line J774 after the stimulation with the peptides, were very similar, there were obtained statistically differences in the superoxide anion production of cells treated with the peptides, and compare with negative control. (Figure 3)

In vivo biological activity assays

Experiments were performed to evaluate the biological effect of the peptide on the growth rate of tilapia larvae. The effect of these peptides on fish growth was determined by measuring the increase in body weight. The tilapia larvae treated with A228 showed a significant increase in body weight compared with the controls as well as the larvae treated with GHRP-6, just 9 days after treatment.

Discussion

In this study, we demonstrate the effect of another peptide molecule having internal cycles and composed solely of L-amino acids that are capable of exerting, due to their chemical structure, similar functions to those attributed to GRLN, des-acyl GRLN, and other peptide GHSs. They are a group of peptide compounds and peptides capable of stimulating production and GH secretion in vitro and in vivo.

There were not differences between both peptides A228 and A233, the structural difference do not interfere on GH secretagogue function at least in this kind of primary culture of pituitary cells experiment.

Our results are similar to those obtained in tilapia (Oreochromis mossambicus), where the effect of GRLN on GH secretion in vitro was dependent on the concentration of the endogenous secre-
Fig. 1. 2D representation of the peptide amide A228 (GKFGDLSPEHQ) and A233 (GKFDLSPHQ), with an internal lactam bond between side chains of underlined amino acids. The lactam bond between Lys and Asp was formed during the synthesis procedure on solid-phase support and is essential for the biological activity of the molecule.

tagogue used (15). Other researchers have reported the stimulatory effect of the synthetic peptides GHRP-6, PACAP, and PACAP-related peptide from *Clarias gariepinus* on the release of GH in vitro by the pituitary gland of tilapia (*Oreochromis niloticus*); (11,19). There should be conducted an experiment using the A228 peptide to study the GH releasing in vivo after the administration of GHS.

It has been reported that the immune system of both fish and mammals possesses both non-specific and specific immune responses with cellular and humoral components. However, fish depends more heavily on non-specific defence mechanisms. The use of immunostimulants given as dietary supplement can improve the innate defense of aquatic animals principally fish and thus providing resistance to pathogens during period of stress, such as grading, sea transfer and vaccination.

Neutrophils and macrophages are known as the phagocytic leucocytes in fish peripheral blood (20). Macrophages play a key role in the host immune system. They are in the first line of defence, participating in detection and identification of potential pathogens. As part of mechanisms involved in the innate system they respond to stimuli activating phagocytosis and releasing reactive oxygen and nitrogen species which destroy microbes. They also initiate the inflammatory response through cytokine production, and furthermore, macrophage acts a link between the innate and adaptive immune responses acting as antigen-presenting cells to prime T cells. (21)

The existence of the binding sites of GH has been shown in both types of leucocytes in the gilthead sea bream *Sparus aurata*. It is known also that the administration in vivo and in vitro GH
stimulates the production of superoxide anion after phagocytosis as a mechanism for elimination of pathogens (2).

In our result we could state the biological action of or peptide in fish and mammals system. There are controversial reports about the effects of ghrelin on phagocytic activity of leukocytes. In fish (rainbow trout), the administration of ghrelin increased phagocytosis and superoxide production in zymosan-stimulated leukocytes, which was abolished by pretreatment of leukocytes with a GHS-R antagonist (2). It was also shown that ghrelin increased mRNA levels of superoxide dismutase and GH in leukocytes, suggesting that the effects of ghrelin was mediated, at least in part, by stimulating GH secretion from leukocytes. On the other hand, ghrelin administration reportedly reduced the elevated phagocytic activity of peritoneal macrophages induced by acute cold-restraint stress in rats (22). In our study we have found an increase of superoxide anion in fish PBL and cell line J774 from murine macrophages after A228 stimulation even has been reported that ghrelin modulates phagocytosis directly or indirectly via GH, but in a different way in different species it should be considered the treatment conditions to evaluate this biological activity.

GHSs are useful molecules as growth enhancement molecules. These synthetic molecules are effective in stimulating production and release of endogenous hormone as a physiological response, with no side effects on the pituitary or toxicity potential; besides their low molecular weight, it makes a better entrance to the organism.

In our laboratory, we have successfully employed the immersion bath technique to study the effects of nutritional supplements and growth factors on growth control and the immune system in fish (12). The immersion bath method used for our studies requires little manipulation and causes minimum stress to fish during treatment. There is evidence suggesting that the gill pillar cells are a possible entry site for some molecules when fish are treated by immersion bath (6).

This study evaluated the biological function of synthetic peptide A228 on weight gain of tilapia (Oreochromis sp.). Tilapia larvae showed a significant increase in growth after 9 days of treatment with peptide A228 (0.1 mg/l). All animals received the same commercial diet, so the increase in weight and height is due to the administration of peptide A228. The positive control group represented by the fish treated with the peptide GHRP6 significantly increased their growth. Similar results were obtained in a former work to administrate A233 peptide, another GHS, to fish larvae enhancing growth and some. Humoral innate immune system parameters (19).

These results are similar to those found in mice where there was an increase in body weight after dosing by s.c. injection of the synthetic peptide GHRP2 (23). In adult rats, GHRP6 also increased body weight (24) as well as other GHSR agonists, like SM-130686 administered orally (25) and BIM-28131 administered by s.c. injection (26). It has been reported that GHSR1a agonist, a pentapeptide with D-amino acids, promotes weight gain in rats, by i.p. administration during 7 days (27). Moreover, the administration, by the same method, of GH tilapia (O. hornorum) secreted into the culture supernatant of yeast Pichia pastorii (28) to larvae of tilapia (Oreochromis sp.), significantly increased growth of these animals. Administration of tilapia recombinant neuropeptide Y (Oreochromis sp.) to larvae of African catfish (C. gariepinus) also produced an increase in animal weight (29).

In addition, treatment with PACAP and PACAP-related peptide from C. gariepinus larvae of African catfish (C. gariepinus), tilapia (O. niloticus), and common carp (Cyprinus carpio) increased body weight and length in three fish species (11).

The application of the peptide during the early stages of fish development should be very important. The peptide designed by modeling bioinformatics from human ghrelin receptor and obtained by chemical synthesis is recognized by the secretagogue GHS-R1a, stimulating the secretion of growth hormone due to the action of growth hormone results in further development of cells and tissues of the larvae. It promotes accelerated growth with increased weight concurrently is recognized by immune system cells and stimulates the innate immune response, the main defense mechanism of fish during the early stages of life as a result you can get higher quality larvae.

To summarize, the action of this GHS has been assessed employing in vitro and in vivo methods, we have demonstrated the effect of a new growth hormone secretagogue, A228, as a growth hormone secretagogue, that elicits GH in tilapia pituitary cell culture and also has stimulatory effect on the superoxide anion production on tilapia PBL and a cell line J774 from mice macrophages. To administrate the molecule using immersion baths, the weight gain is enhanced in tilapia larvae. These results support the interrelation between endocrine and immune system.

![Graph](image)

**Fig. 2.** Effects of A228 and A233 on superoxide anion production by tilapia (Oreochromis sp.) PBLs. Cells were incubated with the peptides for 4 h at 28 °C in 5% CO2. Cells with Hanks’ solution (pH 7.4) were the control. Data are expressed as the absorbance at 620 nm and bars represent S.D. of the mean (S.E.M., n=5). Statistical differences between groups for Newman–Keuls’s test.

![Graph](image)

**Fig. 3.** Effects of A228 and A233 on superoxide anion production in macrophage cell line J774. Cells were incubated with the peptides for 4 h at 37 °C in 5% CO2. Cells with Hanks’ solution (pH 7.4) were the control. Data are expressed as the absorbance at 620 nm and bars represent S.D. of the mean (S.E.M., n=5). Statistical differences between groups for Newman–Keuls’s test.

**References**

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Fig. 4. Growth promotion experiment in Oreochromis sp. larvae immersed in GHRP6 and A228 peptide at a dose of 0.1 mg/l. (A) Effect on length and weight over 9 days of Treatment in fish larvae; samples of 50 animals were weighted at 7 days. The control group did not receive any treatment. Kruskal–Wallis test followed by Dunn’s multiple comparison post test was used for length and body weight comparisons among groups. Different letters represent statistical significance. Data are expressed as mean ± S.D. (n=50).


