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Effects of growth hormone on the salmon pituitary proteome

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ABSTRACT

Growth hormone 1 (GH1), a pituitary hormone, plays a key role in the regulation of growth. Both excess GH1 treatment and overexpression of a GH1 transgene promote growth of salmon, but these animals exhibit physiological abnormalities in viability, fertility and metabolism, which might be related to pituitary function. However, the molecular dynamics induced in the pituitary by excess GH1 remain unknown. In this study, we performed iTRAQ proteome analysis of the amago salmon pituitary, with and without excess GH1 treatment, and found that the expression levels of proteins related to endocrine systems, metabolism, cell growth and proliferation were altered in the GH1-treated pituitary. Specifically, pituitary hormone prolactin (2.29 fold), and somatolactin α (0.14 fold) changed significantly. This result was confirmed by proteome and transcriptome analyses of pituitary from the GH1-transgenic (GH1-Tg) amago salmon. The dynamics of protein and gene expression in the pituitary of GH1-Tg amago salmon were similar to those of pituitary treated with excess GH1. Our findings suggest that not only excess GH1 hormone, but also the quantitative changes in other pituitary hormones, might be essential for the abnormal growth of amago salmon. These data will be useful in future attempts to increase the productivity of fish farming.

1. Introduction

Pituitary, an endocrine organ, synthesizes and secretes various peptide hormones including growth hormone (GH), prolactin (PRL), somatolactin (SL), thyroid-stimulating hormone (TSH), luteinizing hormone (LH) and follicle-stimulating hormone (FSH) [1]. The pituitary also produces adrenocorticotropic hormone (ACTH), melanocyte-stimulating hormone (MSH) and β-endorphin, all of which are generated by proteolytic cleavage of a common pro-hormone, proopiomelanocortin (POMC) [1].

Each pituitary hormone is released into the bloodstream and each acts on different target organs in order to strictly regulate a variety of biological functions [2] such as the stress response [3], appetite [4,5] sexual maturation [2,6], thereby helping to maintain physiological homeostasis, growth and reproduction. Simultaneously, the synthesis and secretion of these hormones are controlled by hormonal feedback regulation systems [7].

Among the pituitary hormones, GH is classified into two divergent paralogs in fish, *GH1* and *GH2* [8]. GH1 has been well studied [7,10–12], though the function of GH2 is not well understood

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[9]. In target tissues, GH1 binds to the GH receptor (GHR), and also acts indirectly through locally GH1-induced IGF-I production [7,10–13], in order to promote proliferation and differentiation of muscle, bone and cartilage cells. Manipulation of GH1 levels would therefore be an important potential means of increasing the productivity of fish farming [14,15].

GH1-treated/GH1 transgenic fish frequently exhibit physiological abnormalities, such as reductions of viability [16], fertility [17], and alteration of metabolism [18,19]. However, the details of the molecular dynamics in GH1-treated fish remain unknown.

In this study, we investigated the effects of excess GH1 on amago salmon pituitary. In order to identify pituitary proteins induced by excess GH1 treatment, we performed comprehensive and quantitative iTRAQ proteome analysis of the pituitary under cultured condition with and without excess GH1. In these experiments, we identified both up-regulated and down-regulated pituitary proteins involved in regulation of endocrine systems, metabolism, cell growth and proliferation. We used bioinformatic analysis to predict the phenotypes that would be affected by alterations in levels of these proteins. Furthermore, in order to confirm the relationship between excess GH1 and the regulation of proteins, we generated GH1-transgenic (GH1-Tg) amago salmon, characterized GH1-Tg phenotypes, and performed comparative quantitative proteome and transcriptome analyses of pituitary from GH1-Tg and non-transgenic (non-Tg) amago salmon. We observed that the dynamics of protein and gene expression in the pituitary of GH1-Tg amago salmon were similar to those observed in pituitary treated with excess GH1.

Based on these results, we hypothesized that not only excess GH1 hormone, but also quantitative changes in levels of the other pituitary hormones, might be important determinants of the abnormal growth of amago salmon. These findings will be important in efforts to increase the productivity of fish farming.

2. Materials and methods

2.1. *Pituitary culture for proteome analysis*

Amago salmon (*Oncorhynchus masou ishikawae*) were grown in circular tanks at 15 °C under a natural light cycle at the Fisheries Research Agency of the National Research Institute of Aquaculture in Japan. For proteome analysis, pituitaries were removed from 20 ice-anesthetized sexually immature amago salmon and immersed in ice-cold RPMI medium containing 20 mM Hepes, 9 mM sodium bicarbonate, 100 U/ml penicillin, 100 U/ml streptomycin, and 0.25 mg/ml fungizone. Next, pituitaries were incubated separately for 4 days at 12 °C in medium for organ culture [RPMI medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 U/ml streptomycin, and 0.25 mg/ml fungizone] with or without excess purified bovine GH1 (12.5 μg/ml, >95% pure) in order to examine the effect of excess GH1 on pituitary proteins dynamics. The method was followed by the previous report for organ culture condition of pituitary [20]. It is well known that mammalian GH1, including bovine, can bind to the fish GH1 receptor, and stimulate the growth of salmon. After culture, each pituitary was washed twice with cold PBS and homogenized in 1 ml lysis buffer [7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 2% (w/v) DTT, 0.05% (w/v) SDS, and protease inhibitor cocktail] using a Polytron homogenizer (Kinematica, Bohemia, NY, USA) on ice, followed by centrifugation at 18,850 g for 30 min at 4 °C. The cleared supernatant was re-centrifuged at 69,600 g for 60 min at 4 °C. The final supernatant was stored at −80 °C until further use.

2.2. *Proteome analysis using iTRAQ reagents*

For iTRAQ labeling, the protein sample buffer was exchanged with 10 mM triethylammonium bicarbonate buffer, pH 8.5 (Sigma-Aldrich, St. Louis, MO, USA) using Microsep™ 3 k Centrifugal Devices (Pall, Port Washington, NY, USA). The protein concentration of samples was determined with a PROTEIN ASSAY kit (Bio-Rad Laboratories, Rockville, MD, USA), using bovine serum albumin as a protein standard. After cysteine blocking with methyl methanethiol sulfonate (MMTS), 32 μg of each protein sample was digested with trypsin. The resultant peptides were labeled with iTRAQ regents (AB-Sciex, Foster City, CA, USA) (Supplementary Fig. 1). After iTRAQ labeling, the samples were combined in a 1:1 ratio (v/v). Half of the combined mixtures of iTRAQ-labeled peptides were subsequently fractionated by a 2D-liquid chromatography system equipped with a strong cation exchange (SCX) column [HiQ Sil SCX, 0.5 mm inside diameter (id)×35 mm, KYA Tech, Tokyo, Japan] in the first-dimension separation, and a reverse-phase (RP) trap column (HiQ Sil C18-3, 0.8 mm id×3 mm, KYA Tech) in the second-dimension separation. The details of fractionation are provided in Supplementary Fig. 1. The fractionated peptides were automatically mixed with the MALDI matrix solution (4 mg/ml α-cyano-4-hydroxycinnamic acid dissolved in 80 μg/ml dibasic ammonium citrate containing 0.1% TFA and 70% acetonitrile), and directly spotted onto four ABI 4800 MALDI plates using a MALDI plate spotter (DiNa Direct Nano-flow LC system; KYA Tech). MS and MS/MS analyses of iTRAQ-labeled peptides were carried out on a MALDI-TOF/TOF mass spectrometer (4800 Proteomics Analyzer; AB-Sciex) in positive ion reflection mode using the 4000 Series Explorer Software (Ver. 3.5; AB-Sciex). The instrument laser power was set to 2800 for MS and 3600 for MS/MS acquisition. Typically, 1000 laser shots were accumulated per well, and MS spectra were acquired from 800 to 4000 Da with a minimum S/N filter of 50 for precursor ion selection. MS/MS analyses were performed for the 10 most abundant precursor ions per well, with an accumulation of 2000 shots for each spectrum. In order to look for less abundant proteins, re-interrogation of the target plates was carried out to acquire the 10 next-most intense peaks (if any were above the S/N threshold of 25). The series of total MS and MS/MS spectrum measurements was performed in duplicate, and these results were combined for data analysis.

MS and MS/MS data were analyzed using the ProteinPilot software ver 3.0 (AB-Sciex), which employs the Paragon algorithm for protein identification and relative quantitation [21]. The database (147,591 entries) consisted of amino acid sequences of fish proteins, retrieved from a subset of the NCBI non-redundant (nr) protein database (accessed April 14 2009) used for this search. The search parameters included iTRAQ labeling at N-terminus and lysine, cysteine modification by MMTS, methionine oxidation, and biological modifications predefined in the software. Other