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Quantitative bioassays for measuring biologically functional gonadotropins based on eel gonadotropic receptors

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ABSTRACT

Significant declines in eel stocks have been noted in many parts of the world. Because eel aquaculture is dependent on wild-caught juveniles, there is a need to achieve artificial reproduction. Adult eel maturation is currently induced by repeated injections of purified gonadotropin (human chorionic gonadotropin [hCG]) or pituitary extract. Thus the determination of the biological efficacy and quantification of internal levels of gonadotropic hormones is important for optimizing artificial reproduction protocols. To quantify the plasma levels of biologically functional gonadotropic hormones, we developed a bioassay for luteinizing hormone (LH) and follicle-stimulating hormone (FSH) based on the stable expression of receptors in HEK293 cells of the Japanese eel *Anguilla japonica* LH (ajLHR) and the European eel *Anguilla anguilla* FSH (aaFSHR), respectively. Such cells also contain a firefly luciferase reporter gene driven by a cAMP-responsive element (CRE-Luc). We found that the obtained stable cells, with ajLHR, responded linearly to a more than 100,000-fold concentration range of hCG diluted in saline. The cells with aaFSHR showed a linear response to a 1000-fold concentration range of salmon pituitary extract mixed with saline. The biological functionality of the LH and FSH bioassays was validated using hCG, human FSH, and pituitary extracts from salmon, carp and eel. Since the toxins in eel plasma damaged the HEK293 cells, the protocol was adapted to selectively inactivate the toxins by heating at 37 °C for 24 h. This process successfully enabled the monitoring of hormone levels in blood plasma sampled from hCG-injected eels. In this paper, we describe the development of gonadotropin bioassays that will be useful for improving reproduction protocols in eel aquaculture.

1. Introduction

The drastic stock decline of eels, particularly in commercially valuable species such as the European, Japanese and American eels, has been a serious issue with respect to biodiversity and sustainable fish resources. Because eel aquaculture is fully dependent on the annual recruitment of natural populations of glass eels into estuaries and rivers, there is a growing need for successful artificial reproduction. Attempts to achieve this objective started with the European eel in the 1930s [5] and markedly-improved protocols and techniques have readily yielded eggs and larvae [11,17,21,23]. Successful development until the juvenile stage has, however, been very limited and a large-scale commercial production of glass eels is not yet available, even for the Japanese eel for which artificial breeding has recently been accomplished [18,36]. Thus, artificial reproduction of eels is still not sufficient for the needs for aquaculture and stock restoration.

Artificial sexual maturation of adult eels is currently induced by repeated injections of human chorionic gonadotropin (hCG) for males, and salmon or carp pituitary extract (SPE and CPE, respectively) for females [5,6,24,26,27]. The injected gonadotropins, which act on the testes and ovaries of eels to promote gametogenesis and reproductive function, can function as the endogenous pituitary glycoprotein hormones, i.e., luteinizing hormone (LH) and follicle-stimulating hormone (FSH). As observed in some fish species such as salmon, these pituitary gonadotropins generally have a temporal secretion pattern during the reproductive cycle. Thus FSH and LH are assumed to differentially regulate gonadal maturation [16]. It has been shown that LH stimulates production of the maturation inducing steroid (17α,20β-dihydroxy-4-pregnen-3-one [DHP]) and final maturation in ovaries of salmonids and red seabream [10,14,29,35] and that FSH is linked to recruitment of oocytes during vitellogenesis in rainbow trout [37] and estradiol secretion from tilapia ovaries [2]. In eels it is generally considered that FSH is concerned with early gametogenetic stages, whereas LH is likely to be crucial for vitellogenesis and the final stage of gonadal development [9,28,32]. Focusing on pituitary

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and plasma levels of these hormones is therefore very important when artificial maturation is employed.

Hormone levels in eel blood plasma have been previously determined using radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) [8,22,25,28]. Investigations into gene expression levels of eel gonadotropins in various tissues have been made use of quantitative real-time PCR [3,9,33,34]. Although the above mentioned methods are highly sensitive, they often require specialized laboratory equipment, the use of radioactive compounds, the development of species-specific antibodies, and sacrificing of animals. Furthermore, ELISA measures the existence of specific peptides, but it does not necessarily produce information on the biological functionality of the peptide under investigation [30]. In this context, a bioassay based on a cloned receptor is more advantageous, particularly for studies on maturation and reproduction of eels, because it only measures the biologically active hormones in a sample.

In this paper, we describe luciferase reporter gene-based bioassays, which allow the quantification of gonadotropic hormone levels in eel blood plasma. A cAMP-mediated luciferase reporter gene assay involves cells that stably express LH or FSH receptors on their membranes and carry a firefly luciferase reporter gene driven by a cAMP-responsive element (CRE-Luc). When the hormone binds to, and then activates, the receptors, the intracellular cAMP level increases and subsequently promotes the production of firefly luciferase, thus enabling quantification of gonadotropic hormones in a sample based on luminescence intensity using a standard curve. We have adapted the protocol of our gonadotropin bioassays to eel blood plasma, so as to monitor hormone levels during maturation without having to sacrifice animals.

2. Materials and methods

2.1. Recombinant DNA constructs

A synthetic cDNA fragment that comprised the complete open reading frame of the *Anguilla japonica* LH receptor (hereafter, ajLHR; NCBI/GenBank/DDBJ accession No. EU635883) was obtained from BaseClear (Leiden, The Netherlands). A cDNA fragment of *Anguilla anguilla* FSH receptor (hereafter, aaFSHR; AB700600) was amplified by reverse transcription polymerase chain reaction (RT-PCR). Total RNA was isolated from the ovary of a European eel using Trizol Reagent and a miRNeasy mini kit, following the manufacturer's protocol (Qiagen, Hilden, Germany). One microgram of total RNA was reverse-transcribed in a 5 μL reaction volume containing 12 μM of random hexamer primers (Roche, Basel, Switzerland) and Moloney murine leukemia virus reverse transcriptase following the manufacturer's protocol (Bio-Labs, Ipswich, MA, USA). Using the synthesized cDNA as template DNA, PCR was performed in a 50 μL of reaction volume containing 400 nM of forward and reverse primers (Aa_FSHR_for, 5′-AGCTAAGCTTGGATCCACCATGACACCTCTGTGGGTCCTCCT-3′; Aa_FSHR_rev, 5′-AGCTGCGGCCGCTCAGTGGGGGTTGTTGATGGGCAC-3′), 5 μL of 10× PCR buffer containing 18 mM of $MgCl_2$, 0.2 mM of dNTP, and 2.5 units of Enzyme Blend (Roche). The primers were designed for the subsequent directional cloning and the initiation of translation via Kozak consensus sequence (ACCATG). The temperature profile was 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 3 min after heating at 95 °C for 2 min. The amplified PCR product was purified using Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Buckinghamshire, United Kingdom). The obtained ajLHR and aaFSHR cDNA fragments were respectively cloned into a pcDNA5-FRT vector (Invitrogen, Paisley, UK) using *Hind*III and *Not*I sites (yielding pcDNA5-FRT-ajLHR and pcDNA5-FRT-aaFSHR, respectively). The insertion was confirmed by DNA sequencing.

2.2. Generating the gonadotropin receptor / luciferase reporter cell lines

Flp-In HEK293 cells were cultured and treated following the manufacturer's protocol (Invitrogen). Flp-In system allows integration of the gene(s) of interest into a specific site in the genome of mammalian cells. Briefly described, the Flp-In cell lines contain a single integrated Flp Recombination Target (FRT) site, which facilitates integration of the gene(s) of interest into a specific site in the genome of mammalian cells. This is achieved by the cloned gene of interest to a pcDNA5/FRT vector followed by co-transfection with pOG44 that expresses Flp recombinase and mediates a homologous recombination event between the FRT sites (the one integrated in the genome of Flp-In cells and the one on pcDNA5/FRT). Accordingly, the pcDNA5/FRT construct is inserted into the genome at the integrated FRT site. This process is accompanied by changes in antibiotics resistances and β-galactosidase activity and results in the expression of the recombinant protein of interest (see the manufacturer's manual for the complete description).

One day prior to transfection, 1.6×10^6 cells were seeded in a 10 cm-dish in 10 mL of the complete medium (Dulbecco's modified Eagle's medium [DMEM] with high glucose [4.5 g/L] containing 10% fetal calf serum, streptomycin [100 μg/mL], penicillin [100 IU/mL]), and incubated at 37 °C overnight. The culture medium was replaced with a serum-free medium just before transfection and pcDNA5-FRT-ajLHR or pcDNA5-FRT-aaFSHR were respectively transfected into HEK293 cells together with pOG44 (Invitrogen) (1:9 in mass), using FuGENE6 Transfection Reagent and following the manufacturer's protocol (Roche). At 8 h after transfection, the medium was replaced with the complete medium (without antibiotics) and incubated at 37 °C overnight. Cells were then placed under hygromycin B-resistance selection (150 μg/mL). The hygromycin B-resistant transgenic cell clones were identified, and each of the isolated clones was subjected to subsequent screening to test Zeocin sensitivity (100 μg/mL) and lack of β-galactosidase activity (*lacZ* staining) (see the manufacturer's instruction, Invitrogen). Clones that showed all the required properties, i.e., hygromycin B-resistance, Zeocin sensitivity and *lacZ* staining-negative, were selected as the cells that carry a single copy of the gonadotropin receptor cDNA (HEK-ajLHR, HEK-aaFSHR). Stable integration and constitutive expression of receptor cDNA were confirmed by genomic PCR and RT-PCR, respectively.

To test the hormone binding ability of eel gonadotropin receptors on the HEK293 cell membrane, a luciferase reporter gene assay was performed, based on transient transfection of pCRE-Luc. To test the ajLHR, 3×10^4 HEK-ajLHR cells were seeded to each well of a 96-well plate and incubated at 37 °C overnight. Co-transfection of pCRE-Luc (Stratagene, La Jolla, CA, USA) and pRL-CMV (Promega, Fitchburg, WI, USA), the latter which carries a cDNA of *Renilla* luciferase, CMV enhancer and early promoter elements to provide high-level expression of *Renilla* luciferase for use as an internal control reporter, was performed (9:1 in mass) using FuGENE6 Transfection Reagent and following the manufacturer's protocol (Roche). At 30 h after transfection, the culture medium was replaced with 72 μL of serum-free DMEM and cells were exposed to ligand for 5 h by adding 8 μL of purified hCG (Sigma–Aldrich, St. Louis, MO, USA) dissolved in saline. A reporter gene assay was performed using Dual-Glo Luciferase Assay System (Promega) following the manufacturer's protocol. To test the aaFSHR, 1×10^6 HEK-aaFSHR cells were seeded to each well in a 6-well plate and incubated at 37 °C overnight. pCRE-Luc (Stratagene) was transfected using XtremeGENE HD DNA Transfection Reagent (Roche), following the manufacturer's protocol. At 24 h after transfection, cells

were trypsinized and 1×10^5 cells were seeded in a well of a 96-well plate and incubated at 37 °C overnight. Culture medium was replaced with 72 μL of serum-free DMEM and cells were exposed to ligand for 5 h. As ligand, SPE (Argent laboratories, Redmond, WA, USA) was mixed with saline (20 mg/mL), of which 8 μL was added to each well. The reporter gene assay was performed using Steadylite plus Reporter Gene Assay System (Perkin Elmer, Waltham, MA, USA) following the manufacturer's protocol. The luminescence signal was measured on either Wallac 1450 Microbeta Jet or Victor^3 multilabel plate reader (Perkin-Elmer). All measurements were performed in duplicate. The most sensitive clone for each receptor was used for subsequent transfection for the stable integration of the luciferase reporter construct.

The construct of a firefly luciferase reporter gene driven by a cAMP-responsive element (pCRE-Luc) was further integrated into HEK-ajLHR and HEK-aaFSHR cells as follows. 1.6×10^6 cells of each line were seeded in a 10 cm-dish in 10 mL of the complete medium, one day before transfection. After replacement with fresh plain DMEM, a mixture of pCRE-Luc and a Neomycin-resistant cassette (2:1 in mass) was transfected into cells using either FuGENE6 Transfection Reagent or XtremeGENE HD DNA Transfection Reagent following the manufacturer's protocols (Roche). The transfection medium was replaced with the complete medium without antibiotics at 8 h after transfection and incubated at 37 °C overnight. Cells were then placed under Neomycin resistance selection (750 μg/mL of G418), and Neomycin-resistant clones were identified and isolated.

The sensitivity of the selected clones that stably expressed eel gonadotropin receptors along with CRE-Luc and Neomycin-resistance gene (named HEK-ajLHR-luc and HEK-aaFSHR-luc) were evaluated by a luciferase assay using hCG, human FSH (3H Biomedical, Uppsala, Sweden; hereafter hFSH), SPE, CPE (Argent laboratories) and eel pituitary (hereafter, EPE). Eel pituitaries were obtained from female European eels with a gonadosomatic index (GSI) of 1.1 (without hormone treatment) and 33.9 after 14 weekly injections of SPE (20 mg/mL/injection). After dissection, EPE was prepared following Adebayo and Fatoyinbo [1]. 3×10^4 HEK-ajLHR-luc or HEK-aaFSHR-luc cells were seeded to each well of a 96-well plate and incubated at 37 °C overnight. The culture medium was replaced with 72 μL of fresh serum-free DMEM and 8 μL of ligand solutions were immediately added to each well. After 5 h of cell stimulation, a luciferase assay was performed using Steadylite plus Reporter Gene Assay System (Perkin Elmer) as described above. All measurements were performed in duplicate or triplicate.

2.3. Adaptations of the bioassay protocols to eel blood plasma

A preliminary analysis in this study found eel blood plasma to be lethal to HEK293 cells. The toxicity of eel blood is well known as it was reported a long time ago [7]. Thus the assay condition needed to be optimized for the use of eel blood plasma as a sample. Since the toxic compound in eel blood has been estimated to be around 100 kDa [39] and known to be inactivated by heating at 58 °C or higher for 15 min [7], we tested both filtration and heat-treatment to remove toxicity from the plasma. As toxicity was observed in blood plasma derived from both male and female eels in our pilot test, we used blood taken from one female eel purchased from a commercial source for this experiment. A plasma sample was prepared by centrifugation at 13,200 rpm at 4 °C for 10 min.

To investigate the effect of the two indicated plasma treatments on subsequent viability of HEK293 cells, one portion of blood plasma was heated at 60 °C for 15 min and another was divided into two fractions by filtration, using a 50 kDa cut-off filter and following the manufacturer's protocol (Amicon Ultra-0.5 50 kDa Ultracel-PL membrane, Millipore, Billerica, MA, USA). HEK-ajLHR-luc cells were seeded in a 24-well plate, incubated at 37 °C overnight and exposed to blood plasma samples (intact, heated, <50 kDa fraction, >50 kDa fraction) at final concentrations of 1%, 5% and 10% of a total volume of culture medium. The conditions of cells were microscopically observed.

The effects of those treatments on the assay result were evaluated. Since heating (as stated above) can affect the properties of toxin proteins as well as the hormone to be measured, the thermal stability of the hormone was first determined using hCG (100 IU/mL, in saline). The solution was heated at 37, 60, 70, 80 or 90 °C for 15 min, immediately cooled and finally incubated at 37 °C for up to 48 h. At multiple time points during this 48-h incubation, aliquots were taken from each sample. The biological activity of heated hCG was measured in a luciferase assay, using HEK-ajLHR-luc cells as described above and converted to percentage of the biological activity of control sample (un-heated hCG). All measurements were done in duplicate.

To test whether the eel toxin could be selectively inactivated by heat-treatment, pure hCG was pre-mixed with intact blood plasma (100 IU/mL), incubated at temperatures ranging from 37 to 90 °C for 15 min, then cooled down to 37 °C and further incubated at 37 °C for up to 48 h, as described above. The methods used for sampling at several time points and undertaking the luciferase assay using HEK-ajLHR-luc cells are described above. All measurements were performed in duplicate.

To observe the effect of filtering, three different mixtures of hCG and eel blood plasma were prepared: Sample i: hCG mixed with the <50 kDa filtrate of blood plasma (10^{-2}–10^3 IU/mL); Sample ii: hCG pre-mixed with intact blood plasma and subsequently filtered using a 50 kDa cut-off filter (1 and 10^2 IU/mL), and Sample iii: hCG pre-mixed with intact blood plasma and subsequently heated at 37 °C for 24 h for a comparison between the effects of filtering and heating. The luciferase assay using HEK-ajLHR-luc cells was performed as described above. All measurements were performed in duplicate.

2.4. Plasma hormone level measurement of hCG injected European male eels

Fourteen male European eels were purchased from a commercial source (Nijvis-Holding B.V., Nijmegen, The Netherlands). Their total length was 39.1 ± 2.0 cm (mean ± SD), and body weight was 114.5 ± 17.5 g. They were kept in seawater in a 7000 L tank which was connected with an 1800 L recirculation system at 21 ± 0.5 °C, and were starved throughout the experiment. After acclimation, they were randomly divided into two groups (seven for each group). One group received a single intra-peritoneal booster injection of hCG (1000 IU in saline) and another received a saline injection as a control. Blood was sampled before (as a control) and one week after injection. Blood plasma was prepared by centrifugation, at 13,200 rpm at 4 °C for 10 min, and stored at −80 °C until hormone measurement by the bioassay. To obtain the standard curve to convert luminescence intensity to the international unit, a serial dilution of purified hCG was made with blood plasma taken from an intact adult eel that had not been treated with injection. A luciferase assay using HEK-ajLHR-luc cells was performed as described earlier. All measurements were performed in triplicate.

3. Results and discussion

3.1. Eel gonadotropin receptor - based bioassays

In the present study, a two-step transfection strategy was used to generate eel gonadotropin receptor-based bioassays. First, a single copy of an ajLHR or aaFSHR cDNA was stably integrated into the

genome of HEK293 cells. Flp recombinase-mediated integration of the ajLHR and aaFSHR expression plasmids into HEK293 cells and subsequent screening through antibiotics selection and β-galactosidase activity resulted in four clonal cell lines for each receptor (named HEK-ajLHR and HEK-aaFSHR). Stable integration and constitutive expression of both gonadotropin receptor cDNAs were confirmed by genomic PCR and RT-PCR, respectively (data not shown). Upon transient transfection with pCRE-Luc and exposure to hCG (10 IU/mL), the stable HEK-ajLHR clones showed a 6–18-fold increase in luminescence intensity. Similarly, the HEK-aaFSHR clones demonstrated a 1.5–9-fold increase in luminescence intensity when they were exposed to SPE (2 mg/mL). These results show the functionality of eel receptors on the HEK293 cell membrane. The most sensitive clones were then used for the second round of transfection to generate cell lines that involve a stable integration of the CRE-Luc reporter gene.

The second round of transfection, to integrate CRE-Luc and Neomycin-resistance genes, resulted in 22 clonal Neomycin-resistant HEK-ajLHR cell lines. Four of these clones showed a significant response to pure hCG (100 IU/mL), indicating that they carry one or more stably integrated copies of the CRE-Luc plasmid (named HEK-ajLHR-luc). The most sensitive clone showed a linear response to a more than 100,000-fold concentration range of pure hCG with, a maximum 42-fold increase in luminescence intensity (Fig. 1a). Twelve Neomycin-resistant clones were isolated from the co-transfection of HEK-aaFSHR cells with CRE-Luc and a Neomycin-resistance gene. Four of those clones demonstrated a significant response to SPE (2 mg/mL) in a luciferase reporter gene assay (named HEK-aaFSHR-luc). Detailed analysis of HEK-aaFSHR-luc cells, using a serial dilution of SPE also demonstrated a high sensitivity ranging from 2×10^{-3} to 2 mg/mL (Fig. 1b). The most sensitive HEK-ajLHR-luc and HEK-aaFSHR-luc clones were used in all subsequent analyses.

The functionality of these bioassays was validated using various ligands. While only ajLHR responded to hCG in a dose-dependent manner (Figs. 1 and 2), both ajLHR and aaFSHR were activated by hFSH (Fig. 2). Pituitary extracts from salmon and carp (SPE and CPE, 200 μg/mL), supposedly containing both LH and FSH, resulted in high fold increases in luminescence intensity with HEK-ajLHR-luc and HEK-aaFSHR-luc cells (42–61 and 42–126-fold increase, respectively, Fig. 3). Furthermore, the pituitary homogenate from an immature eel (GSI = 1.1) specifically induced an increase in luminescence intensity in HEK-aaFSHR-luc cells and not in HEK-ajLHR-luc cells, whereas the homogenate from a mature animal (GSI = 33.9) activated both ajLHR and aaFSH (Fig. 3).

It is generally known that interactions between gonadotropins and their cognate receptors are highly specific in mammals but less so in fish [4,16]. The promiscuous affinity of gonadotropins and their receptors has been reported in various fish species such as

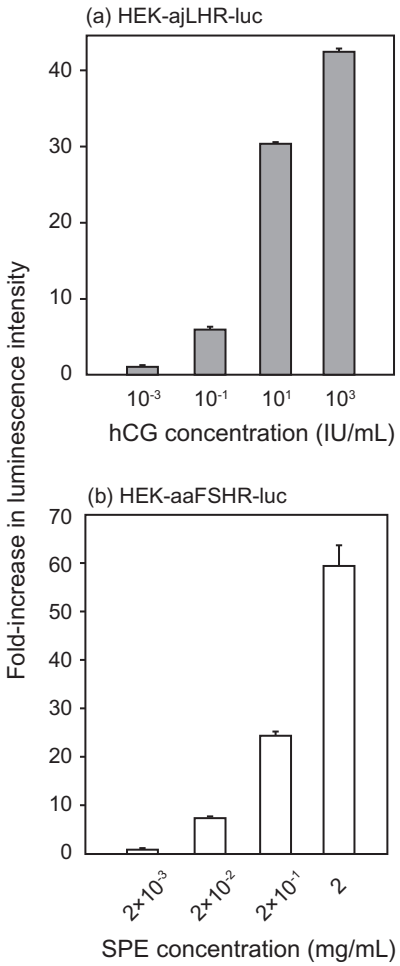

Fig. 1. Dose-dependent responses of ajLHR and aaFSHR expressed on HEK293 cells that also carry stably integrated CRE-Luc (a, HEK-ajLHR-luc; b, HEK-aaFSHR-luc). Serial dilutions of purified hCG and SPE in saline were used as a reference for HEK-ajLHR-luc and HEK-aaFSHR-luc, respectively (indicated as a final concentration). Values are expressed as a fold change relative to a saline control.

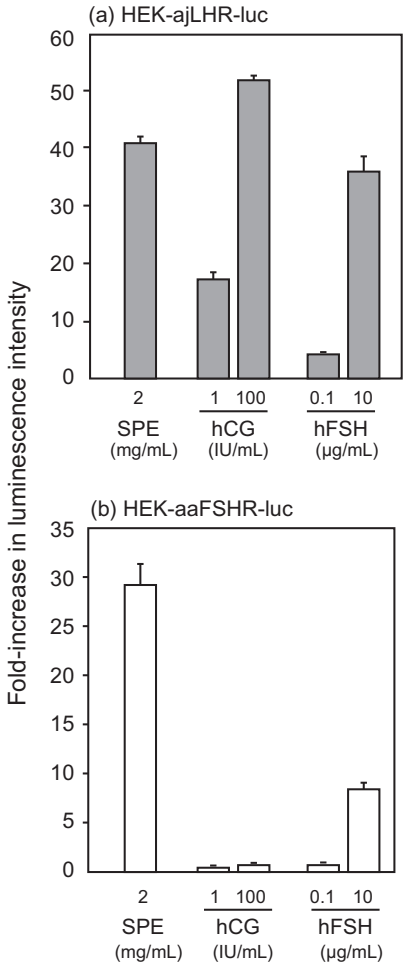

Fig. 2. Functionality of eel gonadotropin receptors to purified human gonadotropins (a, ajLHR; b, aaFSHR). SPE (in saline, 2 mg/mL) was used as a positive control for all assays. Concentrations of ligands are indicated as a final concentration (values expressed as a fold change relative to the saline control).

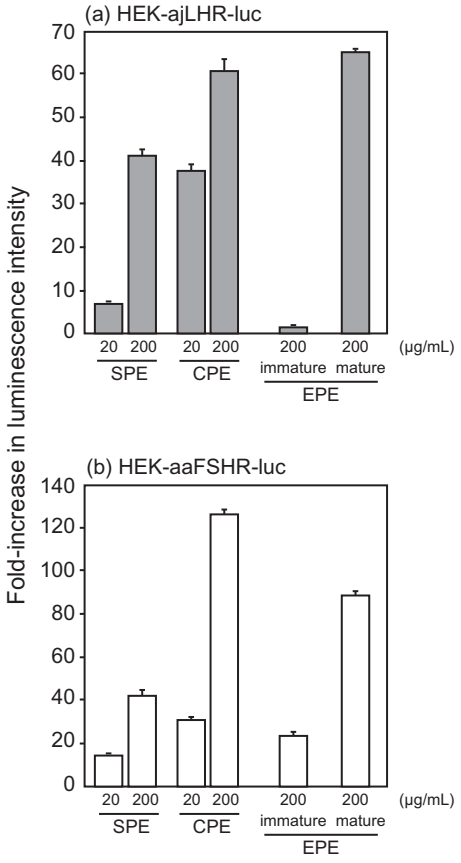

Fig. 3. Functionality of eel gonadotropin receptors to salmon, carp and eel gonadotropins using SPE, CPE and EPE (mixed with saline). Ligand concentrations are indicated as a final concentration. Values are expressed as a fold change relative to the saline control.

African catfish, zebrafish, amago salmon, rainbow trout and sea bass, even when using mammalian gonadotropins [15,19,20,31,38]. In sea bass, for instance, hFSH activates the sea bass LHR in a dose-dependent manner but not the FSHR, whereas hCG activates only the sea bass LHR [19], as observed in eel gonadotropic receptors in this study. In eels, it was recently demonstrated that hCG specifically activates the LHR in the Japanese eel [13]. Moreover, it has been shown that the pituitary of immature eels exclusively contains FSH in the European and Japanese eels [3,32,34], suggesting that the observed fold-increase of luminescence intensity with EPE from an immature animal in this study (Fig. 3) was induced by the interaction between eel FSH and aaFSHR. These observations suggest that the LH and FSH bioassays that we developed are well suited for measuring bioactive cognate hormones.

This validation also demonstrates that human gonadotropins can be used as a reference in an assay, especially hCG for the LH bioassay, which provides an equivalent amount of purified hCG, in the international unit, using a standard curve obtained from its serial dilution. The detection limit of the LH bioassay was estimated to be about 10 mIU/mL of hCG (Fig. 1a), which is slightly higher than that of commercial hCG ELISA kits (e.g., 3–5.3 mIU/mL; BQ kit, Abazyme, Diagnostic Systems Laboratories), although the sensitivities of other methods and our bioassay cannot be compared directly since they measure different molecules. In the case of the FSH bioassay, because the stimulation of the aaFSHR by hFSH seems to be relatively weak compared to that of the ajLHR by hCG (Figs. 1 and 2), we used SPE as a reference in the present study. It should however be noted that an appropriate ligand, such as purified or recombinant eel LH and FSH [12], needs to be used as a reference, depending on the purpose of the assay.

In a previous study, Kazeto et al. [12] developed similar cAMP-mediated bioassays, using Japanese eel gonadotropin receptors based on transient transfection. Their bioassay took four days, involved a higher number of handling steps, and the sensitivity of the method was limited. In the present study, we generated reporter cell lines that stably expressed ajLHR or aaFSHR on a cell membrane and carried a firefly luciferase gene, which showed a wider range of sensitivity. For example, both of our assays responded to a 100-fold concentration range of SPE (Figs. 1–3) whereas the previous study demonstrated a detection limit of a 10-fold range of SPE concentration for both LH and FSH assays [12]. Moreover, the assay procedure of our assays that we developed required only two days, i.e., it seeded reporter cells in a 96-well plate on the first day, and cell stimulation by ligand addition and luminescence measurement were undertaken on the second day. The high sensitivity and easy handling that we achieved in this study are desirable for measurements involving a large number of samples.

3.2. Adaptations of the bioassay protocols to eel blood plasma and plasma hormone level measurement

The two treatment methods, i.e., heating and filtration, to remove toxic compounds from eel blood were first evaluated using intact, heated and filtered blood plasma (<50 and >50 kDa fraction). Heat-treated plasma did not induce death of cells at 1% concentration of the total volume of cell culture medium, throughout the experiment and for up to 30 h after blood plasma addition at 5 and 10%. The filtrate (<50 kDa fraction) did not affect the viability of the HEK293 cells, even after prolonged exposure (48 h) to a high plasma concentration (10%). In contrast, the >50 kDa fraction, which was the concentrated residue of the filtration step and supposedly contained the toxin proteins, killed cells almost instantly at 1% of total culture volume.

Determination of the thermo-stability of hCG through time was based on the biological activity of untreated hCG as a reference, by regarding 100% of biological activity. Treatment that only involved incubation at 37 °C resulted in around 10% decreased biological activity for up to 1 h after heating. This spontaneously returned to 100% of the control value within 4 h after heat-treatment, after which it remained unchanged for at least 48 h. About 90% of hCG activity was detected after heating at 60 °C, while only 20–30% of hCG activity was measured when it was heated at 70, 80 or

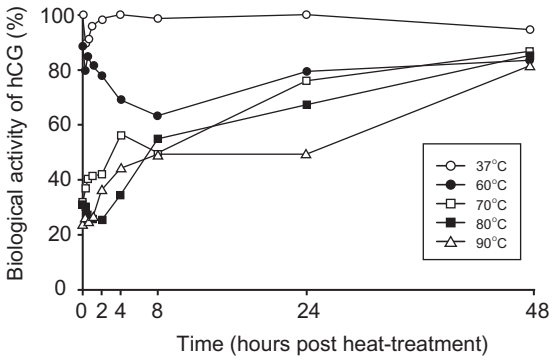

Fig. 4. Temporal change of hCG bioactivity detected in the LH bioassay after heating at various temperatures for 15 min with a test hCG solution of 100 IU/mL in saline. After heat-treatment at the indicated temperature (37, 60, 70, 80 and 90 °C), hCG solutions were cooled and incubated at 37 °C for up to 48 h. Samples were taken at several time points. The luciferase assay was performed on HEK-ajLHR-luc cells. Biological activity of the heated hCG was expressed as percentage of that of unheated hCG solution in saline (100 IU/mL).

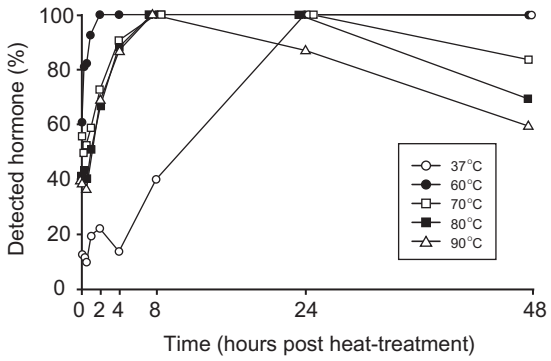

Fig. 5. Temporal change of detectability in the LH bioassay of hCG premixed with eel blood plasma after heating at various temperatures for 15 min. Test hCG was mixed with eel blood plasma at 100 IU/mL). After heat-treatment at the indicated temperature (37, 60, 70, 80 and 90 °C), hCG solutions were cooled and incubated at 37 °C for up to 48 h. Samples were taken at several time points and the luciferase assay was performed on HEK-ajLHR-luc cells. Since unheated blood plasma is lethal to HEK cells (see the text), the response of HEK-ajLHR-luc to hCG solution in saline (100 IU/mL) without heat-treatment was regarded that 100% of hCG in a sample was measured and the values of samples expressed as relative percentages to the reference.

90 °C. Although the biological activity of hCG treated at temperatures at, or above 60 °C gradually regained during the incubation at 37 °C after heating, it did not fully recover within 48 h (maximum levels about 85%) (Fig. 4).

We then tested whether heating would selectively inactivate eel toxin by premixing hCG in blood plasma that was then subjected to heat treatment at various temperatures. When the detected luminescence intensity in non-heated hCG solution in saline was regarded as 100%, less than 20% hCG was detected by HEK-ajLHR-luc cells for up to 4 h after treatment at 37 °C. This was due to the death of cells that had been induced by plasma toxin. However, 100% of hCG activity was detected after 24 h of incubation at 37 °C (Fig. 5). After incubation at 60 °C for 15 min, hCG activity was around 60% just after heating, and it then rapidly recovered to 100% within two hours of incubation at 37 °C. Heating for 15 min at 70, 80 or 90 °C resulted in 40–55% of hormone activity immediately after treatment and full recovery after 8 h at 37 °C. The loss of hormone activity was however observed later on (Fig. 5). These results concur with the previous study that had indicated that heating at temperatures of 60 °C or higher for 15 min removed toxicity, but also showed that the heating strongly affected both the biological activity of hormone (Fig. 4) and the hormone detectability of the assay (Fig. 5). In contrast, we found that incubation at 37 °C alone for 24 h successfully inactivated the toxic compounds in eel plasma without affecting the biological activity of the hormone (Figs. 4 and 5).

The effect of filtering on assay results was investigated. A dose-dependent response was found in Sample i (hCG in pre-filtered blood plasma) and Sample iii (hCG in blood plasma that was heated after mixing) (see Materials and methods for details) (Fig. 6). Observed luminescence intensities of Sample ii (hCG in blood plasma that was filtered after mixing) were about 7.7 and

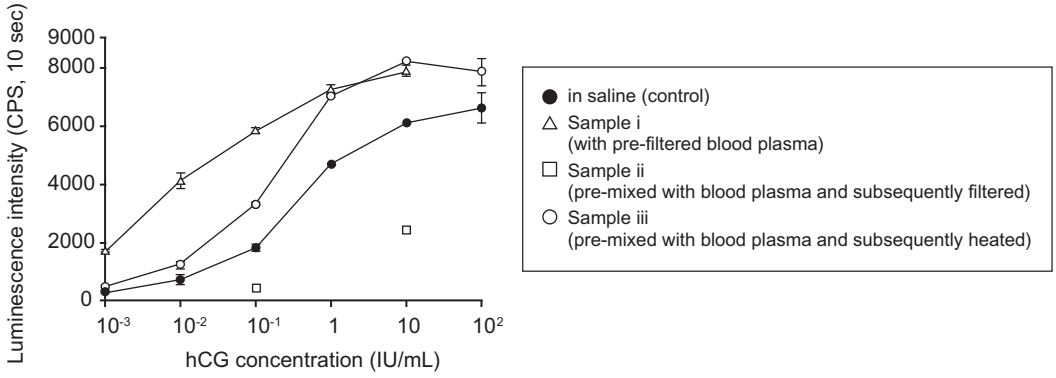

Fig. 6. Effect of filtering and heating treatments to remove toxicity from blood plasma on hormone detection by the bioassay. Closed circles indicate the serially diluted hCG in saline (control). Open circles indicate a serial dilution of hCG mixed with eel blood plasma that was heated at 37 °C for 24 h after mixing (without filtering, Sample iii). Open triangles indicate hCG samples that were mixed with pre-filtered eel blood plasma (without heating, Sample i). Open squares represent hCG that was mixed with blood plasma and subsequently filtered (Sample ii). Treated hormone samples were measured by the LH bioassay. Concentrations of hCG are indicated as final concentrations.

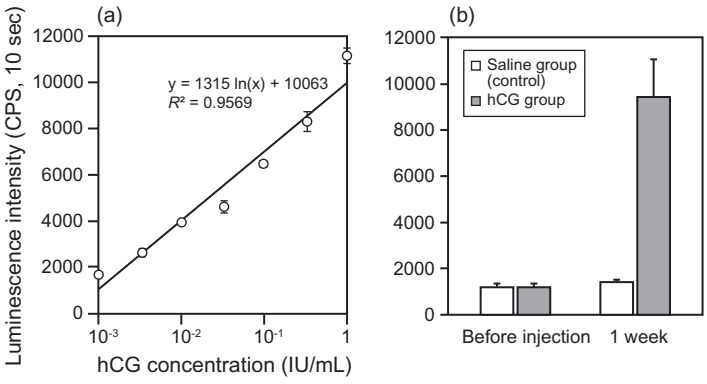

Fig. 7. Relationship between hCG concentrations in blood plasma and luminescence intensities (CPS, 10 s) by HEK-ajLHR-luc cells (a), and luminescence intensities of blood plasma samples taken from eels that received saline (control) and hCG (1000 IU in saline) injections (b). Concentrations of hCG are indicated as final concentrations.

30.5% of those of Samples iii, at 10^{-1} and 10 IU/mL of hCG concentration, respectively (Fig. 6). This indicates that a large amount of hCG that was pre-mixed with intact blood plasma did not become a part of the <50 kDa toxin-free filtrate. Taking all results together, a pre-incubation at 37 °C for 24 h prior to assay is likely to be the best way to prepare eel blood plasma samples for the purpose of measuring its hormone level using the bioassays that we have developed.

We tested the HEK-ajLHR-luc cells so as to directly measure plasma hormone levels in male eels that had received a single hCG injection. The blood plasma samples, together with a serial dilution of hCG dissolved in blood plasma from an intact animal, were pre-heated at 37 °C for 24 h and then analyzed by a luciferase assay. A linear response was observed in a serial dilution of purified hCG in blood plasma taken from an intact eel at concentrations of between 10^{-3} and 1 IU/mL (Fig. 7a). This indicates that the functionality of the LH bioassay is suitable for practical applications. The samples of the hCG-injected group demonstrated high luminescence intensities (mean CPS ± SD = 9506.0 ± 1563.5; 6.74-fold-increase) at one week after injection (Fig. 7b). In order to estimate the hormone level in a sample using a bioassay, it is very important to use a 'true' reference for a standard curve in which a ligand is mixed in an appropriate solvent at various concentrations, since unknown factors may interfere with the interactions between gonadotropic hormones and receptors, which could possibly result in an over- and/or under-estimation when quantifying hormone levels. In the experiment undertaken in the present study, we used pure hCG that was serially diluted in blood plasma of an intact adult eel. Based on the obtained standard curve and regression equation (Fig. 7a) the hormone level in eels of the hCG-injected group was back-calculated to be equivalent to 0.655 IU hCG/mL. This result clearly shows that the adapted protocol of the bioassay is applicable for practical use.

4. Conclusions

In the present study, we developed eel gonadotropin receptor-based bioassays, with the particular purpose of directly measuring levels of gonadotropic hormones in eel blood plasma. Our LH and FSH bioassays indicate high sensitivity allowing for quantification of hormones by using either purified hCG or SPE as references. Moreover, we adapted the bioassay protocol to eel blood plasma by selectively inactivating toxin, which enabled the quantification of plasma levels of biologically functional hormones over time, without having to sacrifice animals. Using our bioassays to monitor hormone levels can provide information on maturation stages of injection-treated adult eels. Optimal timing as well as quantity of injections can be adjusted for each adult. This may also assist in the selection of suitable parents for artificial fertilization. In addition, it was demonstrated that our bioassays are applicable for specification of SPE and CPE, the hormone contents and compositions of which have rarely been compared over different batches. This is very important because the optimal amount of pituitary homogenate, to inject for efficient induction of maturation, can be determined. Consequently, the techniques and protocols used for the artificial maturation of eels can be standardized. The bioassays developed in the present study will help optimize artificial reproduction methods, and will also increase the understanding of the maturation process in the eel species.

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