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In vitro Induction of Luteinizing Hormone Synthesis by Estrogen in Organ-Cultured Pituitary Glands of the Japanese Eel, *Anguilla japonica*

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ABSTRACT—An organ culture method for pituitary glands isolated from immature Japanese eels (*Anguilla japonica*) was developed. This method could conserve the histological features of the pituitary glands for at least 21 days. The ability to synthesize gonadotropic hormone (GTH) in cultured eel pituitary glands was examined by detecting luteinizing hormone (LH) β protein immunohistochemically. In a basal medium (Leibovitz L-15), LH β-immunoreactive cells were very scarce, but after addition of estradiol-17β (E2) a large number of immunoreactive cells appeared, particularly in the proximal pars distalis. The stimulatory effects of E2 on LH β synthesis were dose (1–100 ng/ml)- and time (1.5–7 days)-dependent. Thus, in contrast with previous reports of the lack of a direct effect of E2 on GTH synthesis in primary cultured eel pituitary cells, the present results clearly indicate that E2 can stimulate GTH synthesis in immature eel pituitary glands. This organ culture method is useful to examine the actions of steroids and also other endocrine factors on the eel pituitary gland.

Key words: *In vitro*, luteinzing hormone, estrogen, organ culture, Japanese eel

INTRODUCTION

Gonadotropic hormone (GTH) is a pituitary hormone regulating gonadal development in vertebrates (Shupnik, 1996). In several teleost species, two types of GTH, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), have been isolated and characterized (Suzuki *et al*., 1988; Swanson *et al*., 1991). These GTHs consist of a heterodimer composed of an α subunit, which is common to GTHs and thyroid-stimulating hormone (TSH), and a hormone-specific β subunit. It has been reported that there is a strong positive feedback by sex steroids upon GTH synthesis in juvenile teleosts. For example, administration of testosterone and estradiol-17β (E2) produces a large increase in pituitary LH and/or its mRNA levels in salmonids (Gielen *et al*., 1982; Dickey and Swanson, 1998) and platyfish (Schreibman *et al*., 1986). Such stimulatory effects of sex steroids are attributable, at least in part, to a direct action on the pituitary gland. In fact, many *in vitro* studies have shown that sex steroids directly stimulate LH expression in cultured pituitary glands and/or cells of juvenile rainbow trout (Fåhraeus-van Ree *et al*., 1983; Xiong *et al*., 1994), immature and mature goldfish (Huggard *et al*., 1996), and maturing tilapias (Melamed *et al*., 1998).

Because of its particular life cycle with the arrest of sexual development in fresh water, the eel provides a useful model for the study of the mechanisms of the positive feedback in juvenile teleosts. In the same way as in other teleosts, chronic treatment of female silver European eels *Anguilla anguilla* with E2 and testosterone induces increases in pituitary GTH (Dufour *et al*., 1983; Montero *et al*., 1995; Quérat *et al*., 1991). In accordance with these *in vivo* data, there have been reports that testosterone stimulates LH production in a dose- and time-dependent manner in primary culture of dispersed pituitary cells isolated from juvenile eels (Huang *et al*., 1997). Stimulatory effects are also observed with non-aromatizable androgens such as androstanediol and dihydrotestosterone. However, no direct effect of E2 has been evidenced in the cultured pituitary cells of the eel.

In mammals, organ culture of the pituitary gland is known as a valuable technique for the study of GTH production (Martin and Klein, 1976; Baker and Young, 1979; Guérineau *et al*., 1997). This seems also to be the case in some teleosts such as the rainbow trout (Fåhraeus-van Ree *et al*., 1983) and goldfish (Huggard *et al*., 1996). In the

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present study, we developed an organ culture method for the pituitary gland isolated from the Japanese eel, *Anguilla japonica*, and examined the *in vitro* effect of E2 on the synthesis of a GTH subunit, LH β. Here we report for the first time that E2 stimulates GTH synthesis in the eel pituitary gland *in vitro*.

MATERIALS AND METHODS

Animals and culture of the pituitary gland

Immature male Japanese eels, *Anguilla japonica,* weighing < 500g were purchased from a commercial supplier. They were anesthetized by immersion in ice water, and their pituitary glands were isolated and cultured by the method of Miura *et al*. (1991) with slight modifications. Briefly, isolated pituitaries were floated on elder pith covered with a nitrocellulose membrane in 24-well plastic dishes. Then they were cultured for 3–21 days in humidified air at 20°C. The culture medium consisted of a chemically defined basal medium Leibovitz L-15 (Sigma, St. Louis, USA), supplemented with 0.5% bovine serum albumin fraction V (BSA, Sigma), 7 μg/ml penicillin, 10 μg/ml streptomycin, and 10mM HEPES (pH 7.4). Pituitary glands were also cultured in L-15 without growth factors, and supplemented with either 0.5% BSA, 0.5% BSA + 1 mg/ml bovine insulin, 2% fetal calf serum (FCS, Sigma), or 2% serum substitute (CELOX, USA). E2 (estradiol-17β, Sigma) was dissolved at 1 mg/ml in 100% ethanol and added to the medium at final concentrations of 1–100 ng/ml. The medium was renewed at every 7 days.

Immunohistochemistry of LH β subunit

The cultured pituitary glands were fixed using Bouin-Hollande sublimate. After post-fixation and dehydration in ethanol, they were embedded in paraffin and sectioned sagitally. Deparaffinized sections were treated with 1% hydrogen peroxide in methanol for 30 min to inactivate endogenous peroxidase, and with 10% normal goat serum (DAKO, Glostrup, Denmark) in PBS for 30 min to block nonspecific binding. The sections were incubated with × 1,000 diluted rabbit antiserum against the eel LH β subunit (Ikeuchi *et al*., 1999) for 16 hr at 4°C, and then with a secondary antibody (biotinylated goat anti-rabbit IgG, × 400 dilution, DAKO) for 3 hr at room temperature. After incubation with streptavidin-biotin-horseradish peroxidase complex (DAKO), immunoreactive cells were visualized by incubation with 0.01% diaminobenzidine-0.03% hydrogen peroxide in 50 mM Tris-HCl (pH 7.6) for 3–5 min. Adjacent serial sections were stained with Gomori's aldehyde fuchsin. The number of immunoreactive cells was counted in a 1.4 mm^2 area of the proximal pars distalis (PPD) in three randomly selected sections. The mean of the three sections was expressed as cells/mm^2 in one pituitary gland.

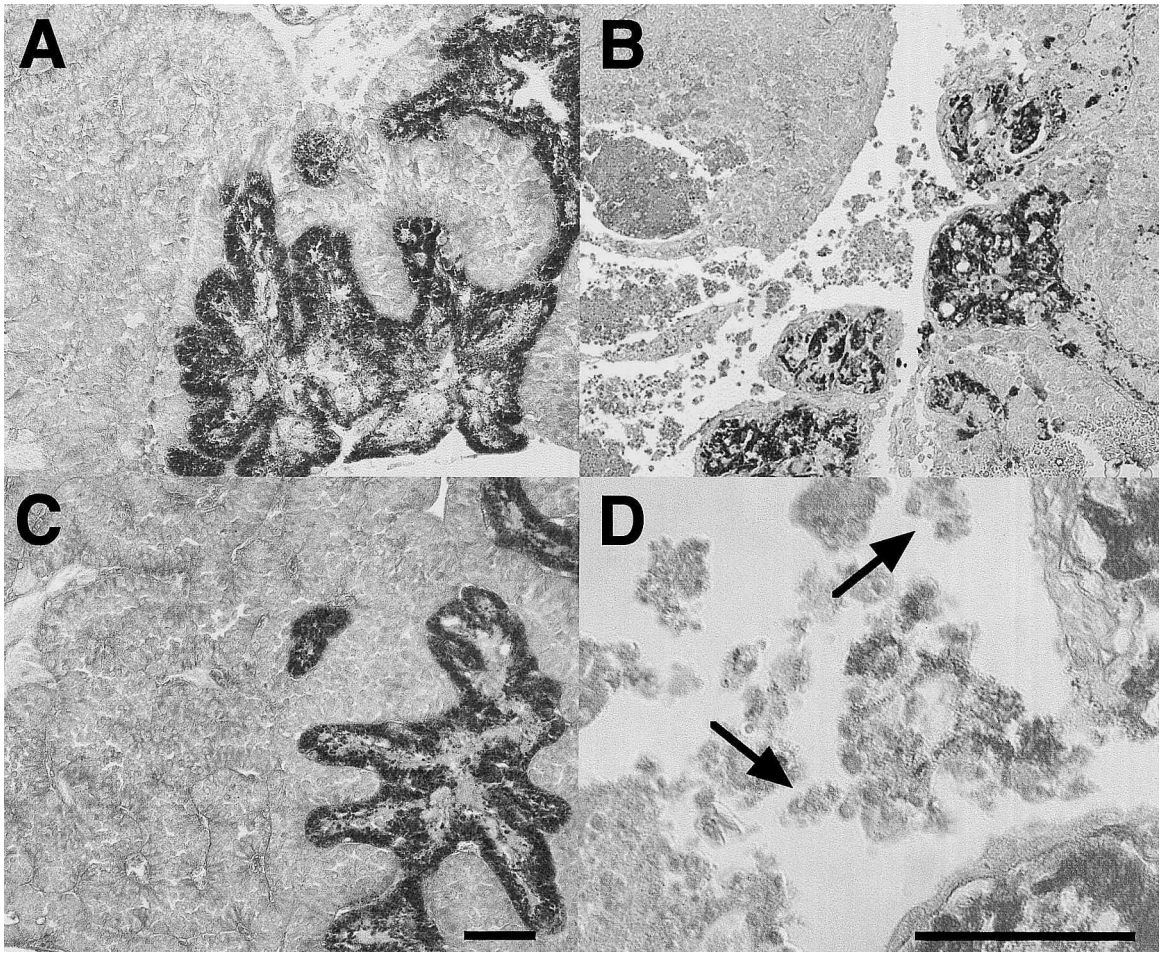


Fig. 1. Histological features of the pituitary gland of the immature male Japanese eel. A: a pituitary gland before culture. B: a pituitary gland cultured for 7 days by the submergence method. C: a pituitary gland cultured for 21 days by the float method. Arrows indicate cells that have degenerated in the central area of the pituitary gland (D). Sagittal sections of the pituitary gland were stained with Gomori's aldehyde fuchsin. Bar 10 μm.

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Statistical analysis

Data are presented as means±SEM for three cultured pituitary glands, and analyzed by the unpaired *t*-test.

RESULTS

Organ culture of eel pituitary glands

To maintain functionally active eel pituitary glands *in vitro*, two methods of organ culture were attempted, a submergence method and a float method. In the former, freshly isolated pituitary glands were totally submerged in the medium, whereas in the latter they were floated on elder pith with a nitrocellulose membrane. As shown in Fig. 1, in the submergence method the central area of the pituitary gland began to degenerate within 3 days of culture and mostly died after 7 days. In contrast, in the float method, the histological features of the tissue seemed not to be altered for at least 21 days.

Effects of growth factors and E2 on LH β subunit protein synthesis

To examine the ability of eel pituitary glands to synthesize GTH *in vitro*, pituitary glands isolated from immature male eels were cultured by the float method, and LH β protein was detected immunohistochemically. In the isolated pituitary glands before culture, LH β-immunoreactive cells were very scarce. When cultured in the absence of E2, immunoreactive cells were also scarce. However, when cultured in the presence of 100 ng/ml E2 for 7 days, a large number of the immunoreactive cell appeared, particularly in PPD (Fig. 2). We have preliminarily reported that the LH β-immunoreactive cell number was also increased when cultured in the presence of testosterone and 11-ketotestosterone (Mitsuhashi *et al*., 2000). Thus the eel pituitary glands cultured by the float method were able to synthesize LH β protein in response to E2 and some other sex steroids.

In the most of the present experiments, we cultured eel pituitary glands in a chemically defined basal medium (Leibovitz L-15) supplemented with 0.5% BSA, in the same way as for organ culture of eel testes (Miura *et al*., 1991). We next examined the effects on LH β synthesis of BSA and some growth factors such as insulin, FCS and serum substitute, a cell multiplication stimulating admixture factors in

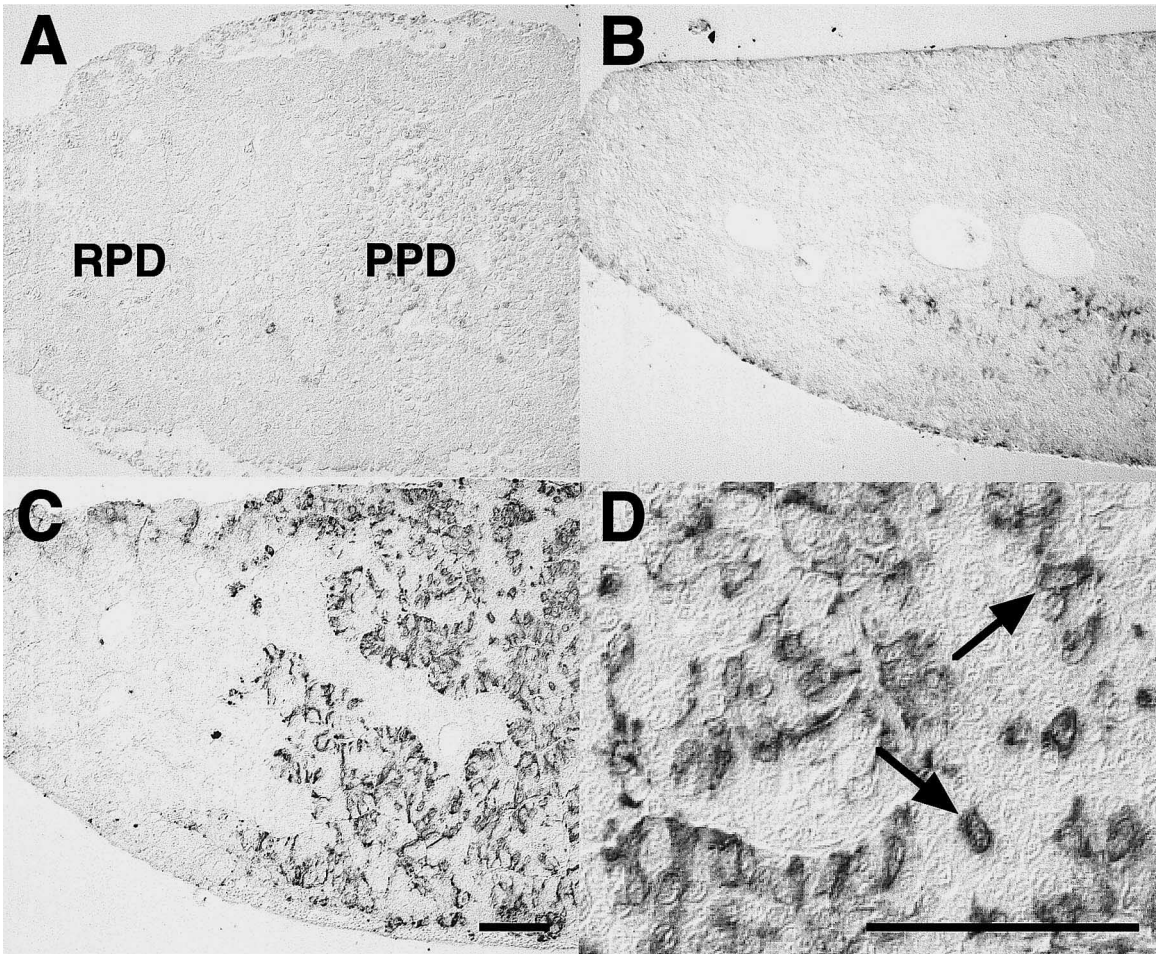


Fig. 2. Immunohistochemical detection of LH β subunit in organ-cultured male eel pituitary glands. Pituitary gland before culture (A), and after 7-day culture in the absence (B) or presence (C, D) of 100 ng/ml estradiol-17β. Sagittal sections of the pituitary glands were stained with an antibody against the eel LH β subunit. Arrows indicate LH β-immunoreactive cells (D). PPD: proximal pars distalis, RPD: rostral pars distalis. Bar 10 μm.

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serum-free medium. To assess the effects of these factors quantitatively, the number of the LH β-immunoreactive cells was counted in a selected area of the PPD. As shown in Fig. 3, neither BSA, insulin, FCS, nor serum substitute gave rise to any noticeable effect on the number of the LH β-immunoreactive cells, regardless of the presence and absence of E2. It was thus confirmed that the cultured pituitary glands maintained the ability to synthesize LH β in the medium without supplementation of any growth factors. Essentially the same results were obtained when cultured for 14 days, and also in the pituitary glands isolated from immature female eels (data not shown).

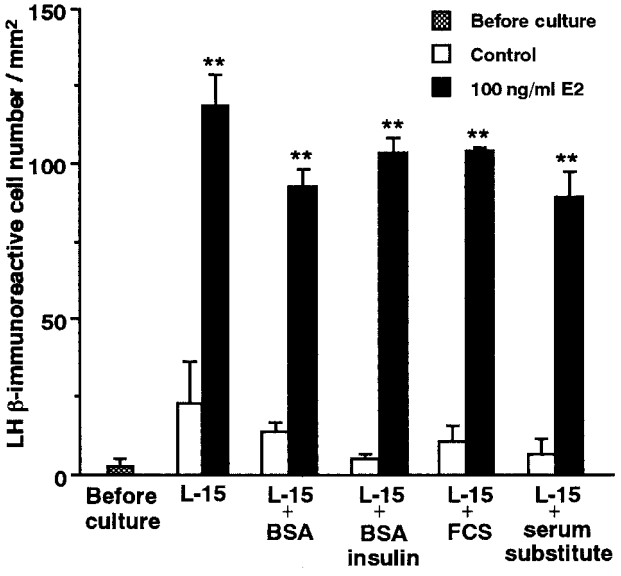


Fig. 3. Effects of growth factors, and estradiol-17β (E2) on LH synthesis in cultured eel pituitary glands. Pituitary glands isolated from immature male eels were cultured in a basal medium (L-15) without growth factors, and supplemented with either 0.5% bovine serum albumin (BSA), 0.5% BSA + 1 mg/ml insulin, 2% fetal calf serum (FCS), or 2% serum substitute and 100 ng/ml E2 for 7 days. The number of LH β-immunoreactive cells detected as in Fig. 2 was counted in 1.4 mm^2 areas of the proximal pars distalis of three randomly selected sections. The mean from the three sections was calculated as cells/mm^2 in one pituitary gland. Values are means±SEM for 3 glands. ** $p<0.01$ vs Control without E2.

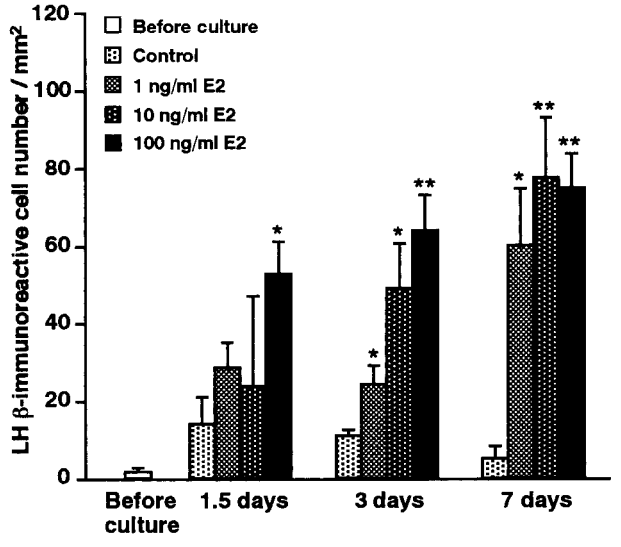


Fig. 4. Dose- and time-dependent effects of estradiol-17β (E2) on LH synthesis in cultured male eel pituitary glands. Pituitary glands were cultured in the presence of 1, 10 or 100 ng/ml E2 for 1.5, 3 or 7 days. The number of LH β-immunoreactive cells is expressed as in Fig. 3. * $p<0.05$ and ** $p<0.01$ vs Control without E2.

Dose- and time-dependent effects of E2 on LH β subunit protein synthesis

Finally, dose- and time-dependent effects of E2 on LH β protein synthesis were examined. As shown in Fig. 4, the immunoreactive cell number was not increased during the 7-day period of culture in the absence of E2, whereas it was markedly increased with increasing doses of E2 (1–100 ng/ml) and with increasing periods of culture (1.5–7 days). Thus, E2 dose- and time-dependently stimulated GTH synthesis in eel pituitaries for at least 7 days *in vitro*.

DISCUSSION

It has been repeatedly reported that *in vivo* administration of E2 and testosterone to juvenile teleosts, including the eel, results in the induction of pituitary GTH. These sex steroids stimulate GTH production in organ-cultured pituitary glands of the rainbow trout and goldfish (Fåhraeus-van Ree *et al.*, 1983; Huggard *et al.*, 1996). Similarly, GTH production in primary culture of eel pituitary cells is stimulated by testosterone and some other steroids, but unexpectedly not by E2 (Huang *et al.*, 1997). In the present study, to examine the possible directly effects of E2 on GTH synthesis in the eel pituitary gland, we developed an organ culture method for the pituitary gland isolated from the immature Japanese eel.

First, we tried to culture pituitary glands by totally submerging them into the medium (submergence method), but found that the central area of the organ degenerated in several days. Thus, we applied a float method previously used for organ culture of eel testes (Miura *et al.*, 1991), where freshly isolated pituitary glands were floated on elder pith covered with a nitrocellulose membrane in plastic dishes and cultured in basal Leibovitz L-15 medium. In the float method, the histological features of the organ seemed not to be altered for at least 21 days, probably because of the sufficient oxygen supply obtained by exposing the organ to air compared with the submergence method.

Using this float method, we examined GTH synthesis in response to E2 by detecting LH β subunit protein immunohistochemically. In the absence of E2, LH β-immunoreactive cells were very scarce, but when cultured in the presence of E2, a large number of positive cells appeared, particularly in PPD. The stimulatory effects of E2 on LH synthesis were dose- and time-dependent. Moreover, the dominant distribution of LH β-immunoreactive cells in PPD was compatible with the previous *in vivo* observations that LH β-immunore-

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active cells were abundant in PPD and TSH β-immunoreactive cells in the rostral pars distalis in eel pituitary glands (Ikeuchi *et al*., 1999). We also confirmed that E2 stimulated LH β mRNA expression in cultured pituitary glands using the float method (Mitsuhashi *et al*., 2000). Collectively, the present results clearly indicated that E2 could stimulate LH synthesis in the eel pituitary gland.

One of the surprising findings in the present study was that LH β synthesis stimulated by E2 was maintained for 7–14 days, even when the pituitary glands were cultured in a basal medium without supplementation of BSA, insulin, FCS, or serum substitute. These results suggested that the present simple culture method for the eel pituitary gland may not need the addition of exogenous growth factors, and hence would be useful to examine the actions of steroid hormones and other factors.

The present results seemed to contradict those for cultured pituitary cells of the European eel (Huang *et al*., 1997), in which E2 did not stimulate GTH synthesis. Although the reasons for their discrepant *in vitro* results in the eel are not clear at present, selective loss of the nuclear E2 receptors during the preparation and culture of pituitary cells may be unlikely, because these cells respond well to other sex steroids. The species difference may also be an unlikely reason, because testosterone stimulates GTH synthesis *in vitro* in both Japanese and European eels (Mitsuhashi *et al*., 2000; Huang *et al*., 1997). Alternatively, it seems more likely that some cell-to-cell interaction in the pituitary gland is important for the stimulatory action of E2. For example, E2-induced GTH synthesis may require some paracrine factors produced by pituitary cells other than GTH-synthesizing cells. Thus further studies are needed to clarify the cellular and molecular mechanisms controlling GTH synthesis in the eel pituitary gland, particularly focusing on GTH induction during different stages of sexual maturation.

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