

IN-VITRO REGULATION OF CORTISSOL AND ACTH OUTPUT
FROM INTERRENAL AND PITUITARY TISSUE
OF GILTHEAD SEA BREAM (*Sparus aurata*)

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Introduction

The involvement of hypothalamus and pituitary in the control of cortisol production has been established for teleost fish, and adrenocorticotrophic hormone (ACTH) has been considered the major factor controlling synthesis and release of cortisol from interrenal cells in the head kidney. The endocrine control of cortisol secretion in teleost is complex, whereas cortisol has been shown to effect self suppression by negative feed-back of its secretion directly at different levels. ACTH has been considered the major factor controlling the synthesis and release which in turn is regulated by hypothalamic factors such as corticotrophic releasing hormone (CRH). However, little is known, specially in marine fish, about the contribution and dynamics of hypothalamic-pituitary hormones on the stress response. We therefore investigated the differences regarding hypothalamus-pituitary-interrenal communication in *Sparus aurata* compared to other fish by testing the *in vitro* responsiveness of interrenal tissue to ACTH and the responsiveness of ACTH producing cells to CRH and by characterising the response using a CRH antagonist.

Material and methods

Animals

Sexually immature gilthead sea bream weighing 75 to 160g were kept under constant conditions of photoperiod (12L:12D), salinity (38‰), temperature ($17\pm 1^\circ\text{C}$) and density (7Kg.m^{-3}). To investigate the responsiveness of ACTH producing cells to CRH, a total number of sixty fish were used, ten groups of six fish were captured and the pituitary glands were excised and superfused. To investigate the responsiveness of interrenal tissue to ACTH, forty five fish were used, nine groups of 5 fish were captured and the head kidneys were excised and superfused. To study the in-vivo and in-vitro effect of cortisol on the sensitivity of cortisol producing tissue to ACTH, fish were pre-treated 36h and 6h with cortisol before sampling. Thus a total of 24 fish (8 controls, 8 cortisol *in-vitro* and 8 cortisol *in-vivo*) were sampled.

Pituitaries and Head Kidneys

Pituitaries and head kidneys were excised. Single pituitaries and head kidneys were superfused with a Hepes (15mM; pH 7.4) buffer solution containing 0.25% (w/v) glucose and 0.03% (w/v) bovine serum albumin. For pituitary glands, medium, either supplemented with CRH competitive antagonist (α -helical CRH (9-41) or not supplemented. The flow was 40 $\mu\text{l}/\text{min}$. After 220 min of superfusion, medium supplemented with different concentrations of CRH was given for 20 min. For head kidney superfusion, tissue was stimulated with ACTH at different concentrations (Fig.1) and at a concentration of 5nM (Table.1) (hACTH₁₋₃₉, Sigma) during 20 minutes. To study the “in-vitro” effect of exogenous cortisol, medium was supplemented with different concentrations of hemisuccinate-cortisol. The maximum cortisol release due to ACTH stimulation was compared with the basal release in order to obtain the stimulation factor of ACTH, (maximum release – basal release) / (basal release).

Biochemical and Statistical analysis

Cortisol and ACTH were measured by well-established and validated radioimmunoassay (RIA) for gilthead sea bream. Results are given as mean \pm SEM for each group. Differences among groups were assessed by means of One-way Analysis of Variance (ANOVA) followed by the Student-Newman-Keuls (SNK) test. The level for accepted statistical significance was (* $p < 0.05$;

** $p < 0.01$, *** $p < 0.001$). The concentration-effect curves were fitted using a non-linear data analysis program describing a sigmoid curve (Jandel Sigma plot, Scientific graphic software, Version 3.0).

Results

In vitro responsiveness of ACTH producing cells to CRH and by characterising the response using a CRH antagonist.

The results show that CRH stimulated the ACTH in a concentration-dependent manner in pituitaries of non-disturbed sea bream. The maximum level of stimulation were about 1000% of basal release for ACTH and the EC50 values defined as the required CRH dose for half-maximal response, were 1.5×10^{-9} M (Fig.1). In the presence of 400nM α -helical CRH (9-41), the ACTH-releasing activity of CRH was greatly reduced. Thus the antagonist blocked the secretion of ACTH that was stimulated by a dose of 1 nM CRH by a 75% and 10nM of CRH by 50%.

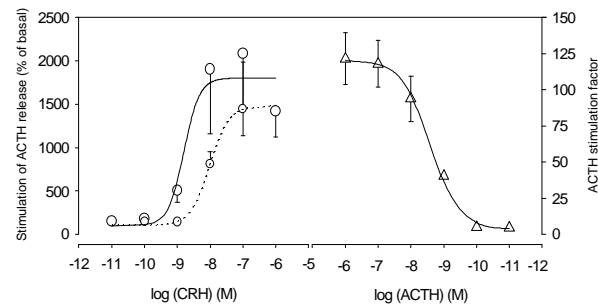


Figure 1. Effect of increasing doses of CRH on the ACTH release from sea bream pituitaries superfused in the presence (dotted line; calculated from (O) or absence (solid line: calculated from (●) of 400nM α -helical CRH (9-41). Data represent mean \pm SEM (n=6). Concentration effect curve showing maximal stimulation of cortisol release during superfusion of control head kidneys (▲). Values are mean \pm SEM; (n=5 for all points).

In vitro responsiveness of interrenal tissue to ACTH and effect of cortisol administration

ACTH stimulated cortisol release in a concentration-dependent manner in interrenal tissue of control fish. The EC50 value was 2.6×10^{-9} M (Fig.1).

Table 1. Sensitivities of interrenal tissues of control to ACTH (expressed as ACTH stimulation factor) under cortisol administration.

	Plasma cortisol (ng.mL ⁻¹)	Medium cortisol (ng.mL ⁻¹)	ACTH stimulation factor
Control	4.2±2	-	79±10
Cortisol "In-vitro"	3.4±1	20 200	62±14 73±11
Cortisol fed	21.2±2*	-	81±12

Table 1 shows the steroidogenic response (expressed as ACTH stimulation factor) of head kidneys from sea bream *in vitro* superfused with a medium either supplemented with cortisol (20 and 200 ng.ml⁻¹) or not supplemented and both stimulated with 5 nM of hACTH₁₋₃₉ for 20 minutes. The ACTH sensitivity of interrenal tissue of non-cortisol-fed sea bream (expressed as ACTH stimulation factor) was 79±10 in the control and was not altered by cortisol administration *in vitro* at the two different concentration tested. In cortisol fed fish, again the ACTH stimulation factor was not different from non-cortisol-fed sea bream.

Discussion

CRH stimulates the pituitary ACTH release in a concentration-dependent manner. Immunocytochemistry studies using antisera directly against mammalian CRH allowed the identification of a CRH-like system in a number of teleosts. In all except one species examined, CRH-like immunoreactivity is

concentrated in the parvocellular and magnocellular areas of the nucleus preopticus (NPO). However, it has been reported (Mancera et al., 1995) that in gilthead sea bream, most of the CRH-like immunoreactivity was found in the nucleus lateralis tuberis (NLT) but not in the NPO. Among the different explanations discussed by the authors it was suggested that perhaps CRH was not a releasing factor for ACTH in this species. Although our *in vitro* approach does not necessarily mimic the *in vivo* situation, the superfusion results do not support that explanation and otherwise they are in accordance with most of the previous works (Balm et al., 1994) showing that CRH is able to stimulate the release of ACTH from sea bream pituitary gland, as in all other fish species studied to date. Hence, when applying the same antagonist (α -helical-CRF(9-41) previously used to antagonise the ACTH-releasing activity of CRH in fish (Weld et al., 1987), a blocking of the ACTH in a dependent CRH concentration is observed. Thus, the superfusion results are consistent with CRH being a physiological regulator of ACTH secretion from pituitary glands of gilthead sea bream, a similar situation than in other teleosts. The ACTH stimulates the interrenal cortisol release in a concentration-dependent manner. Cortisol administration either *in vivo* and *in vitro* had no effect on the sensitivity of the interrenal cells to ACTH (expressed as ACTH stimulation factor. Table 1). Thus, the data suggest that sea bream interrenal cells are relatively insensitive to direct cortisol feedback, which may seem surprising in view of previous “*in vitro*” results on salmon (Bradford et al. 1992). However, this discrepancy could be attributed to the use of the different *in vitro* superfusion systems.

In summary, the present work shows that the results of *in vitro* investigation demonstrate that CRH stimulates the release of ACTH from sea bream pituitaries. Moreover, they demonstrate that α -helical CRH (9-41) antagonises the ACTH-releasing activity of CRH from the sea bream pituitary as it has been shown for another teleost (Weld et al., 1987) and for the mammalian pituitary (Rivier et al., 1984), suggesting that CRH plays important roles in control of the stress response in sea bream. Furthermore, The interrenal ACTH sensitivity in sea bream is probably not regulated by cortisol. Thus, in sea bream the ACTH interrenal sensitivity would be regulated at the hypothalamus-pituitary levels and communicated via circulating ACTH levels, supporting similar mechanisms identified in mammals.

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