Effects of non-steroidal gonadal factors on LH secretion in female common carp during the reproductive cycle

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ABSTRACT: The aim of this study was to evaluate the effects of recombinant human inhibin A, recombinant human activin A and desteroidized ovarian extract on LH secretion *in vitro* and *in vivo* in female common carp during different stages of reproductive cycle. Inhibin stimulated spontaneous as well as GnRH-stimulated LH release *in vitro* in fish during gonadal recrudescence. This hormone did not have an influence on spontaneous LH secretion in the periovulatory period, but had a slightly inhibitory effect on GnRH-stimulated LH release in this stage of gonad maturity. Activin decreased spontaneous LH secretion during gonadal recrudescence and increased LH secretion before ovulation, having no effects on GnRH-stimulated LH release during both stages of gonad maturity. The desteroidized ovarian extract failed to modify spontaneous LH secretion, but decreased GnRH-stimulated LH release during recrudescence and especially before ovulation. It is to conclude that these data suggest the differential role of inhibin/activin as substances in the regulation of LH secretion in common carp females.

Keywords: inhibin; activin; ovarian extract; LH; common carp; aquaculture

The synthesis and secretion of luteinizing hormone (LH) in fish are, similarly like in mammals, controlled also by numerous factors of hypothalamic and gonadal origin. Recently, more attention has been paid to non-steroidal gonadal factors that can affect the release of gonadotropins, especially of activins and inhibins. In fish most data come from studies on cyprinids. In goldfish activin is produced in ovary, testis, brain, pituitary, kidney and liver (Ge et al., 1993; Ge and Peter, 1994). Up to now the specific fish inhibin has not been identified in fish tissues. In mammals activins stimulate FSH release (Vale et al., 1986) but their influence on LH is not so clear. Previous studies showed the absence of activin effects on LH (Vale et al., 1986; Rivier and Vale, 1991), but now it is known that activins can modify LH levels to a certain extent (Stouffer et al., 1993). Contrary to activin, inhibin decreases the secretion of FSH (Dye et al., 1992). There are increasing numbers of data on the role of these hormones in the regulation of FSH and LH synthesis and secretion in fish. While the action of activins and inhibins on FSH is similar to that in mammals, the results concerning LH are often controversial – they show the stimulatory influence of purified porcine activin on LH secretion (Ge et al., 1992), but on the other hand, using recombinant goldfish activin (Ge et al., 1997) they prove its inhibitory role in the synthesis of mRNA for LHβ subunit (Yam et al., 1999).

The aim of the present study was to evaluate the effects of recombinant human inhibin A, recom-

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binant human activin A and desteroidized ovarian extract on *in vitro* and *in vivo* LH secretion in female common carp during different stages of reproductive cycle.

The total of 118 four-years-old female carps originating from the Fishery Station affiliated to the Department of Ichthyobiology and Fisheries at the University of Agriculture in Krakow (Poland) were used at two stages of gonadal maturity: during gonadal recrudescence in December (average body weight – 2.22 ± 0.48 kg) and during the preovulatory period in May (average body weight – 2.96 ± 0.52 kg).

For *in vitro* experiments 12 fishes at the stage of gonad recrudescence and 10 fishes at the preovulatory stage of gonad maturity were used. All cultures were done according to the protocol established by Weil et al. (1986). Briefly, the pituitary glands were aseptically removed, minced using sterile surgical blades and enzymatically dispersed in a Minimum Essential Medium (MEM) solution containing 1% of BSA (Sigma-Aldrich, USA) and 0.1% collagenase A (Boehringer-Mannheim, Germany). After that period the fragments were mechanically dispersed by a sterile 10-ml syringe. The resulting suspension was then subjected to centrifugation for 10 min (200 g at 15°C) and the pellet was washed with MEM-antibiotic-Ultroser SF solution. Finally the cells were placed into Poly-L-Lysine (Sigma-Aldrich, USA) pretreated 96-well microplates (Nunc, Denmark) at the concentration of 50 000 cells per well (under 0.25 ml of MEM-antibiotic-Ultroser SF solution) and incubated for 48 hours at 22°C. After 48-hour preincubation the medium was poured off, and media containing different concentrations of recombinant human activin A (10 and 50 ng/ml; NIBSC, UK), recombinant human inhibin A (10 and 50 ng per ml; NIBSC, UK), sGnRH (10^{-9}, 10^{-8} and 10^{-7}M; Sigma-Aldrich, USA) or combinations of activin or inhibin with sGnRH were added. Each compound or their combination was tested in six replications (wells). After the addition of an incubation medium with tested substances the microplates were sealed and incubated for 24 hours at 22°C. Then the microplates were centrifuged at 200 g, and the resulting supernatant (medium) samples were frozen at –20°C until assayed.

For the purpose of *in vivo* experiments, desteroidized carp ovarian extract was prepared in the following manner: whole ovaries taken from 4-years-old carp females during the preovulatory period were homogenized in 0.6% aqueous solution of NaCl. The homogenate was centrifuged and dextran-coated charcoal (10 mg/ml) was added to the resulting supernatant in order to remove the steroids. After a 3-hour period of stirring at room temperature, the mixture was centrifuged in order to eliminate the charcoal and frozen in aliquots at –20°C. The levels of testosterone, 17β-estradiol and 17α, 20β-dihydroxyprogesterone were measured before and after dextran-coated charcoal treatment to ensure that the steroids were removed. Prior to each *in vivo* experiment fish were netted from a pond and transferred to concrete basins (2 m^3 each) with regulated water circulation and simulated natural photoperiod. Before all manipulations fish were anaesthetized with Propiscin (0.3 ml/l; IRS, Zabieniec, Poland). After 2 days of adaptation to the above-mentioned conditions fish were injected intraperitoneally with different doses of desteroidized ovarian extract and/or sGnRH analogue (Des-Gly^9, D-Arg^6, Pro-NHEt^9-LHRH, Bachem, Switzerland) as shown in Table 1. Just before and 3, 12 and 24 hours after injection the blood was sampled from each fish from the caudal vein. The blood samples were then centrifuged, and the re-

Table 1. Experimental groups in *in-vivo* experiments (for each group n = 8)

No.	Group name	Compounds and doses
1	control (saline)	0.6% NaCl solution 1 ml/kg b.w.
2	OE1	desteroidized ovarian extract (OE) 1 mg/kg b.w./1 ml solution
3	OE10	desteroidized ovarian extract (OE) 10 mg/kg b.w./1 ml solution
4	sGnRH-A	sGnRH analogue 5 μg/kg b.w./1 ml solution
5	sGnRH-A/OE1	sGnRH analogue 5 μg/kg b.w./1 ml solution + desteroidized ovarian extract (OE) 1 μg/kg b.w./1 ml solution
6	sGnRH-A/OE10	sGnRH analogue 5 μg/kg b.w./1 ml solution + desteroidized ovarian extract (OE) 10 mg/kg b.w./1 ml solution

sulting blood plasma was frozen and kept at –20°C until assayed.

The levels of LH were measured using the homologous ELISA assay according to the protocol established by Kah et al. (1989). The standard LH hormone and specific antibody were kindly donated by Dr. Bernard Breton (INRA, France). The levels of steroid hormones were measured using the ELISA technique (Szczerbik, 2008).

The LH concentrations were expressed as mean ± standard error of the mean (SEM). The results were subjected to one-way ANOVA followed by Tukey's post-test. The differences were considered significant at $P \leq 0.05$.

In common carp females at the time of gonadal recrudescence only the higher concentration of inhibin (50 ng/ml) induced a statistically significant increase in LH levels *in vitro*. The combined treatment with different concentrations of sGnRH and inhibin also evoked a significant stimulation of LH secretion in comparison with sGnRH-alone treated pituitary cells. In the case of the smallest sGnRH concentration (10^{-9}M) inhibin used at both concentrations significantly increased LH secretion. In the presence of higher sGnRH concentrations (10^{-8} and 10^{-7}M), only inhibin at a concentration of 10 ng/ml significantly increased LH levels in comparison with sGnRH treated cells (Figure 1a). In female common carp during the preovulatory period inhibin did not have a significant influence on the spontaneous LH secretion. Neither was LH secretion in the presence of sGnRH at concentrations of

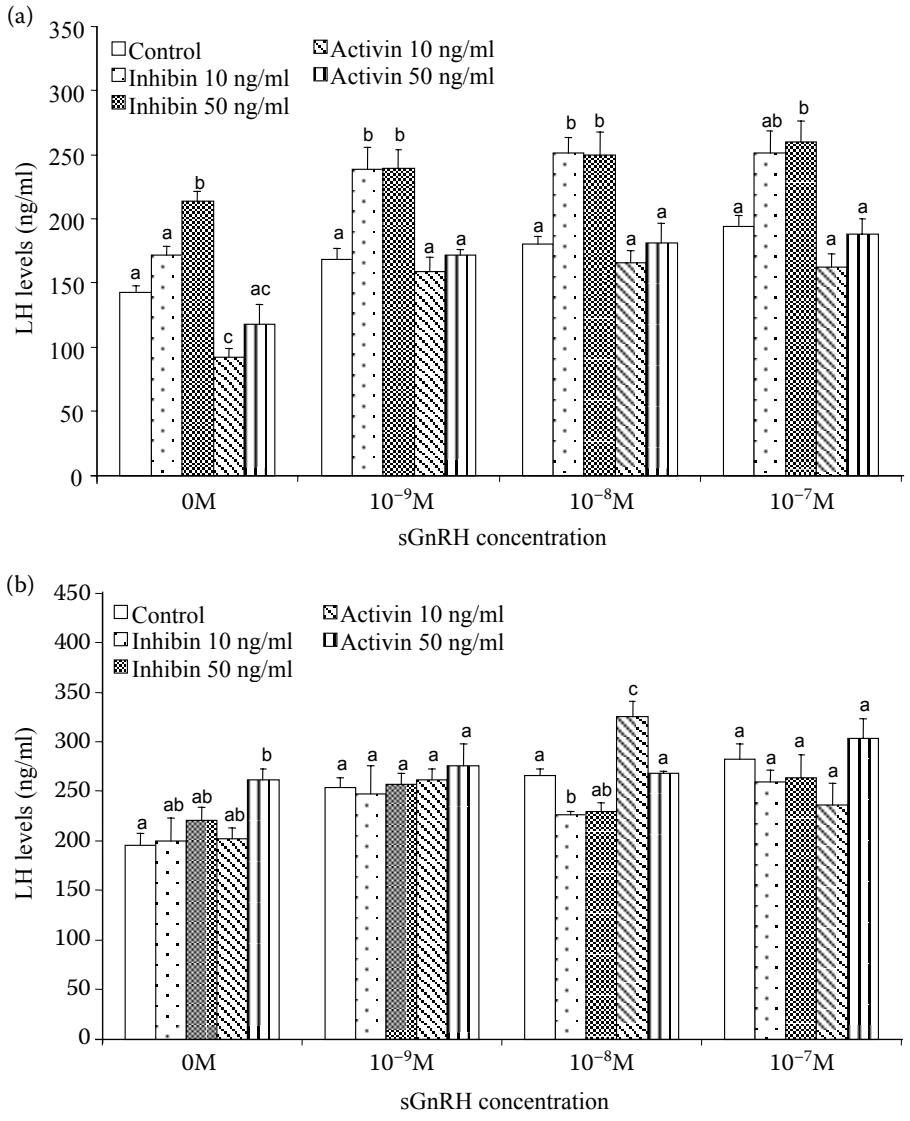

Figure 1. Effects of recombinant human inhibin A and recombinant human activin A on spontaneous as well as sGnRH-stimulated LH secretion from dispersed pituitary cells of female common carp during gonadal recrudescence (a) and during the periovulatory period (b); results are expressed as mean ± SEM. Columns bearing the same letter are not significantly dif-ferent

10^{-9}M and 10^{-7}M generally affected by both concentrations of inhibin. The only significant impact was observed after the treatment of dispersed cells with a combination of sGnRH 10^{-8}M and inhibin at a dose of 10 ng/ml which caused a decrease in LH levels compared to sGnRH alone (Figure 1b).

The stimulatory, dose-dependent effects of inhibin on spontaneous *in vitro* LH secretion in recrudescent female carp are consistent with the results obtained in goldfish by Ge et al. (1992). There are no available studies on the effects of inhibin on GnRH-stimulated LH secretion in cyprinid fish, however Chyb (2004) showed that inhibin stimulated sGnRH-induced LH secretion in rainbow trout until the beginning of vitellogenesis, but later it was unable to modify LH release under the influence of gonadotropin-releasing hormone. Our results seem to be in accordance with the latter study.

In the recrudescent common carp females, activin only at a concentration of 10 ng/ml significantly decreased spontaneous *in vitro* LH secretion. The treatment of dispersed pituitary cells with the combination of activin and different doses of sGnRH did not modify sGnRH-induced LH secretion (Figure 1a). During the periovulatory period activin at a concentration of 50 ng/ml significantly stimulated spontaneous LH secretion in fish, but the lower concentration of this hormone did not have any significant effects. In most cases activin was unable to modify sGnRH-induced LH release, except the significant stimulation of LH levels by

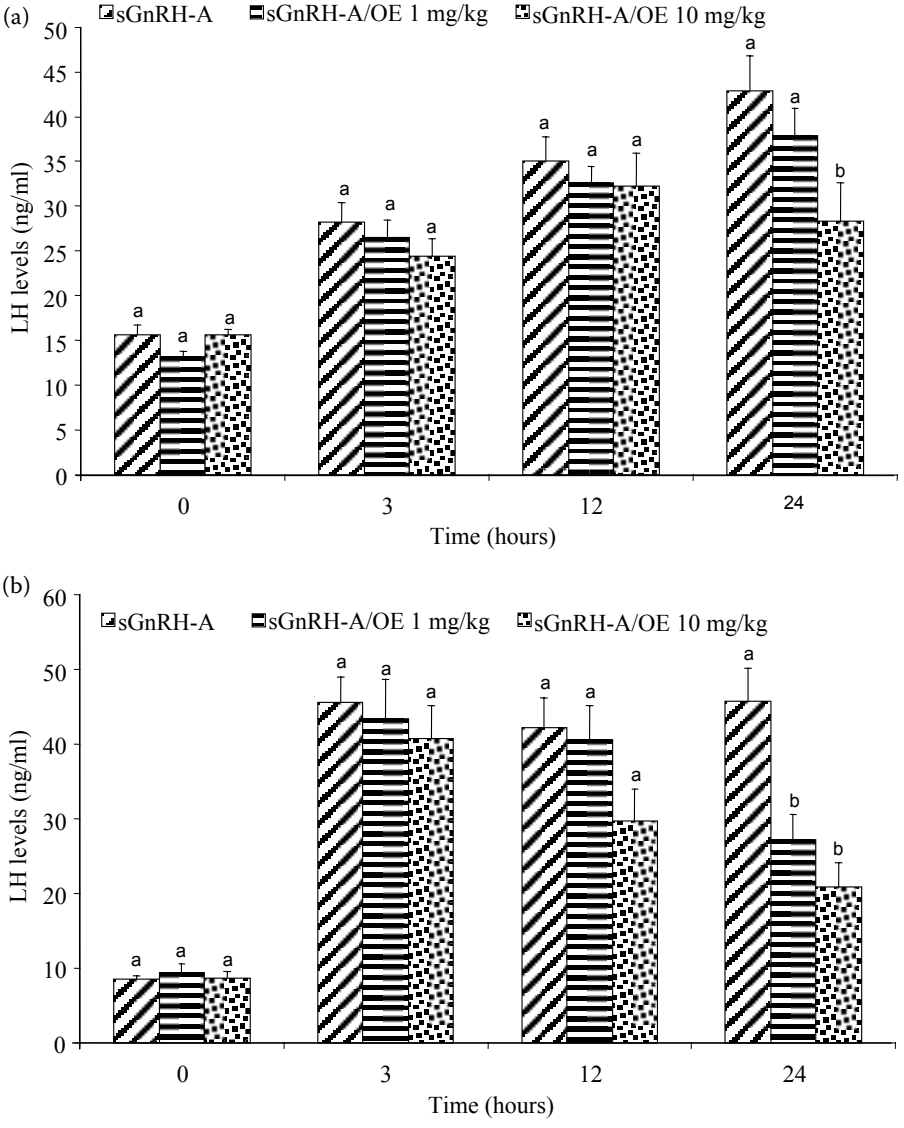

Figure 2. Influence of desteroidized ovarian extract (OE) at doses of 10 and 50 mg/kg b.w. on sGnRH-A-stimulated LH release *in vivo* in female common carp during gonadal recrudescence (a) and periovulatory period (b); results are expressed as mean ± SEM. Columns bearing the same letter within the same sampling time are not significantly different

activin at a concentration of 10 ng/ml in the presence of 10^{-8}M GnRH (Figure 1b).

These results are in contrast to previous studies on the impact of activin on LH release (Ge et al., 1992) that demonstrated the stimulatory influence of purified porcine activin on the spontaneous LH release in goldfish during regression and gonadal recrudescence. However, more recent work of Yam et al. (1999) showed that recombinant goldfish activin decreased LHβ subunit synthesis in goldfish during gonadal recrudescence. The stimulation of LH release by a high concentration of activin during the preovulatory period, observed in our experiments, is somehow surprising in the light of its inhibitory influence during gonadal recrudescence, but it is known that in goldfish approaching the ovulatory period the inhibitory influence of activin on LHβ synthesis becomes weaker and activin tends to elevate LHβ synthesis in the preovulatory period (Ge, personal communication). Until now there have been no data on the combined action of activin and GnRH on LH release in cyprinids, but in female rainbow trout recombinant human activin A had a very weak influence on GnRH-stimulated LH release – it had an inhibitory impact on LH release only in immature fish, having no effects in the later stages of ovarian development (Chyb, 2004). This seems to be consistent with our studies.

Our *in vivo* experiments have shown that the intraperitoneal injections of ovarian extract at both doses did not modify spontaneous LH secretion in female common carp during recrudescence (results not shown) but the ovarian extract used at a dose of 10 mg/ml significantly decreased sGnRH-stimulated LH secretion 24 h after injection (Figure 2a). The ovarian extract did not modify *in vivo* LH levels in fish during the periovulatory period in comparison with the control group (results not shown). On the other hand, both doses of the extract significantly decreased sGnRH-stimulated LH release 24 hours after injections (Figure 2b).

Interestingly, the inhibition of GnRH-A-induced LH secretion *in vivo* was observed in both stages of gonadal development, being more pronounced before ovulation. It is very doubtful that the gonadal steroids could be responsible for this inhibition, because the measurements of steroids clearly showed an important decrease in steroid levels in the ovarian extract after desteroidization (results not shown). Thus it is highly probable that non-steroidal gonadal factors could be engaged in this phenomenon.

In conclusion, this study demonstrates the evidence that inhibins and activins play a role in the control of LH release during the reproductive cycle in female common carp. The results of *in vitro* experiments show that inhibin and activin act in the opposite manner on spontaneous LH secretion in common carp females, having a more pronounced action during the recrudescence: this differential impact of activin and inhibin on LH is also present in the case of GnRH-stimulated LH release to a certain extent. Moreover, our results show that both inhibin and activin can influence LH release, contrary to mammals, where no impact on LH is generally observed. It also clearly shows that the effects of non-steroidal gonadal factors on LH release in common carp depend on the stage of reproductive cycle.

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