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LH BIOSYNTHESIS AND SECRETION IN RAT ANTERIOR PITUITARY CELL CULTURES: STIMULATION OF LH GLYCOSYLATION AND SECRETION BY GNRH AND AN AGONISTIC ANALOGUE AND BLOCKADE BY AN ANTAGONISTIC ANALOGUE

S. Azhar*, J.R. Reel†, C.A. Pastushok† and K.M.J. Menon*
*Endocrine Laboratory, Departments of Obstetrics and Gynecology and Biological Chemistry, University of Michigan Medical School, Ann Arbor, Michigan 48109

and

†Department of Pharmacology, Warner-Lambert/Parke-Davis Pharmaceutical Research Division, Ann Arbor, Michigan 48106

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SUMMARY: In rat anterior pituitary cell cultures GnRH (1nM) stimulated a progressive increase in LH release into the medium from 1 to 8 h of incubation, while cellular LH showed a corresponding decrease. GnRH (1nM) neither modified the uptake nor the incorporation of [^{3}H]-glucosamine and [^{3}H]-proline into total protein. The incorporation of [^{3}H]-proline into cellular LH also was unaffected by GnRH. In contrast, GnRH stimulated a 3 to 4-fold increase in [^{3}H]-glucosamine incorporation into cellular LH. The agonistic analogue, [des GlyNH_2^{10}]-LHRH ethylamide, mimicked the GnRH effects and was 5 to 6 times more potent than GnRH. The antagonistic analogue, [D-Phe^2, D-Phe^6]-LHRH blocked the GnRH-stimulated effects. These results suggest that GnRH and agonistic analogues may preferentially regulate turnover or synthesis of the carbohydrate moiety of LH.

It has been demonstrated that both natural and synthetic GnRH preparations stimulate the release of gonadotropins from rat pituitaries *in vivo* and *in vitro* (1-7). Further studies revealed that GnRH increases the accumulation of total radioimmunoassayable LH (8-10), suggesting the possibility that it may regulate, not only the release but also the synthesis of LH. Currently there are reports in favor (11-14) and against (15, 16) the involvement of GnRH in protein synthesis in the rat anterior pituitary. The present studies have attempted to resolve these conflicting results by investigating the direct effects of GnRH and peptide analogues in a rat anterior pituitary cell culture system.

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*To whom correspondence should be addressed.

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MATERIALS AND METHODS

Preparation of anterior pituitary cell cultures, incubation with labeled precursors, and extraction and immunoprecipitation of LH.

Anterior pituitaries were obtained from diestrous Charles-River CD rats (200-250 g) and pooled glands were dispersed with collagenase and hyaluronidase followed by Viokase® as described by Vale et al. (17) and Baker et al. (18). Cells were seeded at 1.5 x 10^6 cells per flask (Falcon Plastics, 75 cm^2) and grown in monolayer culture for 4 days using Dulbecco's modified Eagle's medium (DME, Grand Island Biological Co.) supplemented with 10% horse serum and 2.5% fetal calf serum. Following the removal of the growth medium and washing twice with serum-free DME, the 4-day cultures were employed in the experiments described. Ten ml of serum-free DME containing 20 µCi of either D-[6-^{3}H(N)]-glucosamine HCl (10.1 or 20.7 Ci/mmole, New England Nuclear Corp.) or L-[2,3-^{3}H]-proline (30 Ci/mmole, New England Nuclear Corp.) without or with appropriate concentrations of GnRH or peptide analogs were added to each flask. The uptake and incorporation of radioactive glucosamine and proline were allowed to proceed for 4 and 2 hr, respectively. At the end of the incorporation period the cultures were washed 3 times with ice-cold phosphate-buffered saline (PBS) and then dissolved in 1 ml of 0.1N NaOH. The dissolved cells were frozen and thawed twice, centrifuged at 4°C for 30 min at 1000 x g and each supernatant fraction decanted into dialysis tubing. The cell extracts were dialyzed twice against 50mM Tris HCl, pH 9.5 and twice against 0.1 M PBS, pH 7.5, with a volume ratio of 200:1 and a total time of 24 h at 0-4°C.

Non-specifically bound radioactivity was reduced by treating 1 ml of the dialyzed cell extract with 10 ul of undiluted normal rabbit serum (NRS) and incubating at 23°C for 1 h. The NRS was precipitated with goat anti-rabbit γ-gobulin by incubation overnight at 4°C followed by centrifugation. The supernatant fraction was used for specific LH immunoprecipitation. Immunoprecipitation of LH was carried out by a modified procedure of Burek and Frohman (19) as described by Chowdhury and Steinburger (20).

Uptake and incorporation of [^{3}H]-glucosamine and [^{3}H]-proline into the total cell fraction, acid-soluble pool, and acid-precipitable proteins.

At the end of the radiolabeling period, the cell cultures were washed 3 times with ice-cold PBS and then dissolved in 1 ml of 0.1 N NaOH. An aliquot (0.5 ml) of dissolved cells was neutralized with 0.25 ml of 0.2 N HCl, then 0.25 ml of γ-globulin (4%) and a mixture of 20% trichloroacetic acid and 2% phosphotungstic acid containing 1 mg/ml of the corresponding unlabeled precursor was added (21). After 10 min on ice this suspension was thoroughly mixed and the radioactivity in a 0.1 ml aliquot was determined after solubilization in 0.5 ml of NCS. This fraction was designated the total cell fraction. The remainder of the suspension was centrifuged at 7000 rpm for 10 min in a Sorvall centrifuge fitted with an SS34 rotor. An aliquot of the supernatant fraction was counted to determine the radioactivity in the acid-soluble pool. The pellet was washed 3 times by dissolving in 0.2 ml of 1 N NaOH followed by precipitation with 0.2 ml of 1 N HCl (22). The resulting acid-precipitable protein was dissolved in 1 ml of NCS tissue solubilizer (Amersham/Searle) and counted in 10 ml of scintillation fluid (23) using a Beckman model LS230 liquid scintillation spectrometer.

Radioimmunoassay of LH.

LH in the medium or dialyzed cell extract was measured by the double-antibody radioimmunoassay method (24) and the values expressed as NIH-LH-S1 equivalents (0.033 x NIAMDD rat LH-RP1 equivalents).

Peptides.

The GnRH and the three analogs employed in this study were synthesized and provided to us by Warner Lambert/Parke-Davis chemists. These synthetic peptides were as follows: GnRH, p-Glu-His-Try-Ser-Tyr-Gly-Leu-Arg-Pro-GlyNH_2 (acetate-hydrate; M.W. 1329); Agonist, [des-Gly$NH_2{}^{10}$]-GnRH ethylamide (acetate-hydrate, M.W. 1363); Antagonist, [D-Phe^2,D-Phe^6]-GnRH (hydrochloride; M.W. 1382); and inactive analogue, p-Glu-His-Tyr-Ser-Tyr-Gly-Leu-ArgNH_2 (acetate-hydrate; M.W. 1139).

RESULTS

Time course of GnRH-stimulated LH release.

GnRH (1nM) stimulated a progressive increase in LH release into the medium from 1 to 8 h of incubation, while cellular LH showed a corresponding decrease (Fig. 1). In addition, the total LH content in the culture system (medium plus cells) remained unchanged for both GnRH-treated and untreated cells.

Effect of GnRH on [^{3}H]-glucosamine and [^{3}H]-proline uptake and incorporation into total protein and cellular LH.

GnRH (1nM) neither affected the uptake of [^{3}H]-glucosamine and [^{3}H]-proline into the total cell fraction or acid-soluble pool nor incorporation into total protein. In addition, GnRH did not alter the incorporation of [^{3}H]-proline into cellular LH. In contrast, GnRH stimulated a 3-fold increase in the incorporation of [^{3}H]-glucosamine into cellular LH (Table 1). In an additional experiment, GnRH (1nM) had no effect on [^{35}S]-methionine incorporation into immunoprecipitable LH after 1, 2, 4 or 8 h of incubation (not shown).

Dose-response relationships: Effects of GnRH and an agonistic analogue on LH secretion and glycosylation.

GnRH at concentrations ranging from 10^{-11} to $^{-9}$ M stimulated a progressive increase in LH secretion. The agonistic analogue, [des-Gly$NH_2{}^{10}$]-GnRH ethylamide, exhibited a five-fold greater potency than GnRH in stimulating LH release over the same range of peptide concentrations. That is, the concentration of agonist stimulating half-maximal (ED_{50}) LH release (70 ng LH/ml) was 4.6 x 10^{-11}M, whereas that for GnRH was 2.3 x 10^{-10}M. Maximal LH release occurred at about 10^{-9}M for GnRH and 3 x 10^{-10}M for the agonist. As expected, the N-terminal octapeptide of GnRH was inactive over the same concentration range (Fig. 2A).

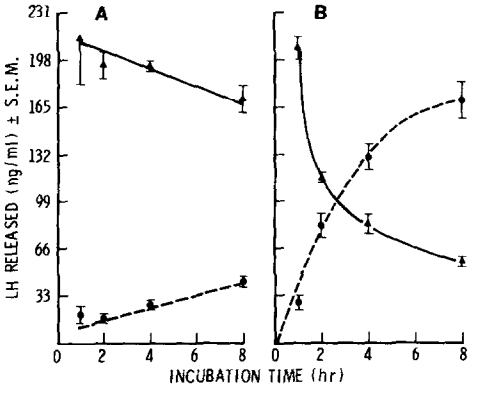

Fig. 1. Effect of GnRH on the Time Course of LH Release by Cultured Pituitary Cells.

Incubation conditions are described in "Materials and Methods". [A] Control; [B] GnRH (10^{-9}M); ▲—▲—▲ cells; ● -●-·-● medium.

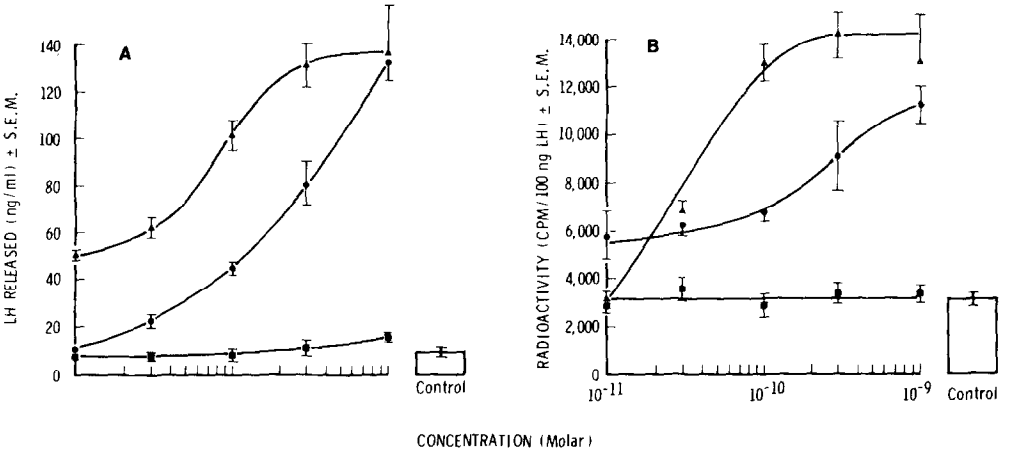

Fig. 2. Dose-Response Relationships: Effects of GnRH and an Agonostic Analogue on LH Secretion and Glycosylation.

Incubation conditions were same as described in "Materials and Methods". ▲—▲—▲, agonistic analogue; ●—●—●, GnRH; ■—■—■, inactive analogue. A = LH released into medium; B = Radioactivity into immunoprecipitable LH.

The stimulation of LH glycosylation by GnRH and the agonist showed a similar dose-response relationship to that observed for LH release. The ED_{50} for the stimulation of LH glycosylation was 3.8×10^{-11}M for the agonist and

Table 1

Effect of GnRH on the Uptake and Incorporation of [^{3}H]-Glucosamine and [^{3}H]-Proline into Total Protein and Cellular LH in Rat Pituitary Cell Cultures

Incubation conditions were as described under "Materials and Methods".

	Radioactivity (CPM/100µg Protein ± SEM)			(CPM/100ng cell LH ± SEM)
Additions	Total Cell Fraction	Acid-Soluble Pool	Acid precipitable Protein	Immunoprecipitable LH
I. [^{3}H] Glucosamine				
None	3035 ± 248	1412 ± 127	830 ± 58	771 ± 180
GnRH (10^{-9}M)	3748 ± 357	1548 ± 75	1007 ± 30	2304 ± 288
II. [^{3}H] Proline				
None	18818 ± 477	6635 ± 477	4306 ± 167	891 ± 33
GnRH (10^{-9}M)	19434 ± 128	6548 ± 444	4165 ± 257	780 ± 80

2.4 x 10^{-10}M for GnRH. Thus, by this endpoint, the agonist was 6 times more potent than GnRH. Maximal LH glycosylation occurred at 10^{-9}M for GnRH and 3 x 10^{-10}M for the agonist. Again, the N-terminal octapeptide of GnRH was inactive (Fig. 2B).

Blockade of GnRH-stimulated LH secretion and glycosylation by an antagonistic analogue.

At a molar concentration of 1000 times that of GnRH the antagonistic analogue, [D-Phe^2, D-Phe^6]-GnRH, effectively blocked GnRH-stimulated LH release and glycosylation (Fig. 3A and 3B). In addition, the antagonist (10^{-6}M) showed no intrinsic agonistic activity by either endpoint.

DISCUSSION

These results indicate that exposure of rat pituitary cell cultures to to GnRH (10^{-9}M) neither modified amino acid or glucosamine uptake nor incorporation into total protein. Furthermore, GnRH (10^{-9}M) did not alter the incor-

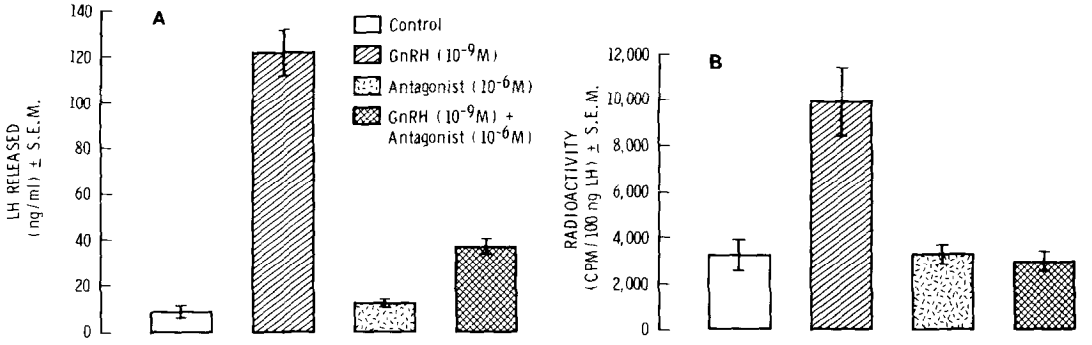

Fig. 3. Blockade of GnRH-stimulated LH Secretion and Glycosylation by a GnRH Antagonistic Analogue.

Incubation conditions are described in "Materials and Methods".

poration of [^{3}H]-proline or [^{35}S]-methionine into cellular LH. In contrast, during a 4 h incubation, GnRH (10^{-9}M) stimulated an apparent 3 to 4-fold increase in the incorporation of [^{3}H]-glucosamine into cellular LH. The interpretation of this latter effect was complicated by differences in the cell to medium distribution of total LH in GnRH-treated as compared to untreated cultures. That is, cells incubated for 4 h with 10^{-9}M GnRH contained only about 20 to 40% as much LH as untreated cells. Hence, if GnRH-treated and untreated cells released only preexistent unlabeled LH into the medium during the 4 h [^{3}H]-glucosamine incorporation period then the reduction of total LH in GnRH-treated cells could account for the increased specific activity of cellular LH. This possibility seems unlikely in view of the fact that the specific activity of cellular LH did not change in GnRH-treated cells during 1 to 8 h periods of labeled amino acid incorporation. To resolve this dilemma it will be necessary to determine the radioactive LH released into the medium during the precursor incorporation period. Thus far, technical difficulties have prevented us from making these determinations.

The hormonal specificity of the stimulatory effect of GnRH on LH release and the increase in [^{3}H]-glucosamine incorporation into cellular LH was evident from the following observations: (1) The agonistic analogue, [des $GlyNH_2^{10}$]-

LHRH ethylamide, (25-27) mimicked the GnRH effects and was 5 to 6 times more potent than GnRH; (2) the antagonistic analogue, [D-Phe^2, D-Phe^6]-LHRH, (28, 29) blocked the GnRH-stimulated effects; and (3) the N-terminal octapeptide of LHRH (30) was inactive.

The effects of GnRH on [^{3}H]-glucosamine incorporation into LH in part confirm the previous results of Liu, Jackson, and Gorski (13) and Liu and Jackson (31). These workers incubated quartered or halved rat pituitaries *in vitro* and found that GnRH stimulated the incorporation of labeled glucosamine into total LH but had no effect on the incorporation of radioactive amino acid into LH. In addition, during a 4 h incubation, GnRH stimulated the release of LH labeled either with radioactive glucosamine or amino acid. Collectively these data along with those obtained in the present study suggest that GnRH may preferentially stimulate the turnover of or incorporation of glucosamine into the carbohydrate portion of LH. LHRH stimulation of LH glycosylation might result from either a direct enzymatic activation or through increased synthesis or decreased degradation of glycosyltransferases. Further studies will be required to determine which of these mechanisms may be operative.

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