Gonadotropin-releasing peptide from human follicular fluid: Isolation, characterization, and chemical synthesis

(Choh Hao Li*, K. Ramasharma, Donald Yamashiro, and David Chung)

Laboratory of Molecular Endocrinology, University of California, San Francisco, CA 94143

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ABSTRACT A gonadotropin-releasing peptide has been isolated from human follicular fluid. Its amino acid composition and sequence are completely different from the hypothalamic lutropin-releasing hormone. It is designated human follicular gonadotropin-releasing peptide and abbreviated as hF-GRP. The primary structure of this peptide (H-Thr-Asp-Thr-Ser-His-His-Gln-Asp-His-Pro-Thr-Phe-Asn-OH) has been confirmed by chemical synthesis. In the mouse pituitary incubation assay, the ED50 value for follitropin or lutropin release is estimated to be 1.2-1.6 nM.

It has been reported that seminal and follicular fluid have stimulating activity for the secretion of pituitary gonadotropins (1, 2) but the chemical structure of the active substance(s) is not known. We report here the isolation of a 14-amino acid peptide from human follicular fluid with in vitro gonadotropin-releasing activity. The amino acid composition and sequence of the peptide are completely different from the hypothalamic lutropin-releasing hormone (LHRH) (3). The peptide is designated human follicular gonadotropin-releasing peptide (hF-GRP).

MATERIALS AND METHODS

Follicular fluid was obtained from women undergoing fertility examination at the Department of Obstetrics, Gynecology, and Reproductive Sciences on this campus. After removal of insoluble material by centrifugation (30 min; 10,000 rpm), the supernatant was acidified to 0.01% trifluoroacetic acid and stored at 0°C. Carboxypeptidase Y was obtained from Pierce, human a1-antitrypsin was from Sigma, and chloromethyl resin was from Lab Systems (San Mateo, CA).

Exclusion chromatographies were carried out with a Sephadex G-100 column (2.2 × 45 cm) with 0.01 M NH4OAc (pH 4.6) or a Sephadex G-10 column (1.5 × 22 cm) with 0.1 M HOAc. HPLC was performed on a Vydac 218TP104 or 218TP1010 column (4.5 or 10 × 250 mm; Western Analytical Products; Temecula, CA) using a dual pump system from Laboratory Data Control (Riviera Beach, FL) with UV detection at 210 nm. The solvents used were 2-propanol in 0.1% trifluoroacetic acid or acetonitrile in 25 mM H3PO4.

Paper electrophoresis was on Whatman 3MM paper in γ-collidine/HOAc/H2O, pH 7 (8.9:3.1:988, vol/vol) at 400 V for 2 hr and detection was by the Pauly reagent. Amino acid analysis was performed in an automatic amino acid analyzer (Beckman model 119 C) as described (4). The NH2-terminal residue was determined by the dansyl-Edman analysis (5). For COOH-terminal residue analysis, carboxypeptidase Y digests were carried out in 1 M pyridine acetate (pH 5.5) for 4 and 16 hr at 37°C with an enzyme/substrate molar ratio of 1:7. Automatic Edman sequence analysis was carried out on a model 470A sequencer (Applied Biosystems, Foster City, CA) in the Biomolecular Resource Center of this campus. Gonadotropin-releasing activity was assayed by the in vitro mouse pituitary procedure as described (6, 7). The follicle-stimulating hormone (FSH) and luteinizing hormone (LH) released into the medium was estimated by specific radio-receptor assays (6) using highly purified ovine gonadotropins prepared in our laboratory as standards.

RESULTS

The acidified follicular fluid (50 ml) was added to 350 ml of rapidly stirred cold ethanol and then kept at 0°C for 16 hr. After centrifugation, the supernatant was dried by rotary evaporation in vacuo, and the resulting residue was dissolved in 200 ml of distilled water and lyophilized to yield 3.2 g of white powder. This ethanol-soluble fraction was separated into four components in a Sephadex G-10 column as shown in Fig. 1. The bioactive material was located mainly in component 3, which was desalted on Sephadex G-10. From 50 ml of follicular fluid, ~10 mg of crude bioactive peptide was recovered. The desalted peptide was submitted to further purification on HPLC, and the bioactive fraction was eluted at 17 min as a single peak (Fig. 2). The content in the single peak from 1 mg of desalted peptide was lyophilized to yield 30 μg of hF-GRP. Thus, 50 ml of follicular fluid yielded ~0.3 mg of hF-GRP.

A sample of hF-GRP (5 μg) was shown to be homogeneous by HPLC using a gradient of acetonitrile in 25 mM H3PO4 (see Fig. 4B). Paper electrophoresis of the purified peptide revealed a single band. The dansyl–Edman analysis (5) showed that threonine was the only NH2-terminal residue. Carboxypeptidase Y digestion of hF-GRP did not release any amino acids. Amino acid analysis after a 22-hr analysis in constant boiling HCl gave the following residues (sequence values in parenthesis): Asp3,70 (4); Thr5,64 (3); Ser,y,09 (1); Glu1,06 (1); Pro1,08 (1); Phe0,83 (1); His2,51 (3). Thus, the peptide consists of 14 amino acid residues with a calculated molecular weight of 1624.8. Automatic sequence analysis of hF-GRP gave the following result: H-Thr-Asp-Thr-Ser-His-Asp-Asp-His-Pro-Thr-Phe-Asn-OH.

Synthesis was performed by the solid-phase method (8). i-Butoxycarbonyl asparagine (Boc-Asn-OH) was esterified with chloromethyl resin by the CsHCO3 procedure (9) with KI catalysis to give a load of 0.145 mmol/g. Synthesis was carried out in a Beckman model 990 peptide synthesizer with couplings being effected with preformed symmetrical anhydrides (10) in CH2Cl2 except for glutamine, which was incorporated by the 1-hydroxybenzotriazole method (11).

Abbreviations: hF-GRP, human follicular gonadotropin-releasing peptide; LH, lutropin or luteinizing hormone; LHRH, LH-releasing hormone; FSH, follitropin or follicle-stimulating hormone.

*To whom reprint requests should be addressed: Laboratory of Molecular Endocrinology, Room C-440, University of California, San Francisco, CA 94143-0642.
Side-chain protecting groups were: benzyl for threonine, aspartic acid, and serine, and 2-bromobenzyloxycarboxyl for histidine. (The preparation and use of $N^\omega$-$t$-butoxycarbonyl-$N^\alpha$-$t$-butoxycarbonyl-2-bromobenzyloxycarboxyl)histidine [Boc-His(2-BrZ)-OH] in peptide synthesis will be described elsewhere.) Removal of all protecting groups and cleavage from the resin were performed in 10% $p$-cresol in liquid HF for 1 hr at 0°C (12) followed by gel filtration on Sephadex G-10 in 25 mM NH$_4$HCO$_3$ and preparative HPLC on a Vydac 218TP1010 column with a gradient of 0–25% (vol/vol) acetonitrile in 25 mM H$_3$PO$_4$ (2.5 ml/min) over 60 min. After final gel filtration on Sephadex G-25 (25 mM NH$_4$HCO$_3$), the product was isolated in ≈40% yield based on starting resin. It was homogeneous in HPLC on a Vydac 218TP104 column in two solvent systems [0–20% 2-propanol in 0.1% trifluoroacetic acid (Fig. 3) and 0–20% acetonitrile in 25 mM H$_3$PO$_4$ (Fig. 4A)] and in paper electrophoresis (data not shown). Amino acid analysis after a 24-hr hydrolysis in constant boiling HCl was: Asp$_{10}$; Thr$_{3.92}$; Ser$_{1.00}$; Glu$_{1.05}$; Pro$_{0.97}$; Phe$_{1.04}$; His$_{2.92}$.

Fig. 5 and Table 1 present the in vitro bioassay data for synthetic hF-GRP in stimulating FSH and LH release in mouse pituitary incubation system. Detailed studies on the biological property of synthetic hF-GRP will be reported elsewhere.

**DISCUSSION**

Various investigations have been attempted to isolate from gonadal fluid active peptides or proteins that are different from that of hypothalamic LHRH. Ying et al. (13) believed their purified peptides from rat follicular fluid with gonado-
Control, stimulating LHRH-like activity fluid. Preliminary Manjunath herein a seminal plasma. rat whole observation). Published of the 6. FIG. 5. Dose-response relationship of synthetic hF-GRP in stimulating mouse pituitary FSH (A) and LH (B) secretion in vitro. Control, hatched bar; LHRH (10 ng), open bar. n = 5; mean ± SEM.


tropin-releasing activity to have molecular weight <3500. Manjunath (2) reported three forms of acidic proteins with LHRH-like activity and molecular weight =20,000 from bull seminal plasma. Rivier et al. (14) described the existence of FSH-releasing activity in bovine follicular fluid. We report herein a 14-amino acid peptide (hF-GRP) with gonadotropin-releasing activity in an in vitro assay from human follicular fluid. Preliminary data showed that hF-GRP is active in the whole rat pituitary incubation system and anterior pituitary cell culture assay (M. R. Sairam, K.R., and C.H.L., unpublished observation).

It is interesting to note that hF-GRP is located in residues 11–24 of the primary structure of human α1-antitrypsin (15, 16) as shown in Fig. 6. hF-GRP-(10–14) was also found in the amino acid sequence of bovine papilloma virus (17). In addition, hF-GRP-(1–3), (6–8), and (10–12) are tripeptide sequences in the baboon α1-antitrypsin structure (18). Since there is only a single lysine in residue positions 10 and 25 of both human (15, 16) and baboon α1-antitrypsin (18) sequences, it is possible that they are precursors for hF-GRP. However, it is generally accepted that cryptic domains of precursors for the formation of peptide hormones are flanked by pairs of basic amino acids (19–21). It may also be noted that α1-antitrypsin does not exhibit gonadotropin-releasing activity (Table 1).

Table 1. Effects of synthetic hF-GRP, α1-antitrypsin, and LHRH on FSH and LH release in the mouse pituitary incubation assay

<table>
<thead>
<tr>
<th>Polypeptide</th>
<th>Dose, μg</th>
<th>Gonadotropin release, ng/ml*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>FSH</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>5.6 ± 0.7</td>
</tr>
<tr>
<td>hF-GRP</td>
<td>0.12</td>
<td>6.0 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>1.11</td>
<td>13.6 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>3.33</td>
<td>32.0 ± 8.6</td>
</tr>
<tr>
<td>LHRH</td>
<td>0.01</td>
<td>36.3 ± 2.3</td>
</tr>
<tr>
<td></td>
<td>0.025</td>
<td>68.9 ± 5.2</td>
</tr>
<tr>
<td>α1-Antitrypsin</td>
<td>40.0</td>
<td>6.4 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>200.0</td>
<td>4.3 ± 0.6</td>
</tr>
</tbody>
</table>

*Mean ± SEM (n = 5).

Peptides with primary structure different from that of hypothalamic LHRH exhibit gonadotropin-releasing activity. Millar et al. (22) found that a segment of human LHRH precursor (23) [pHLHRH-(14–26)] is capable of stimulating gonadotropin release from human and baboon pituitary cells. Ying et al. (24) showed that the transforming growth factor type β is a potent stimulator of FSH-release in rat pituitary cells. Two laboratories (25, 26) described independently purification and characterization of FSH-releasing proteins from porcine follicular fluid: one is a homodimer of two inhibin β subunits linked by disulfide bonds (25), and the other is a heterodimer of the β subunits from two forms of inhibin (26). It is, therefore, of interest to note that the 14-amino acid hF-GRP from human follicular fluid with gonadotropin-releasing activity has an amino acid sequence completely different from that of LHRH, pHLHRH-(14–26) (Fig. 7), transforming growth factor type β, and FSH-releasing proteins.

Fig. 6. Comparison of hF-GRP primary structure with α1-antitrypsin (human and baboon) and bovine papilloma virus amino acid sequences.

Fig. 7. Comparison of hF-GRP primary structure with LHRH and pHLHRH-(14–16).
In comparison with the hypothalamic LHRH, hF-GRP is considerably less active in releasing gonadotropins in the mouse pituitary incubation assay (Table 1 and Fig. 5). The ED90 value for FSH release was estimated to be 2.0 μg (1.2 nM), and for LH release was estimated to be 2.6 μg (1.6 nM) per tube per ml. Since hF-GRP is a simple peptide with only 14 amino acid residues, it may not be difficult to design analogs as potent agonists or/and antagonists.

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