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Human seminal α-inhibins: Detection in human pituitary, hypothalamus, and serum by immunoreactivity

(follitropin/radioimmunoassay/immunoblot analysis/exclusion chromatography)

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ABSTRACT **An antiserum generated in rabbits against human seminal α-inhibin-52 has been used to develop a sensitive radioimmunoassay for the detection of α-inhibins. The α-inhibin-52 antiserum reacts with α-inhibin-92 and α-inhibin-31 with equal avidity. These peptides were found to be present in human pituitary, hypothalamus, and serum. In exclusion chromatography on Sephadex G-100, the immunoreactive material eluted in a large molecular size region. Immunoblot analysis of column-derived fractions of these extracts revealed the presence of α-inhibin-92. The mean concentrations of immunoreactive α-inhibin were found to be 7.2 ng/ml in normal adult male serum, 70.3 ng/g (wet weight) of pituitary, and 12.9 ng/g (wet weight) of hypothalamus. This communication reports on the evidence for the existence of gonadal peptides in the brain.**

Evidence emerging from various laboratories suggests that gonads secrete biologically important proteins and peptides (1–3). Among these molecules, inhibin is most important because it is involved in the feedback regulation of follitropin (FSH) secretion (3–6). Several investigators (7–11) have reported the isolation and primary structure of inhibin-type molecules from gonadal fluids. These molecules were shown to affect pituitary follitropin secretion in a number of model systems. Furthermore, it has been suggested the hypothalamus is an additional site of inhibin action (12). We describe herein a sensitive radioimmunoassay (RIA) for α-inhibin, and the detection of immunoreactive α-inhibin in human pituitary, hypothalamus, and serum.

MATERIALS AND METHODS

Materials. α-Inhibin-52 and α-inhibin-92 were isolated from human seminal plasma as described (9). Freunds adjuvant was obtained from GIBCO, lactoperoxidase from Calbiochem-Behring, $Na^{125}I$ from New England Nuclear, sheep anti-rabbit gamma globulin from Antibodies, Inc., bovine serum albumin (BSA) from Sigma, and Trans-Blot cell from Bio-Rad. Fresh human pituitary glands were supplied by the National Hormone and Pituitary Program (National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases), and human hypothalami were from the Multiple Sclerosis Human Neurospecimen Bank (Veterans Administration Wadsworth Medical Center, Los Angeles, CA).

RIA. Two male New Zealand white rabbits were injected subcutaneously in small volumes (50 μl) with 1 mg of α-inhibin-52 in saline/Freunds complete adjuvant (2 ml). The booster injections consisting of the incomplete Freunds adjuvant emulsified with 1 mg of α-inhibin-52 in saline were given after 4 weeks. The animals were bled after 2 weeks, and antibody titers were determined.

α-Inhibin-92 was iodinated by the lactoperoxidase method (13), and the double-antibody procedure (14) was employed for RIA as described (15). The total incubation volume was 0.80 ml per tube. Radioactivity was counted in a Beckman gamma spectrometer. The data were evaluated using a nonlinear least-squares curve-fitting program, which was adopted from published methods (16, 17).

Tissue Extractions. Extractions were carried out at 4°C. Tissues were cut into small pieces and homogenized in cold 0.1 M HCl (1 g/5 ml) with a Brinkmann polytron (setting 10) for 10–15 s, and the suspensions were centrifuged in a Sorvall RC-5B centrifuge with an *SS34* rotor (10,000 rpm; 10 min). The clear supernatant was lyophilized and stored at −70°C.

Exclusion Chromatography. Chromatography was performed on a Sephadex G-100 column (1.3 × 65 cm) at 4°C. The column was equilibrated in 0.01 M NH_4OAc of pH 4.6 and 2-ml fractions were collected. Human pituitary or hypothalamic extract (100 mg) or 3 ml of lyophilized human serum (acidified) were dissolved in 2 ml of buffer; insoluble material was separated by centrifugation, and clear supernatant was applied to the column. Column fractions were stored at −20°C prior to monitoring immunoreactive material by RIA.

Immunoblot Analysis. Immunoreactive fractions (150 μg) obtained by gel filtration of human pituitary, hypothalamus, or serum were fractionated on $NaDodSO_4$/PAGE as described (18). The gel was either immediately fixed and stained in Coomassie blue (R-250) or used for immunoblot analysis. The electrophoretic transfer procedure has been adapted as described (19). Immediately following transfer, the nitrocellulose sheet was immersed in a 0.02 M sodium phosphate/0.15 M NaCl, pH 7.4 (PBS), containing 3% (wt/vol) BSA for 30 min with constant shaking. Following two washes in the same buffer, the nitrocellulose sheet was soaked in the milk-based cocktail (20) for 1 hr. The sheet was washed three times with PBS and incubated with primary antibody (1:1000 dilution) in PBS with 3% (wt/vol) BSA overnight with constant shaking. Then the sheet was washed four times with PBS containing 0.01% Tween 80 followed by two washes with PBS/3% (wt/vol) BSA and incubated in PBS/3% (wt/vol) BSA containing ^{125}I-labeled sheep anti-rabbit gamma globulin for 4 hr at 22°C. The nitrocellulose was washed thoroughly three times with PBS/3% (wt/vol) BSA and PBS containing Tween 80. The sheet was dried on a gel dryer for 10 min and exposed to XAR-5 film (Eastman Kodak, Rochester, NY) for 48 hr at −70°C.

RESULTS

Antiserum of high titer (final dilution, 1:40,000) was obtained from one of the two rabbits that had been immunized with α-inhibin-52. It cross-reacted with α-inhibin-31 and α-inhibin-92 with equal affinity. The antiserum showed no cross-reaction to any of the known pituitary or hypothalamic

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Abbreviations: RIA, radioimmunoassay; BSA, bovine serum albumin.

peptides. The radioiodinated α-inhibin-92 was stable for 4 to 5 weeks when stored at 4°C. The nonspecific binding ranged 4–6% during this period.

A typical standard curve is shown in Fig. 1. The effective dose, 50% (ED_{50}), and the slope of 10 different assays (mean ± SE) ranged 2.23 ± 0.1 ng per tube and 0.57 ± 0.03, respectively. The minimal detectable dose was found to be 0.1–0.2 ng of inhibin per tube. The recovery of added α-inhibin-92 to the blank serum at two concentrations (10 and 50 ng) was 85.5% (n = 20) in three different experiments. The intra- and interassay coefficient of variations for a pool of unidentified human serum were 9.49 and 14.0%, respectively. The pool of human serum showed a dose-dependent response as shown in Fig. 1. Human pituitary and hypothalamic extracts showed concentration-dependent response; the dose–response curve for the pituitary extract shifted to the left after fractionation on a Sephadex G-100 column. The amounts of immunoreactive material in human pituitary, hypothalamus, and serum were estimated to be as follows: pituitary, 70.3 ± 3.9 ng per g (wet weight) (n = 10); hypothalamus, 12.9 ± 1.8 ng per g (wet weight) (n = 15); and serum (normal adult male), 7.2 ± 1.1 ng/ml (n = 6).

When the human serum sample was fractionated on a Sephadex G-100 column, the immunoreactive material eluted earlier than the authentic α-inhibin-92 or α-inhibin-52 (Fig. 2*A*). When α-inhibin-92 was mixed with nonimmune human serum and chromatographed on same column, the immunoreactivity again appeared in the void volume (Fig. 2*B*). Under identical chromatographic conditions, the immunoreactive material in the human pituitary and hypothalamic extracts was also found in the high molecular size region (Fig. 2 *C* and *D*).

The immunoreactive fraction, obtained from gel filtration and further fractionated by $NaDodSO_4$/PAGE, was resolved into several bands. These bands were electrophoretically transferred to nitrocellulose sheets. As shown in Fig. 3, immunoreactive material of the column eluent of human pituitary extract appeared to behave as α-inhibin-92 and α-inhibin-52. The hypothalamic extract and human serum showed α-inhibin-92-related material. All these extracts contained a high molecular size protein that showed very little migration into the gels.

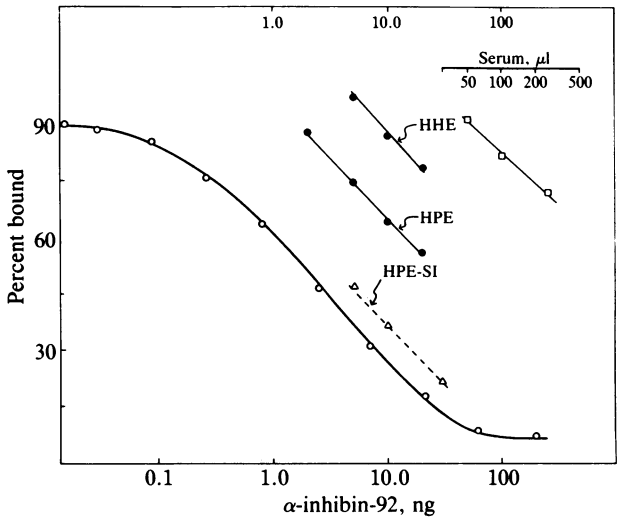

FIG. 1. Displacement of ^{125}I-labeled α-inhibin-92 in RIA by different concentrations of unlabeled α-inhibin-92. The dose–response relationship of pooled human serum (□) and crude extracts (●) of human pituitary (HPE) and human hypothalamus (HHE). HPE-SI (△) indicates the Sephadex G-100 fraction of HPE.

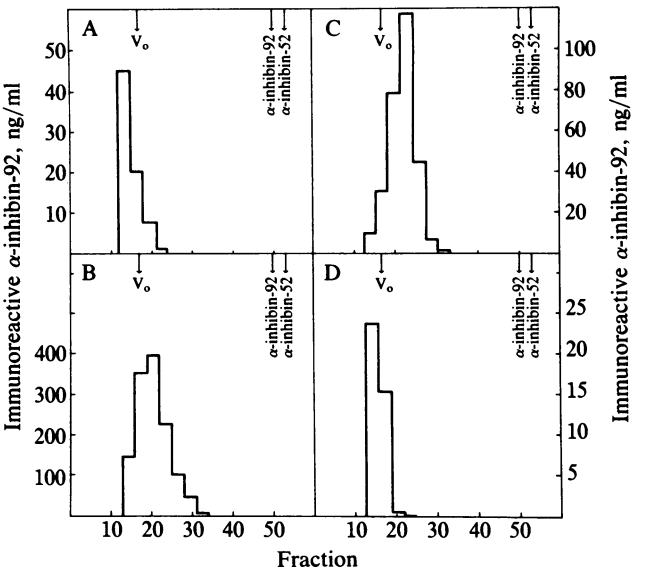

FIG. 2. Immunoreactive α-inhibin-related material from Sephadex G-100 column fractions. Acidified human serum (*A*). A mixture of α-inhibin-92 and a blank serum (*B*). Human pituitary (*C*). Human hypothalamus (*D*). Arrows indicate the position of elution of radiolabeled α-inhibin-92 and α-inhibin-52. V_o, Void volume.

DISCUSSION

The RIA method for α-inhibin described herein is specific, sensitive, and applicable for identification of immunoreactive α-inhibin-related peptides. To clarify whether the immunoreactivity measured in RIA is related to α-inhibin-92 or α-inhibin-52, the serum sample was chromatographed on a Sephadex G-100 column. The immunoreactivity was found in the void volume in contrast to the authentic peptide markers (Fig. 2*A*). In addition, when a mixture of α-inhibin-92 (1 μg/ml) and 3 ml of nonimmune serum was chromatographed on the same column, immunoreactive α-inhibin-92 appeared in the void volume (Fig. 2*B*). These data suggest that α-inhibin peptides are not free in the circulating serum but

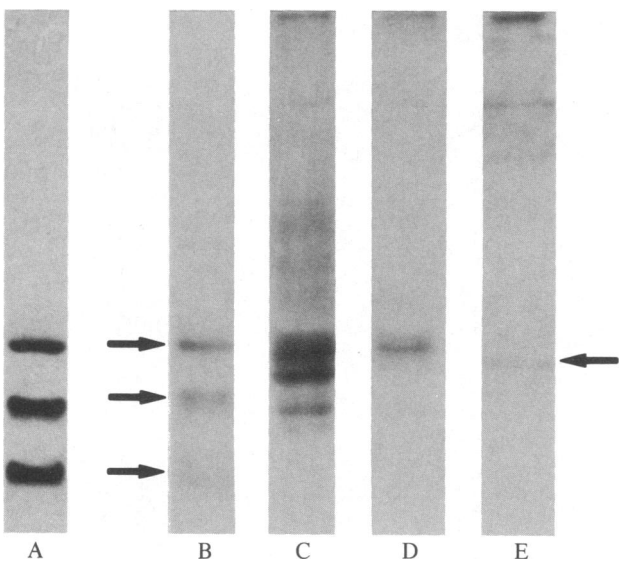

FIG. 3. Immunoblot analysis of α-inhibin-related peptides. Lane A, Coomassie blue-stained α-inhibin-92, -52, and -31. Lane B, immunoblot of α-inhibin peptides (50 ng). Lanes C, D, and E, Sephadex G-100 column-derived fractions of human pituitary, hypothalamus, and serum.

bind to proteins of high molecular weight. However, the blotting analysis results give evidence of the presence of immunoreactive α-inhibin-92 in the column-derived serum fraction.

The surprise finding was that pituitary and hypothalamic extracts contain immunoreactive α-inhibin-related peptides. Preliminary studies showed the existence of α-inhibin in sheep and rat pituitary glands as examined by RIA. When these extracts were subjected to gel filtration, the immunoreactivity appeared mostly in the high molecular size region. However, the blotting analysis clearly indicates the presence of immunoreactive α-inhibin-92 and α-inhibin-52. Significant amounts of immunoreactivity were found in the anterior pituitary but not in the posterior lobe. The amount of α-inhibin immunoreactivity found in the hypothalamus is lower than that in the pituitary. The functional significance of α-inhibin peptides in the pituitary and hypothalamus remains to be investigated.

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