High Molecular Weight Forms of Immunoreactive ACTH in a Human Pituitary and Ectopic ACTH-Producing Tumors

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Abstract

High molecular weight forms of immunoreactive ACTH (IR-ACTH) were studied in a human pituitary gland and 4 ectopic ACTH-producing tumors in man. Both the pituitary and tumor extracts contained “big” IR-ACTH, which eluted near the void volume, and a small amount of “intermediate” IR-ACTH components, which eluted between the void volume and $^{125}\text{I-ah}^{1-39}\text{ACTH}$, in addition to “little” ACTH which coeluted with $^{125}\text{I-ah}^{1-39}\text{ACTH}$ by Sephadex G-100 gel filtration. A significant amount of the “big” IR-ACTH applied bound to the concanavalin A-agarose column and was eluted with 0.2 M $\alpha$-methyl-D-mannopyranoside, indicating the glycoprotein content of “big” IR-ACTH fractions. When the “big” and “intermediate” fractions were further analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, they were resolved into 4 molecular forms of IR-ACTH with apparent molecular weights of 37,000, 24,000, 18,000 and 4,500, respectively.

These results indicate that 3 high molecular forms of IR-ACTH are present in the human pituitary and the ectopic ACTH-producing tumors.

High molecular weight forms of immunoreactive ACTH (IR-ACTH) have been observed in pituitary glands of several species including man and in human ectopic ACTH-producing tumors (Yalow & Berson, 1971; Yalow & Berson, 1973; Coslovsky & Yalow, 1974; Gewirtz et al., 1974; Scott & Lowry, 1974; Eipper & Mains, 1975; Hirata et al., 1975; Lowry et al., 1976; Hirata et al., 1976; Himsworth et al., 1977). However, no detailed biochemical characterization of these peptides had been attempted until the demonstration of the glycoprotein nature of the high molecular weight forms of IR-ACTH with molecular weights of 31,000, 23,000 and 13,000 produced by a mouse pituitary tumor cell line (ArT-20/D-16v), using sodium dodecyl sulfate(SDS)-polyacrylamide gel electrophoresis, among which the first was shown to be a biosynthetic precursor for other molecular forms of ACTH (Mains & Eipper, 1976; Eipper et al., 1976). Mains et al. (1977) and Roberts & Herbert (1977) further identified the IR-ACTH with a molecular weight of 31,000 as the common precursor to $\beta$-LPH/$\beta$-endorphin as well as ACTHs. Recently, human ectopic ACTH-producing tumors were also shown to contain the common precursor to ACTH and $\beta$-LPH/$\beta$-endorphin (Orth & Nicholson, 1977; Orth et al., 1978; Bertagna et al., 1978.)

In the present study attempts were made to define the molecular characteristics of IR-ACTH in a human pituitary gland and 4 ectopic ACTH-producing tumors using Sephadex G-100 gel chromatography and SDS-polyacrylamide gel electrophoresis.
Materials and Methods

A human pituitary and 4 ectopic ACTH-producing tumors (2 malignant thymomas, a pheochromocytoma and a small cell carcinoma of the lung) were obtained at autopsy or surgery and stored at -20°C until extracted with glacial acetic acid according to a modification (Imura et al., 1973) of the method of Payne et al. (1950). The extract in powdered form was reconstituted in 2 to 5 ml of 0.1 M acetic acid at 4°C by homogenizations and heated in boiling water for 5 min. After centrifugation at 3,000 rpm for 15 min, the supernatants were subjected to gel filtration at 4°C on a Sephadex G-100 column (1.4 x 90 cm) which was equilibrated and eluted with 0.1 M acetic acid at a flow rate of 14 ml/hr. Each 2 ml was collected and stored at -20°C until assayed. "Big" IR-ACTH fractions which emerged near the void volume, "intermediate" IR-ACTH which eluted between the void volume and 125I-ahl-39ACTH (Takeda Pharmaceutical Co., Osaka), and "little" IR-ACTH fractions, which coeluted with 125I-ahl-39ACTH, were pooled and lyophilized in preparation for concanavalin A affinity chromatography and SDS polyacrylamide gel electrophoresis.

Concanavalin A affinity chromatography was performed as described by Eipper et al. (1976). The lyophilized pools of "big", "intermediate" and "little" IR-ACTH fractions were dissolved in 10 mM Tris-HCl buffer (pH 7.4) containing 1 mM MgCl2, 1 mM CaCl2, 1 mM MnCl2, 0.1% bovine serum albumin, 1 M NaCl and 0.1% Triton X-100, and then applied at room temperature to the concanavalin A-agarose (Sigma) column (0.6 x 5.5 cm) which had been equilibrated with the same buffer. The column was then "washed" with approximately 30 column volumes of the buffer at a flow rate of 5 ml/hr. Specifically bound IR-ACTH was eluted with 10 ml of the buffer containing 0.2 M α-methyl-D-mannopyranoside (Sigma). Tightly bound IR-ACTH was "purged" with 0.1 M acetic acid containing 0.1% bovine serum albumin.

SDS polyacrylamide gel electrophoresis (10% acrylamide, 0.4% N, N'-methylenebisacrylamide) was performed according to a modification (Nakamura et al. 1978) of the method of King and Laemmli (1971). The lyophilized "big" or "intermediate" IR-ACTH fractions were dissolved in 0.1 M Tris-HCl buffer (pH 6.8) containing 3.3% SDS, 17% glycerol, 5% 2-mercaptoethanol, and 0.025% bromphenol blue as the marker dye. After heating in boiling water for 3 min, samples were applied to the gel. Electrophoresis was carried out at 20 mA per gel and at room temperature for approximately 3 hr until bromphenol blue reached to the bottom of the gel. After electrophoresis, gels were cut into 2 mm slices and eluted into 5 mM NaHCO3 containing 0.05% SDS at 37°C for 8 hr. The eluate was removed and assayed for IR-ACTH. The following materials were used as molecular weight markers: bovine serum albumin (molecular weight 68,000), ovalbumin (molecular weight 43,000), aldolase (molecular weight 40,000), lactate dehydrogenase (LDH) (molecular weight 36,000), chymotrypsinogen (molecular weight 25,700), myoglobin (molecular weight 17,200) and human ACTH (molecular weight 4,500).

A rat anterior pituitary was also extracted and analyzed as described above except for concanavalin A affinity chromatography.

IR-ACTH was measured according to the method reported previously by Hirata et al. (1975) using the antiserum directed mainly to the 18-24 amino acid sequence of the ACTH molecule, except that synthetic ah-ACTH (Takeda Pharmaceutical Co.) was used for iodination and standard in the present study. ACTH radioimmunoassay was unaffected by the concentrations of SDS as high as 0.05 mg/ml, so it was possible to carry out radioimmunoassay of ACTH in the eluate except for occasional samples described later.

Results

The elution profiles on Sephadex G-100 gel filtration of the extracts of a human pituitary and 4 ectopic ACTH-producing tumors are shown in Fig. 1. The two distinct peaks of IR-ACTH were observed in each elution profile; the one eluted near the void volume (Vo) and designated "big" IR-ACTH fraction, and the other in a 125I-ahl-39ACTH position and therefore designated a "little" IR-ACTH fraction accord-
canavalin A-agarose. A significant portion (10 to 20%) of “big” IR-ACTH fractions of the extracts of the pituitary and the tumors bound specifically to concanavalin A and were eluted with mannopyranoside, indicating the glycoprotein content of the “big” IR-ACTH fractions (Fig. 2). “Intermediate” and “little” ACTH fractions from each extract of the pituitary and the tumors did not significantly bind to the lectin column (data not shown). When a tracer of \(^{125}\)I-hTSH was applied to this column, not less than 80% of the radioactivity bound to the lectin and was eluted with mannopyranoside (Fig. 2) whereas \(^{125}\)I-\(\alpha\)-\(^{1-39}\)ACTH was incapable of binding to concanavalin A-agarose (data not shown).

In order to further define the molecular sizes of “big” and “intermediate” IR-ACTH, both fractions were analyzed by SDS polyacrylamide gel electrophoresis, which was shown to give a linear relationship between the relative mobilities and the logarithm of the marker polypeptide molecular weights ranging from 68,000 (bovine serum albumin)
Fig. 3. SDS polyacrylamide gel electrophoresis of "big" (○——○) and "intermediate" (●——●) IR-ACTH fractions of the extracts obtained by Sephadex gel filtration. The migrated positions of bovine serum albumin (molecular weight 68,000), ovalbumin (molecular weight 43,000), aldolase (molecular weight 40,000), LDH (molecular weight 36,000), chymotrypsinogen (molecular weight 25,700), myoglobin (molecular weight 17,200) and human ACTH (molecular weight 4,500) are shown on top.

to 17,200 (myoglobin). After electrophoresis, "big" fractions of IR-ACTH in the extracts of the pituitary and the tumors were resolved into 4 forms of IR-ACTH with apparent molecular weights of 3,7000, 24,000, 18,000 and 4,500 ("little" ACTH), according to the calibration (Fig. 3). The gel electrophoresis of the "intermediate" fractions of IR-ACTH from the extracts of the human pituitary and a thymoma gave patterns similar to those of "big" fractions, except for the relative decrease in the amount of higher molecular weight ACTH (Fig. 3). Serial dilutions of IR-ACTHs with apparent molecular weights of 37,000, 24,000 and 18,000, thus obtained from the extracts of both the pituitary and the tumors by gel electrophoresis, were found to be parallel to the standard curve for αh-ACTH whereas those of "little" ACTH with a molecular weight of 4,500 were not, indicating that IR-ACTHs with higher molecular weights are immunologically undistinguishable from αh-ACTH while "little" ACTH seems to be immunologically rather different from authentic αh-ACTH.

When the extract of rat pituitary was fractionated on a Sephadex G-100 gel column, 3 distinct peaks of IR-ACTH; "big", "intermediate" and "little" ACTH fractions, were observed (Fig. 1). The "big" IR-ACTH fractions thus obtained were further resolved into 4 forms of IR-ACTH with apparent molecular weights of 31,000, 24,000, 13,000 and 4,500 after SDS-polyacrylamide gel electrophoresis (Fig. 3).

Discussion

Since the first report of Yalow & Berson (1971) on the existence of "big" IR-ACTH in plasma and in extracts of pituitary glands and an ectopic ACTH-producing thymoma in man, high molecular forms of IR-ACTH were repeatedly demonstrated in the pituitary of several species and other ectopic
ACTH-producing tumors in man (Yalow & Berson, 1973; Coslovsky & Yalow, 1974; Gewirz et al., 1974; Scott & Lowry, 1974; Eipper & Mains, 1975; Hirata et al., 1975; Lowry et al., 1976; Hirata et al., 1976; Himsworth et al., 1977). Recently, Mains & Eipper (1976) and Eipper et al. (1976) reported on the glycoprotein nature of the high molecular weight forms of IR-ACTH produced by mouse AtT-20 tumor cells, consisting of 3 forms of IR-ACTH with apparent molecular weights of 31,000, 23,000 and 13,000, based on SDS polyacrylamide gel electrophoresis. Subsequently Orth & Nicholson (1977) demonstrated 3 fractions of IR-ACTH ("big", "intermediate" and "little" IR-ACTH) in plasma and in extracts of pituitary glands and ectopic ACTH-producing tumors in man after Sephadex G-100 gel filtration and, furthermore, confirmed the glycoprotein nature of the "big" and "intermediate" IR-ACTH using affinity chromatography on concanavalin A-agarose. The present finding is essentially in agreement with that of Orth & Nicholson (1977) who, however, observed a more significant portion (29–61%) of "big" IR-ACTH and a smaller but significant portion of "intermediate" IR-ACTH bound specifically to the lectin in contrast to our data of a rather small portion (10 to 20%) of "big" IR-ACTH and no significant amount of "intermediate" IR-ACTH which bound specifically to the column. The reduced binding of high molecular IR-ACTHs to the lectin observed in the present study could be in part explained by our use of acid for the extraction of ACTH from tissues, instead of the use of water as by Orth & Nicholson (1977) in view of the possible cleavage of carbohydrate side chains under an acidic condition (Reinhold, 1968), thus decreasing the affinity for the lectin.

Of particular interest is our finding that "big" and "intermediate" forms of the human pituitary and the ectopic ACTH-producing tumors were separated into 4 forms of IR-ACTH with apparent molecular weights of 37,000, 24,000, 18,000 and 4,500, according to the calibration based on the relative mobilities on SDS acrylamide gel electrophoresis. The apparent molecular weight (37,000) of this largest molecular IR-ACTH in man corresponds to that of ACTH produced by human ectopic ACTH-producing pulmonary small cell carcinoma (molecular weight 15,500–40,000, Bertagna et al., 1978) and by bovine pituitary gland (molecular weight 35,000, Nakaniishi et al., 1976). The apparent molecular weights of two other large IR-ACTHs (molecular weight 24,000 and 18,000) in man seem to be slightly larger than those obtained from mouse pituitary tumor (molecular weight 23,000 and 13,000, Mains & Eipper, 1976 or 20,000 to 23,000 and 13,000, Allen et al., 1978) and from rat pituitary gland (molecular weight 24,000 and 13,000, the present study). In order to determine the precise molecular weights of large molecular IR-ACTHs, however, the analyses of the aminoacid sequences and the carbohydrate chains of these glycopeptides would be required. The "big" and "intermediate" IR-ACTHs unexpectedly contained "little" ACTH besides the high molecular weight forms of IR-ACTH after gel electrophoresis. This finding could be explained by the size-heterogeneity of peptides probably due to aggregation and nonspecific binding to larger proteins even after gel chromatography, and/or the nonspecific interference in the radioimmunoassay since the high concentration of SDS contained in 3.3% SDS-Tris-HCl buffer used for dissolving the samples was found to migrate to the bottom of the gel with ACTH during electrophoresis and elution. No parallelism was noticed between serially diluted "little" ACTH and standard ah-ACTH in displacing a labelled tracer from an anti-ACTH serum as described before. These findings rather indicate that most of the "little" ACTH observed after gel electrophoresis of the high mole-
cular forms of IR-ACTHs could be due to the nonspecific interference by concentrated SDS in the present assay system.

Mains et al. (1977) and Roberts & Herbert (1977) proposed that IR-ACTH with an apparent molecular weight of 31,000 in a mouse pituitary tumor is a single precursor to β-LPH/β-endorphin as well as various forms of ACTH. Very recently, Nahanishi et al. (1979) determined the complete amino acid sequence of the common precursor in the bovine pituitary confirming the presence of β-LPH/β-endorphin and ACTH in the molecule.

It was further demonstrated that ACTH, β-LPH and β-endorphin immunoreactivities also were present in "big" fraction of a human ACTH-producing pancreatic islet cell carcinoma (Orth et al., 1978) and a human pulmonary small cell carcinoma in culture (Bertagna et al., 1978). These findings including our data suggest that the human pituitary gland and nonpituitary tumors biosynthesize various forms of ACTH and other related peptides in a manner quite similar to that proposed in mouse (Mains et al., 1977; Allen et al., 1978) and rat pituitary gland (Crine et al., 1978).

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References