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ACTH IMMUNOREACTIVITIES PREDOMINATING IN NORMAL HUMAN PLASMA ARE NOT ATTRIBUTABLE TO THE HUMAN $ACTH_{1-39}$ MOLECULE

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SUMMARY

Using reversed-phase high performance liquid chromatography, it was demonstrated that immunoreactive ACTH in normal human plasma is composed of various components. These components were partly of lower hydrophobicity, but to a large extent, they were of a higher hydrophobicity similar, though not identical, to that of human $ACTH_{1-39}$, which itself only contributes to a minor extent to total ACTH immunoreactivity. The relative distribution between these compounds varied greatly among plasmas. The non-destructive character of the analytical procedure applied was documented by tracer experiments with pg-amounts of non-labeled and radio-iodinated human $ACTH_{1-39}$. It is concluded that the components related immuno logically to $ACTH_{1-39}$ originate from peripheral metabolic processes.

INTRODUCTION

In human circulation, many of the peptide hormones are present in several different 'forms' whether they represent precursor molecules of the predominant cellular hormonal product (1) or its degradation products resulting from peripheral metabolism (2). Several forms of corticotropin (ACTH) differing in molecular size have been detected in normal human pituitary tissue (3,4), in human pituitary tumors (5), in extracts of tumors associated with ectopic ACTH secretion (3) and in plasma from patients with Nelson's syndrome and the ectopic ACTH syndrome (6). In normal human plasma, ACTH has been reported to be present in only one immunoreactive form eluting chromatographically in the position of purified human $ACTH_{1-39}$ (6,7). In the rat, considerable amounts of ACTH fragments have been shown to occur in peripheral plasma within a short time after administration of ^{3}H-labeled synthetic $ACTH_{1-24}$ (8). As is widely known for other peptide hormones, the kidney, along with other body tissues (8), plays a predominant role in this fragmentation process (9). The question whether the

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kidney or other tissues exert a regulatory function by the rate of catabolism and/or by cleavage synthesis of biologically activated fragments, has yet to be answered. We assessed the potential presence of such fragments or other molecules related immunochemically to ACTH in human plasma using reversed-phase high-performance liquid chromatography (HPLC), the separating potential of which, in contrast to the classical techniques of peptide separation (1-7), is focused more on smaller peptides than on forms with larger molecular weight.

MATERIALS AND METHODS

Blood from normal subjects was withdrawn into cooled tubes containing EDTA, aprotinin and mercaptoethanol as stabilizing agents (3). Peptides were extracted from 4 ml of plasma on a 1 x 0.5 cm column of octadecasilyl-bonded silica (10) using 0.05 M trifluoroacetic acid (TFA)/acetonitril (20/80). The lyophilizates of the extracts were redissolved in 150 µl of 0.05 m TFA and subjected to HPLC (11) using octadecyl-coated silica (Lichrosorb RP-18, Knauer, Ltd., Berlin), 0.05 M TFA/acetonitril and gradient elution. A Hewlett-Packard chromatograph (Model 1084 b) equipped with a variable volume injector and a variable wavelength detector was used. 1-min or 0.2-min fractions were lyophilized and redissolved in 450 µl of 0.01 HCl containing 5% bovine serum albumin. 200 µl in duplicate, were assessed for ACTH immunoreactivity by radioimmunoassay (RIA). An anti-porcine-$ACTH_{1-39}$ antiserum (CEA, Gif-sur-Yvette) directed to the N-terminal part of the ACTH molecule, ^{125}I-human-$ACTH_{1-39}$ and human-$ACTH_{1-39}$ (kindly supplied by Ciba, Basel) as standard were used in the RIA. The sensitivity of the standard curve (2 S.D. of zero bound) amounted to 1.8 ± 0.7 (S.D.) pg and the 50%-intercept to 17.6 ± 3.4 (S.D.) pg. Human $ACTH_{1-39}$ was radio-iodinated according to the method of Greenwood and Hunter (12).

RESULTS

In contrast to gel filtration or gel electrophoresis techniques which have commonly been applied for peptide separation (3,4,7), the reversed-phase HPLC-technique applied in the present approach provides effective separation of peptides differing only slightly in molecular structure (Fig. 1a). For example, the exchange of Ser in human-$ACTH_{1-39}$ against Leu in porcine-$ACTH_{1-39}$ at position 31 of the molecule is distinctly recognized by the chromatographic system.

The chromatograms of ACTH-immunoreactivities arising in normal human plasma samples (Fig. 1b and 1c) reveal seemingly broad peaks of ACTH-like compounds. Taking into account that one definite molecule elutes within 0.6 min (Fig. 1a), it must be stated that several molecules contribute to the total amount of plasma ACTH-immunoreactivities. The sum of immunoreactivities separated by

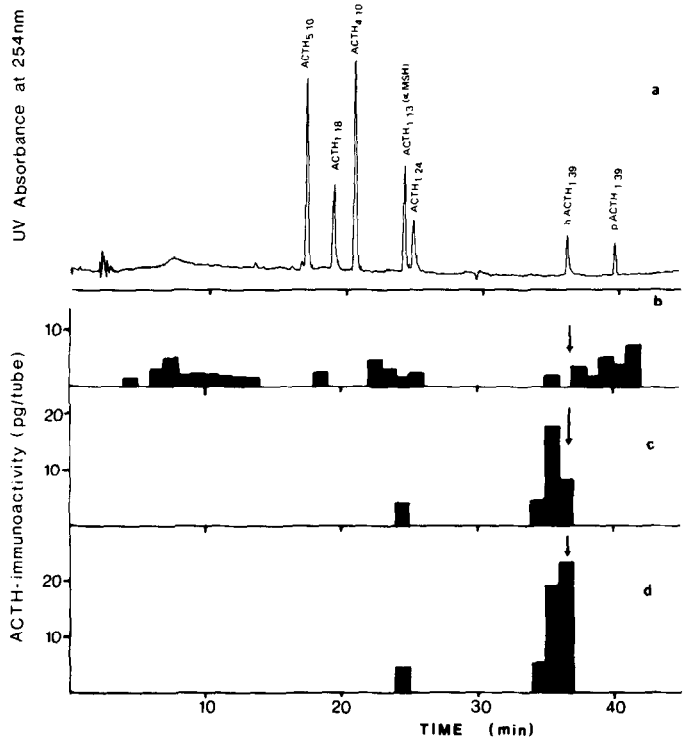

Figure 1. High-performance liquid chromatography of human and porcine ACTH standards (5µg), of ACTH fragments (Fig. 1a) and of ACTH-like immunoreactivities arising in 4 ml of two different normal human plasma samples (Fig. 1b and 1c). Fig. 1d shows the chromatogram of the plasma sample presented in Fig. 1c, to which 100 pg of synthetic human $ACTH_{1-39}$ (Ciba) had been added prior to assay. Extracts of plasma samples were applied to a column (4.6 x 250 mm) of octadecyl-coated silica (5µm). A stepwise linear gradient from 0.05 M trifluoroacetic acid (TFA): acetonitril (95:5 by vol) to 0.05 M TFA: acetonitril (10:90 by vol) at a flow rate of 1.3 ml/min was used. Temperature of solvents and oven was 40°C. Fractions in Fig. 1b-d were collected at intervals of 1 min. The elution position of synthetic human $ACTH_{1-39}$ established by calibration with UV-visibel standard (Fig. 1a) and with pg-amounts in charcoal-stripped serum is indicated by arrows (Fig. 1b-d).

HPLC was equal to the total immunoreactivity measured without HPLC. In control experiments assessing plasma samples from which peptides had been stripped by charcoal, no significant immunoreactivities were assessable throughout the complete chromatogram. Furthermore, when pg-amounts of synthetic human-$ACTH_{1-39}$ (Ciba) were added to the plasma sample prior to the assay, the additional immunoreactivity of intact peptide eluted as a single sharp immunoreactive peak at a retention time of about 36 min (Fig. 1d), as did UV-visible amounts (µg-range) of the synthetic human-$ACTH_{1-39}$ standard

(Fig. 1a). The residual immunoreactive background remained unaltered (Fig. 1d), thus demonstrating that immunoreactive molecules other than the $ACTH_{1-39}$ molecule are not artefact products arising during the extracting or chromatographic procedures.

Although the chromatographic profile of the ACTH-immunoreactivities as well as the magnitude of single peaks differed significantly between individual plasma samples, the general profile was similar to those shown in Fig. 1b and 1c, consisting of a very small group of immunoreactivities eluting in the more hydrophilic part and a larger group eluting in the more hydrophobic part of the chromatogram. The more hydrophobic molecules exhibited a chromatographic behavior very similar but not identical to that of the synthetic human-$ACTH_{1-39}$ molecule. Thus, even the profile of the sample (Fig. 1c) showing a single immunoreactivity peak apparently identical to the human-$ACTH_{1-39}$ molecule proved to consist of two adjacent peaks if fractionated at 0.2-min intervals (Fig. 2b). The one which eluted later represented a smaller amount of total immunoreactivity and was attributable to the human-$ACTH_{1-39}$ molecule, as demonstrated by tracering the same sample with synthetic human-$ACTH_{1-39}$ (Fig. 2c).

The non-destructive character of the present procedure was further demonstrated in tracer experiments with ^{125}I-$ACTH_{1-39}$. The HPLC profile of crude radio-iodinated ^{125}I-ACTH seemingly exhibited one broad peak (Fig. 3a), which, however, proved to consist of at least 4 individual radioactive peptide molecules if the interval of fractionation was reduced to 0.2 min (Fig. 3b). A single radioactive component ran true on refractionation whether the component was directly subjected to HPLC (Fig. 3c) or had been added to a plasma sample processed according to the outlined extraction procedure (Fig. 3d).

DISCUSSION

From data recently obtained in ACTH-catabolism experiments in the rat (8), it was concluded that the initial, obviously unspecific peptidase attack on the $ACTH_{1-24}$ molecule was concentrated around the two termini of the molecule, and the cleavage sites identified could all be explained in terms of attack by three main types of peptidase, namely aminopeptidase (residues 1-2 and 2-3), trypsin-like endopeptidase (residues 8-9 and the basic region 15-21) and

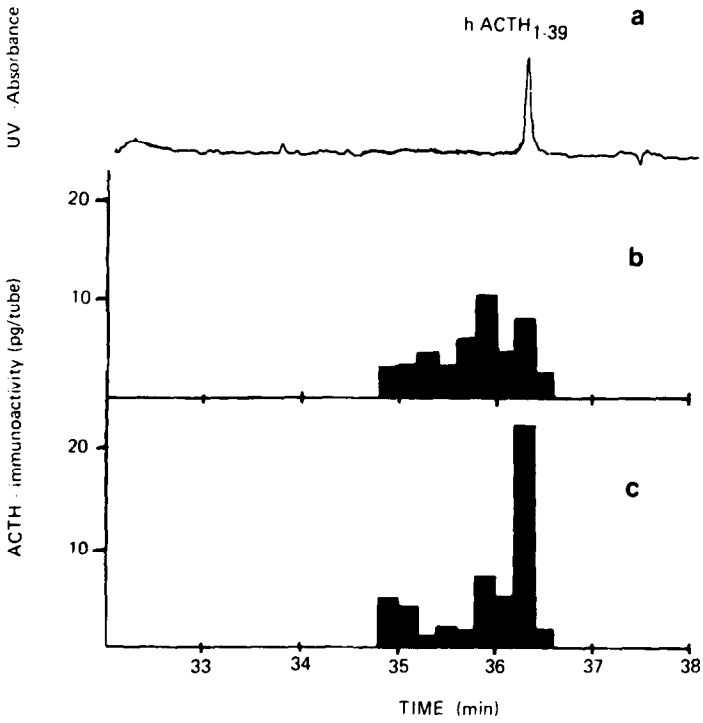

Figure 2. Immunochromatogram of the normal human plasma sample apparently displaying a single ACTH-like immunoreactive peak (see Fig. 1c). Interval of fractionation was 0.2 min. Fig. 2c shows the chromatogram of the same sample after addition of 100 pg of human $ACTH_{1-39}$ prior to assay. Position of synthetic human $ACTH_{1-39}$ is displayed in Fig. 2a. HPLC conditions were as described in Fig. 1.

secondary carboxypeptidase activity (in the basic region). Extrapolating these findings to the present data, the more hydrophilic ACTH-immunoreactivities may tentatively be attributed to low-molecular-weight ACTH fragments resulting from endopeptidase cleavage at the basic region of the molecule. Assuming that this is valid and taking into account that the cross-reactivity of the antiserum applied was relatively low against low-molecular fragments, one has to conclude that actual concentrations of these fragments are much higher than expressed by the amount of $ACTH_{1-39}$ equivalents used for quantitation here. The more hydrophobic immunoreactivities are potentially attributable to fragments resulting from N-terminal cleavage, thus yielding fragments with molecular weights very similar to that of the native $ACTH_{1-39}$ molecule. These fragment molecules differ from the $ACTH_{1-39}$ molecule by only one or two amino acids. It is, therefore, quite conceivable that

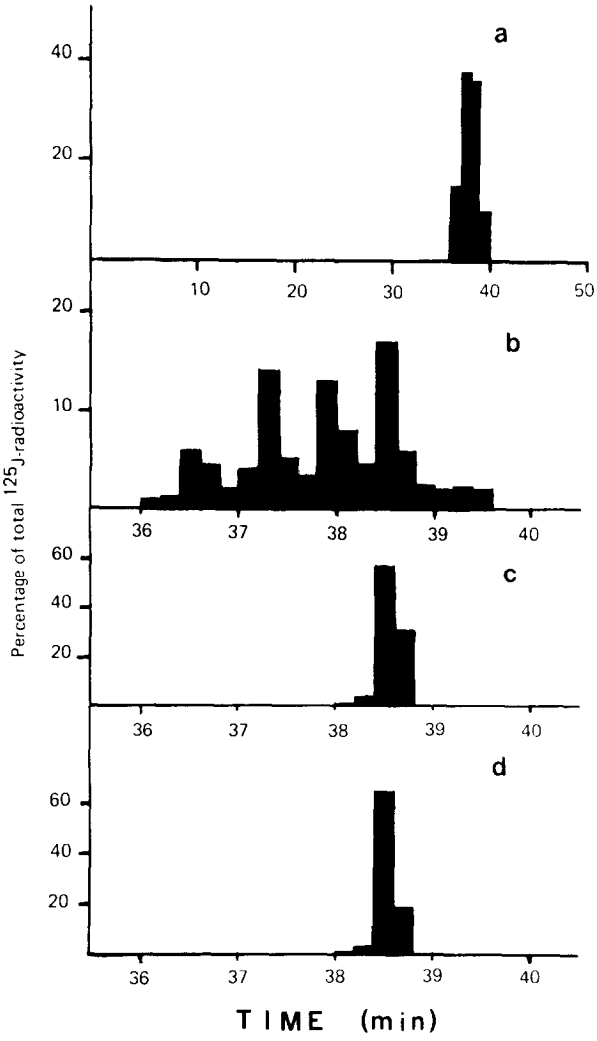

Figure 3. HPLC-profiles of crude, radio-iodinated, synthetic human ACTH$_{1-39}$ fractionated at 1-min intervals (a) and at 0.2-min intervals (b). Refractionation of a single radioactive component subjected directly to HPLC (c) and after being processed in a plasma sample (d). Values are depicted as percentage of total radioactivity (sum of all fractions).

they exert almost complete ACTH-like immunoreactivity but are potentially devoid of steroidogenic bioactivity as shown for the corresponding ACTH$_{1-24}$ fragments (13). However, a contribution by large-molecular-weight precursor molecules, which, in reversed-phase HPLC, reveal chromatographic properties similar to those of human-ACTH$_{1-39}$ (14), cannot be excluded at this time.

The seeming discrepancy between the present findings and those of previous reports (3,7) may be explained by the different chromatographic features applied to study this topic. The gel filtration techniques applied in previous studies (3,7) are not well suited for monitoring potential low-molecular fragmental forms of ACTH, since these are eluted in the very late part of the chromatogram, resulting in broad peaks and low-concentration fractions. Furthermore, using gel filtration, fragments produced by terminus-cleavage of the ACTH molecule can scarcely be distinguished from the native molecule due to the minimal differences in molecular weight. In contrast, these chromatographic challenges are well met by the HPLC technique.

At this point, at least two issues must be considered with regard to the immunochemical heterogeneity of ACTH fragments in the normal human circulation. Firstly, estimates of total ACTH-immunoreactivity in human plasma may be the result of different secreting or metabolizing mechanisms and thus do not necessarily reflect activity of pituitary ACTH secretion. Potentially, dissociation between bioactive and immunoreactive ACTH estimates (15) may be explained by this phenomenon. In any case, in diagnostic or scientific issues, one should be cautious in drawing conclusions based on total ACTH-immunoreactivities. Secondly, the nature and potential physiological significance of the circulating fragments have yet to be defined. If all fragments generated are biologically inactive, then the cleavage process simply reflects catabolic degradation of the hormone. On the other hand, if one or more of the fragments are biologically active, the potential physiological meaning of such active fragments is rather fascinating. Thus, such a postulated active fragment would constitute the dominant active molecular species of the pituitary ACTH molecule or a form of the hormone whose spectrum of action is different from that of the intact molecule.

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