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Circulating Corticotropin Releasing Factor-Like Activity in Man[1] (39970)

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Although corticotropin releasing factor (CRF) was one of the first hypothalamic releasing factors proposed (1), its isolation and characterization have yet to be achieved. Nonetheless, a considerable amount of physiologic data has been complied regarding its secretion in animals under normal circumstances (2, 3) as well as during stress (4) and steroid administration (5). In addition, a number of studies have provided evidence that a CRF is detectable in the peripheral circulation of animals (6-9).

Human hypothalamic fractions have been shown to elicit ACTH secretion *in vitro* (10), and normal subjects release cortisol in response to partially purified hypothalamic CRF (11), indicating that a human CRF most likely exists. The presence of CRF in the peripheral circulation in humans has not been firmly established, however, a preliminary report by Yasuda *et al.* suggests that human plasma contains CRF-like activity (12). It is the purpose of this communication to report the results of studies designed to demonstrate corticotropin releasing material in human plasma.

Materials and methods. Preparation of plasma extracts. Blood was obtained from three normal subjects in heparinized tubes at 0800 in amounts of 75-100 ml. Following immediate centrifugation at 4°, the plasma was extracted with QUSO G-32 microfine silica particles to remove ACTH (13). This technique removes >90% of added ACTH in our hands. To further assess the possibility of adding exogenous ACTH in the plasma extracts, a control experiment was performed wherein ^{125}I-radiolabeled α_hACTH was added to an aliquot of QUSO-extracted normal human plasma and subjected to the methanol extraction procedure. The plasma was then methanol-extracted by the method of Malacara *et al.* (14). Following the final extraction, aliquots were air-dried and frozen. For *in vitro* studies, the plasma extracts were reconstituted in incubation media such that 1 ml was equivalent to 20 ml of original plasma.

ACTH radioimmunoassay. A modification of the method of Galskov (15) was employed for the radioimmunoassay of ACTH. The standard and tracer was synthetic α^{1-24}ACTH, and iodination was achieved with a chloramine-T method (16). An antibody against synthetic α^{1-24}ACTH was produced in our laboratory using the method of Rose and Newsome (17). The antibody has the highest affinity for α^{1-24}ACTH, but crossreacts with α^{1-16}ACTH, α_hACTH, and α^{11-24}ACTH in decreasing order, while no significant cross-reactivity occurs with α^{17-39}ACTH. Serial dilutions of a homogenate of rat anterior pituitary tissue in the assay buffer cross-reacted in parallel, suggesting that our system qualitatively measures rat ACTH. The assay has a sensitivity of 50-100 pg of α^{1-24}ACTH. In this assay, a 100-μl volume of test substance is added to each assay tube, and each sample is assayed in triplicate. The incubation medium samples were diluted 1/10 and 1/100 prior to assay, and the pituitary homogenates were diluted 1/500 and 1/1000.

In vitro studies. Anterior pituitary tissue was obtained from 150- to 200-g male Holtzman rats. Anterior pituitaries were removed within 3 min of sacrifice by decapitation, weighed, sectioned into four pieces, and then placed in the incubation flask. For each experiment, two anterior pituitary glands (eight pieces) were incubated per flask in a total volume of 5 ml. The basic medium was Ringer's lactate solution with glucose added to a cencentration of 1 mg/ml. The electrolyte concentrations

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(milliequivalents per liter) in this solution are: Na, 130; K, 4; Ca, 3; Cl, 109; and lactate, 28; and these were not altered significantly by the addition of plasma extract to incubation flasks. The pH of the medium varied from 6.8 to 7.0 during incubation experiments. Following placement of the pituitaries in the flasks, they were gently agitated for 30 sec, and a baseline sample was obtained from the medium. The incubations were carried out for 2 hr at 37° in a metabolic shaker, and samples were obtained at 1 and 2 hr, following which the pituitary tissue was homogenized and diluted in the ACTH RIA buffer. The amount of ACTH released in each experiment was calculated by subtracting the baseline value from the 1- and 2-hr values.

Following an initial six experiments, wherein the incubations were performed in incubation medium alone, two additional sets of studies were performed. The first set involved 11 experiments with 100 μl of the plasma extract per flask, nine experiments with 250 μl, six with 500 μl, and four with 750 μl. In each case, an equivalent amount of buffer was omitted to maintain a volume of 5 ml per flask. In the second set of studies, NIAMDD rat hypothalamic extract (HE-RP-1) was used. Five experiments were performed with 0.5 hypothalamic equivalents per flask, five experiments with 1, five with 2, five with 5, and six with 10. Again, the incubation volume was maintained at 5 ml per flask.

An aliquot of plasma extract was subjected to ultrafiltration across an Amicon UM-10 filter, and a portion of the filtrate was used for incubations and the remainder filtered across a UM-2 filter. The UM-10 and UM-2 filtrates were each used for three incubations with the dose equivalent to the filtrate of 500 μl of plasma extract. These incubations were then compared to the studies with 500 μl of plasma extract added directly to the incubation medium.

A set of control studies was performed, including three experiments wherein 5 hypothalamic equivalents in 500 μl of Ringer's lactate solution were subjected to the methanol extraction procedure to determine the efficacy of the extraction technique with a known source of CRF. In three additional experiments, 5 hypothalamic equivalents in 500 μl of Ringer's lactate solution containing human serum albumin at a concentration of 3.5 g/100 ml were methanol-extracted in order to simulate plasma and assess any interference by large proteins. These sets of experiments were compared to the incubations with 5 hypothalamic equivalents added directly to the incubation flasks.

Where statistical comparisons between groups were performed, Student's *t* test for unpaired observations was employed. In all experiments, the data have been expressed as percentage release (amount released/total pituitary ACTH content × 100).

Results. Plasma extract experiments. The control experiment with radiolabeled ACTH in plasma resulted in 13.5% of added ACTH recovered in the final methanol extract. The control value for percentage release of ACTH in incubation medium was 3.3 ± 0.8% (mean ± SEM) at 2 hr, and this value is used for statistical comparison with all other studies. In this and all other experiments, the basal ACTH in the medium was approximately 2.8–6.4 ng/ml, and with the 2-hr incubations in medium alone, the ACTH content was increased by a factor of 1.5–2.5. The release in all other experiments was greater, with the highest values reaching 10 times the basal release. The time course of ACTH release in response to 100-, 250-, 500-, and 750-μl doses of plasma extract is plotted in Fig. 1 and compared to control. ACTH release with all doses of plasma extracts was significantly greater than control ($P < 0.01$), and the

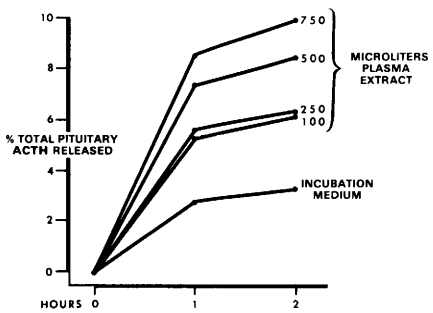

FIG. 1. The percentage release of ACTH over 2 hr in incubation medium and with increasing doses of plasma extract.

release with the 750-μl dose significantly exceeded that obtained with 250 μl ($P < 0.01$). The log dose-response relationship for ACTH release with plasma extracts is shown in Fig. 2.

Hypothalamic extract experiments. The studies incorporating rat hypothalamic extract at doses of 0.5, 1, 2, 5, and 10 hypothalamic equivalents per flask are summarized in Table I. A stepwise increase in ACTH release occurred, with 2, 5, and 10 hypothalamic equivalents eliciting greater ACTH release than control ($P < 0.001$). Other statistical differences are indicated in the table.

Ultrafiltration experiments. The incubations with UM-10 and UM-2 ultrafiltrates of plasma extract resulted in less ACTH release than the incubations with 500 μl of plasma extract directly added to the medium. Both ultrafiltrate experiments resulted in ACTH release comparable to that obtaining in incubation medium control experiments (Table II).

Control experiments. The experiments with 5 hypothalamic equivalents per flask ($n=5$) resulted in 13.5 ± 0.8% ACTH release (mean ± SEM); the experiments with methanol extracts of 5 hypothalamic equivalents in Ringer's lactate solution alone, 17.4 ± 0.8% (mean ± SEM); and with added human serum albumin, 19.5 ± 1.8% (mean ± SEM). Both of these latter experiments resulted in significantly more ACTH release than obtained with the unextracted hypothalamic material ($P < 0.05$).

Discussion. The data reported herein suggest the presence of a peripherally circulating corticotropin releasing factor-like activity in man. The difficulty in measuring CRF

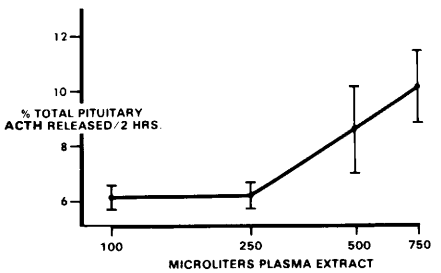

FIG. 2. The log dose-response of percentage ACTH release with plasma extract. Vertical bars indicate SE.

TABLE I. EFFECT OF RAT HYPOTHALAMIC EXTRACTS ON ACTH RELEASE FROM RAT PITUITARIES *in vitro*.

No. of hypothalamic equivalents/flask	No. of experiments	Percentage total pituitary ACTH released mean ± SE	
		1 hr	2 hr
Control	6	2.8 ± 0.7	3.3 ± 0.8
0.5	5	3.7 ± 0.7	4.3 ± 0.5
1	5	3.9 ± 1.0	5.0 ± 0.5
2	5	10.7 ± 1.1	12.3 ± 1.9*, **
5	5	12.5 ± 1.4	13.5 ± 0.8*
10	6	16.8 ± 1.8	20.9 ± 2.3*, ***

* $P < 0.001$ as compared to control.
** $P < 0.01$ as compared to 1 hypothalamic equivalent.
*** $P < 0.02$ as compared to 5 hypothalamic equivalents.

TABLE II. EFFECT OF ULTRAFILTRATES OF HUMAN PLASMA EXTRACT ON ACTH RELEASE FROM RAT PITUITARIES *in vitro*.

Incubation medium	No. of experiments	Percentage total pituitary ACTH released mean ± SE	
		1 hr	2 hr
Control	6	2.8 ± 0.7	3.3 ± 0.8
500-μl of plasma extract	6	7.4 ± 2.0	8.5 ± 1.6
UM-10 filtrate	3	3.6 ± 1.2	4.0 ± 0.4*
UM-2 filtrate	3	2.6 ± 0.8	2.7 ± 0.9*

* No significant difference from control.

has been reviewed extensively by Vernikos-Danellis and Marks (18), and it appears that methods measuring ACTH directly by radioimmunoassay have an advantage over tandem bioassay methods where ACTH is assessed by the adrenal corticosterone response. Rees *et al.* have utilized a radioimmunoassay system recognizing the 1-24 subunit of ACTH for measuring rat ACTH (19), and with a similar method demonstrating parallel immunoreactivity of rat pituitary homogenates, our assay should allow valid estimates of ACTH concentration.

In 1962, Brodish and Long reported that rat plasma contained corticotropin releasing activity and suggested that it may be a hypothalamic neurohumor (7). Yasuda *et al.* (12) recently reported the existence of a heat-stable corticotropin releasing material in rat and human whole plasma which stimulates cultured rat anterior pituicytes. In

additional reports from the same laboratory, rat hypothalamic extracts have been shown to stimulate ACTH from cultured rat anterior pituitary tissue (20), and rat plasma and human urine caused ACTH release from the same system (9). Kendall *et al.* recently reported that rat plasma contains a CRF which elutes in the void volume during Sephadex G-75 chromatography and is retained by an ultrafilter with an average molecular weight retention of 15,000 daltons (8). Our data similarly indicate that human plasma CRF is a material which is excluded by an ultrafilter with a retention of approximately 10,000 daltons. Thus, CRF may be larger than the established releasing factors which are small peptides, or alternatively, CRF may be bound to a larger moiety.

The linear log dose–response relationship over a threefold increase in concentration of the plasma extract suggests a physiologic mechanism eliciting ACTH release rather than artifactual hormone release. This is further supported by the similar pattern of ACTH release in response to rat hypothalamic extracts in our *in vitro* system, although the log dose relationship for hypothalamic extracts is not parallel to plasma extracts (data not plotted in Fig. 2). This observation is similar to that reported by Yasuda and Greer (9), wherein the log dose ACTH release with hypothalamic extracts was not parallel to release with extrahypothalamic sources of CRF.

The fact that methanol extracts the corticotropin releasing substance(s) from plasma would suggest a compound or compounds not unlike the currently established hypothalamic releasing factors, many of which are methanol soluble (21), such as LHRH, which also appears to be extractable from peripheral human plasma (14). We have recently reported that a prolactin releasing factor other than TRH is contained in methanol-extracted human plasma (22). Our control studies indicate an increased ACTH response to the methanol extract of hypothalami when compared to the hypothalami added directly to the incubation medium. This demonstrates that CRF is methanol soluble and, in addition, suggests that methanol extraction of hypothalami might remove some unknown inhibitors of ACTH release in our system. Furthermore, with the low extraction of ACTH by methanol (13.5%), these results mitigate against any significant effect of ACTH added with the hypothalami, as a greater ACTH release was noted with methanol-extracted hypothalami.

Extrahypothalamic sources for CRF are recognized (23), and the possibility exists that the human CRF detected in our studies is extrahypothalamic. Physiologic validation of the significance of the circulating CRF-like material awaits appropriate measurements following maneuvers known to effect ACTH secretion.

Summary. In order to investigate the possible existence of corticotropin releasing factor (CRF)-like activity in human plasma, methanol extracts of plasma were incubated with rat anterior pituitary tissue *in vitro* and ACTH release was determined. The extracts were reconstituted so that 1.0 ml was equivalent to 20 ml of original plasma, and the following doses in 5.0-ml incubation volumes elicited a stepwise increase in ACTH release: 100, 250, 500, and 750 μl. The 250-, 500-, and 750-μl doses resulted in a linear log dose–response. Parallel experiments with 0.5, 1, 2, 5, and 10 rat hypothalamic equivalents in 5.0-ml incubation volumes resulted in a similar stepwise increase in ACTH release. Control experiments indicated a significantly greater ACTH release when pituitary tissue was incubated with methanol extracts of rat hypothalami in comparison to hypothalami added directly *in vitro*, validating the methanol extraction technique with a known source of CRF. Ultrafiltration experiments with the human plasma CRF-like activity suggest a molecular weight larger than 10,000 daltons.

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