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Quantitative Determination of Lymphocyte $ACTH_{1-39}$

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Abstract—A method was developed for quantitative measurement of $ACTH_{1-39}$, produced by human lymphocytes. It was shown that pH adjustment to 2 was essential for processing of the precursor molecule proopiomelanocortin (POMC). Linearity between time of incubation and ACTH production was shown. The existence of specific endopeptidases in lymphocytes for processing of the POMC molecule remains doubtful.

Introduction

It has long been recognized that cells can direct particular proteins to different intracellular organelles for processing and storage or secrete the products without prolonged storage in the cell (1). The functional distinction between basal secretion and stimulated secretion has also been appreciated for a long time. Most, if not all, secretory tissues have a measurable rate of secretion in the absence of secretagogues (basal rate), and their secretory rate can be increased substantially in response to appropriate stimuli (stimulated rate). The terms "constitutive" and "regulated" pathways have been widely used to describe the cellular entities leading to basal and stimulated secretion (2, 3). In this scheme, newly synthesized proteins proceeding through the Golgi apparatus and destined for secretory granules are "sorted" away from other proteins which become part of the constitutive pathway (2, 4). The constitutive pathway has been postulated to involve post-Golgi vesicles distinct from secretory granules (5, 2, 4).

It is clear that some intracellular compartments along the biosynthetic pathway of many secretory cells are acidic; there is a gradual acidification from the cis to the trans face of the Golgi stack, and mature secretory granules are even more acidic (pH 5.5-6.0) than the Golgi compartments (4, 6, 7, 8).

The evidence that cells of the immune system synthesize hormones that are classically associated with the neuro-endocrine system was obtained when it was observed that, during production of interferon (IFNα), human peripheral leukocytes coordinately expressed a peptide that was antigenically related to corticotropin (ACTH) (9). The ACTH bioactivity in human leukocyte IFNα preparations was demonstrable only after pepsin or acid treatment (10). The ACTH derived from

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human leukocyte IFNα by pepsin digestion comigrated with a purified ACTH standard in $NaDodSO_4$/polyacrylamide gel electrophoresis (10).

Peripheral leukocytes secrete ACTH and other proopiomelanocortin (POMC)-derived peptides in the presence of applicable neurochemical stimuli (11). The immunoreactive ACTH (ir-ACTH) identified in human peripheral leukocytes after virus-infection or endotoxin treatment (10) was found to be similar to pituitary-derived ACTH with respect to its biological activity, molecular weight, antigenicity, and retention time on a reverse phase high pressure liquid chromatography column (10). This ir-ACTH also possesses remarkable steroidogenic activity when injected into hypophysectomized mice.

In the absence of neurochemical stimuli, human peripheral leukocytes synthesize little ACTH in culture and the incubation medium is almost devoid of ir-ACTH (11).

Previously leukocyte-derived ACTH was measured in a mouse adrenal tumor (Y-1) cell rounding assay (12, 13). These cells round in proportion to the amount of ACTH present. This assay could detect 10^{-4} unit (about 0.5 ng) of ACTH.

In this study, a method was developed for quantitative measurement of ACTH produced by human lymphocytes.

Materials and Methods

For the preparation of pure lymphocyte suspensions, the method of Boyum (14) was used with minor modifications (15, 16). Blood was drawn in Li-heparin (Sigma) containing polypropylene syringes (130 USP Li-heparin/10 ml blood), diluted (1:1) with sterile filtered phosphate buffered saline (PBS; pH 7.3), well mixed, and layered onto 4 ml Lymphoprep (Nyegaard & Co. AS, Oslo, Norway; density 1.077 ± 0.001 g/ml [20°C]) in 15 ml tubes. The tubes were centrifuged (400 g; 20 min; 20°C) in a Beckman J-6M/E centrifuge. The mononuclear cells at the interphase were aspirated and diluted with an equal volume of PBS containing 2% (v/v) foetal calf serum (FCS; Gibco Limited, Paisley, Scotland). The suspension was centrifuged (250 g; 15 min; 20°C) and the supernatant aspirated. The pellet was resuspended in 2% (v/v) FCS/PBS and centrifuged (350 g; 10 min; 20°C). This step was repeated and the pellet resuspended in 10% (v/v) FCS/RPMI-1640 medium (Gibco Limited, Paisley, Scotland; pH 7.3) containing 0.6% (v/v) antibiotics (Novopen 10^3 U/ml + Novostrep 10^3 U/ml dissolved in 100 ml sterile distilled water). The cell suspension was transferred to a petri dish and incubated in a Hot-pack CO_2-incubator for 30-45 min (37°C; 5% CO_2; 95% humidity). The supernatant was aspirated and the concentration of lymphocytes determined with a Coulter-counter.

Aliquots of the lymphocyte suspension (1×10^6 cells) were incubated in 1 ml incubation medium (10% v/v FCS/RPMI-1640; pH 7.3; 0.6% antibiotics) in a CO_2-incubator (37°C; 5% CO_2; 95% humidity). Synthesis of POMC was terminated by adjusting the pH to pH2. Processing of POMC was carried out at 37°C for 24 hours. The released ACTH was subsequently measured quantitatively using a standard ACTH (^{125}I) Irma adrenocorticotropin-hormone kit (Euro-Diagnostics BV, Apeldoorn, Holland). The sensitivity of the assay is 8 pg/ml as judged by a 2.5 SD change of duplicate standards from zero values. Both inter- and intra-assay variability was less than 10%. It should be mentioned that this kit is highly specific for $ACTH_{1\text{-}39}$ and that ACTH fragments 1-24, 1-32 and 18-39 do not interfere with the assay.

Results

Initial measurements in medium containing lymphocytes showed absence of ACTH. From this observation it was deduced that ACTH, produced by lymphocytes, was still an integral part of the precursor molecule, POMC. Methods were evaluated whereby ACTH could be released from the POMC molecule.

After the initial culture period, the lymphocyte culture was treated with 3% (m/v) pepsin and the pH adjusted to pH2 for optimal conditions of pepsin. From Table 1 it is clear that this step was necessary for the release of ACTH from the precursor-molecule since the pepsin-treated cultures showed statistically significant increased ACTH levels ($p < 0.05$; Mann-Whitney U test).

Investigations into the possibility that pH alone

Table 1 Effect of pepsin treatment on the processing of lymphocyte POMC and release of ACTH

Treatment	*n*	*pg ACTH/10^6 cells* *Exp. 1* *mean ± SD*	*Exp. 2* *mean ± SD*	*Exp. 3* *mean ± SD*
No pepsin; no pH adjustment	4	44.25 ± 6.6	18.00 ± 0.0	30.59 ± 1.0
3% pepsin; pH2	4	60.87 ± 3.1	79.50 ± 5.0	70.78 ± 4.7
% increase		37.56	341.67	131.38

could be sufficient for releasing ACTH led to the observation that this treatment of lymphocyte cultures was satisfactory in producing measurable quantities of ACTH. Differences between cultures treated with pepsin and those in the absence of pepsin were not statistically significant (Mann-Whitney U-test; results not shown).

Aliquots of suspensions containing 1 × 10^6 cells/ml were also incubated for different times before pH adjustment. From Figure 1 the linearity between ACTH production and time of incubation is clear. In addition, it was found that ACTH production increased linearly with increasing concentrations of lymphocytes (Fig. 2).

Lymphocytes of 5 male controls (mean age ± SD = 33.2 ± 8.5 yr) displayed ACTH levels of 97.4 ± 31.2 pg/10^6 cells/24 hour, while those of 10 female controls (mean age ± SD = 34.0 ± 9.6 yr) showed levels of 110.3 ± 45.8 pg ACTH/10^6 cells/24 hour. No statistically significant difference between the two sexes could be detected (Table 2; Mann-Whitney U-test).

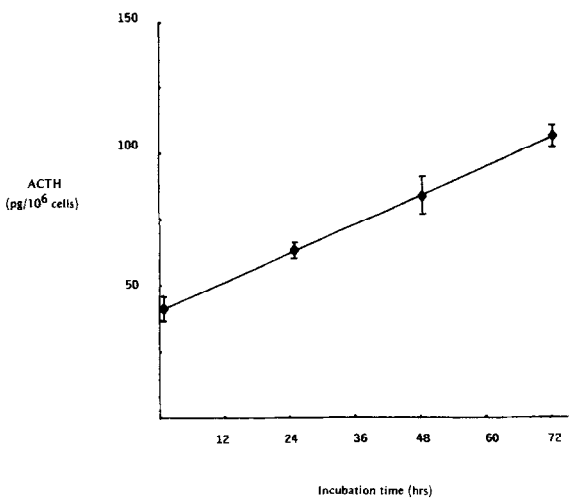

Fig. 1 A representative graph of the effect of the course of incubation on the production of ACTH by human lymphocytes in vitro.

Discussion

Initial ACTH measurements on lymphocyte cultures revealed the absence of this neuropeptide in the medium when the culture was not treated with pepsin. Pepsin treatment at the pH-optimum for pepsin, however, caused the release of ACTH from the precursor molecule, POMC. The above-mentioned finding is in agreement with previous investigations on pituitary cells, where it was found that basal secretion of neuropeptides was enriched with precursor molecules while stimulated secretion was enriched with product peptides (17, 18, 19, 20).

Subsequent investigations revealed that adjustment to pH2 alone was sufficient to produce ACTH in quantifiable amounts. This could be the result of stimulation of specific endopeptidases involved in the processing of the POMC-molecule, similar to those found in pituitary cells (21). Although the pH-optima for two specific endopeptidases (21) that have been isolated from pituitary cells were 4-5 and 9, respectively, it is still uncertain which endopeptidases, if any, are involved in the processing of POMC by the human lymphocyte.

Incubation of lymphocytes in culture medium for specific time periods, followed by pH adjustment as described above, revealed a linearity between time of incubation and the basal measurement of ACTH. It was shown by Smith

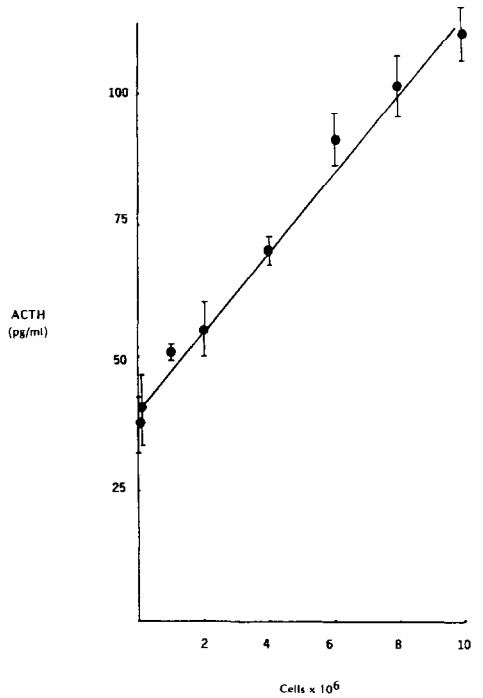

Fig. 2 A representative graph of the effect of number of cells on the production of ACTH by human lymphocytes in vitro.

and Blalock (10) that lymphocytes, although unstimulated by factors like CRF, vasopressin or mitogens, continuously synthesize ACTH in the form of its precursor molecule. This is in agreement with results observed in this study. In the present study it was also shown that ACTH production increased linearly with increasing concentrations of lymphocytes.

In a study by Harbour et al. (22) it was established that the immune system processes POMC products differently depending on the stimulus for induction. For example, the B lymphocytes from C3H/HeJ (LPS-resistant) mice expressed but did not process POMC after LPS or CRF treatment, nor did their B cells express an enzymatic activity at a certain pH that cleaved ACTH into a smaller $ACTH_{1\text{-}23\ to\ 26}$ (22). From this study it was also concluded that mice, resistant to the in vivo effects of LPS, did not appear to express the enzymes necessary to process the POMC molecule in the lymphocyte system.

In conclusion, a method was developed by which ACTH, produced by human lymphocytes, may be quantitatively measured. This study will be extended to investigate the biological activity of this neuropeptide by incubating lymphocyte secreted ACTH in the presence of human adrenal fasciculata cells. By doing so, it would be expected that cortisol secretion by these cells in the presence of $ACTH_{1\text{-}39}$ will increase.

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Table 2 ACTH determinations on lymphocytes of normal healthy controls

		Age (years)	*ACTH (pg/24 hours/ 10^6 cells)*	
Group	*n*	*Mean ± SD*	*Mean ± SD*	*Median*
♂ Controls	5	33.2 ± 8.5	97.4 ± 31.2	100.0
♀ Controls	10	34.0 ± 9.0	110.3 ± 45.8	102.6

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