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Human lymphocytes produce urocortin, but not corticotropin-releasing hormone

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Abstract

Hypothalamic corticotropin-releasing hormone (CRH) is the principal regulator of the hypothalamus-pituitary-adrenal axis in mammals. In addition, immunoreactive CRH is also present at peripheral sites, where it is thought to act as a proinflammatory peptide. However, the source of peripheral CRH has remained obscure. Human lymphocytes were shown to produce immunoreactive CRH, yet the data on CRH mRNA expression in these cells are equivocal. More recently, Vaughan et al. discovered a new member of the CRH family, termed urocortin. Urocortin was shown to act through the same receptors as CRH. The current study was designed to investigate both mRNA and protein expression of CRH and urocortin in human lymphocytes. Using a commercial CRH(1-41) radioimmunoassay, we demonstrate that normal human lymphocytes and Jurkat T lymphoma cells produce significant amounts of immunoreactive peptide. However, no CRH mRNA was detectable by RT-PCR in these cells. In contrast, a band of the correct size and sequence was amplified with urocortin-specific primers. Immunocytochemical analysis of human lymphocytes using antibodies that could distinguish between CRH and urocortin revealed significant expression of urocortin but not of CRH, consistent with our RT-PCR data. We conclude that human lymphocytes produce urocortin, but not CRH.

Introduction

Corticotropin-releasing hormone (CRH) is the key regulator of the hypothalamic-pituitary adrenal axis in mammals (1-5). By stimulating adrenocorticotropic hormone (ACTH) and, thus, cortisol secretion, centrally produced CRH exerts profound immunosuppressive effects (5). CRH has also been shown to be present at peripheral sites where it may act as a proinflammatory peptide (6, 7). In support of this concept, Karalis et al. demonstrated that the inflammatory response could be markedly inhibited by administration of anti-CRH antibodies (6).

More recently, a new member of the CRH family, termed urocortin, was cloned and shown to be expressed in the central nervous system of rats (8) and humans (9). The urocortin peptide shares 45 % sequence homology with CRH and can bind to both CRH_1 and CRH_2 receptors (8-13).

Several studies suggest that lymphocytes are an important source of immunoreactive CRH (14-16). However, the presence of CRH mRNA in human lymphocytes has never been proven unequivocally. Furthermore, the expression of urocortin in human lymphocytes has not been investigated as yet.

In an effort to clarify this matter, we analyzed the expression of CRH and urocortin in normal human lymphocytes and Jurkat T lymphoma cells both at the mRNA and protein level. Our data clearly indicate that human lymphocytes produce urocortin, but not CRH.

Materials and Methods

Cell culture

Normal peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood from healthy volunteers, using Ficoll-Isopaque density centrifugation (Pharmacia, Uppsala, Sweden). Cells were cultivated in RPMI 1640 supplemented with 10 % FCS and antibiotics. After 20 h in culture, the lymphocyte-enriched population of nonadherent cells was collected and used for stimulation experiments. Cells were stimulated for 16 h with either control medium or medium containing phorbol ester (TPA, 0.5×10^{-7} M, Sigma, Deisenhofen, Germany), ionomycin (1 µg/ml, Sigma), and, in some experiments, forskolin (25 µM, Sigma). Cells were harvested for RNA extraction, and supernatants were collected for CRH radioimmunoassay.

Similar experiments were performed with human Jurkat T lymphoma cells (ATCC, Rockville, MD).

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Radioimmunoassay

To measure CRH-like immunoreactivity in cell culture supernatants, we employed a rabbit antiserum against full-length human CRH(1-41) as part of a commercial CRH radioimmunoassay (RIA) kit (Peninsula Lab., Belmont, CA), following the instructions of the manufacturer. The standard curve was created with human CRH(1-41) peptide. In addition, human urocortin peptide (Bachem Biochemica, Heidelberg, Germany) was used to detect potential crossreactivity of the anti-CRH antiserum.

RT-PCR, Southern hybridization, and DNA sequencing

Total RNA from normal human lymphocytes, Jurkat cells, and human 3rd trimester placenta (positive control) was extracted by standard methods and quantified by UV-absorption. Complementary DNA was synthesized from 5 μg total RNA with 100 U SuperScript polymerase (Life Technologies, Gaithersburg, MD), using oligo(dT) primers (Life Technologies). PCR was carried out with 10 pmol 5'- and 3'-primers, 0.2 mM dNTPs, 0.5 U of *Pfu* polymerase (Stratagene, Heidelberg, Germany) in a reaction volume of 50 μl. Initial denaturation was performed for 2 min at 95 °C, followed by two cycles each at 68, 66, 64, and 62, and 35 cycles at 60 °C (annealing temperature). Primer sequences were as follows: human urocortin sense primer 5'-CAGGCGAGCGGCCGCG-3', human urocortin antisense primer 5'-CTTGCCCACCGAGTCGAAT-3', human CRH sense primer 5'-GCACCCGGCTCACCTGCGAA-3' (exon 1), human CRH antisense primer 5'-CTTGCCCACCG AGTCGAAT-3' (exon 2). GAPDH-specific primers were used as positive control and to exclude contamination with genomic DNA. PCR products were electrophoresed in a 1.5 % agarose gel and visualized by UV light.

Southern blotting of the urocortin RT-PCR products was performed by standard methods, using a Hybond N+ membrane (Amersham, Buckinghamshire, England). Blots were hybridized with a digoxigenin-labeled urocortin-specific cDNA probe, which was generated by PCR, using the Boehringer DIG-PCR-Labeling Kit (Boehringer Mannheim, Germany). Signals were visualized by incubation with anti-digoxigenin alkaline phosphatase-conjugated antibodies (Boehringer), subsequent application of CDP-Star luminescence substrate (Boehringer), and exposure to Fuji RX film (Fuji, Tokyo, Japan).

After subcloning into the PCR-Scipt vector (Stratagene, Heidelberg, Germany), the urocortin-specific PCR product was further analyzed by DNA sequencing, using the ABI PRISM Dye Terminator System (Perkin Elmer, Foster City, CA).

Immunocytochemistry

Immunocytochemistry of normal human lymphocytes was performed as previously described (17). Urocortin and CRH immunoreactivity was detected with polyclonal, non-crossreactive urocortin- and CRH-specific antibodies, respectively (Santa Cruz Biotechnology, Santa Cruz, CA). The specificity of the antibodies was confirmed by dot blot experiments using human sequence urocortin and CRH, respectively (not shown).

Results

CRH-like immunoreactivity in normal human lymphocytes and Jurkat T lymphoma cells

The results of our RIA experiments are shown in Fig. 1. Both normal lymphocytes and Jurkat cells produced comparable amounts of CRH-like immunoreactivity. Combined treatment with TPA, ionomycin, and forskolin caused a small increase in the release of CRH-like reactivity. However, human sequence urocortin was also detected by the anti-CRH(1-41) antiserum, indicating significant crossreactivity (Fig. 2).

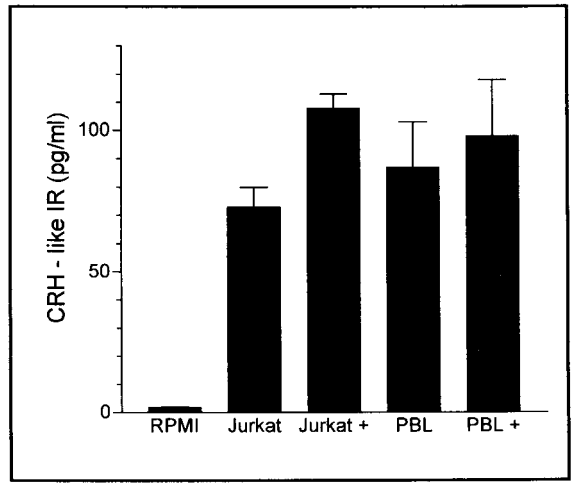

Fig. 1. Detection of CRH-like immunoreactivity (IR) by RIA in unstimulated and TPA/ionomycin/forskolin-stimulated (+) Jurkat T lymphoma cells and lymphocyte-enriched human peripheral blood mononuclear cells (PBL).

Expression of urocortin mRNA, but not of CRH mRNA in normal human lymphocytes and human Jurkat T lymphoma cells

Expression of CRH and urocortin mRNA in human peripheral blood lymphocytes and Jurkat T lymphoma cells was analyzed by RT-PCR, using human CRH- and urocortin-specific primers, respectively. Human placenta cDNA served as a positive control for both CRH (18) and urocortin (19) expression. A specific band of the predicted length (145 bp) was obtained with urocortin-specific

primers in unstimulated and stimulated human lymphocytes, as well as in Jurkat cells (Fig. 3A). No band was obtained with the CRH-specific primers, indicating that neither normal human lymphocytes nor Jurkat T lymphoma cells express the CRH gene. All urocortin PCR products specifically hybridized with the urocortin cDNA probe (Fig. 3B). The urocortin PCR product was further analyzed by DNA sequencing. The obtained sequence showed no difference to the human urocortin cDNA sequence (9).

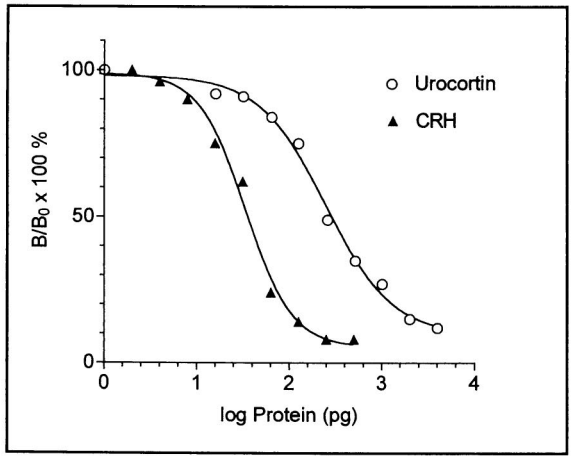

Fig. 2. Both human sequence CRH and, less sensitively, human sequence urocortin are detected by the commercial anti-CRH(1-41) RIA. B = specific binding, B_0 = total binding.

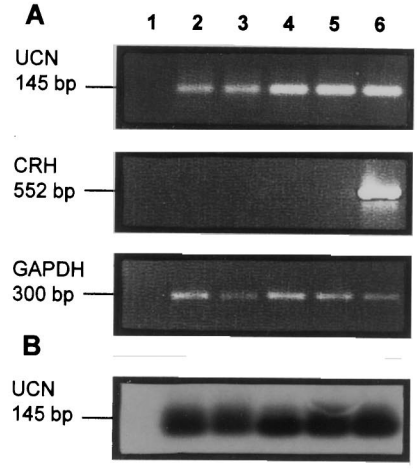

Fig. 3. (A) Expression of urocortin (UCN) mRNA (upper panel), but not of CRH mRNA (lower panel) in human lymphocytes and Jurkat T lymphoma cells. (B) Southern hybridization of the urocortin PCR products with a urocortin-specific cDNA probe. Lanes: 1=water, 2=unstimulated lymphocytes, 3=TPA/ionomycin-stimulated lymphocytes, 4=unstimulated Jurkat cells, 5=TPA/ionomycin-stimulated Jurkat cells, 6=3rd trimester placenta.

Human lymphocytes and Jurkat T lymphoma cells express the urocortin, but not the CRH peptide

Normal human lymphocytes were further analyzed by immunocytochemistry, using CRH- and urocortin-specific antibodies. In these experiments, no CRH was detectable. In contrast, specific staining was seen with the urocortin-specific antibody (Fig. 4).

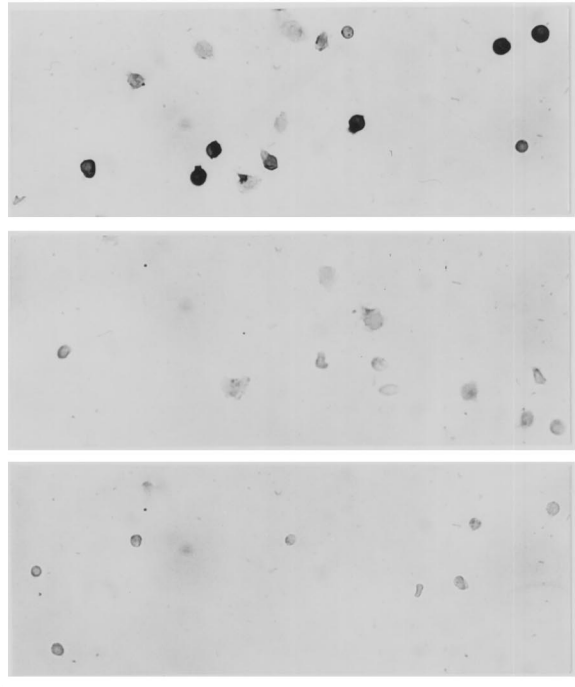

Fig. 4. Specific expression of urocortin (upper panel), but not of CRH (middle panel) in normal human lymphocytes. Lower panel = negative control (normal lymphocytes incubated with preimmune serum).

Discussion

In the present study, we show for the first time that normal human lymphocytes and Jurkat T lymphoma cells express urocortin mRNA and peptide. Furthermore, we demonstrate that neither CRH mRNA nor peptide are expressed by these cells. Finally, we provide evidence that a polyclonal anti-CRH(1-41) antiserum can crossreact with urocortin.

In addition to its expression throughout the central nervous system, immunoreactive CRH was also found at numerous peripheral sites, e.g. placenta (18), ovary (20), and endometrium (21). So far, peripheral expression of urocortin mRNA and peptide has only been shown in the human placenta and fetal membranes (19).

An important pathophysiological role for peripheral CRH was suggested by Karalis et al., who demonstrated the

presence of CRH-like immunoreactivity at sites of chemically induced inflammation (6). Furthermore, it was shown that local administration of anti-CRH antibodies or a CRH receptor antagonist markedly reduced the degree of edema in the inflamed skin, indicating a proinflammatory role of CRH (6, 22). In support of this concept, increased levels of immunoreactive CRH were also found in the synovial fluid of patients with rheumatoid arthritis (23).

Despite the obvious pathophysiological relevance of 'immune' CRH, there are few data concerning the exact source of CRH and the actual expression levels of the CRH gene at inflammatory sites. Expression studies in mice revealed that the CRH gene is actively transcribed in splenic T lymphocytes (15), indicating that immune cells might be an important source of CRH production during the immune/inflammatory response. However, Karalis et al. could not demonstrate CRH mRNA expression in acute inflammatory sites (6), while Crofford et al. only detected it in chronic inflammatory responses (23). Using an anti-CRH(1-41) antiserum, two groups previously demonstrated the production of CRH-like immunoreactivity by cultured human T lymphocytes (14, 16). Our results indicate that this type of antiserum can detect both human CRH and human urocortin. Furthermore, our immunocytochemistry experiments employing a CRH-specific antibody clearly showed lack of significant CRH production in normal human lymphocytes. Finally, CRH mRNA was not found to be expressed in these cells. As opposed to CRH, both urocortin mRNA and peptide were expressed by human lymphocytes. Therefore, part of the previously described peripheral CRH-like immunoreactivity may have been produced by other cell types, such as peripheral nerves (24), and/or may have been urocortin.

The functional role of peripheral urocortin has yet to be determined. Since urocortin was previously shown to exert its effects through the same receptors as CRH (8, 9), and since CRH binding sites are present on immune cells (25), one can assume that 'peripheral' urocortin has predominantly immunoenhancing/proinflammatory effects. To dissect the roles of CRH and urocortin in the immune/inflammatory response is, thus, an exciting goal of future research.

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