Corticosteroid-independent inhibition of tumor necrosis factor production by the neuropeptide urocortin

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1Mario Negri Institute for Pharmacological Research, 20157 Milan; 2Department of Pharmacology, Dompe SpA Research Center, 67100 L'Aquila; and 3Consiglio Nazionale delle Ricerche-Cellular and Molecular Pharmacology Center, 20129 Milan, Italy

Agnello, Davide, Riccardo Bertini, Silvano Sacco, Cristina Meazza, Pia Villa, and Pietro Ghezzi. Corticosterone-independent inhibition of tumor necrosis factor production by the neuropeptide urocortin. Am. J. Physiol. 275 (Endocrinol. Metab. 38): E757–E762, 1998.—Urocortin (UCN) is a neuropeptide homologous with corticotropin-releasing factor (CRF), which has anti-inflammatory activities not all mediated by corticosteroids. In mice, UCN (1 µg/mouse sc) significantly reduced lipopolysaccharide (LPS)-induced serum tumor necrosis factor (TNF) and interleukin (IL)-1β levels in vivo but did not affect serum IL-6. These effects were paralleled by a rise in corticosterone (CS) levels. Blockade of the CS increase by cyanoketone did not prevent TNF inhibition by UCN, suggesting the neuropeptide has anti-inflammatory mechanisms independent of the hypothalamus-pituitary-adrenal axis. In fact UCN had a direct inhibitory effect on LPS-induced TNF in rat Kupffer cells at concentrations between 10−10 and 10−16 M, and this effect was related to increased cAMP levels. However, the in vivo inhibition of LPS-induced IL-1β by UCN was reversed by cyanoketone, indicating that the increase of endogenous glucocorticoids might be more important in IL-1β inhibition than in TNF inhibition by UCN.

inflammation; cytokines; corticotropin-releasing factor; lipopolysaccharide; hypothalamus-pituitary-adrenal axis; interleukin-1; interleukin-6; Kupffer cells

UROCORTIN (UCN), a recently described neuropeptide detected in rat brain and 45% homologous with corticotropin-releasing factor (CRF) (28), is an endogenous ligand for the CRF receptor. The main physiological role of CRF is activation of the hypothalamus-pituitary-adrenal axis (HPAA), where it stimulates ACTH production by the pituitary and, thus, ultimately increases the release of corticosteroids. CRF reportedly acts by binding to two different receptors. CRF receptor type 1 is expressed mainly in the brain and the pituitary (18), whereas CRF receptor type 2 is expressed in brain and peripheral tissues but not in the pituitary (15). The distribution of UCN is not the same as CRF and correlates with the distribution of the CRF receptor type 2 but not type 1 (28); UCN also has higher affinity for receptor type 2 than CRF itself (28).

Because corticosteroids are potent anti-inflammatory agents, UCN and CRF can be viewed as anti-inflammatory mediators. In fact, immunosuppressive or anti-inflammatory effects of exogenously administered CRF have been described (6, 13, 17, 31), and CRF transgenic mice have an immunosuppressive phenotype that is reversed by adrenalectomy (3). The mechanism of anti-inflammatory action of CRF is unclear, and it may have anti-inflammatory activities independent of endogenous corticosteroids (12, 17).

UCN is also reported to have anti-inflammatory activity (27) and to inhibit experimental autoimmune encephalomyelitis (17) by a glucocorticoid-independent mechanism. Its anti-inflammatory activity may be mediated by CRF receptor type 2 (27).

Various cytokines are considered important pathogenic mediators of inflammation. Tumor necrosis factor (TNF) is particularly important, as demonstrated by the protective effects of anti-TNF antibodies or inhibitors of TNF synthesis in animal models of arthritis (32) and experimental autoimmune encephalomyelitis (22) and by the clinical efficacy of anti-TNF antibodies in rheumatoid arthritis (8).

Corticosteroids are potent inhibitors of TNF synthesis, so activation of the HPAA amounts to a negative-feedback mechanism that limits TNF synthesis (5, 11). In fact, activation of the HPAA by cytokines or stress inhibits TNF production (10), whereas its blockade by adrenalectomy or glucocorticoid antagonists or inhibitors upregulates TNF production and worsens TNF-mediated diseases (2, 11).

To better define the effects of these neuropeptides in inflammation, we studied the effect of UCN on TNF, using a model where lipopolysaccharide (LPS) is administered to mice to induce circulating TNF. We also investigated the levels of two other inflammatory cytokines, interleukin (IL)-1α and IL-6.

Because these experiments showed that UCN had an inhibitory effect on TNF and IL-1β levels, we investigated whether this was a direct or HPAA-mediated effect, using cyanoketone to prevent the rise of blood corticosterone (CS) (10). Finally, we investigated whether UCN directly affected TNF levels in mouse blood or rat Kupffer cells in vitro.

MATERIALS AND METHODS

Materials. Synthetic rat UCN was synthesized and kindly provided by Dr. Nicholas Ling of Neurocrine Biosciences (San Diego, CA). Synthetic rat-human CRF and LPS (Escherichia coli 055:B5) were from Sigma (St Louis, MO). Cyanoketone was a kind gift from Sanofi Research Division (Malvern, PA).

Animals and treatments. Male Crl:CD-1 (ICR) BR mice (25 g body wt) were used for in vivo studies. Male Crl:CD (SD) BR rats (200–250 g body wt) were used for preparation of

UCN was reconstituted by adding 0.1 ml of 1 N acetic acid to 1 mg of peptide. CRF was dissolved in sterile, pyrogen-free water to a concentration of 1 mg/ml. Both peptides were then diluted in phosphate-buffered saline (PBS) and injected at the dose of 1 µg/mouse sc. Control mice received PBS alone. One hour later, LPS was injected at the dose of 100 ng/mouse iv. Serum TNF and IL-6 were determined at 1.5 h, and serum IL-1β was determined at 3 h after LPS injection. These schedules were chosen on the basis of the kinetics of cytokine induction after LPS (4, 24) and of preliminary experiments. CS was determined at the times of LPS injection (1 h after UCN and CRF) and cytokine determination (1.5 and 3 h after LPS) on different groups of animals. Control mice received saline alone. Blood was obtained from the retroorbital plexus, and serum was prepared and stored frozen until it was used for cytokine and CS assays.

In some experiments, cyanoketone was given twice, 24 h and 1.5 h before the experiment, at the dose of 100 mg/kg ip in corn oil (10). Control mice received the same amount of corn oil.

Whole blood cultures. For whole blood cultures, heparinized (14 U/ml; Liquemin, Roche, Milan, Italy), freshly obtained whole blood was plated in 96-well tissue culture plates (100 µl/well) and incubated for 4 h at 37°C in 5% CO2 with 1 µg/ml LPS and with and without different concentrations of UCN (added 1 h before LPS). At the end of the incubation, blood was diluted 1:1 (vol/vol) with cold RPMI 1640 medium and centrifuged, and the supernatant was collected for TNF determination.

Kupffer cell cultures and cAMP determination. Kupffer cells were prepared according to the method of Smedsrod and Pertoft (25) after perfusion and digestion of rat liver. Cells were seeded at 0.2 × 10^6/well for TNF experiments and at 4 × 10^6/well for cAMP and were used after 48 h of culture in Williams’ medium E at 37°C in a 5% CO2-humidified atmosphere.

For TNF determination, the cells were incubated at 37°C for 30 min with either control medium or UCN at different concentrations and then stimulated with 0.1 µg/ml of LPS. After 4 h, the supernatants were collected for TNF assay.

For cAMP determination, the cells were incubated at 37°C for 30 min with 1 mM 3-isobutyl-1-methylxanthine (Sigma) and for another 30 min with either control medium or UCN at different concentrations. The cells were then placed on ice, and the medium was removed. Cultures were washed twice with cold PBS and scraped into 10 ml of cold 65% (vol/vol) ethanol. After centrifugation, the supernatants were dried under a stream of nitrogen at 60°C, and the dried extracts were dissolved in 1 ml of assay buffer for cAMP determination with cAMP ELISA from Amersham International (Little Chalfont, UK), according to the instructions of the manufacturer.

Cytokine determination. TNF was measured by cytotoxicity on L929 cells as previously described (1), using recombinant TNF-α as a standard (specific activity 0.6 × 10^7 U/mg; a kind gift from BASF, Ludwigshafen, Germany). The sensitivity of the assay was 0.1 U/ml. IL-6 was measured as hybridoma growth factor with TTD1 cells (a kind gift from Dr. van Snick) as previously described (23). IL-6 activity is expressed as

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Fig. 1. Effect of urocortin (UCN) and corticotropin-releasing factor (CRF) on tumor necrosis factor (TNF; A), interleukin (IL)-1β (B), and IL-6 (C) levels. Mice were treated with UCN or CRF (1 µg/mouse sc). Control mice received PBS alone. Lipopolysaccharide (LPS; 100 ng/mouse iv) was given 1 h later. Serum TNF and IL-6 were measured 1.5 h after LPS; serum IL-1β was measured 3 h after LPS. Data are means ± SD (5 mice/group). * P < 0.05, ** P < 0.01 vs. PBS alone by Duncan's test.
costimulatory units per milliliter, using recombinant IL-6 as a standard. The sensitivity of the assay was 50 U/ml. IL-1β was measured with an ELISA kit (Amersham International). The sensitivity of the assay was 3 pg/ml.

CS determination. CS was measured by a competitive-binding RIA, with an antiserum for corticosterone RIA obtained from Sigma (C-8784), as previously described (10). [3H]corticosterone was purchased from Amersham International.

RESULTS

As shown in Fig. 1A, UCN or CRF at the dose of 1 µg/mouse, 1 h before LPS, significantly reduced serum TNF levels (85 and 75% inhibition, respectively). UCN also lowered serum levels of IL-1β by 65%, whereas CRF had no effect (Fig. 1B). IL-6 was unaffected by either UCN or CRF (Fig. 1C). No TNF, IL-1β, or IL-6 was detected in serum of control mice (data not shown).

We also evaluated the effect of UCN or CRF on serum CS levels, which were increased 1 h after UCN or CRF (Fig. 2), in agreement with previous results (21). LPS induced CS at higher levels than UCN or CRF alone, and pretreatment of mice with these neuropeptides did not further increase the response to LPS (Fig. 2).

To investigate whether TNF and IL-1β inhibition by UCN was fully mediated by the increase of glucocorticoids, we inhibited UCN- and LPS-induced CS synthesis by pretreating mice with cyanoketone (10) (Figs. 3B and 4B). As shown in Figs. 3A and 4A, UCN significantly reduced TNF levels but did not affect IL-1β production in cyanoketone-pretreated mice.

To study the direct effects of UCN on TNF-producing cells, we performed a series of in vitro experiments on whole mouse blood and rat Kupffer cell cultures. On whole mouse blood at UCN concentrations between 10⁻² and 10⁻¹⁴ M, we never observed more than 30% inhibition, which was not statistically significant (data not shown). However, UCN significantly inhibited TNF production by rat Kupffer cells with an inverted, bell-shaped dose-response curve. The effective concentrations ranged between 10⁻¹⁰ and 10⁻¹⁶ M (Fig. 5).

To further investigate the CS-independent mechanism for UCN inhibition of TNF production, we also measured cAMP levels in rat Kupffer cells after exposure to different UCN concentrations. The neuropeptide significantly raised cAMP levels at the concentrations that inhibited TNF production (Table 1).

DISCUSSION

The present study showed that UCN, like CRF, inhibits LPS-induced TNF in vivo. This effect seems to be largely independent of endogenous CS. In fact, UCN also inhibited TNF levels in cyanoketone-pretreated mice, although to a slightly lesser extent than in intact mice (in three independent experiments, UCN inhib-
ited TNF production by 85, 83 and 76%; in two experiments with cyanoketone-pretreated mice, inhibition amounted to 70 and 57%).

The corticosteroid-independent immunosuppressive action of UCN is in agreement with a report that this neuropeptide can protect adrenalectomized animals from experimental autoimmune encephalomyelitis (17).

A direct effect on TNF-producing cells might be the cause of UCN inhibition of TNF production, although this does not rule out the possibility that other mediators produced by the HPAA reported to inhibit TNF production, such as the peptide melanocyte-stimulating hormone (14), might be important too.

We focused our attention on the first hypothesis and tried to demonstrate a direct effect on TNF production in vitro in mouse blood and rat Kupffer cells. In our experimental conditions, we observed no real effect of UCN in whole blood. However, UCN significantly inhib-

ited TNF production in rat Kupffer cells, which constitute the main macrophage population of the body. This observation is in agreement with the reported presence of CRF receptors on macrophages (7).

We also investigated the possibility that cAMP was involved in the mechanism of TNF inhibition by UCN. UCN, like CRF (30), has been reported to raise cAMP levels (28), and cAMP is a potent inhibitor of TNF production (20, 26). There was an increase in the amount of cAMP in Kupffer cells after pretreatment with UCN at concentrations that lower TNF levels. Further studies are needed to see whether this increase in cAMP is the only mechanism responsible for the inhibition of TNF production.

We have also shown that UCN inhibits IL-1β production, but this effect is reversed by cyanoketone. The increase of endogenous glucocorticoids might therefore be more important in IL-1β inhibition by UCN than in TNF inhibition, and the increase in cAMP levels might be less important than in the case of TNF inhibition. In fact, although there is no doubt about the effects of cAMP on TNF production, conflicting reports have been published regarding the inhibition of IL-1 production by agents that increase cAMP levels. Some authors reported that IL-1 production was only slightly or not at all inhibited in human monocytes or mouse macro-

Fig. 4. Effect of cyanoketone on UCN inhibition of IL-1β (A) and CS (B) levels. Mice were treated with UCN (1 µg/mouse sc). Control mice received PBS alone. Cyanoketone was given intraperitoneally at the dose of 100 mg/kg, 24 h and 1.5 h before UCN (or PBS). LPS (100 ng/mouse iv) was given 1 h after UCN or PBS. Serum IL-1β and CS were measured 3 h after LPS. Data are means ± SD (5 mice/group). *P < 0.05, **P < 0.01 vs. PBS control by Duncan’s test.

Table 1. Effect of UCN on the amount of cAMP in rat Kupffer cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>cAMP, fmol/4 × 10⁶ cells</th>
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<tbody>
<tr>
<td>Control medium</td>
<td>583 ± 173</td>
</tr>
<tr>
<td>UCN</td>
<td>882 ± 358</td>
</tr>
<tr>
<td>10⁻⁸ M</td>
<td>2,195 ± 185*</td>
</tr>
<tr>
<td>10⁻¹⁰ M</td>
<td>1,641 ± 280*</td>
</tr>
</tbody>
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Data are means ± SD from triplicate wells. Experiments were done as described in MATERIALS AND METHODS. UCN, urocortin. *P < 0.01 vs. control medium.
phages pretreated with phosphodiesterase inhibitors (9, 16, 19), whereas others reported that rolipram reduced IL-1β secretion from human monocytes but increased IL-1α mRNA (29). In a preliminary experiment, we did not see any inhibition of IL-1 secretion from Kupffer cells pretreated with UCN at different concentrations (data not shown).

Finally, we report that UCN does not affect IL-6. This is consistent with previous data showing that IL-6 levels in vivo are not inhibited and may be even increased by cAMP-elevating agents (33, 34). IL-6 production in vivo is also resistant to the inhibitory effect of glucocorticoids, whereas TNF production is extremely sensitive even to low doses of glucocorticoids (24).

In conclusion, we have shown that UCN is a potent inhibitor of TNF production in vivo and in vitro and also inhibits production of IL-1β in vivo, an observation that supports the concept of an anti-inflammatory activity of this neuropeptide, through corticosteroid-independent and -dependent mechanisms.

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