Peripheral corticotropin-releasing factor mediates the elevation of plasma IL-6 by immobilization stress in rats

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Ando, Tetsuya, Jean Rivier, Hitoshi Yanaihara, and Akira Arimura. Peripheral corticotropin-releasing factor mediates the elevation of plasma IL-6 by immobilization stress in rats. Am. J. Physiol. 275 (Regulatory Integrative Comp. Physiol. 44): R1461–R1467, 1998.—We previously reported the elevation of plasma interleukin (IL)-6 activity in response to immobilization stress in rats. To investigate the role of peripheral corticotropin-releasing factor (CRF) in this response, we examined the effects of CRF antagonists on immobilization-induced IL-6 response. Intravenous pretreatment with either [D-Phe12,Nle21,38,C-MeLeu37]-anti-human rat (h/r) CRF 12—41 (1.5 mg/kg) or cyclo(30—33)[D-Phe12,Nle21,38,Glu30,Lys33]-h/rCRF 12—41 (Astressin, 0.5 mg/kg) attenuated the IL-6 response to immobilization, which confirmed our previous finding that systemic administration of an antisem against CRF blocked this response. In addition, an intraperitoneal injection of h/rCRF (100 µg/kg) or rat urocortin (10 and 100 µg/kg) increased the plasma IL-6 activity, mimicking the response to immobilization. An intravenous injection of h/rCRF (100 µg/kg) also elevated plasma IL-6 in adrenalectomized rats. These findings suggest that peripheral CRF mediates the plasma IL-6 elevation in response to immobilization.

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METHODS

Animals. Male CD rats (Charles River, Wilmington, MA) weighing 350-400 g were used. The animals were housed in a room maintained at ~25°C with illumination from 0800 to 2000. Standard Purina laboratory chow and water were available ad libitum. The experimental protocol was approved by the Tulane University Medical School Advisory Committee for Animal Resources (protocol no. 1783–3–03–105).

Cannulation into the jugular vein. To implant blood samples for plasma IL-6 and ACTH measurement, we implanted the animals with an indwelling jugular venous catheter filled with heparinized saline solution (50 U/ml). Surgery was performed 2 days before the experiment under ketamine (100 mg/kg ip) and xylazine (10 mg/kg ip) anesthesia.

Immobilization stress. The animals were adapted to the experimental conditions by daily handling for 7 days to avoid manipulative stress. On the day of experiment, the cages containing the rats were moved to a laboratory and left there for at least 2 h to acclimate to the experimental environment. The rats were restrained on a board in a supine position by taping the limbs to holders for 60 min without anesthesia. They were then released and returned to their home cages.

Drugs. d-Phe-CRF12–41, a CRF antagonist, and Astressin, a CRF antagonist, were synthesized by one of the authors (J. Rivier), and rat urocortin was provided by Dr. N. Yanaihara (Yanaihara Institute). h/rCRF1–41 was purchased from American Peptide (Sunnyvale, CA). The CRF antagonists and h/rCRF were dissolved in 0.04 M phosphate-buffered saline, pH 7.4, containing 0.1% bovine serum albumin and 0.01% ascorbic acid. The injection volume was 1 ml for both the intravenous and intraperitoneal injections.

Blood sampling. Blood samples (0.7 ml) for determination of plasma IL-6 and ACTH levels were taken through the jugular vein catheter 10 times (at ~30, 0, 15, 30, 60, 90, 120, 180, 240, and 300 min) before, during, and after immobilization stress. Each sample was collected in an ice-chilled tube containing Trasylol (aprotinin; 500 KIU, Mobay), and EDTA (2 mg, Mallindckrodt). After centrifugation of the blood samples, the plasma (0.3 ml) was removed and frozen at ~80°C. The blood cells were resuspended in physiological saline (0.3 ml) and injected through the same catheter after each blood collection to prevent loss of blood volume.

Plasma IL-6 concentration. Plasma IL-6 activity was measured using an IL-6-dependent murine hybridoma subclone B9 cell line. B9 cells were maintained in RPMI 1640 with 25 mM HEPES containing 10% heat-inactivated (56°C, 30 min) fetal bovine serum and a 1% antibiotic-antimycotic solution (GIBCO, Baltimore, MD) in the presence of 20 hybridoma growth units/ml recombinant human (rh) IL-6, where 1 unit was defined as the amount that caused half-maximal B9 cell proliferation. A 2-µl plasma sample was placed in a single well of a 96-well plate containing a 198-µl culture medium and was serially diluted to 1:32 the original concentration of the first well. In addition, each plate had a standard diluted line containing the rhIL-6, in which the first well was 1 U (100 µl). After five washes, 10 µl/m B9 cells in culture medium were added to each well (100 µl) and the cells were incubated in 5% CO2 at 37°C for 72 h. IL-6 activity was measured colorimetrically using thiazolyl blue tetrazolium bromide. The minimum detectable concentration was 15 U/ml in plasma. The inter- and intra-assay variations were <5 and 10%, respectively. The B9 cell assay is commonly used for measurement of plasma IL-6 levels by many investigators (16, 36). Although the rhIL-6 stimulated the proliferation of B9 cell in a dose-dependent manner, other cytokines, such as rh-tumor necrosis factor-β, rhIL-1α, rhIL-1β, rhIL-2, rh-interferon (IFN)-α, recombinant mouse IFN-γ, rhFN-β, rhIL-3, rhIL-4, rh-granulocyte/macrophage-colony stimulating factor (CSF), and rh-macrophage-CSF, did not. In this assay, CRF, urocortin, d-Phe-CRF12–41, and Astressin (10 pg/ml–1 µg/ml) did not affect B9 cell growth in vitro.

Radioimmunoassay. Plasma ACTH and corticosterone levels were determined by RIA using kits from the National Hormone and Pituitary Program of the National Institute of Diabetes and Digestive and Kidney Diseases and ICN Biochemicals (Irvine, CA), respectively.

Adrenalectomy. Animals were adrenalectomized bilaterally under ketamine-xylazine anesthesia 10 days before immobilization. Each adrenalectomized animal was implanted with a corticosterone pellet (10 mg; Innovative Research of America, Toledo, OH) to maintain normal basal serum levels of corticosterone and provided with drinking water containing 0.45% NaCl.

Statistical analysis. All values are given as means ± SE. A two-way ANOVA with a split-plot design was used to test the interaction effect of the treatment and the time over plasma IL-6 or ACTH levels. When a significant interaction was found, a repeated-measures ANOVA and Dunn’s test were performed within each group to compare values at individual time points with the basal level. Student’s t-test or a one-way ANOVA and Dunn’s test were used to determine statistical differences among groups at individual time points. The significance level was adjusted by the Bonferroni procedure in repetitive comparisons. P < 0.05 was regarded as significant.

RESULTS

Plasma IL-6 and ACTH responses to immobilization. Figure 1A shows the plasma IL-6 response to 1-h immobilization. The values of each group were averaged and plotted against the individual times. Basal levels of plasma IL-6 were 21.8 ± 4.3 U/ml in the immobilized group and 30.3 ± 7.5 U/ml in the nonstressed control group; there was no significant difference between the two. Plasma IL-6 levels started to increase 30 min after the initiation of the immobilization, peaked at 90 min (1,989.6 ± 507.5 U/ml), quickly dropped at 120 min, and then gradually returned to the basal level after more than 300 min. There was a significant interaction effect of the immobilization and time over plasma IL-6 activity (two-way ANOVA, split-plot design, F = 11.671, P = 0.0021). The levels of plasma IL-6 in the immobilized animals at several time points were significantly higher than the basal level (60 and 90 min, repeated-measures ANOVA (F = 11.917, P = 0.0076) followed by Dunn’s test, P < 0.01) or those in the control animals (30, 90, 120, 180, and 300 min, Student’s t-test, P < 0.005). There was no significant change of IL-6 activity in the control animals over time (F = 2.286, P = 0.1364).

Figure 1B shows plasma ACTH levels immediately before immobilization and at 15 min after the initiation of the stress. There was a significant interaction effect of the immobilization and time over plasma ACTH concentration (F = 16.574, P = 0.0022). Immobilization increased plasma ACTH concentrations at 15 min significantly as compared with the basal (P < 0.01) and the control (P < 0.01).
Blockade of plasma IL-6 and ACTH response to immobilization by CRF antagonists. To investigate the possible involvement of peripheral CRF in immobilization-induced plasma IL-6 elevation, a CRF antagonist, either D-Phe-CRF_{12-41} (1.5 mg/kg) or Astressin (0.5 mg/kg), was injected intravenously 5 min before the initiation of immobilization. Pretreatment with either D-Phe-CRF_{12-41} (Fig. 2A) or Astressin (Fig. 3A) significantly attenuated the plasma IL-6 responses to immobilization compared with the vehicle (F = 5.965, P = 0.0156, and F = 3.680, P = 0.0329, respectively). Comparison at individual time points revealed that the IL-6 level at 90 min in the D-Phe-CRF_{12-41}-treated group was significantly lower than that in the vehicle group (254.9 ± 81.4 vs. 1,512.7 ± 238.1 U/ml, Student’s t-test, P = 0.0011). Astressin also reduced the average of peak IL-6 levels at 90 min (1,032.2 ± 151.6 vs. 2,104.7 ± 388.1 U/ml) (P = 0.0244).

Both antagonists attenuated immobilization-induced plasma ACTH elevation (D-Phe-CRF_{12-41}: F = 29.466, P = 0.0006; Astressin: F = 14.435, P = 0.0029) (Figs. 2B and 3B). The ACTH concentration at 15 min in either the D-Phe-CRF_{12-41} or Astressin-treated group was significantly lower than that in corresponding vehicle control (P < 0.05).

Plasma IL-6 response to intraperitoneal injections of CRF and urocortin. To further investigate a possible involvement of peripheral CRF in the immobilization-induced IL-6 elevation, either rat CRF (10 and 100 µg/kg) or rat urocortin (10 and 100 µg/kg) was injected intraperitoneally and we examined the changes in plasma IL-6 and ACTH concentrations.

An intraperitoneal injection of CRF increased plasma IL-6 in a dose-dependent manner (Fig. 4). The peak effect was observed at 90 min (173.0 ± 50.6 U/ml at 10 µg/kg and 626.4 ± 142.4 U/ml at 100 µg/kg) after the injection. The IL-6 concentration returned to baseline levels after more than 300 min. There was a significant interaction effect of CRF treatment and time over plasma IL-6 activity (F = 7.547, P = 0.0002). CRF at 100 µg/kg increased IL-6 activity significantly compared with the basal level (F = 3.669, P = 0.1019, and F = 2.891, P = 0.1386, respectively). CRF at 100 µg/kg differed from the vehicle control at 90 and 120 min (1-way ANOVA, P < 0.005 followed by Dunn’s test, P < 0.05).

An intraperitoneal injection of urocortin, a CRF-related peptide, also increased plasma IL-6 significantly (F = 3.27, P = 0.0471), peaking at 120 min.
Urocortin at 10 and 100 µg/kg increased plasma IL-6 activity significantly compared with the basal level (F = 6.368, P = 0.0470, and F = 7.754, P = 0.0398, respectively) (Fig. 5A).

An intraperitoneal injection of either CRF or urocortin elevated the plasma ACTH concentration significantly (F = 6.177, P = 0.0143, and F = 13.598, P = 0.0008, respectively). Either CRF or urocortin at 100 µg/kg increased ACTH levels significantly from the basal levels or the vehicle at 30 min (P < 0.05). At 10 µg/kg, ACTH levels remained unchanged (P > 0.05).

Plasma IL-6 response to intravenous injections of CRF in adrenalectomized animals. In contrast to intraperitoneal injection, intravenous injection of CRF (100 µg/kg) does not cause significant changes in plasma IL-6 levels in intact rats (3). Intravenous injection of CRF quickly increases plasma ACTH and subsequently corticosterone levels, and high levels of corticosterone are known to suppress the IL-6 response to immobilization (29) and hemorrhagic shock (14). Thus, to eliminate the suppressive influence of the HPA axis, we examined the effect of an intravenous injection of CRF using adrenalectomized rats that had received corticosterone pellets subcutaneously to maintain normal levels of plasma corticosterone. Figure 6 shows the effect of intravenous injection of CRF (2–100 µg/kg) on plasma IL-6 levels. The basal corticosterone concentrations were 3.6 ± 0.9 to 4.0 ± 0.6 µg/dl, and there was no significant difference among groups.

There was a significant overall effect of intravenous CRF administration on plasma IL-6 activity (F = 4.142, P = 0.0214), although the interaction effect of the CRF treatment and time was not statistically significant (F = 2.341, P = 0.0941). The total mean of IL-6 activities in CRF 100 µg/kg group was significantly higher than that in the vehicle group (1,102.5 ± 283.8 vs. 56.2 ± 6.3 U/ml, P < 0.05; Fig. 6). However, lower doses of CRF (2 and 20 µg/kg) did not change the plasma IL-6 levels as compared with the vehicle control (P > 0.05).

**DISCUSSION**

The present data show that an intravenous pretreatment with a specific CRF receptor antagonist, d-Phe-CRF$_{12-41}$ (1.5 mg/kg) or Astressin (0.5 mg/kg), significantly attenuates plasma IL-6 response to immobilization.
This is consistent with our previous finding that an intravenous injection of 0.5 ml rabbit antiserum to rat CRF suppressed the IL-6 response (3).

The potent inhibiting action of these CRF antagonists on ACTH secretion in vivo has already been reported. A bolus intravenous pretreatment with 1.7 mg/kg D-Phe-CRF_{12-41} and 0.1 mg/kg Astressin inhibited ACTH secretion in the adrenalectomized rats for more than 60 and 90 min, respectively (9, 11). Intravenous pretreatment with Astressin at 0.3 mg/kg blocked electric shock-induced ACTH secretion for at least 30 min in rats (9). In the present study, treatments with these CRF antagonists also inhibited the ACTH response to 1 h immobilization, proving their effectiveness in blocking the actions of endogenous CRF induced by this stressor.

It is unlikely that the attenuation of IL-6 response was brought about by this ACTH blocking action, because either hypophysectomy or adrenalectomy enhances the IL-6 response to immobilization (29). Although it is not known whether peripherally administered CRF antagonists can suppress the action of CRF acting as a neurotransmitter/modulator in the brain, our previous studies showed that an intracerebroventricular injection of a CRF antagonist, α-helical CRF (25 µg over 5 min followed by 25 µg over 60 min), failed to block stress-induced IL-6 elevation (3). Thus the action of peripheral CRF, rather than the central CRF, seems essential for the immobilization-induced plasma IL-6 elevation. This is supported by the present findings that an intraperitoneal injection of either CRF (100 µg/kg) or urocortin (10–100 µg/kg) elevated plasma IL-6 levels with a time course similar to that observed after immobilization.

In contrast to an intraperitoneal injection, an intravenous injection of a high dose of CRF (a bolus injection of 50 µg/kg followed by an infusion of 50 µg/kg over 30 min) was not found to cause significant changes in plasma IL-6 levels in intact rats (3). The present study revealed that an intravenous administration of CRF could increase plasma IL-6 levels in adrenalectomized animals. Therefore, the ineffectiveness of intravenous CRF in normal rats might be attributed to the rapid activation of the HPA axis by CRF, which is known to suppress IL-6 production (14, 29). Another possible reason for the discrepancy of effectiveness between intravenous and intraperitoneal CRF is that the site of CRF action regarding the IL-6 response may be within the peritoneal cavity. In fact, the existence of CRF or CRF-like immunoreactivity has been reported in many abdominal organs, including the liver (28), pancreas (26), stomach (23, 33), and colon (13). Further study is necessary to determine the peripheral site of CRF action on IL-6 production and/or secretion during stress.

Our previous studies demonstrated that both central and peripheral catecholamines are involved in the immobilization-induced plasma IL-6 response (29). CRF has been detected in sympathetic ganglia in monkeys (32), as well as in neurons in spinal intermediolateral cell column in rats (20), and its role in modulating the release of catecholamine has been postulated (32). Therefore, it is possible that intraperitoneal injection of CRF stimulates peripheral sympathetic systems, thereby increasing plasma IL-6. However, the exact relationship between the sympathetic nervous system and the peripheral CRF in the stress-induced IL-6 elevation remains to be investigated.

**Fig. 5.** Plasma IL-6 (A) and ACTH (B) responses to intraperitoneal injections of urocortin (Uro). Urocortin (10 or 100 µg/kg) or vehicle was injected intraperitoneally into rats at time 0. Values are means ± SE. *Significantly different from basal level. #Significantly different from vehicle.

**Fig. 6.** Plasma IL-6 responses to intravenous injections of CRF in adrenalectomized rats. Rats were adrenalectomized 10 days before experiment. CRF (2, 20, or 100 µg/kg) or vehicle was injected intravenously into rats at time 0. Values are means ± SE. #Significantly different from vehicle.
The role of CRF as a local proinflammatory agent has also been suggested. Treatment with an anti-CRF antiserum suppressed carrageenan-induced inflammation, and a high concentration of CRF was detected in the inflamed tissue (12). CRF was present in the joints and surrounding tissues of Lewis rats with streptococcal cell wall- and adjuvant-induced arthritis, and CRF mRNA was expressed in the inflamed synovia (5). CRF was also reported to stimulate leukocytes to secrete cytokines such as IL-1, -2, and -6 (17, 30).

CRF receptors are divided into two subtypes, designated type 1 and type 2 receptors. The type 2 receptor has two splice variants, type 2α and type 2β, with type 2β being the dominant subtype in peripheral tissues (4, 18). Neither Astressin nor o-Phe-CRF$_{12-41}$ is selective to a particular subtype of CRF receptors (19). Urocortin is reported to have ~40 times higher affinity for the type 2β receptors than CRF (35). We observed that urocortin at 10 μg/kg elevated plasma IL-6 activity significantly, whereas CRF at the same dose did not.

This finding may be related to the different affinities of these agonists to peripheral CRF receptors.

The elevation of plasma IL-6 concentration caused by psychological stressors is considered to be one of the most valuable models for studying the brain’s control over immune functions and host defense mechanisms. We conclude from this present study, together with our previous findings, that peripheral CRF is involved in stress-induced plasma IL-6 elevation. Further studies may reveal the underlying mechanisms and suggest the physiological and pathological roles of IL-6 elevation by stress.

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