Mechanisms underlying the anti-inflammatory actions of central corticotropin-releasing factor

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1Gastroenterology Department, Institut Clínic de Malalties Digestives, Hospital Clinic, University of Barcelona, and 2Digestive System Research Unit, Hospital Vall d’Hebron, 08035 Barcelona, Spain; and 3Discovery Research, Pharmacia-Upjohn Laboratories, Kalamazoo, Michigan 49007

Casadevall, Maria, Esteban Saperas, Julián Panés, Azucena Salas, Donald C. Anderson, Juan R. Malagelada, and Josep M. Piqué. Mechanisms underlying the anti-inflammatory actions of central corticotropin-releasing factor. Am. J. Physiol. 276 (Gastrointest. Liver Physiol. 39): G1016–G1026, 1999.—Immune activation of hypothalamic corticotropin-releasing factor (CRF) provides a negative feedback mechanism to modulate peripheral inflammatory responses. We investigated whether central CRF attenuates endothelial expression of intercellular adhesion molecule 1 (ICAM-1) and leukocyte recruitment during endotoxemia in rats and determined its mechanisms of action. As measured by intravitral microscopy, lipopolysaccharide (LPS) induced a dose-dependent increase in leukocyte rolling, adhesion, and emigration in mesenteric venules, which was associated with upregulation of endothelial ICAM-1 expression. Intracisternal injection of CRF abrogated both the increased expression of ICAM-1 and leukocyte recruitment. Intravenous injection of the specific CRF receptor antagonist astressin did not modify leukocyte-endothelial cell interactions induced by a high dose of LPS but enhanced leukocyte adhesion induced by a low dose. Blockade of endogenous glucocorticoids but not α-melanocyte-stimulating hormone (α-MSH) receptors reversed the inhibitory action of CRF on leukocyte-endothelial cell interactions during endotoxemia. In conclusion, cerebral CRF blunts endothelial upregulation of ICAM-1 and attenuates the recruitment of leukocytes during endotoxemia. The anti-inflammatory effects of CRF are mediated by adrenocortical activation and additional mechanisms independent of α-MSH.

inflammation; intercellular adhesion molecule 1; endotoxin;

THE DEVELOPMENT OF AN inflammatory response triggers the activation of the neuroendocrine system via hypothalamic corticotropin-releasing factor (CRF) (9). A growing body of evidence indicates that immune activation of the neuroendocrine system provides a counter-regulatory mechanism that critically modulates inflammatory events (9, 60). CRF-mediated activation of the pituitary-adrenal axis and the consequent hypersecretion of glucocorticoids provide a major anti-inflammatory mechanism at multiple levels (4). However, other stress hormones activated by CRF, such as the proopiomelanocortin-derived α-melanocyte-stimulating hormone (α-MSH) (7, 39, 53), or catecholamines (41, 55, 65), which have complex interactions with the cytokine network, may also contribute to finely orchestrate the inflammatory process.

Up to now, evidence for the anti-inflammatory role of cerebral CRF is derived from studies showing the attenuation of final biological or macroscopic findings of inflammatory injury. Infiltration of tissues by leukocytes is the main cellular event of inflammatory responses, resulting from the interaction between circulating leukocytes and endothelial cells. Recruitment of leukocytes is a multistep process involving rolling, firm adhesion, and emigration into the target tissue, which involves a number of endothelial and leukocyte adhesion molecules (6, 21, 58). Among them, endothelial intercellular adhesion molecule 1 (ICAM-1) has become increasingly recognized as a key molecule for leukocyte recruitment during endotoxemia (30, 44, 67). Thus the aims of the present study were 1) to investigate whether intracisternal injection of CRF modulates both leukocyte-endothelial cell interactions on mesenteric venules and ICAM-1 expression in different organs during endotoxemia in rats and, if so, 2) to characterize the mechanisms mediating the anti-inflammatory action of cerebral CRF.

MATERIALS AND METHODS

Animals

Experiments were performed on male Sprague-Dawley rats (125–150 g; Charles River Laboratories, Saint Aubin les-Elbeuf, France) that were fasted overnight but allowed water ad libitum up to the onset of experiments. All studies were performed according to the Guifor the Care and Use of Laboratory Animals [DHHS Publication No. (NIH) 85-23, Revised 1985, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20205].

Intravitral Microscopy

Intravitral microscopy was used to assess leukocyte-endothelial cell interactions. Using this technique, we determined the number of rolling, adherent, and emigrated leukocytes. Rats were placed in a right lateral decubitus position on an adjustable microscope stage, and the mesentery was prepared for microscopic observation, as previously described (1, 20). Briefly, the mesentery was extended over a nonautofluorescent coverslip that allowed observation of a 2-cm2 segment of tissue. Mesentery was superfused constantly with bicarbonate-buffered saline (pH 7.4 at 37°C). An inverted microscope (Nikon Diaphot 300, Tokyo, Japan) with a chromatic free fluor ×40 objective lens (Nikon) was used to observe the mesenteric vessels. The preparation was transilluminated with a 12-V, 100-W, direct current-stabilized light source. A charge-coupled device camera (model D XC-930P, Sony, Tokyo, Japan) mounted on the microscope projected the

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image onto a color monitor (Trinitron KX-14CP1, Sony), and the images were recorded using a videocassette recorder (SR-S368E, JVC, Tokyo, Japan).

Single, unbranched venules with diameters ranging from 25 to 35 µm in diameter were studied. Venular diameter was measured on-line using a video caliper, and centerline red blood cell velocity was measured using an optical Doppler velocimeter (Microcirculation Research Institute, Texas AM University, College Station, TX). Venular blood flow was calculated from the product of mean red blood cell velocity ($V_{\text{mean}}$ = centerline velocity/1.6) (14) and microvascular cross-sectional area, assuming cylindrical geometry. Venular wall shear rate (34) was calculated using the Newtonian definition: $8V_{\text{mean}}$/venular diameter. The number of adherent and emigrated leukocytes and the leukocyte rolling velocity were determined off-line after playback of videotapes. A leukocyte was considered to be adhered to the vessel wall if it was stationary for more than 30 s. Leukocyte rolling velocity was determined as the time that it took for a single leukocyte to get through a given length of venule. In each animal, four to six unbranched mesenteric venules were examined, and values for leukocyte rolling, adhesion, and emigration were obtained by calculating the mean of each parameter in the venules examined.

Quantification of ICAM-1 Expression

Monoclonal antibodies. Monoclonal antibodies (MAbs) used for the in vivo assessment of ICAM-1 expression were 1A29, a mouse IgG1 against rat ICAM-1 (62), and P-23, a nonbinding murine IgG1 directed against human (but not rat) P-selectin (37). These MAbs were scaled up and purified by protein A/G chromatography at Upjohn Laboratories (Pharmacia & Upjohn, Kalamazoo, MI). To test whether changes in accumulation of 1A29 after treatment with endotoxin were related to differences in endothelial surface area induced by treatment, this parameter was measured by means of accumulation of MAb 9B9 (Chemicon International, Temacula, CA), a mouse IgG1 against human angiotsin-converting enzyme (ACE) that cross-reacts with rat and monkey ACE (13).

Radioiodination of MAbs. The binding MAb directed against ICAM-1 and the nonbinding MAb (P-23) were labeled with 125I and 131I (Amersham, Barcelona, Spain), respectively. Radioiodination of the MAbs was performed by the iodogen method (19). Briefly, 250 µg of protein were incubated with 250 µCi of Na125I and 125 µg of iodogen at 4°C for 12 min. After the radioiodination procedure, the radiolabeled MAbs were separated from free 125I by gel filtration on a Sephadex PD-10 column (Pharmacia LKB, Uppsala, Sweden). The column was equilibrated with phosphate buffer containing 1% BSA and was eluted with the same buffer. Two fractions of 2.5 ml each were collected, the second of which contained the labeled antibody. Absence of free 125I or 131I was ensured by extensive dialysis of the protein-containing fraction. Less than 1% of the activity of the protein fraction was recovered from the dialysis fluid. Labeled MAbs were stored in 500-µl aliquots of cell-free plasma and immediately frozen in liquid nitrogen.

Preparation of nuclear extracts. Samples from small intestine were washed in Dulbecco's PBS without Ca2+ and Mg2+ and immediately frozen in liquid nitrogen. Extraction of nuclear protein was performed using a modification of the original method (44) to correct the tissue accumulation of the radiolabeled MAbs. The accumulated activity of each MAb in an organ was expressed as the percentage of injected dose (%ID) per gram of tissue. The formula used to calculate ICAM-1 expression was as follows: ICAM-1 expression = (%ID/g for 125I) – (%ID/g for 131I) × (%ID/g 125I in plasma)/ (%ID/g 131I in plasma). This formula was modified from the original method (44) to correct the tissue accumulation of nonbinding MAb for the relative plasma levels of both binding and nonbinding MAbs (29). An identical procedure was used to estimate the relative endothelial surface area of the organs based on the accumulation of 9B9 MAb.

Eletrophoretic Mobility Shift Assays

Preparation of nuclear extracts. Samples from small intestine were washed in Dulbecco's PBS without Ca2+ and Mg2+ and immediately frozen in liquid nitrogen. Extraction of nuclear protein was performed using a modification of the previous published protocol (38). Intestinal biopsies were homogenized in a Dounce homogenizer at 4°C in homogenization buffer (buffer A) with 0.1% Nonident P-40 (NP-40, Igepal, Sigma Chemical, Madrid, Spain); buffer A contained 10 mM NaHEPES, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 1/1,000 vol of 1M dithiothreitol (DTT), and 1/200 vol of 200 mM phenylmethylsulfonyl fluoride (PMSF) (in isopropanol). Samples were transferred to a centrifuge tube and kept in ice for at least 15 min but no longer than 1 h. Then tubes were centrifuged at 1,100 g at 4°C for 10 min. Supernatants were discarded, and pellets were resuspended in buffer A without NP-40 and centrifuged again. Supernatants were discarded and resuspended in buffer C (25% vol/vol) glycerol, 20 mM HEPES, pH 7.9, 0.42 M NaCl, 1.5 mM MgCl2, 0.2 mM NaEDTA, pH 8.0, DTT, and PMSF). Preparations were rocked at 4°C for 30 min, and nuclear protein was pelleted by centrifugation at 13,000 g at 4°C for 10 min. Supernatants were aliquoted and stored at −70°C until used. Protein concentrations were determined by the Bradford method using BSA as a standard.

Gel shift assays. Double-stranded nuclear factor-κB (NF-κB) consensus oligonucleotide probe (5'-AGTTGAGGGAGCTTTCCAGG-3'; Promega, Madison, WI) was labeled with [γ-32P]ATP (50 µCi at 222 TBq/mmol, Amersham Life Science, Buckinghamshire, UK) at the 5' end with T4 kinase (Pharmacia Biotech). Binding reactions were performed by mixing 5 µg of nuclear protein, 1 µg of poly(dI-dC) (Pharmacia Biotech), 2 M MgCl2, 0.5 M NaEDTA (pH 8.0), 1 M DTT, 5 M NaCl, and 2 M Tris (pH 7.5) that was then incubated at 4°C for 10 min. Then 35 fmol of [γ-32P]ATP oligonucleotide were added to the mixture and incubated for 20 min at room temperature. Proteins were separated through nondenaturing 4% PAGE in Tris-borate-EDTA buffer at 100 V for 3 h. Gels were then vacuum dried and exposed overnight to X-ray film (Kodak Diagnostic Film MRE-1, Eastman Kodak, Windsor, CO) at −70°C.
G1018  CENTRAL CRF INHIBITS LEUKOCYTE RECRUITMENT

Measurement of Plasma Corticosterone Levels

All rats were killed between 3:00 PM and 5:00 PM to avoid the circadian rhythm on corticosterone levels. Trunk blood was collected into prechilled tubes containing EDTA. Blood samples were centrifuged immediately at 1,000 g (4°C) for 10 min, and plasma was stored at –70°C until assayed. Plasma corticosterone levels were determined by RIA using an 125I-labeled corticosterone kit (DRG Instruments, Marburg, Germany). Each sample was diluted 1:200, and 100-µl aliquots were assayed in duplicate. Results were expressed as nanograms per milliliter of plasma. The sensitivity of the assay was 25 ng/ml. Intra- and interassay coefficients of variation were 7.1 and 7.2%, respectively.

Drugs and Treatments

Lipopolysaccharide (LPS) from Escherichia coli serotype 055:B5 (Sigma) was freshly dissolved in 0.9% saline and injected intravenously at doses of 10 µg/kg or 1 mg/kg in a volume of 1 ml/kg. CRF (Sigma) was dissolved in 0.9% saline, and aliquots containing 10 µg were injected intracisternally in a volume of 10 µ1. α-MSH (Sigma) was dissolved in 0.9% saline, and aliquots containing 10 µg were stored at –20°C until use. Freshly prepared dilutions (1 µg/10 µl) were injected intracisternally. The specific CRF receptor antagonist, a-MSH (kindly provided by Dr. J. Rivier, Clayton Foundation Laboratory, Salk Institute, La Jolla, CA), was dissolved in 0.9% saline, and aliquots containing 10 µg were injected intravenously in a volume of 1 ml/kg. Metyrapone (Sigma), hexamethonium (Sigma), and chlorisondamine (Ecolid, kindly provided by Ciba-Geigy, Summit, NJ) were dissolved in 0.9% saline and administered intraperitoneally in 0.5 ml volume. The α-MSH antagonist [d-Trp5,Ala8,D-Phe11]-α-MSH (6–11)-amide (Bachem, Bubendorf, Switzerland) was dissolved in 0.9% saline (25 µg/0.5 ml) and injected subcutaneously.

Experimental Design

Intravenous injection of LPS or vehicle and intracisternal injection of peptides were performed in rats under light ketamine anesthesia (100 mg/kg im) (Ketolar, Parke Davis). Leukocyte-endothelial cell interactions and endothelial ICAM-1 expression, determined by intravital microscopy and the double radiolabeled MAb technique, respectively, were assessed under anesthesia with thiobutabarbital (100 mg/kg ip) (Inactin; Research Biochemicals International, Natick, MA) 2.5 h after treatments.

Effects of intracisternal injection of CRF on LPS-induced endothelial ICAM-1 expression and leukocyte-endothelial cell interactions. In preliminary experiments, we determined the effects of intravenous administration of LPS (10 µg/kg and 1 mg/kg) on both endothelial expression of ICAM-1 and leukocyte-endothelial cell interactions. We then determined the effects of intracisternal injection of CRF on both ICAM-1 expression and leukocyte-endothelial cell interactions induced by LPS. For that purpose, three groups of rats (n = 5–6) were treated with vehicle (0.9% saline), LPS (1 mg/kg iv), or CRF (10 mg ic) immediately before administration of LPS (1 mg/kg iv). To test whether the inflammatory response elicited by LPS was ICAM-1 dependent, an additional group of rats was pretreated with an anti-ICAM-1 MAb (1A29, 2 mg/kg iv) before administration of LPS.

LPS and cytokines are known to activate the transcription factor NF-κB (2, 48). In addition, previous reports have identified putative NF-κB-like binding sequences in the promoter region of the ICAM-1 gene (24). To assess the implication of the transcription factor NF-κB on the anti-inflammatory effects of intracisternal CRF, we performed analysis of nuclear extracts from intestinal tissue obtained 1 h after treatments from rats treated with vehicle (0.9% saline), LPS (1 mg/kg iv), or CRF (10 mg ic) immediately before administration of LPS (1 mg/kg iv).

RESULTS

Effect of Intracisternal Injection of CRF on LPS-Induced Leukocyte-Endothelial Cell Interactions and ICAM-1 Expression

Intravenous administration of LPS caused a dose-dependent increase in leukocyte-endothelial cell interactions, compared with control rats treated with vehicle. A low dose of LPS (10 µg/kg) increased the
number of adherent leukocytes in mesenteric venules (P < 0.01) without affecting the number of rolling and emigrated leukocytes (Table 1). A higher dose of LPS (1 mg/kg) induced a further increase in the number of adherent leukocytes and significantly increased numbers of rolling and emigrated leukocytes (P < 0.05) (Table 1). Compared with control animals, red blood cell velocity and shear rate were not modified by the low dose of LPS but were decreased by the high dose (P < 0.05) (Table 1). Arterial blood pressure was not modified by any dose of LPS (control = 105 ± 7 mmHg, LPS at 10 µg/kg = 103 ± 6 mmHg, LPS at 1 mg/kg = 103 ± 6 mmHg). LPS (1 mg/kg iv) increased endothelial ICAM-1 expression both in systemic (lung, heart, and brain) and splanchnic (mesentery and small intestine) organs (Fig. 1), compared with control animals (P < 0.01).

Increased expression of ICAM-1 after 1 mg/kg LPS was not the result of increased endothelial surface area, since accumulation of the anti-ACE MAb 9B9 (a measure of endothelial surface area (54)) was very similar at baseline conditions or after treatment with 1 mg/kg LPS in all organs studied, including lung (12.27 ± 1.32 vs. 11.92 ± 0.93%), heart (0.041 ± 0.004 vs. 0.036 ± 0.006%), brain (0.021 ± 0.001 vs. 0.021 ± 0.001%), small intestine (0.097 ± 0.008 vs. 0.096 ± 0.007%), and mesentery (0.065 ± 0.004 vs. 0.062 ± 0.004%).

Immunoneutralization of endogenous ICAM-1 by administration of a MAb before treatment with LPS abrogated leukocyte adhesion and emigration in mesenteric venules normally observed 2.5 h after LPS administration (Table 1). Treatment with the anti-ICAM-1 MAb also resulted in a significant decrease in the number of rolling leukocytes observed after exposure to LPS. This is an unexpected finding, since previous evidence has ruled out a role for ICAM-1 as a molecular determinant of rolling. This decrease might be related to a lower production of leukocyte-derived products that activate endothelial cells as a result of decreased leukocyte infiltration.

Intracisternal injection of CRF (10 µg) abrogated LPS-induced leukocyte-endothelial cell interactions in mesenteric venules; numbers of rolling, adherent, and emigrated leukocytes in this group were similar to those observed in the control group and significantly lower than those of the group treated with LPS alone (Fig. 2). In contrast, LPS-induced reductions in leukocyte rolling velocity and shear rate were not modified by intracisternal CRF injection (Table 2). Central administration of CRF also abrogated LPS-induced upregulation of endothelial ICAM-1 in lung, heart, brain, and mesentery and significantly attenuated upregulation of this molecule in small intestine (Table 1). In animals not receiving LPS, intracisternal injection of CRF did not have any significant effect on the number of rolling (1.32 ± 0.25 vs. 1.72 ± 0.63 cells/100 µm), adherent (3.73 ± 0.52 vs. 3.33 ± 0.71 cells/100 µm), or emigrated (2.49 ± 0.79 vs. 2.83 ± 0.52 cells/field) leukocytes.

Analysis of nuclear extracts from intestinal tissue revealed that treatment with LPS results in the translocation to the nucleus of a complex that binds the NF-κB consensus oligonucleotide, which is demonstrated in the gel retardation assay shown in Fig. 3. Pretreatment with intracisternal CRF had no detectable effect on the NF-κB activation elicited by LPS.

Effect of Peripheral CRF Receptor Blockade by Astressin on LPS-Induced Leukocyte-Endothelial Cell Interactions

Peripheral administration of astressin (10 µg iv) did not modify the inflammatory response induced by the high dose of LPS (1 mg/kg). However, astressin (10 µg iv) significantly increased the number of adherent leukocytes induced by a low dose of LPS (10 µg/kg) (P < 0.05), albeit it did not modify numbers of rolling or emigrated leukocytes (Fig. 4). This increased leukocyte adhesion was not a result of a reduction in dispersal forces, since shear rate in mesenteric venules was not significantly modified by injection of astressin before low-dose LPS (control = 549 ± 23 s⁻¹, LPS at 10 µg/kg = 525 ± 28 s⁻¹, astressin + LPS at 10 µg/kg = 507 ± 40 s⁻¹). The decrease in leukocyte rolling velocity induced by low-dose LPS was not modified by pretreatment with astressin (control = 36 ± 3.1 mm/s, LPS at 10 µg/kg = 27 ± 1.7 mm/s, astressin + LPS at 10 µg/kg = 25 ± 2.2 mm/s; P < 0.01 vs. control).

Effect of Metyrapone on the Anti-Inflammatory Action Induced by Intracisternal CRF During Endotoxemia

Pretreatment with metyrapone blocked the LPS-induced corticosterone surge. Increased plasma corticosterone levels during endotoxemia (385 ± 57 ng/ml) were reduced by metyrapone (129 ± 19 ng/ml) to levels similar to those found in control rats (161 ± 30 ng/ml) (P < 0.05).

Inhibition of endogenous glucocorticoid synthesis by metyrapone blocked the anti-inflammatory action afforded by intracisternal CRF in response to LPS, as

| Table 1. Effect of different doses of intravenous LPS on leukocyte-endothelial cell interactions and hemodynamic parameters of mesenteric venules |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | Adhesion,       | Emigration,      | Number of Rollers, | V<sub>W</sub>, |
|                 | cells/100 µm    | cells/field      | µm/s             | V<sub>R</sub>, |
|                 |                 |                 |                  | mm/s            |
|                 |                 |                 |                  | s⁻¹             |
| Control         | 3 ± 0.2         | 3 ± 1.0         | 1.0 ± 0.2        | 36 ± 3.1        | 2.7 ± 0.2       | 549 ± 23 |
| LPS (10 µg/kg)  | 7 ± 1.0*        | 3 ± 0.4         | 1.4 ± 0.3        | 27 ± 1.7*       | 2.8 ± 0.2       | 525 ± 28 |
| LPS (1 mg/kg)   | 10 ± 1*†        | 5 ± 0.6*†       | 2.8 ± 1.1*       | 22 ± 1.8*       | 2.2 ± 0.2*†     | 395 ± 32*† |
| LPS (1 mg/kg) + anti-ICAM-1 MAb | 4 ± 0.6†‡     | 1 ± 0.4‡       | 0.5 ± 0.2‡       | 28 ± 0.6*       | 2 ± 0.4*‡       | 400 ± 56*‡ |

Values are means ± SE. V<sub>W</sub> and V<sub>R</sub>, velocities of white and red blood cells, respectively; LPS, lipopolysaccharide; ICAM-1, intercellular adhesion molecule 1; MAb, monoclonal antibody. *P < 0.05 compared with controls; †P < 0.05 compared with LPS (10 µg/kg); ‡P < 0.05 compared with LPS (1 mg/kg).
shown by a significant increase in the number of adherent leukocytes compared with animals treated with intracisternal CRF (Fig. 5). The numbers of adherent and emigrated leukocytes in rats pretreated with metyrapone before CRF plus LPS were similar to those observed with LPS alone (Fig. 5). The number of rolling leukocytes after injection of LPS was similar to that in rats treated with metyrapone plus intracisternal CRF and to that in rats receiving only intracisternal CRF; in both groups, the number of rolling leukocytes was significantly lower than that observed in the group treated with LPS alone (Fig. 5). Metyrapone did not modify the decrease in shear rate or leukocyte rolling velocity induced by LPS (Table 2).

Metyrapone pretreatment had no effect on CRF-induced inhibition of endothelial ICAM-1 upregulation during endotoxemia, since no significant differences in ICAM-1 expression were observed in any organs between rats with or without glucocorticoid synthesis inhibition that were treated with LPS plus intracisternal CRF (Fig. 6).

Role of the α-MSH Pathway on the Action of CRF

Intracisternal injection of α-MSH (1 µg/10 ml) before LPS treatment elicited anti-inflammatory effects similar to those induced by CRF, as shown by attenuation of leukocyte adhesion and emigration (Fig. 7) and inhibition of LPS-induced endothelial ICAM-1 upregulation in nonsplanchnic organs (Fig. 8). However, levels of ICAM-1 in mesentery and small intestine were similar to those observed in the LPS-treated group (Fig. 8).

Pretreatment with an α-MSH receptor antagonist did not modify the inhibitory effects of CRF on the LPS-induced inflammatory response. In rats treated...
Table 2. Leukocyte rolling and hemodynamics of mesenteric microvessels of control and LPS (1 mg/kg)-treated rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>V_{RBC}, µg/s</th>
<th>V_{RBC}, mm/s</th>
<th>Shear Rate, s⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>36 ± 0.6</td>
<td>2.7 ± 0.2</td>
<td>549 ± 22</td>
</tr>
<tr>
<td>LPS</td>
<td>22 ± 2.0*</td>
<td>2.2 ± 0.2*</td>
<td>395 ± 32*</td>
</tr>
<tr>
<td>LPS + intracisternal CRF</td>
<td>21 ± 2.0*</td>
<td>2.0 ± 0.3*</td>
<td>388 ± 44*</td>
</tr>
<tr>
<td>Adrenalectomy + intracisternal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRF + LPS</td>
<td>27 ± 2.5</td>
<td>2.1 ± 0.2*</td>
<td>472 ± 47*</td>
</tr>
<tr>
<td>α-MSH receptor antagonist +</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>intracisternal CRF</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRF + LPS</td>
<td>28 ± 1.7*</td>
<td>2.0 ± 0.5*</td>
<td>410 ± 60*</td>
</tr>
<tr>
<td>Exogenous α-MSH + LPS</td>
<td>21 ± 7.0*</td>
<td>1.5 ± 0.3*</td>
<td>301 ± 49*</td>
</tr>
<tr>
<td>Systemic CRF</td>
<td>24 ± 3.1*</td>
<td>2.3 ± 0.4</td>
<td>463 ± 46*</td>
</tr>
</tbody>
</table>

Values are means ± SE. CRF, corticotropin-releasing factor; α-MSH, α-melanocyte-stimulating hormone. *P < 0.05 vs. control.

with the α-MSH receptor antagonist before CRF, numbers of adhered and emigrated leukocytes during endotoxemia were similar to those in rats treated with CRF alone or vehicle, and these figures were lower than those observed in the LPS-treated group (Fig. 7). The decrease in shear rate and leukocyte rolling velocity induced by LPS was not modified by the α-MSH receptor antagonist (Table 2). Treatment with the α-MSH receptor antagonist did not modify the attenuation of LPS-induced ICAM-1 caused by intracisternal CRF (Fig. 8).

**Effect of Intravenous Injection of CRF on Leukocyte-Endothelial Cell Interactions and ICAM-1 Expression**

Intravenous injection of CRF (10 µg) induced a marked increase in the number of adherent leukocytes, similar to those observed in response to LPS (Fig. 9). The mean number of emigrated leukocytes was higher than that observed in controls, although the difference did not reach statistical significance (Fig. 9). These changes were accompanied by a significant decrease in shear rate and leukocyte rolling velocity (Table 2) and by an increase in the number of rolling leukocytes (Fig. 9).

Intravenous CRF did not change endothelial ICAM-1 expression in any of the organs studied compared with control animals (Table 3).

**DISCUSSION**

The results of the present study demonstrate that attenuation of endothelial-leukocyte interactions is a crucial mechanism by which cerebral CRF modulates peripheral inflammatory responses. As previously established, LPS elicited a marked inflammatory response characterized by increased numbers of rolling, adher-
ent, and emigrated leukocytes and a decrease in venular shear rate in mesenteric postcapillary venules (17, 40). We have now shown that intracisternal injection of CRF attenuates cellular inflammatory response induced by LPS, as shown by a decrease in numbers of rolling, adherent, and emigrated leukocytes. This effect was unrelated to reversal of LPS-induced decreased venular shear rate, which would be expected to increase dispersal forces. Conversely, it was associated with suppression of the increased ICAM-1 expression in all organs studied. Although the activation of many LPS-induced genes has been associated with activation of the transcription factor NF-κB (48), intracisternal injection of CRF before LPS did not alter nuclear translocation or binding to the consensus oligonucleotide, indicating that CRF exerts its anti-inflammatory action by effects at a later stage of the process or by modifying activation of transcription factors other than NF-κB that have transactivating potential on this gene. Recruitment of leukocytes into inflammatory sites is a complex multistep process orchestrated by adhesion molecules in both endothelial cells and leukocytes (6, 21, 58). We previously showed that ICAM-1 is constitutively expressed in the vascular endothelium and is upregulated during endotoxemia in rats (44). Direct evidence for the relevance of this adhesion molecule in leukocyte traffic during endotoxemia is provided in the present study by the observation that immunoneutralization of ICAM-1 abrogates LPS-induced leukocyte adhesion and emigration in mesenteric venules. Our findings confirm those of previous studies showing that blockade of ICAM-1 or inhibition of its expression by antisense oligonucleotides resulted in reduced neutrophil emigration and organ dysfunction in this setting (17, 30). Likewise, ICAM-1-deficient mice are resistant to the lethal effects of high doses of

Fig. 6. Endothelial ICAM-1 expression in nonsplanchnic and splanchnic organs of rats treated with LPS (1 mg/kg) alone, in rats pretreated with either metyrapone or vehicle before icCRF + LPS (1 mg/kg). Results are expressed as means ± SE. *P < 0.05 vs. LPS.

Fig. 7. Leukocyte rolling, adhesion, and emigration in rats treated with LPS (1 mg/kg) alone, in rats pretreated with either vehicle or α-melanocyte-stimulating hormone (α-MSH) antagonist before icCRF + LPS (1 mg/kg), and in rats pretreated with α-MSH before LPS (1 mg/kg). Results are expressed as means ± SE. *P < 0.05 vs. LPS.
LPS (67). Thus our results suggest that inhibition of
the upregulation of endothelial ICAM-1 contributes to
the attenuation of leukocyte-endothelial cell interactions elicited by CRF.

In contrast, CRF given intravenously at the same
dose as that injected intracisternally increased the
number of rolling and adhered leukocytes in postcapil-
lar venules, indicating that peripheral CRF exerts a
local proinflammatory action. Cellular response caused
by CRF was comparable to that induced by LPS but was
independent of ICAM-1 upregulation. Our observation
is in agreement with the immunostimulatory action of
CRF in vitro (32, 33, 56, 57), as well as with in vivo
studies that showed the generation of CRF in various
inflamed tissues of rats (11, 26, 66) and humans (10, 27)
and that immunoneutralization of CRF reduces the
cellular inflammatory response induced by carrageenin
in rat air pouches (26).

Hypothalamic CRF is now recognized as the major
physiological regulator of pituitary adrenocorticotropic
hormone (ACTH) secretion (52). Besides its hypophysio-
tropic actions, CRF acts within the central nervous
system to initiate coordinated behavioral, autonomic,
and visceral responses to stress (5, 18, 31). A physiologi-
cal role of hypothalamic CRF to modulate inflamma-
tory responses is supported by immunohistochemical
as well as in situ hybridization studies showing that
immune challenge or cytokines activate CRF neurons
and increase the expression of CRF mRNA in the
paraventricular nucleus of the hypothalamus (25, 49,
50). Direct evidence for the anti-inflammatory role of
hypothalamic CRF has been provided by studies show-
ing an enhanced susceptibility of genetically CRF-
hyporesponsive Lewis rats to inflammatory injury (59).
Central administration of CRF mimics many of the
effects induced by inflammatory stress through interac-
tions with CRF receptors on cerebral areas reached by
the peptide. Although CRF delivered intracisternally
may act preferentially on the dorsal vagal complex,
previous studies have shown its ability to gain access
Table 3. Endothelial ICAM-1 expression in splanchnic and nonsplanchnic organs of control animals and animals treated with intravenous CRF

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Intravenous CRF</th>
<th>LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small intestine</td>
<td>0.12 ± 0.02</td>
<td>0.10 ± 0.01</td>
<td>0.20 ± 0.01†</td>
</tr>
<tr>
<td>Mesentery</td>
<td>0.14 ± 0.01</td>
<td>0.13 ± 0.02</td>
<td>0.21 ± 0.01†</td>
</tr>
<tr>
<td>Lung</td>
<td>3.4 ± 0.16</td>
<td>3.8 ± 0.14</td>
<td>5.3 ± 0.87*</td>
</tr>
<tr>
<td>Heart</td>
<td>0.17 ± 0.02</td>
<td>0.14 ± 0.01</td>
<td>0.46 ± 0.05†</td>
</tr>
<tr>
<td>Brain</td>
<td>0.02 ± 0.00</td>
<td>0.02 ± 0.00</td>
<td>0.03 ± 0.004†</td>
</tr>
</tbody>
</table>

Values are means ± SE in units of percentage of injected dose. Results of ICAM-1 expression after stimulation with LPS are included for comparison. *P < 0.05 vs. control; †P < 0.05 vs. control and intravenous CRF.

Indeed, in the present study, blockade of endogenous ACTH, is a potent modulator of inflammation by mechanisms independent of glucocorticoids (35). This peptide is predominantly produced by the pituitary gland, but it is also generated in the central nervous system and peripheral tissues. Neuroanatomic and functional evidences suggest that CRF and prodopomelanocortin systems may be functionally related not only in the pituitary but also in the central nervous system. In fact, the release of α-MSH in the septum of the brain induced by interleukin (IL)-1 is paralleled by a decreased CRF in the paraventricular nucleus (36). In addition to its antipyretic action, central administration of α-MSH attenuates skin inflammation induced by intradermal injection of proinflammatory mediators such as IL-1β, IL-8, leukotriene B4, and platelet-activating factor (7) or irritants (35). Systemic administration of this peptide also reduces some biological responses of cytokines (53) and inhibits neutrophil migration in vitro and in various animal models of inflammation (8, 39, 46). In the present study, α-MSH injected intracisternally attenuated LPS-induced leukocyte rolling, adhesion, and emigration in rat mesenteric vessels and reduced the upregulation of ICAM-1 in most of the organs studied. Similar central actions of α-MSH and CRF on sleep and pyrogenic responses induced by IL-1 have been previously reported in rabbits (42, 43). Because both peptides exerted similar anti-inflammatory activities, we investigated whether α-MSH contributed to the anti-inflammatory action of CRF. This possibility, however, was ruled out because pretreatment with a competitive α-MSH antagonist, at a dose capable of blocking both central and peripheral actions of endogenous α-MSH (47), did not modify the anti-inflammatory effects of CRF in terms of inhibiting leukocyte recruitment or blunting endothelial ICAM-1 upregulation.

Central nervous system activation of the sympathetic nervous system, which is accompanied by increased circulating levels of catecholamines (5), is another mechanism that may contribute to the anti-inflammatory action of intracisternal CRF. The potential role for catecholamines is supported by studies showing that production of inflammatory cytokines such as tumor necrosis factor and IL-1 induced by LPS in vitro (28, 55) and in vivo (41) is prevented by sympathomimetic stimuli. Likewise, infusion of epi- nephrine before LPS attenuates the production of the proinflammatory cytokines and simultaneously potentiates the production of the anti-inflammatory cytokine IL-10 in humans (16, 65). In addition, activation of the sympathetic nervous system appears to mediate peripheral immunosuppression induced by the central action of CRF or IL-1, since it was reversed by ganglionic...
blockade with chlorisondamine (23, 61). However, in our model, ganglionic blockade during endotoxemia induced marked hypotension that made our preparations unsuitable for study. This hemodynamic effect may be explained by the additive hypotensive effects of ganglionic blockade (39, 64) to the reduction of peripheral vascular resistance caused by LPS. Therefore, the present study cannot clarify the potential role of the sympathetic nervous system to the anti-inflammatory action of intracerestral CRF.

In conclusion, our results indicate that the anti-inflammatory action of intracerestral CRF involves downregulation of endothelial ICAM-1 and attenuation of ICAM-1-dependent leukocyte-endothelial cell interactions. This anti-inflammatory action is mediated by glucocorticoids and additional mechanisms independent of α-MSH.

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