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# A possible role of endogenous central corticotrophin releasing factor in lipopolysaccharide induced thymic involution and cell apoptosis: Effect of peripheral injection of corticotrophin releasing factor

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ABSTRACT

The aim of the study was to investigate the role of endogenous peripheral and central corticotrophin-releasing factor (CRF) following lipopolysaccharide (LPS) challenge on thymic involution and apoptosis. Administration of LPS (100 μg/mouse, ip) led to thymic involution, to a decrease of CD4 + CD8 + thymocyte subset, and to fragmentation of thymic DNA. Pretreatment of LPS challenged mice with intracerebroventricular α-helical CRF (a CRF antagonist) attenuated the effect of LPS however, intraventricular administered α-helical CRF failed to affect LPS response on thymus. Moreover, the effects of LPS on thymus, examined on 1, 7 and 14 days were wholly abrogated by prior administration of intraventricular CRF (10 μg/animal). The plasma corticosterone levels were found to be decreased with single dose of peripheral CRF in LPS challenged mice. These findings indicate that central endogenous CRF involved in LPS induced thymic atrophy. However, peripheral CRF offers protective effect on LPS induced thymic involution and cell apoptosis.



## 1. Introduction

Lipopolysaccharide (LPS) causes apoptotic death in a variety of organs and tissues including the thymus, a phenomenon that has been linked to immune dysfunction and poor survival during sepsis (Hotchkiss et al., 1997; Wang et al., 1994). This is accompanied by a classical stress response characterized by activation of the hypothalamic–pituitary–adrenal (HPA) axis (Beishuizen and Thijs, 2004). During HPA axis activation, there is enhanced synthesis and release of hypothalamic corticotrophin-releasing factor (CRF), a forty one amino acid hypothalamic peptide (Vale et al., 1981) resulting in a systemic rise in glucocorticoid and proinflammatory cytokine cascade, and should be contributing to acute thymic involution (Bateman et al., 1989; Weiss et al., 1989). CRF also affects the immune system in a direct manner without involving HPA axis. Since, high expression levels of CRF, urocortin, and their receptors CRHR1 and CRHR2 have been detected in the spleen, the thymus, and activated macrophages, where it may exert paracrine/autocrine effects (Karalis et al., 1991; Webster et al., 1998; Kalantaridou et al., 2007). Indeed, both pro- and anti-inflammatory local paracrine actions of CRF were reported at the site of inflammation.

Importantly, there are mediators of the immune system like, interleukin 1 (IL-1), tumor necrosis factor α (TNF-α) and interleukin 6 (IL-6) known to stimulate HPA axis (Sapolsky et al., 1987; Besedovsky et al., 1991). It is reported that secretion of brain CRF is stimulated by increases in endogenous IL-1 in the brain, resulting in peripheral immune suppression (De Souza, 1993). Furthermore, IL-1 has been shown to stimulate CRF secretion from the hypothalamus directly whereas, there is lack of responsiveness of immune cell CRF to IL-1 (Tsagarakis et al., 1989).

Endotoxin stress induced thymus atrophy is an excellent example of the immune endocrine relationship, and a useful model to study the mechanisms involved therein. Currently, there are no treatments available to protect against acute thymic atrophy or accelerate recovery, thus leaving the immune system compromised during acute stress events. Interesting findings suggest that CRF inhibits proinflammatory cytokine dependent monocyte activation during endotoxin shock (Correa et al., 1997). Previous report also suggests a beneficial effect of peripheral CRF in LPS induced pulmonary vascular leak (Kelley et al., 1994).

On the basis of the above reports, CRF appears to play an important role in LPS induced immune response. There are neither experimental nor clinical data evaluating CRF interaction on thymopoiesis, and there is a paucity of data relating the action of hypothalamic CRF in stress related thymus involution. However, a growing body of experimental evidence suggests that an extra-pituitary immune CRF plays a role in the programming of the immune system. Thus, the first part of the present work consists of experiments examining the role of peripheral and central CRF response on LPS induced thymic involution. For this purpose, intracerebroventricular (icv) (2.5 μg/kg) or intraventricular (iv) (25 μg/kg) α-helical CRF, a CRF antagonist pretreatment given in LPS

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challenged mice. Subsequently a phenotypic analysis and the parameters such as relative weight, cellularity and DNA integrity of thymus were done. Moreover, CRF antagonistic activity was confirmed by determination of corticosterone levels. Taking into consideration the sexual dimorphism, which is very well established; studies involved both male and female mice. We next investigated the influence of peripheral CRF by single dose administration of r/hCRF (10 μg/animal, iv) on LPS induced thymic atrophy model, particularly because exogenous CRF reported to inhibit the secretion of IL-1 from mature neutrophils purified from spleens of mice injected with LPS (Rmadulovic et al., 1999). It has been proposed that CRF may exert a protective effect in sepsis by acting as a negative feedback to limit IL-1β secretion from CRF-R1+ neutrophils. Furthermore, endotoxin evoked monocytic IL1, IL-6 production from human mononuclear cells was inhibited by locally administered CRF (Hagan et al., 1992).

## 2. Materials and methods

### 2.1. Animals

Inbred Swiss albino mice (6–8 weeks) of either sex weighing about 18–20 g, born and reared in the Animal House of Department of Pharmaceutical Sciences, Nagpur, were used for the present study. The animals were housed in a group of six per cage under a standard light (12:12 h light/dark cycle) and controlled conditions of temperature and humidity (25 ± 2 °C, 55–65%). Animals received standard rodent chow (Poshak Livestock Services, Nagpur, India) and water ad libitum. Studies were approved by the Institutional Animal Ethics Committee, constituted for the purpose of control and supervision of experimental animals by the Ministry of Environment and Forests, Government of India, New Delhi, India. Earlier immunological studies employed Swiss albino mice (Vi´ctor et al., 2003; Gomaa et al., 2007; Talmadge et al., 2007), and hence the same has been used in the present study.

### 2.2. Drugs

CRF antagonist α-helical CRF, rat/human CRF (r/hCRF), and *Escherichia coli*-derived LPS (L-2880) were obtained from Sigma-Aldrich (St Louis, MO, USA). For icv administration, α-helical CRF was dissolved in artificial cerebrospinal fluid (aCSF) of the following composition: 140 mM NaCl, 3.35 mM KCl, 1.15 mM $MgCl_2$, 1.26 mM $CaCl_2$, 1.2 mM $Na_2HPO_4$ and 0.3 mM $NaH_2PO_4$. LPS challenged mice received 100 μg LPS intraperitoneally (ip), which was reconstituted at 1 mg/ml in PBS. All drug solutions were prepared fresh.

### 2.3. Surgical methods

#### 2.3.1. icv cannulation in mice

The icv cannulations in mice were carried out as described earlier (Akwa et al., 2001). In brief, mice were anesthetized with ketamine, xylazine combination (100 mg/kg and 5 mg/kg respectively, i.m.) and stainless steel cannulae (Becton Dickinson, India, 24 gauge) were stereotaxically implanted with coordinates from Paxinos and Franklin {Mice: [AP −0.82 mm; ML +1 mm and DV +2.0 mm; related to bregma]}. Guide cannulae were secured to the skull using mounting screws and dental cement (Dental Products of India, Mumbai). A stainless steel dummy cannula was used to occlude the guide cannula when not in use. The animals were then allowed to recover for a week under antimicrobial cover of cefotaxime (50 mg/kg/day, s.c.), during which they were habituated to the experimental protocols to minimize non-specific stress. Injections were made using a Hamilton microliter syringe (Hamilton, Nevada, USA) connected to an internal cannula (31 gauges) by polyethylene tubing with a volume of 2.0 μl administered over a period of 1 min into the right lateral ventricle. The injection cannula was left in place for a further 1 min before being slowly withdrawn to avoid back flow. At the end of all icv experiments, a dilute India ink was injected (2 μl, icv) and animals were euthanized by pentobarbitone overdose. Only data from animals showing uniform distribution of ink into lateral ventricles were used for statistical analysis.

### 2.4. Treatments

#### 2.4.1. Effect of central and peripheral administration of α-helical CRF on thymic involution, apoptosis and serum corticosterone levels in LPS challenged mice

A group of mice received vehicle (iv or icv) or α-helical CRF (2.5 μg/mouse, icv) or α-helical CRF (25 μg/mouse, iv) 5 min prior to the administration of a single dose of vehicle (10 ml/kg, ip) or LPS (100 μg/mouse, ip). Blood was obtained from the retro-orbital venous plexus at sequential time points (1, 2, 4, 6, 12 h) during the first 12 h after administration of LPS with or without a single dose of α-helical CRF or rhCRF (n = 3–4/group) for each time point. The animals were then sacrificed by cervical dislocation after 24 h and thymus glands were removed for determination of relative weight, cellularity, DNA integrity, and phenotypic analysis of the thymus. For each of the treatment a separate group of six or seven mice (male: n = 3–4 and female: n = 3) was employed.

#### 2.4.2. Effect of peripheral administration of r/hCRF on thymic involution, apoptosis and serum corticosterone levels in LPS challenged mice

To demonstrate the effects of r/hCRF in endotoxin stress on thymus homeostasis, we first determined the kinetics of LPS induced thymic atrophy. A group of mice was given vehicle (ip) or LPS (100 μg/mouse, ip), and sacrificed by cervical dislocation on days 1, 3, 7, 10, 14, 20, and 30. Thymus glands were removed for phenotypic analysis, and determination of the parameters such as relative weight, cellularity and DNA integrity of thymus (n = 3).

A separate set of mice received vehicle (iv) or r/hCRF (10 μg/mouse, iv) 5 min prior to the administration of a single dose of vehicle (10 ml/kg, ip) or LPS (100 μg/mouse, ip). Blood was obtained from the retro-orbital venous plexus at sequential time points (1, 2, 4, 6, 12 h) during the first 12 h after administration of LPS with or without a single dose of r/hCRF (n = 3–4). The animals were then sacrificed by cervical dislocation at 1, 7 and 14 days and thymus glands were removed for determination of relative weight, cellularity, DNA integrity, and phenotypic analysis of thymus. For each of the treatment a separate group of six or seven mice (male: n = 3–4 and female: n = 3) was employed.

### 2.5. Experimental methods

#### 2.5.1. Relative thymus weight and cellularity

The mice were euthanized by cervical dislocation after injections. Thymus glands were isolated, weighed, and placed in cold medium (Hanks' Balanced Salt Solution, HBSS), and passed through a wire mesh to obtain a single cell suspension. Cells were washed in HBSS and their viability measured by trypan blue exclusion. Their weights of thymus were expressed as relative organ weights (mg/g). Thymus of saline treated group served as a control.

#### 2.5.2. Hormone determinations

Blood was obtained from the retro-orbital venous plexus, animals showing anemia were discarded, and the remaining were used for statistical analysis. Serum was isolated by centrifugation for 20 min at 2000 ×*g* and stored at −20 °C until thawed for analysis of corticosterone. Serum corticosterone levels were determined using a corticosterone ELISA kit purchased from R&D Systems.

#### 2.5.3. Flow cytometry

After washing the thymocytes in cold phosphate-buffered saline (PBS), direct immunofluorescence staining was performed with anti-mouse directly conjugated monoclonal antibodies (Ab): anti-CD4 PE