PSYCHOSOCIAL STRESS AFFECTS THE INVOLVEMENT OF PROSTAGLANDINS AND NITRIC OXIDE IN THE LIPOPOLYSACCHARIDE-INDUCED HYPOTHALAMIC-PITUITARY-ADRENAL RESPONSE

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The role of prostaglandins and nitric oxide (NO), generated after peripheral lipopolysaccharide (LPS) administration, in the adaptation of hypothalamic-pituitary-adrenal (HPA) axis under stressful circumstances remains to be elucidated. The aim of the present study was to assess the effect of chronic repetitive restraint or social crowding stress on the involvement of nitric oxide and prostaglandins in the LPS-induced pituitary-adrenocortical response. Male Wistar rats were restrained in metal tubes 2x10 min/day or crowded in cages for 7 days prior to treatment. All compounds were injected i.p., cyclooxygenase (COX) and nitric oxide synthase (NOS) inhibitors 15 min before LPS. Two hrs after injection LPS induced a significant increase in ACTH and corticosterone secretion. Repeated restraint impaired more potently than crowding stress the LPS-induced HPA-response. Indomethacin, a non-selective COX inhibitor, considerably reduced the LPS-induced HPA response in non-stressed rats and to a lesser extent diminished this response in repeatedly restrained or crowded rats. Neuronal NOS inhibitor, Nω-nitro-L-arginine decreased the LPS-induced HPA response, more potently in control than crowded rats. Aminoguanidine, an iNOS inhibitor, diminished the LPS-elicited ACTH response in crowded rats. These results indicate that prostaglandins and NO generated by neuronal and inducible NOS are involved in the LPS-induced HPA axis response under basal conditions and during its adaptation to chronic social stress circumstances.

Key words: social crowding stress, repeated restraint, lipopolysaccharide, ACTH, corticosterone, prostaglandins, nitric oxide, L-NNA, aminoguanidine.
INTRODUCTION

Immune stimulation by peripheral administration of the bacterial endotoxin lipopolysaccharide (LPS) elicits extensive changes in pituitary hormone secretion (1-3). The activation of the hypothalamic-pituitary-adrenal (HPA) axis by LPS is primarily mediated by pro-inflammatory cytokines (4-6), interleukin (IL)-1, IL-6 and tumor necrosis factor α (TNF-α) (7). These three cytokines can activate the HPA axis at different levels but the acute-phase ACTH response is mainly mediated by the hypothalamic release of corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP), two major secretagogues of ACTH (8). Cytokines generated after peripheral LPS as large polypeptides do not cross the blood-brain barrier (BBB) readily, but may cross this barrier through fenestrated capillary endothelium of the circumventricular organs or via limited transport system. Peripheral LPS may also alter pituitary function by stimulating the secretion of neurotransmitters, which in turn modulate the secretion of pituitary hormones (9). Systemic LPS may also induce cytokines production by cells of the central nervous system itself (10). Centrally-produced IL-1β activates the HPA axis by stimulating neurons in the hypothalamic paraventricular nucleus (PVN) to release CRH which then stimulates secretion of adrenocorticotropin hormone from the anterior pituitary gland.

Exposure to different kind of stressful stimuli alters peripheral and central proinflammatory cytokine expression (11-13). Stressful stimuli evoke a series of neuroendocrine responses that activate the HPA axis. The bilateral communication between the immune and neuroendocrine systems plays an essential role in modulating the adequate response of the HPA axis to the stimulatory influence of interleukins and stress-related mediators.

Prostaglandins (PGs) are known to be expressed by LPS in the brain. Systemic administration of bacterial lipopolysaccharide evokes inducible cyclooxygenase (COX-2) expression in neurons of several brain regions. Marked expression of COX-2 mRNA appeared at 1/2 h in perivascular cells and increased to a peak at 2 h after LPS injection (14). Inducible cyclooxygenase converts arachidonic acid to prostaglandins, which are thought to mediate various peripheral lipopolysaccharide-induced central effects, including activation of the HPA axis (15). Prostaglandins are critically involved in the LPS-induced ACTH and corticosterone secretion under basal conditions (16). We have shown that PGs generated by both constitutive cyclooxygenase (COX-1) and inducible cyclooxygenase are involved to a different extent, in the CRH- and vasopressin-induced HPA axis stimulation during social crowding stress. (17,18).

Nitric oxide (NO) plays a pivotal role in central neuroendocrine outputs within the hypothalamic paraventricular nucleus (19). Nitric oxide affects ACTH secretion and the transcription of genes encoding CRH and AVP in the hypothalamus of the rat. One of the most highly expressing neuronal nitric oxide synthase (nNOS) cell populations in the rat brain have been identified within the hypothalamus. Strong endothelial NOS (eNOS) and nNOS immunoreactivity is detected in the vascular
tissue and at the immediate proximity of the neuroendocrine nerve terminals of the external zone of the median eminence (20). Lipopolysaccharide and proinflammatory cytokines also induce nitric oxide synthase (iNOS) which generates NO, an important modulator of HPA axis activity.

Lipopolysaccharide induced COX-2 and iNOS isoenzymes which generate the synthesis of PG and NO are essential regulators of the HPA axis activity particularly under stress conditions. However, the significance and functional relations of these regulators in the stimulation of HPA axis by immune insults have not been clarified. The objective of the present study was to determine the effect of repeated restraint stress and social crowding stress in the hyporesponsiveness of HPA axis to LPS and to compare the significance of PGs and NO in this response under basal and psychosocial stress circumstances.

MATERIALS AND METHODS

**Animals**

Male Wistar rats weighing 180-210 g were maintained under standard 12:12 h light-dark cycle and temperature 21±2°C, with free access to rodent food and tap water. Rats were housed 6 per cage for at least one week before experimental period. All animal procedures were approved by the Institutional Animal Care and use Committee.

**Experimental procedures**

Rats were randomly assigned to three experimental groups: control rats left undisturbed and rats restrained for 10 min twice a day for 7 days and crowded for 7 days. All rats from a given cage were assigned to the same experimental group, control and restraint or crowding. At the end of experiment rats were rapidly decapitated without stress within 10 sec after they had been removed from the animal box or tube. Trunk blood was collected in plastic tubes on ice-cold bath, and plasma obtained after centrifugation was frozen at -80°C until hormone assays were performed. All experiments were performed between 9.00 a.m. and 12.00 a.m. and all decapitations between 12 a.m. and 13 a.m.

**Restraint**

The animals were individually placed inside metal perforated cylinders and both ends were closed. Control rats remained undisturbed throughout the stress period. Stressed and non-stressed rats were tested simultaneously on a given test day.

**Social crowding stress**

The control rats were housed in groups of 7 to a cage (52x32x20 cm) and they remained in their home cages until scheduled for treatment. The stressed rats were crowded in groups of 21 per a cage of the same size for 7 days, since after that time we found the most potent and significant impairment of the HPA responsiveness to the central neurotransmitter and neuropeptide receptor stimulation (17, 18).

**Bacterial lipopolysaccharide administration**

The rats were injected i.p. with either vehicle (sterile physiological saline) or 1 mg/kg of bacterial lipopolysaccharide Escherichia coli serotype 026:B6 in sterile physiological saline. The
The dose of LPS selected was based on previous studies that used the acute i.p. administration (16). The time selected for blood sampling 2 h after acute i.p. LPS administration was based on previous studies on the cytokine and HPA axis hormone profile in rats receiving LPS (2, 14, 16). Dilutions were prepared immediately before use. The required doses of drugs or solvents were injected i.p. in a volume of 2 ml/kg. The doses of drugs used are expressed in terms of salts.

**COX and NOS inhibitors administration**

Indomethacin a non-selective COX inhibitor was prepared by sonification in 1% Tween solution and injected i.p. 30 min before LPS. Neuronal NOS inhibitor Nω-nitro-L-arginine (L-NNA) and inducible NOS inhibitor aminoguanidine (AG) were dissolved in sterile physiological saline and injected i.p. 15 min before LPS. All drugs were purchased from Sigma Chemicals Co.

Plasma ACTH concentrations were measured using the double antibody $^{125}$I radioimmunoassay obtained from CIS Bio International and calculated as pg/ml of plasma. The concentration of serum corticosterone was measured fluorometrically and expressed as µg/100ml.

**Statistics**

The results were calculated as a group mean ± standard error of the mean. Statistical evaluation was performed by an analysis of variance, followed by individual comparisons with Duncan’s test. P values less than 0.05 were taken to indicate statistical significance.

**RESULTS**

**ACTH and corticosterone response to LPS in repeatedly restrained and crowded rats**

In rats restrained 10 min 2x/day for 7 days LPS injected 24 h after the last restraint evoked considerably lower increase in plasma ACTH level (155.5 pg/ml) than in control non-restrained rats (365.5 pg/ml). Likewise, the LPS-induced corticosterone response was significantly lower in repeatedly restrained than in control rats, 17.6 vs. 33.6 µg/dl, respectively (**Fig. 1**). Crowding stress for 7 days induced markedly weaker impairment in LPS-induced ACTH response, from 403.9 pg/ml to 290.7 pg/ml (-28%) than chronic restraint stress, (-57.5%). The diminution by crowding stress of the LPS-induced corticosterone response from 35.8 µg/dl to 19.9 µg/dl (44.4%) did not differ from a similar decrease by restraint stress (46.6%) (**Fig. 1**).

**Crowding stress impairs the effect of indomethacin on the LPS-induced ACTH and corticosterone responses**

In control, non-stressed rats, indomethacin given i.p. 30 min prior to LPS significantly and to the same extent reduced the LPS-induced ACTH and corticosterone response (43.4% and 44.1%). Crowding stress for 7 days weakened (-18.6%) the inhibitory effect of indomethacin on the LPS-induced ACTH secretion or reversed (+43%) the inhibition of corticosterone secretion (**Fig. 2**).
Effect of L-NNA on the LPS-induced ACTH and corticosterone response in crowded rats

Pretreatment of non-stressed rats with neuronal NOS inhibitor L-NNA significantly impaired the LPS-induced increase in ACTH and corticosterone secretion (34.7% and 34.5%, respectively). In rats crowded for 7 days L-NNA moderately diminished the LPS-induced ACTH secretion (14.8%) and did not substantially alter (-5.0%) corticosterone secretion. (Fig.3). These results suggest that social crowding stress was unable to markedly alter the LPS-induced hormone secretion diminished by iNOS antagonist.

Effect of aminoguanidine on the LPS-induced HPA axis response in crowded and restrained rats

Aminoguanidine, a fairly selective iNOS inhibitor, moderately decreased the LPS-elicited ACTH secretion and did not alter corticosterone secretion in non-stressed rats. In rats crowded for 7 days this iNOS blocker further markedly diminished the LPS-induced ACTH secretion (28.3%) but did not substantially affect corticosterone levels induced by LPS. (Fig.4)
A repeated restraint stress for 7 days considerably reduced the LPS-induced ACTH and corticosterone response, by 57.5% and 47.4%, respectively in comparison with the response in non-stressed rats. Pretreatment with aminoguanidine markedly prevented this inhibitory effect on ACTH secretion (-32.7%) and totally abolished the stress induced inhibition of corticosterone response. (Fig.5)

**DISCUSSION**

In the present experiment psychosocial stress of crowding and mainly psychological stress of repeated restraint for 7 days significantly impaired the
LPS-induced ACTH and corticosterone response. Repeated restraint stress was more potent in diminishing ACTH response (-57.5%) than chronic crowding stress (-28%) and both types of stress induced almost identical decrease in corticosterone response 46.6% and 44.4%, respectively. This finding indicates that some of central multifactorial mechanisms involved in the immunostimulatory effect of LPS on ACTH secretion are more potently impaired by repeated restraint than crowding stress during 7 days.

After peripheral administration, LPS was found in the general circulation within 15 min, whereas elevation of plasma adrenocorticotropic hormone and corticosterone concentrations appeared after 90 min. Increased levels of plasma hormones were associated with elevated levels of the LPS indicating that circulating endotoxin is required for the activation of the HPA axis (21). In our experiment considerable elevation of plasma ACTH and corticosterone levels appeared 2 h after peripheral
endotoxin administration i.e. when IL-1β messenger RNA expression in brain structures and anterior pituitary lobe was at the peak level (14). It was found that LPS highly stimulated the transcription of the gene encoding the CRH type 1 receptor involved in CRH secretion in the parvocellular division of the paraventricular nucleus, and magnocellular PVN and in the supraoptic nucleus which releases vasopressin (22). Therefore, LPS administered systemically may stimulate the nuclei which generate two major secretagogues of ACTH (8). Lipopolysaccharide or LPS-induced proinflammatory cytokines like IL-1β, TNFα and IL-6 may transmit stimulatory signals to the brain either by passing the leaky barrier of circumventricular organs or may affect brain functions indirectly by inducing COX-2 and prostaglandin E₂ generation in cerebral endothelial cells which may stimulate HPA axis centrally (15). Cytokines might also be produced by neurons and glial cells within the brain structures involved in the complex neuroendocrine responses to different LPS-induced neurotransmitters and cytokine stimulation (9,10).
The induction of central and peripheral cytokines by stressors remains controversial and the pattern of their induction by different stressors is not consistent (11). Considerable controversy exists regarding the ability of different stressors to induce hypothalamic IL-1 expression. Deak at al. (13) reported that restraint stress for 2 h failed to alter hypothalamic IL-1 levels despite robust activation of the HPA response, but footshock led to a two-fold increase in the hypothalamic mRNA for IL-1. Cytokines have a direct or indirect role in stress-related HPA axis response. Exposure to acute stressors induces the expression of cytokines at the periphery which can penetrate the blood-brain-barrier and activate potently the HPA axis (12).

In the present experiment a non-selective cyclooxygenase blocker, indomethacin given 30 min earlier significantly impaired the LPS-induced ACTH and corticosterone response. Compound NS 398, a selective COX-2 inhibitor, elicited markedly stronger reduction of ACTH secretion than indomethacin or piroxicam, a preferential COX-1 antagonist (16). This indicates that PGs,
particularly generated by COX-2, are significantly involved in the LPS-induced HPA response under basal circumstances. A striking enhancement in the expression of inducible cyclooxygenase by LPS may be elicited by a concomitant increase in IL-1β and IL-6 expression (23). However, in the present experiment indomethacin moderately deepened (-18.6%) the LPS-elicited ACTH secretion reduced by crowding stress. This observation may suggest that heterologous desensitization of PGs system during chronic social stress was not complete and remaining functional PG activity stimulating ACTH secretion was abrogated by pretreatment with indomethacin. The reversal by indomethacin of the LPS-induced corticosterone response (+43%) in stressed rats indicates a clear dissociation of the central mechanisms inducing pituitary ACTH response and the adrenal cortex response to stimulation with LPS (24). A similar dissociation between ACTH and corticosterone response, also encountered by other authors during peripheral LPS stimulation of HPA axis in rats (3), may suggest an altered adrenal cortex sensitivity to ACTH.

The present data revealed that in non-stressed rats neuronal NOS inhibitor L-NNA markedly decreased the LPS-induced ACTH and corticosterone secretion, which suggests that NO generated by constitutive nNOS is significantly involved in the LPS-induced HPA response under basal conditions. In rats crowded for 7 days neuronal NOS blocker did not substantially alter the LPS-induced ACTH and corticosterone secretion significantly impaired by stress itself. This finding suggests that chronic social stress inhibits the nNOS activity involved in the HPA response to LPS under basal conditions. Nitric oxide generated by inducible NOS appeared to be not substantially involved in the ACTH response to immune stimulation by LPS. In non-stressed rats iNOS antagonist aminoguanidine moderately diminished the LPS-induced ACTH response (-15.7%) and did not alter corticosterone response. However, this antagonist further significantly decreased (-28.3%) the LPS-induced ACTH response, already impaired by crowding stress, without influencing corticosterone response. Nitric oxide generated by nNOS but not iNOS in mediation of the LPS-induced ACTH secretion under basal conditions. Likewise, NO generated by iNOS is not substantially involved in the HPA axis response to neurotransmitters under basal circumstances (25). Although following systemic administration LPS increases mRNA expression of both nNOS and iNOS, and augments NO production in the hypothalamic PVN (26,27) our results suggest a more pronounced involvement of nNOS than iNOS in the LPS-induced ACTH response.

The present study shows that prostaglandins and NO generated by nNOS are significantly involved in the LPS-induced HPA axis activation under basal conditions. Psychological and psychosocial stress considerably impairs the HPA axis response to bacterial endotoxin stimulation. Psychosocial stress desensitizes the prostaglandin systems involved in the LPS-elicited axis response. Chronic social stress inhibits nNOS activity involved in the LPS-induced HPA axis response under basal conditions.
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