We investigated the role of nitric oxide (NO) in the interleukin 1β (IL-1β) and nicotine induced hypothalamic-pituitary-adrenal axis (HPA) responses, and a possible significance of CRH and vasopressin in these responses under basal and social stress conditions. Male Wistar rats were crowded in cages for 7 days prior to treatment. All compounds were injected i.p., nitric oxide synthase (NOS) inhibitors, α-helical CRH antagonist and vasopressin receptor antagonist 15 min before IL-1β or nicotine. Identical treatment received control non-stressed rats. Plasma ACTH and serum corticosterone levels were measured 1 h after IL-1β or nicotine injection. L-NAME (2 mg/kg), a general nitric oxide synthase (NOS) inhibitor, considerably reduced the ACTH and corticosterone response to IL-1β (0.5 µg/rat) the same extent in control and crowded rats. CRH antagonist almost abolished the nicotine-induced hormone responses and vasopressin antagonist reduced ACTH secretion. Constitutive endothelial eNOS and neuronal nNOS inhibitors substantially enhanced the nicotine-elicited ACTH and corticosterone response and inducible iNOS inhibitor, aminoguanidine, did not affect these responses in non-stressed rats. Social stress significantly attenuated the nicotine-induced ACTH and corticosterone response. In crowded rats L-NAME significantly deepened the stress-induced decrease in the nicotine-evoked ACTH and corticosterone response. In stressed rats neuronal NOS antagonist did not alter the nicotine-evoked hormone responses and inducible NOS inhibitor partly reversed the stress-induced decrease in ACTH response to nicotine. These results indicate that NO plays crucial role in the IL-1β-induced HPA axis stimulation under basal and social stress conditions. CRH and vasopressin of the hypothalamic paraventricular nucleus may be involved in the nicotine induced alterations of HPA axis activity. NO generated by eNOS, but not nNOS, is involved in the stress-induced alterations of HPA axis activity by nicotine.

Key words: social crowding stress, interleukin-1β, nicotine, ACTH, corticosterone, nitric oxide, NOS antagonists
INTRODUCTION

Pro-inflammatory cytokines are able to induce the release of corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) from the hypothalamic paraventricular nucleus (PVN) and augment neurochemical stimulation of the pituitary gland (1, 2). Blood-borne cytokines can convey information to the central nervous system by several mechanisms. As large polypeptides (17-26 kDa) cytokines do not cross the blood-brain barrier (BBB) readily. However, they may enter the brain via the fenestrated capillary endothelium of the circumventricular organs or at sites of increased BBB permeability (3). Interleukin-1α (IL-1α) and IL-1β may also be transported to the brain either by a single transport system or by specific, saturable systems (4). It is possible that in the intact animal, the major or initial effect of IL-1 is mediated through the hypothalamus. The presence of IL-1β mRNA, protein and receptors in the brain suggests that it may not be necessary for IL-1 to cross the BBB to induce effects in the central nervous system. The presence of IL-1 receptors on pituitary cells and the fact that ACTH release can be PGE₂ and catecholamine independent may suggest that IL-1 may have a direct effect on the pituitary in vivo.

Apart from stress response triggered by immune-inflammatory stimuli, cytokines may be involved in mediating hypothalamic responses to restraint or exercise stress and nociceptive stimuli (3, 5). It is not clear whether different stressors are able to induce hypothalamic IL-1 expression (6). Despite strong stimulation of the HPA response forced swim stress failed to alter hypothalamic IL-1 levels (7), while chronic mild stress in mice decreased expression of peripheral IL-1β and IL-6 (8). Various forms of psychological stress increased hypothalamic IL-1β levels and enhanced circulating level of IL-1β. Inescapable tailshock raised IL-1β and its protein levels in hypothalamus and pituitary (9). These acute stressors are known to induce release of the hypothalamic CRH and AVP which stimulate ACTH secretion.

It is known that peripherally administered nicotine can penetrate into the brain and act on central nervous system, inducing neurotransmitters and neuropeptides release. Nicotine activates the HPA axis indirectly by stimulation the release of CRH from both the parvocellular part of the hypothalamic PVN, and from CRH containing neurons in the median eminence (10). In vivo acetylcholine stimulate the CRH gene expression in the hypothalamic PVN (11). Nicotine stimulates the HPA axis also by activation of the brainstem catecholaminergic neurons and release of noradrenaline in the PVN in rats (12). Neuronal nicotinic presynaptic receptors influence many neurotransmitter systems including catecholaminergic transmission (13).

Nitric oxide (NO) as a signaling molecule facilitates crosstalk among various cell types and systems serving also as a neuronal messenger molecule (14, 15). As a potential nonsynaptic modulator NO may have an important role in the regulation of monoaminergic and neurotransmitter systems (16). Nitric oxide plays
physiological role in regulation of the HPA axis. Blockade of NO generation by nitric oxide synthase (NOS) antagonists markedly affects ACTH and corticosterone response to cholinergic agonists and neuropeptides (17, 18). Nitric oxide may be involved in the stress response. Under diverse stressors increase in the NOS activity and/or expression have been reported in the limbic-hypothalamic-pituitary-adrenal axis (19, 20). Nicotine increases the activity of endothelial eNOS more than that of neuronal nNOS and has no effect on inducible NOS. Although nicotine causes an overall increase in NOS positive neurons, the stress arising from chronic applications reduces the number of NOS-positive neurons (21).

In the present study we investigated the involvement of nitric oxide in the interleukin 1β- and nicotine-induced HPA axis response under social stress conditions. Further purpose of these studies was to investigate a possible participation of CRH and AVP in the nicotine-induced stimulation of HPA axis during social stress.

MATERIALS AND METHODS

Animals

Rats weighing 190-220 g were used in these studies. The animals were kept 7 per cage and were provided with unlimited access to commercial food and tap water. The animal room was maintained on a 12-hour light-dark cycle beginning at 7.00 a.m. All animals were given a one-week acclimation period before the onset of experimentation. The experiments procedures were performed in accordance with bioethical requirements and were approved by the Institutional Ethics Committee.

Experimental design

The rats were randomly assigned to one of two experimental groups: control and social stress of crowding. Control rats were housed 7 per cage (52x32x20 cm) and remained in their home cages until scheduled for treatment. Stressed rats were crowded in groups of 24 per cage of the same size for 7 days since after that time we found a potent impairment of the HPA responsiveness to some monoaminergic and cholinergic stimulation. Social crowding stress for 7 days, induced a significant reduction in the HPA axis response to acute 10 min restraint stress, equal to the reduction induced by repeated daily restraint stress during the same time period (22).

Experiments were carried out in three separate series. First series of experiments was performed to determine the effect of general NOS blocker L-NAME on the IL-1β-induced pituitary-adrenocortical stimulation in non-stressed and crowded rats. Control rats, intact or crowded, were injected i.p. with saline (0.9% NaCl 2 ml/kg). Second series of experiments was performed to determine the effects of CRH-and AVP-antagonists on the nicotine-induced ACTH and corticosterone secretion in non-stressed rats. Third series of experiments was performed to determine the effect of endothelial (eNOS), neuronal nitric oxide synthase (nNOS) and inducible nitric oxide synthase (iNOS) antagonists on the HPA axis stimulation by nicotine. In this series of experiment rats were pretreated 15 min before nicotine with Nω-nitro-L-arginine methyl ester (L-NAME 5.0 mg/kg i.p.), a non-selective cNOS blocker, or with Nω-nitro-L-arginine (L-NNA 2 mg/kg) preferentially nNOS blocker and with aminoguanidine (AG, 100 mg/kg) an iNOS blocker. One hour after the last injection the rats were decapitated immediately after their removal from the cage and their trunk blood was collected.
Drugs and solution

Drugs used in this study were: interleukin-1β (IL-1β) mouse recombinant, expressed in E. coli, (-)-nicotine 9[-]-1methyl-2-[3-pyridyl]pyrrolidine hydrogen tartrate salt, Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME), N-nitro-L-arginine (L-NNA), aminoguanidine, corticotropin releasing factor antagonist (α-Helical CRF[9-41]) and vasopressin receptor antagonist[β-Mercapto-β, β-cyclopentamethylene-propionyl1, 0-Me-Tyr2-Arg8]-vasopressin, all from Sigma. The drugs were dissolved in a sterile saline solution. Dilutions were prepared immediately before use. The required doses of drugs or solvents were injected i.p. in a volume of 2 ml/kg.

ACTH and corticosterone determinations

Trunk blood samples were collected on ice to plastic conical tubes containing 200 µl of a solution of 5 mg/ml EDTA and 500 TIU of aprotinin (Sigma). Control rats were decapitated concurrently with the experimental group. Plasma was separated by centrifugation in a refrigerated centrifuge within 30 min and frozen at -20°C until the time of assay. Plasma ACTH concentrations were measured using the double antibody 125I 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 per 100 ml. To avoid circadian variability, all experiments were performed between 10 - 11 a.m. and all decapitations between 11 - 12 a.m., when plasma hormones are at a relatively low levels.

Statistics

The effects of nitric oxide synthase blocker on the interleukin-1β- and nicotine-induced ACTH and corticosterone responses in crowded rats were compared with the respective effects in control animals. 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 comparision with Duncan’s test. The results were considered to be significantly different when p<0.05.

RESULTS

Effect of L-NAME on IL-1β-induced HPA axis stimulation

IL-1β (0.5 µg/rat) given i.p. significantly increased plasma ACTH levels, from 98.2 to 521 pg/ml, and serum corticosterone levels from 8.1 to 34.1 µg/ml, respectively, 1 h after administration. Pretreatment of non-stressed rats with L-NAME a non-selective constitutive, preferentially e-NOS inhibitor, considerably reduced the IL-1β-induced ACTH response, from 521 to 185.8 pg/ml, and to a lesser extent, the corticosterone response from 34.1 to 24.2 µg/ml (Fig. 1). In rats exposed to crowding stress the IL-1β-induced ACTH response was diminished by 18.8% and corticosterone response by 32.6% in comparison with the responses in non-stressed rats. However, crowded rats treated with IL-1β retained full responsiveness to the NOS-inhibitor L-NAME which decreased the IL-1β-induced ACTH response by 60% and corticosterone response by 42.6% in comparison with respective inhibition by 64.3 and 29% in non-stressed rats (Fig. 1).
Involvement of CRH and AVP in the nicotine-induced HPA response

In order to determine a possible involvement of corticotropin-releasing hormone and vasopressin, physiological neuropeptides stimulating anterior pituitary corticotrops, in the HPA response to nicotine, antagonists of these neuropeptide hormones were administered i.p. 15 min prior to nicotine given by the same route. CRH antagonist considerably reduced the nicotine-induced ACTH and corticosterone response, by 63.9 and 67.5%, respectively. Likewise, AVP antagonist significantly diminished the nicotine-induced ACTH response, by 59.9%, but did not alter increased corticosterone secretion after nicotine (Fig. 2). These results suggest a significant and equipotent involvement of both CRH and AVP in the central stimulatory effect of nicotine.

Fig. 1. Effect of L-NAME given 15 min earlier on the interleukin 1β (IL-1β)-induced ACTH and corticosterone secretion in control and crowded rats. In Fig. 1-5 rats were crowded for 7 days before treatment. Rats were decapitated 1 h after agonist treatment. Values represent the mean ± SEM of rats. ++ p<0.01 vs. vehicle treated group; ^^^p<0.01 vs. IL-1β treated group; *p<0.05, and **p<0.01 vs. IL-1β treated non-stressed group.
Crowding stress impairs the nicotine-induced HPA response

Crowding stress for 7 days markedly diminished the nicotine-induced ACTH and corticosterone response, by 36.2 and 30.3%, respectively, in comparison with the response in non-stressed controls (Fig. 3).

Effect of NOS antagonists on the nicotine-induced HPA response in control and crowded rats

In non-stressed rats, L-NAME, a preferential e-NOS inhibitor and L-NNA, neuronal NOS inhibitor given i.p. 15 min before nicotine markedly augmented the nicotine-induced ACTH response and inducible NOS antagonist AG, did not substantially alter the nicotine-induced ACTH response (Fig. 4).

Fig. 2. Effect of CRH- and vasopressin (AVP)-antagonists on the nicotine-induced ACTH and corticosterone secretion in control and crowded rats. ++p<0.01 vs. saline treated group. ##p<0.01 vs. nicotine treated group.
In rats crowded for 7 days the nicotine-evoked ACTH and corticosterone response were significantly attenuated. In stressed rats all the NOS inhibitors used in the present study considerably diminished the nicotine-induced ACTH response, L-NAME by 66.3%, L-NNA by 46% and AG by 22.6% in comparison with the respective levels after the same treatment in non-stressed rats (Fig. 4). The constitutive NOS antagonists were more potent than inducible NOS inhibitor in decreasing the nicotine-induced ACTH response in crowded rats. These NOS inhibitors also markedly enhanced the nicotine-induced corticosterone responses in non-stressed rats (14.1 to 33.4%) (Fig. 5). Crowding stress significantly impaired the nicotine-induced corticosterone response (by 30.4%). In stressed rats L-NAME evoked the most potent impairment of the nicotine-induced corticosterone response (-60.3%) in comparison with the response in non-stressed rats.

![Fig. 3. The inhibitory effect of crowding stress on the nicotine-induced ACTH and corticosterone secretion in control and crowded rats. ++p<0.01 vs. saline treated group; **p<0.01 vs. nicotine treated non-stressed group.](image-url)
group. The neuronal NOS inhibitor L-NNA diminished the nicotine-induced corticosterone secretion by 40%, and iNOS antagonist AG lowered the corticosterone response to nicotine by 33.7% as compared with respective changes in non-stressed rats (Fig. 5).

DISCUSSION

In the present study IL-1β (0.5 µg/rat) considerably increased the plasma ACTH and serum corticosterone levels 1 h after systemic administration. Different findings indicate that circulating IL-1β is able to convey information to
the brain and induce long-lasting HPA sensitization via enhanced hypersecretion of CRH and AVP (4, 23, 24). Considerable stimulation of ACTH secretion by i.p. IL-1β in the present experiment suggests that circulating cytokine elicited the stimulation of CRH containing hypothalamic PVN neurons.

IL-1 is known to induce CRH secretion in vivo (25) and inhibitors of cyclooxygenase signaling pathway block this cytokine mediated effect. The increase of ACTH secretion after i.p. IL-1β may also, in part, result from a direct stimulation of the pituitary gland via the signal transducing subunit gp 130 and paracrine communication between the non-secretory folliculostellate cells and the ACTH-producing corticotrops (3).
In control, non-stressed rats L-NAME, a general constitutive NOS inhibitor, induced a striking decrease in the IL-1β-induced ACTH secretion (-64.3%) and significant, though somewhat less, diminution of corticosterone secretion (-29%). This finding suggests that NO plays a critical role in the manifestation of the HPA response to systemic administration of IL-1β in rats under basal conditions. Inhibitor of cNOS was also able to block the IL-1β-induced CRH and ACTH release in rat hypothalamic and anterior pituitary cell cultures (26). Our present data show that social crowding stress for 7 days markedly diminishes in the IL-1β-induced ACTH response (-18.8%) and corticosterone response (-32.6%). In the rat increases in ACTH secretion evoked by systemic injections of IL-1β are reduced by pretreatment with antisera to CRH-41 and AVP (1) indicating the involvement of these peptides in the IL-1β induced stimulation. Although IL-1β increases the release of CRH and AVP from the hypothalamic PVN, the involvement of these neuropeptides in the diminution of the IL-1β-induced ACTH secretion in rats after 7 days of social stress may not be similar. Vasopressin is known to be preferentially expressed over CRH in parvocellular neurons of the hypothalamic PVN and pituitary AVP receptors are upregulated and hyperresponsive during stress (27). We found that crowding stress considerably impairs the AVP-induced ACTH and corticosterone secretion but does not markedly affect the CRH-induced hormone responses. Moreover, crowding stress did not affect the nNOS inhibitor L-NNA-evoked increase in AVP-elicited hormone responses, but it abolished the CRH-induced ACTH secretion (28). A moderate impairment of the IL-1β-induced ACTH and corticosterone response by chronic crowding stress in the present experiment may be connected with more pronounced desensitization of the AVP-induced stimulation (29, 30).

Our data indicate that NO is a critical, significant mediator of the IL-1β-induced HPA response under both basal and social stress conditions. Pretreatment of rats with L-NAME, a general constitutive NOS inhibitor, considerably diminished the IL-1β-induced ACTH and corticosterone secretion, in crowded rats (60% and 42.6%, respectively) i.e. to a similar extent as in control, non-stressed animals. Therefore, social crowding stress does not markedly affect the activity of constitutive NO systems involved in the IL-1β-induced HPA axis stimulation under basal conditions.

In the present study CRH antagonist given 15 min prior to nicotine almost totally abolished the nicotine-induced ACTH and corticosterone secretion, indicating strong involvement of the hypothalamic CRH in the nicotine-elicited HPA activation in vivo. Pretreatment of rats with AVP antagonist also considerably reduced the nicotine-induced ACTH response indicating the mediation of AVP in the nicotine-evoked ACTH secretion. Also effect of nicotine on ACTH and cortisol in men may be partly mediated by vasopressin (31).

Under basal conditions constitutive NOS blockers L-NAME and L-NNA markedly enhanced the nicotine-induced ACTH response, by 25.3% and 20.7%,
respectively. This result confirms our and other earlier findings indicating that, NO is an inhibitory modulator of the nicotine-induced ACTH secretion. Aminoguanidine, an inducible NOS blocker, did not substantially alter the nicotine-induced increase in ACTH secretion. This suggests that NO generated by iNOS does not participate in the nicotine-elicited ACTH secretion in rats under basal conditions. In rats exposed to crowding stress L-NAME further significantly impaired the nicotine-induced ACTH response, in comparison with the respective levels in non-stressed rats. This result agrees with the data indicating that nicotine increases the activity of eNOS more potently than of nNOS and has no effect on iNOS (32). Predominantly neuronal NOS blocker L-NNA, did not alter the nicotine-elicited ACTH response in stressed rats and inducible NOS antagonist AG significantly prevented (+32%) the nicotine-induced decrease in ACTH secretion evoked by crowding stress. These results suggest that NO generated by iNOS may be responsible for the stress-induced impairment of the nicotine-induced ACTH secretion, whereas NO generated by nNOS has not any marked effect in this regulation.

In non-stressed rats constitutive NOS blockers, L-NAME and L-NNA enhanced the nicotine-evoked increase in corticosterone secretion to a similar extent like they affected ACTH secretion. Chronic social stress reversed the stimulatory action of cNOS blocker L-NAME, but not nNOS or iNOS blockers, on the nicotine-induced corticosterone secretion observed in non-stressed rats.

In conclusion, the present results indicate that NO plays crucial role in the IL-1β-induced HPA axis response to nicotine under basal and social stress conditions. CRH and vasopressin released by the hypothalamic paraventricular nucleus may be involved in the nicotine-induced stimulation of the HPA axis under basal and chronic stress conditions. NO derived by eNOS is mainly involved in the stress-induced alterations of HPA axis response to nicotine.

*Acknowledgements:* This study was supported by KBN grant for statutory activity for Institute of Pharmacology, Polish Academy of Sciences.

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Received: July 8, 2005
Accepted: September 5, 2005

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