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THE INVOLVEMENT OF NITRIC OXIDE AND PROSTAGLANDINS IN THE CHOLINERGIC STIMULATION OF HYPOTHALAMIC-PITUITARY-ADRENAL RESPONSE DURING CROWDING STRESS

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The aim of the present study was to determine the effect of social crowding stress and significance of nitric oxide (NO) and prostaglandins (PG) generated by constitutive and inducible nitric oxide synthase (NOS) and cyclooxygenase (COX) in the stimulation of hypothalamic-pituitary-adrenal (HPA) axis by cholinergic muscarinic receptor agonist carbachol. Inhibitors of neuronal NOS (nNOS) L-NNA, general NOS L-NAME and inducible NOS (iNOS) aminoguanidine, as well as inhibitors of COX-1, piroxicam, and COX-2, compound NS-398 were administered 15 min prior to carbachol to control or crowded rats (24 rats in cage for 7, during 3 and 7 days). In stressed rats L-NAME, L-NNA and aminoguanidine significantly intensified the carbachol-induced ACTH and corticosterone secretion, like in control rats. Piroxicam, markedly decreased the carbachol-induced ACTH and corticosterone response under either basal or stress conditions. Compound NS-398 did not markedly alter the carbachol-induced HPA response in control and stressed rats. Crowding stress (3 days) significantly impaired the i.c.v. prostaglandin E_2-induced ACTH response. Corticotropin releasing hormone (CRH) receptor antagonists, α-helical CRH [9-14], given i.c.v. did not alter the PGE_2-evoked corticosterone response in either control or stressed rats, indicating that hypothalamic CRH is not involved in the PGE_2-induced central stimulation of HPA axis. In control rats L-NAME considerably enhanced, while L-arginine, a physiological NOS substrate, abolished the PGE_2-induced ACTH and corticosterone response. In stressed rats this NOS blocker significantly increased and L-Arg reduced the stimulatory effect of PGE_2 on ACTH and corticosterone secretion. The carbachol-induced corticosterone response was significantly increased by pretreatment with nNOS inhibitor L-NNA and was considerably reduced by indomethacin, a general COX inhibitor. Pretreatment with both antagonists left the carbachol-induced corticosterone level unchanged, suggesting an independent and reciprocal effect of NO and PG in the cholinergic stimulation of pituitary-adrenocortical response.
These results indicate that in the stimulatory action of muscarinic agonist, carbachol, NO is an inhibitory transmitter under basal and crowding stress conditions. This

psychosocial stress does not functionally affect the NOS/NO systems. Prostaglandins are involved in the cholinergic muscarinic-induced stimulation of HPA response to a significant extent in non-stressed rats. PGE_2 may be involved in the carbachol-elicited HPA response under basal and stress conditions. Prostaglandins released in response to muscarinic stimulation did not evoke the hypothalamic CRH mediation. NO significantly impairs and PG stimulates the carbachol-induced HPA response in rats under basal and social stress conditions.

Key words: *social stress, nitric oxide, prostaglandins, carbachol, HPA axis adaptation, ACTH, corticosterone*

INTRODUCTION

Acetylcholine (ACh) is known to stimulate the hypothalamic-pituitary-adrenal (HPA) axis through a central release of corticotrophin releasing hormone (CRH). Cholinergic muscarinic and nicotinic receptors are widely distributed in the central structures regulating the activity of HPA axis (1). Hypothalamic CRH neurons receive stimulating innervation from the cholinergic systems and central muscarinic receptors play a significant role in this stimulation (2). Hippocampal cholinergic system mediates activation of the HPA axis by a significant involvement of bed nucleus of the stria terminalis. It sends axonal projections into neuroendocrine cell regions of the hypothalamic PVN (3). Carbachol which in some reactions in brain structures acts as a muscarinic agonist, is fairly selective cholinergic muscarinic receptor agonist in the stimulation of HPA axis in vivo (4, 5). Muscarinic receptors also play an important role in cytokine-induced CRH release (6). In rat thalamus slices and hippocampus carbachol mediates increase in extracellular inositol phosphate formation through muscarinic receptor activation. One of intracellular mediators of muscarinic receptor action is mobilization of cystolic Ca^{2+} which probably contributes to activation of phospholipase A_2, which generate arachidonic acid and prostaglandins. The cholinergic system comprise the acetylcholine gene, which codes for acetylcholine and its partner, the acetylcholinesterase gene, which codes for the enzyme that destroys the function of acetylcholine excitatory neurotransmission in the central nervous system (7).

Acute stress activates not only the HPA axis but also the septo-hippocampal cholinergic system in male rats. Acute and chronic stress can induce a series of changes in cholinergic gene expression that alter the normal balance of acetylcholine and acetylcholinesterase metabolism and can influence the structures regulating the HPA axis activity. Restraint stress significantly enhances the ACh release in the hippocampus and increases corticosterone release in female rats (8).

Nitric oxide is regarded as a major and ubiquitous modulator of a variety of physiological reactions (9-11). In the central nervous system, NO easily diffuses intracellularly and acts as a signaling molecule. Nitric oxide is formed

intracellularly from L-arginine through the action of respective NO synthase (NOS), neuronal nNOS, endothelial eNOS or inducible iNOS (12-14). The production of NO is a calmodulin-dependent process, preceded by the elevation of intracellular Ca^{2+} concentration. In cholinergic neurons in the rat brain NOS gene expression implies a role for NO in cholinergic neurotransmission (15).

Prostaglandins regulate central cholinergic receptor sensitivity and may be involved in the stimulation of HPA axis at the level of hypothalamic CRH, pituitary corticotrophs or in the rat adrenal glands (16). Cholinergic system may also directly affect the rat and bowine adrenal cortex function since cholinergic nerve fibres were demonstrated in the capsula and zona glomerulosa cells (17). Prostaglandins and nitric oxide generated by COX and nNOS and iNOS are involved in the immune stimulation of HPA axis by peripheral administration of the bacterial endotoxin lipopolysaccharide (LPS) under basal conditions (18, 19) and during its adaptation to chronic stress circumstances (20, 21).

The purpose of the present study was to determine the involvement of endogenous nitric oxide and prostaglandins in the cholinergic-induced HPA axis response in rats exposed to social crowding stress. Furthermore, the involvement of CRH and NOS inhibitors in the PGE_2-induced HPA axis response was investigated.

MATERIALS AND METHODS

Male Wistar rats weighing 190-220 g were housed 6 per cage under standard laboratory conditions 12/12 h light-dark cycle (lights on at 7.00 a.m.) at an ambient temperature 20±2°C for a week before the experiment. The rats were allowed free access to a standard diet and to drinking water. The experiments procedures followed the guidelines for the care and use of laboratory animals, and they were approved by the Institutional Ethics Committee.

Experimental design

Experiments were carried out in rats randomly assigned to non-stressed control and crowding stress groups. 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 3 and 7 days, since after that time we found a potent impairment of the HPA responsiveness to cholinergic muscarinic receptors stimulation. In the first series of experiment the effects of cyclooxygenase blockers on the carbachol-induced ACTH and corticosterone responses in crowded rats were compared with the effects in control animals. For this purpose both control and crowded rats were pretreated with piroxicam, a preferentially selective constitutive cyclooxygenase (COX-1) blocker (2 mg/kg i.p.) and compound NS-398, a selective inducible cyclooxygenase (COX-2) blocker (2 mg/kg i.p.) 15 min before carbachol (0.2 mg/kg i.p.). In the second series control and crowded rats were pretreated with constitutive non-selective NO synthase (cNOS) blocker, L-NAME (2 and 10 mg/kg), the neuronal NOS blocker L-NNA, or inducible iNOS blocker aminoguanidine (100 mg/kg) blocker 15 min before carbachol and the secretion of ACTH and corticosterone was compared. In the third series of experiment the effect of PGE_2 given i.c.v. on HPA axis response in controls and rats crowded for 3 days was examined. Also the effect of inhibition or increase in the synthesis of NO by cNOS blocker L-NAME or L-

Arginine, endogenous NO substrate, were determined. In the fourth series of experiment a possible involvement of CRH in the PGE_2-induced HPA response was determined by i.c.v. pretreatment of rats with CRH antagonist, α helical CRH 9-41 (0.1 and 10 μg) 15 min before i.c.v. PGE_2. In the last series of experiments the effects of non-selective COX blocker, indomethacin, and neuronal NOS blocker, L-NNA, on the carbachol-induced ACTH and corticosterone response were compared.

Required doses of the drugs were dissolved in saline immediately before use and injected i.p. in volume of 0.2 ml/kg, or they were administered into the right lateral cerebral ventricule in a volume of 10 μl of saline or solvent to rats whose skulls were prepared 24 h earlier, under light ether anesthesia, for free-hand i.c.v. injections. 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 relatively low levels.

Preparation of drugs

Drugs used in this study were: carbamylcholine hydrochloride (Carbachol), indomethacin, piroxicam hydrochloride, prostaglandin E_2 (Sigma), corticotropin releasing factor antagonist (α-Helical CRF [9-14] (Sigma), L-Arginine hydrochloride (L-Arg), N-ωnitro-L-arginine methyl ester hydrochloride (L-NAME), L-nitro-L-arginine (L-NNA) and aminoguanidine (AG), all from Sigma and compound NS-398 from Cayman Chemical Co. Piroxicam and indomethacin were prepared for injection by sonication in 1% Tween solution, NS-398 was dissolved in dimethyl sulfoxide (DMSO, Sigma) and remaining drugs were dissolved in saline. The doses used are expressed in terms of salts. Solutions were prepared immediately before use.

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 ^{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 per 100 ml.

Analysis of data

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

RESULTS

Carbachol stimulates ACTH and corticosterone secretion by central muscarinic receptors

Carbachol is considered to stimulate both muscarinic and nicotinic cholinergic receptors in different functional responses. We found that in unanesthetized rats i.c.v. administered carbachol activates HPA axis in a major part via stimulation of

muscarinic cholinergic receptors. A selective nicotine receptor antagonist mecamylamine given i.c.v. 15 min earlier did not markedly diminish the carbachol-induced ACTH and corticosterone response whereas pretreatment with atropine almost totally abolished these responses (4).

Effect of NOS antagonists and L-Arg on basal plasma ACTH and corticosterone levels

A broad spectrum of NOS antagonist L-NAME (2-10 mg/kg) and predominantly neuronal NOS inhibitor L-NNA (2-5 mg/kg) given i.p. did not significantly alter the basal plasma ACTH and corticosterone levels. Likewise, these NOS antagonists given alone i.c.v. to non-anesthetized rats left these basal hormone levels unaffected. Also the physiological NO substrate L-Arginine (120-300 mg/kg) given i.p. did not affect markedly the resting plasma ACTH and corticosterone levels (data not shown).

Effect of NOS inhibitors on the carbachol-induced HPA response

Carbachol (2 μg) given i.c.v. to conscious non-stressed rats induced a significant increase in ACTH and corticosterone secretion 1h later. L-NNA, a preferentially neuronal NOS inhibitor, given both i.c.v. (10 μg) or i.p. (10 mg/kg) 15 min earlier significantly intensified the carbachol-evoked ACTH and corticosterone secretion *(Fig. 1)*. Pretreatment with L-NAME (5 mg/kg i.p.) elicited more pronounced increase in the carbachol-induced ACTH and corticosterone response (by 35 and 47%, respectively) than L-NNA (2 mg/kg i.p.) (*Fig. 2*).

Effect of NOS antagonists on the carbachol-induced HPA response in stressed rats

Carbachol (0.2 mg/kg i.p.) significantly enhanced the ACTH and corticosterone responses in non-stressed rats. The ACTH response was markedly diminished by crowding stress for 7 days. Constitutive NOS inhibitors, L-NAME (5 mg/kg) and L-NNA (2 mg/kg) and inducible NOS inhibitor aminoguanidine (100 mg/kg), given i.p. in effective doses, significantly increased the i.p. carbachol-evoked ACTH and corticosterone responses in stressed rats *(Fig. 2)*.

Effect of COX inhibitors on the carbachol-induced HPA response in stressed rats

We have shown that PG system is involved in the carbachol-induced HPA response (4). Under basal conditions piroxicam (2 mg/kg i.p.) a COX-1 blocker, significantly impaired the carbachol-evoked ACTH and corticosterone secretion, while compound NS-398, (2 mg/kg i.p.), a selective COX-2 blocker, did not alter the carbachol-induced ACTH secretion and moderately decreased corticosterone secretion.

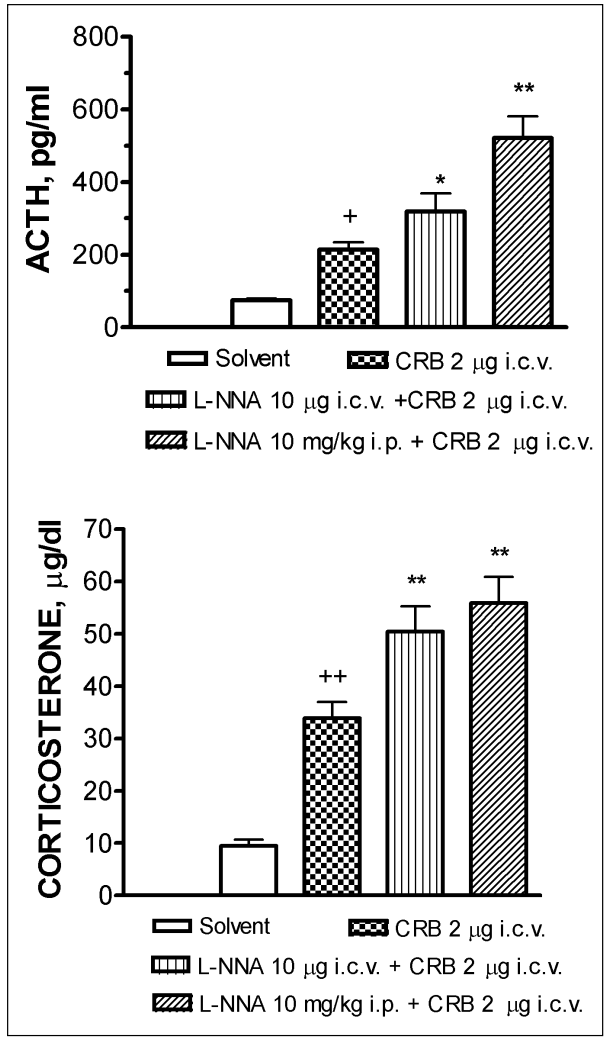

Fig 1. The increase in the i.c.v.-carbachol-induced plasma ACTH and serum corticosterone levels by nNOS antagonist, L-NNA, under basal conditions. In *Fig. 1-7* the rats were decapitated 1 h after the last agonist injection. Values represent the mean ± SEM of 5-7 rats. +$p<0.05$ and ++$p<0.01$ vs. saline control; $_{*}$ $p<0.05$ and $_{**}$ $p<0.01$ vs. carbachol (CARB) - treated group.

In rats crowded for 7 days, piroxicam and compoud NS-398, substantially diminished the carbachol-induced ACTH and corticosterone response *(Fig. 3)*.

Effect of social stress on the PGE_2-induced HPA response

Prostaglandin E_2 (10 μg) administered i.c.v. considerably increased plasma ACTH and corticosterone levels in non-stressed rats. Prior crowding stress for 3 days considerably impaired the PGE_2-induced ACTH response by 30%, and did not alter significantly corticosterone secretion as compared with the responses in non-stressed rats *(Fig. 4)*.

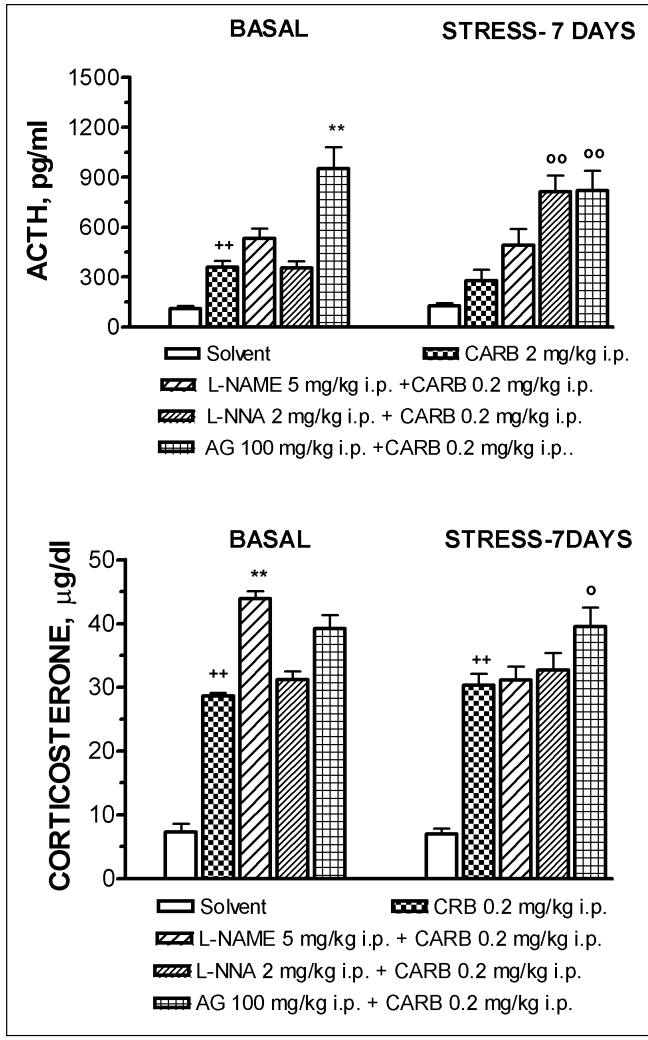

Fig. 2. The stimulatory effects of NOS blockers, L-NAME, L-NNA and aminoguanidine (AG) given 15 min. before CARB on the CARB-induced ACTH and corticosterone secretion. ++$p<0.01$ vs. saline control; ** $p<0.01$ vs. CARB -treated group; o $p<0.05$ and oo $p<0.01$ vs. CARB-treated stressed group;

Effect of CRH antagonist on the PGE_2-induced HPA response under basal and stress conditions

In order to elucidate if CRH is involved in the activation of HPA response to i.c.v. administered PGE2, CRH antagonist αhelical CRH 9-41 was given i.c.v. 15 min prior to PGE2. Under basal conditions CRH antagonist (0.1 and 10 μg) did not alter the PGE2-induced corticosterone secretion (Fig. 5). Crowding stress for 3 days markedly diminished (18.4%) the corticosterone response to i.c.v. PGE_2. In these rats CRH antagonist did not alter the PGE2-induced serum corticosterone levels (Fig. 5). These results suggest that hypothalamic CRH is not involved in the PGE_2-induced central stimulation of HPA axis in either basal or social stress conditions.

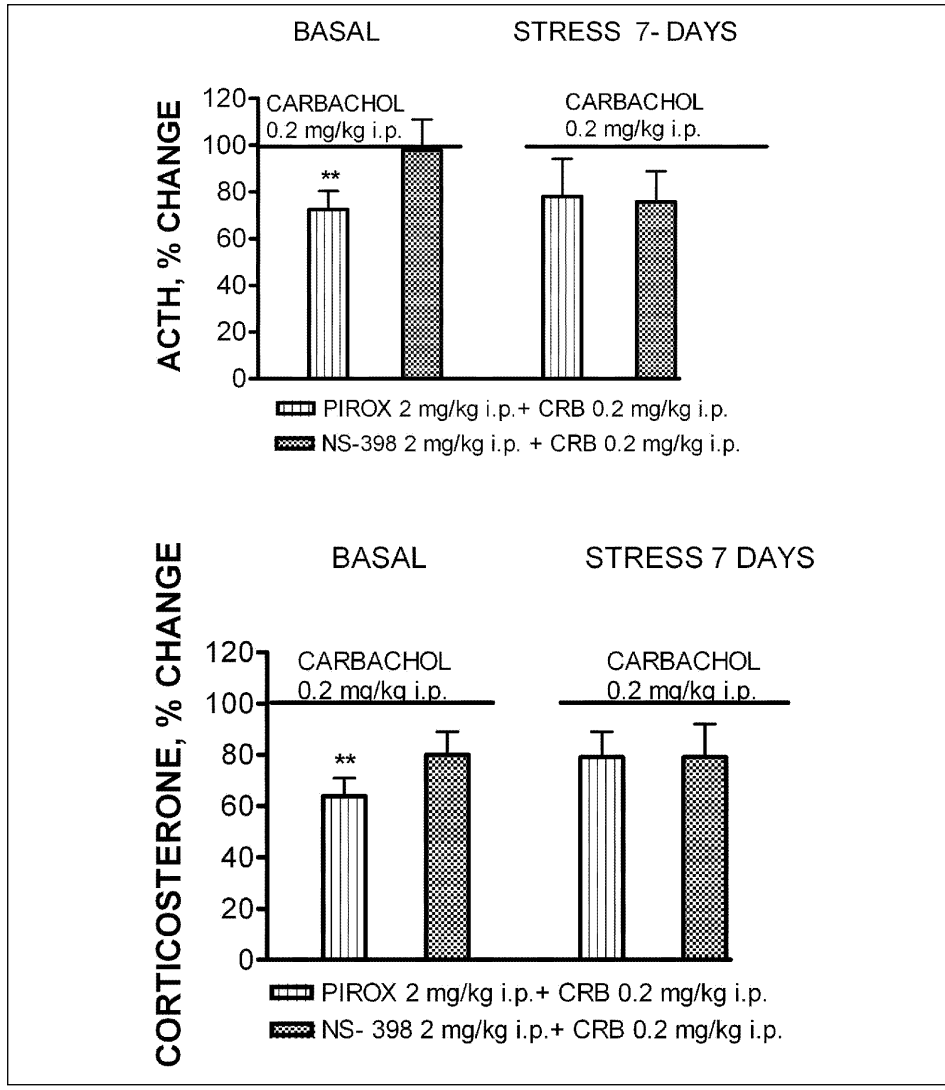

Fig. 3. Effect of piroxicam (PIROX) and compound NS-398 on the CARB-induced ACTH and corticosterone secretion in control and crowded rats. PIROX and NS-398 was injected 15 min. before CARB; ** $p<0.01$ vs. CARB -treated group.

Endogenous NO affects the PGE_2-induced responses under basal and stress conditions

In order to determine if, and to what extent, NO may influence the PGE_2-induced increase in the HPA activity, endogenous NO level was altered by either the inhibition or the increase its synthesis by pretreatment with general NOS

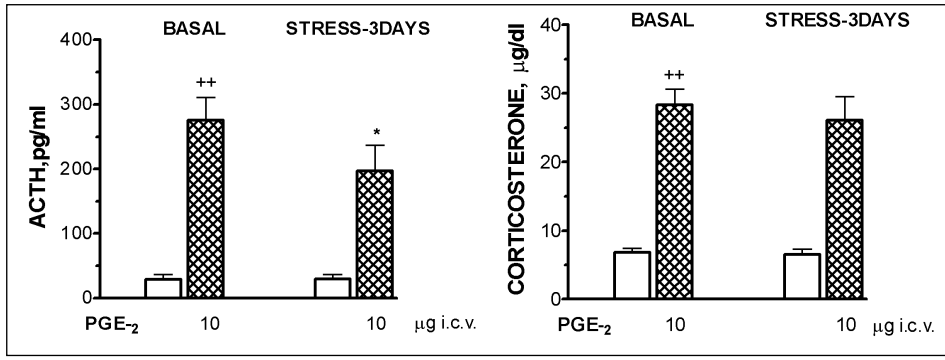

Fig. 4. Effect of i.c.v. prostaglandin E_2 (PGE_2) on ACTH and corticosterone secretion in control and crowded rats. ++$p<0.01$ vs. saline-treated group and * $p<0.05$ vs. PGE_2-treated control, non-stressed group.

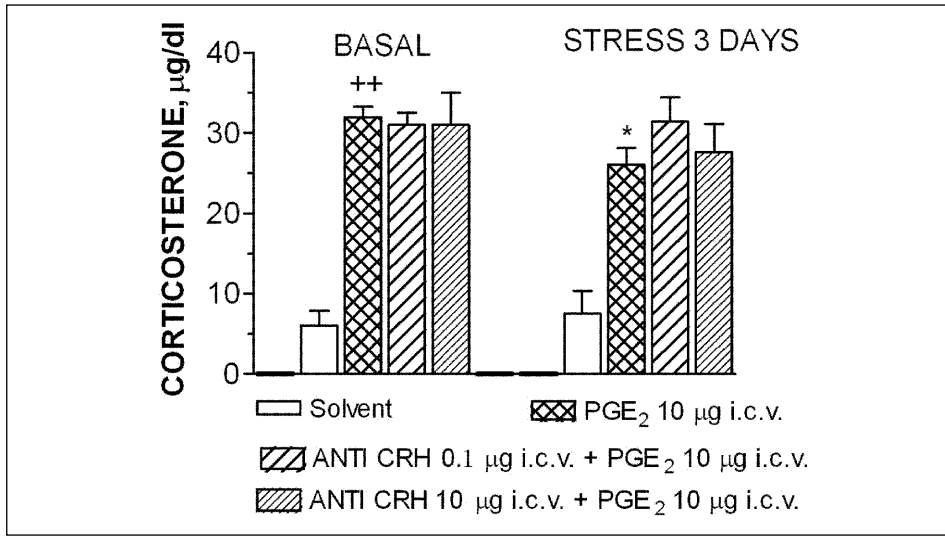

Fig. 5. Effect of i.c.v. CRH antagonist (ANTI CRH) on the i.c.v. PGE_2-induced corticosterone secretion in control and crowded rats (3 days). ++$p<0.01$ vs. saline-treated group; * $p<0.05$ vs. PGE_2-treated control group.

inhibitor, L-NAME (10 μg i.c.v.) or L-Arginine (120 mg/kg i.p.) respectively, before PGE_2 (10 μg i.c.v.). Under basal conditions L-NAME considerably intensified the PGE_2-induced significant increase in ACTH and corticosterone secretion. The stimulatory effect of PGE_2 on ACTH and corticosterone secretion in control rats was greatly reduced by pretreatment with L-Arginine (120 mg/kg i.p.) the endogenous substrate of NO synthesis. In crowded rats the PGE_2–induced

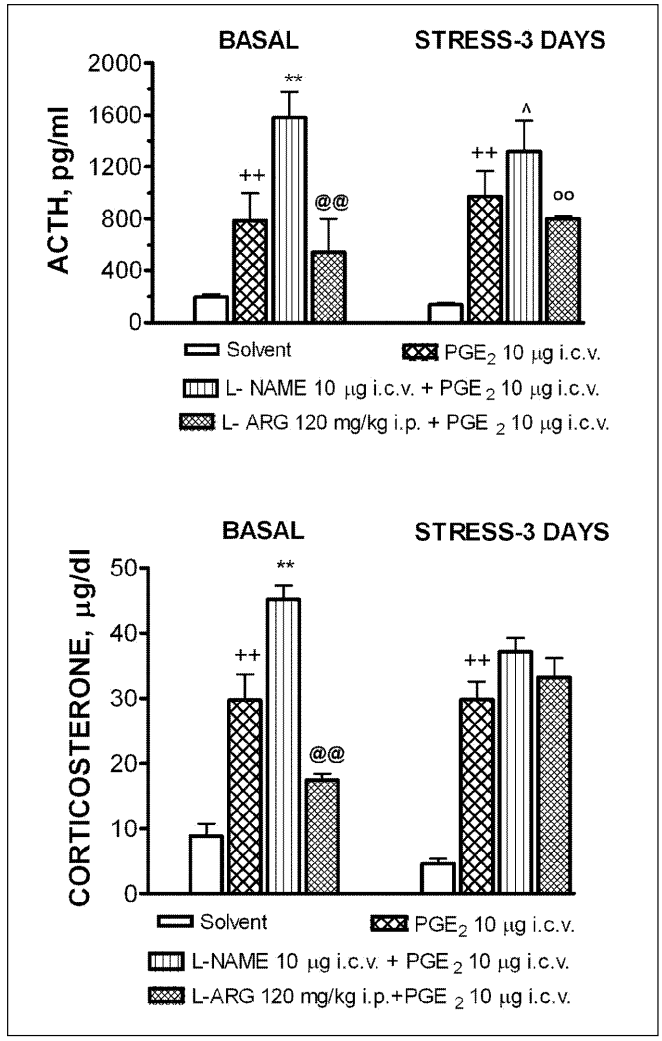

Fig. 6. Effects of general NOS antagonist i.c.v., L-NAME, and NO substrate, L-Arginine (L-ARG), on the i.c.v. prostaglandin E_2-induced ACTH and corticosterone secretion in control and crowded rats. L-NAME was given 15 min prior and L-ARG 30 min before PGE_2. ++$p<0.01$ vs. saline-treated group; ** $p<0.01$ vs. PGE_2-treated group; @@ $p<0.01$ vs. PGE_2-treated group; ^ $p<0.05$ vs. PGE_2-treated stressed group; and oo $p<0.01$ vs. PGE_2-treated stressed group.

ACTH response was significantly decreased by L-Arg and corticosterone response remained unchanged *(Fig. 6)*.

Effect of L-NNA and indomethacin on the carbachol-evoked corticosterone response

The carbachol-induced significant increase in corticosterone secretion was considerably intensified by i.c.v. pretreatment with nNOS inhibitor, L-NNA (10 μg i.c.v.) and was significantly reduced by pretreatment with indomethacin (2 mg/kg i.p.). After combined treatment with L-NNA and indomethacin the

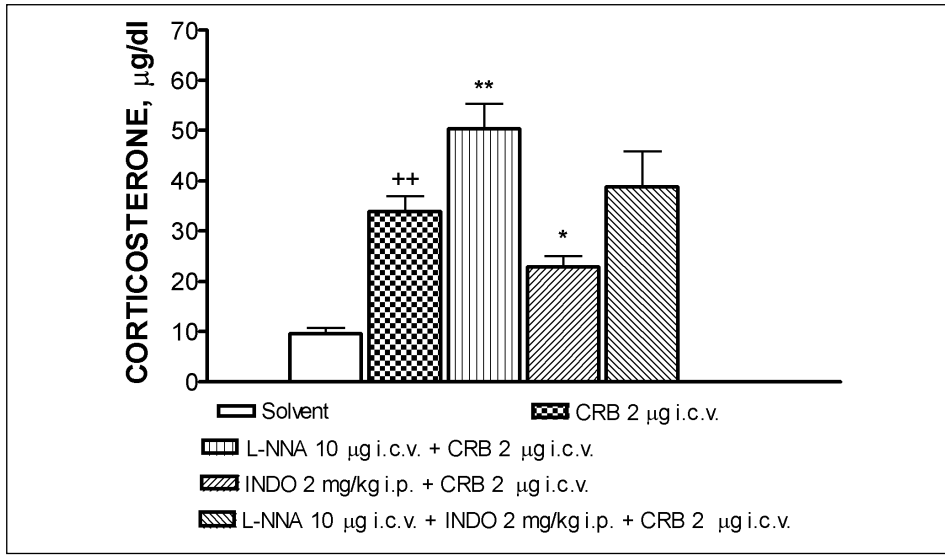

Fig. 7. Effect of L-NNA and indomethacin (INDO) separately and concurrently on the i.c.v. carbachol-induced corticosterone secretion. ++$p<0.01$ vs. saline-control group; * $p<0.05$ and **$p<0.01$ vs. carbachol-treated group.

carbachol-induced corticosterone levels did not markedly differ from the carbachol alone-evoked level *(Fig. 7)*.

DISCUSSION

We have shown earlier that carbachol administered into the lateral cerebral ventricle, stimulates central HPA responses via selective activation of cholinergic muscarinic receptors (4). Carbachol does not stimulate cholinergic nicotinic receptors that activate central structures involved in activation of HPA axis (5). Central nervous system cholinergic activity is stimulated in human by centrally active cholinesterase inhibitor physostigmine (22) which enhances ACTH and cirtisol response. Neostigmine administered into the third cerebral ventricle stimulates ACTH secretion directly throught central cholinergic muscarinic receptors and may also affect hypothalamic neuronal monoaminergic systems (23).

The present results show that nitric oxide is involved in the carbachol-induced HPA response. Under basal in vivo conditions in rats neither nitric oxide synthase inhibitors nor endogenous NO synthesis substrate L-arginine, given alone, exerted any marked effect on plasma ACTH and serum corticosterone levels (data not shown). The present data show a significant involvement of endogenous NO in central cholinergic muscarinic stimulation of HPA axis by i.c.v. administration

of carbachol. Constitutive, endothelial and neuronal NO synthase blockers used in the present study were able to significantly increase the carbachol-induced ACTH and corticosterone response, similarly like they increased the nicotine-induced responses (24, 25). In the present experiment preferentially neuronal NOS blocker, L-NNA given i.c.v. or i.p., significantly enhanced the carbachol-induced HPA response. Acetylcholine-induced CRH release from the hypothalamus and the amygdala, the structures known to contain high levels of CRH (26), is antagonized by cholinergic blockers atropine and mecamylamine and also by preferentially general NOS blocker, Nω– methyl-L-arginine, indicating the involvement of NO-mediated signaling in the acetylcholine-induced CRH release in central structures regulating HPA axis activity.

In the rat brain NOS gene is expressed in cholinergic neurons and colocalized with choline acetyltransferase (15). The inhibitor of neuronal NOS, L-NNA diminishes the release of ACh from the perfused basal forebrain. Nitric oxide is evidently involved in the cholinergic neurotransmission in multiple brain regions. The presence of NOS in perikarya of the hypothalamic nuclei closely associated with regulation of the pituitary activity, as well as in the median eminence and the pituitary itself, indicate that NO might play a physiological role in regulating the secretion of hypothalamic and pituitary hormones.

In our experiment constitutive and inducible NOS antagonists increased the carbachol-induced ACTH and corticosterone response in rats crowded for 7 days like in non-stressed animals. This finding suggests that social crowding stress did not substantially affect the NOS/NO system stimulated by cholinergic muscarinic receptors under basal conditions. Aminoguanidine, an iNOS blocker, induced pronounced increase in the carbachol-evoked ACTH and corticosterone secretion in stressed rats, suggesting that iNOS sytem was not markedly altered by crowding stress in the present experiment. Nitric oxide has been shown to activate soluble guanylate cyclase, leading to increases in cyclic GMP levels and cyclooxygenase enzymes, resulting in an increase in prostaglandins synthesis. In particular, NO mediates the release of prostaglandin E_2 induced by noradrenaline in the hypothalamus. The CRH-releasing activity of interleukin-1 in the hypothalamus has been reported to involve prostaglandins action (27, 28). Therefore the CRH secretory action of interleukin-1 might involve the production of NO either as an independent/parallel mechanism of prostaglandin activation or as a previous step mediating prostaglandin formation.

Crowding stress may evoke desensitization of intracellular signal transduction activated by muscarinic receptors by their phosphorylation. The phosphorylation of acetylcholine receptor by the various protein kinases: C, A and tyrosine kinase, appears to be the major regulator of the desensitization of these receptors to their agonists (29). In the present experiment crowding stress for 3 days significantly impaired the i.c.v. PGE_2-induced ACTH response suggesting marked desensitization of central components in the PGE_2-induced stimulation of HPA axis. The magnitude of this impairment was comparable to that induced by stress

in the carbachol-treated rats suggesting the involvement of PGE_2 in desensitization of HPA to the carbachol-induced response in stressed rats.

In our experiment α-helical CRH 9-41, a CRH receptor antagonist (0.1-10 μg i.c.v.) did not alter the PGE_2-induced corticosterone response in rats under either basal or social stress conditions. This suggests that CRH does not mediate the PGE_2-induced HPA response. Intracerebroventricular injection of α-helical CRH 9-41 (10 μg), significantly attenuated a mild cage-switch stress-induced circulatory responses but it did not affect these responses induced by central PGE_2 or i.p. IL-1β administration (30).

Interleukin 1β and IL-6 produce increases in the release of PGE_2 from hypothalamic tissue *in vitro* and *in vivo* which, on a temporal basis, parallel the release of CRH and AVP. We found that the IL-1β-induced increase in ACTH and corticosterone response was considerably dependent on the inhibition of endogenous NO synthesis by L-NAME both under basal conditions and after 7 days of crowding stress (31). In the rat increases in ACTH secretion evoked by systemic injections of IL-1β are effectively abrogated by pretreatment with antisera to CRH which indicates the involvement of CRH in that stimulation. At the hypothalamic level PG exert a powerful stimulatory influence on the release of CRH *in vitro* (32). Also *in vivo* acetylcholine stimulates the CRH gene expression in the hypothalamic PVN (23) and NO appears to have enhancing effect on CRH expression.

In the present experiment the i.c.v. PGE_2-induced increase in ACTH and corticosterone secretion under basal conditions was considerably intensified by i.c.v. pretreatment with L-NAME (10 μg). By contrast, systemically administered L-Arginine (120 mg/kg) significantly lowered the i.c.v. PGE_2-induced increase in ACTH secretion, and moderately decreased corticosterone secretion. This finding suggests that NO in brain structures involved in HPA axis regulation is markedly involved in the PGE_2-induced increase in HPA response. This indicates that under basal conditions NO impairs the PGE_2–evoked stimulation of HPA axis. Crowding stress (3 days) moderately diminished both the inhibitory action of NO and reciprocal effect of L-Arginine on the PGE_2-induced ACTH and corticosterone secretion. These results suggest that the role of NO in central stimulation of HPA axis by PGE_2 resembles similar effect of NO in the stimulation of HPA axis by muscarinic receptor agonist carbachol. Furthermore, they indicate the involvement of prostaglandins in the stimulatory action of HPA axis by carbachol, and the inhibitory effect by nitric oxide.

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