The present study was designed to determine the involvement of nitric oxide (NO) and prostaglandins (PG) in the stimulatory action of clenbuterol, a selective β₂-adrenergic receptor agonist on hypothalamic-pituitary-adrenal (HPA) axis under basal and social crowding stress conditions. Clenbuterol given i.c.v. (10 µg) or i.p. (0.2 mg/kg) considerably increased ACTH and corticosterone secretion. A selective β₂-receptor antagonist compound ICI 118551 and non-selective β-receptor antagonist propranolol given by either route reduced the stimulatory action of clenbuterol. Crowding stress (21 rats in a cage for 7) for 3-7 days significantly reduced the i.c.v. clenbuterol-induced ACTH and corticosterone secretion and i.p. clenbuterol-elicited ACTH secretion. L-NAME, mainly endothelial nitric oxide synthase (NOS) blocker, stronger than L-NNA, a neuronal NOS blocker, reduced the clenbuterol-evoked ACTH and corticosterone secretion in control rats but did not significantly alter this secretion already reduced by crowding stress. Piroxicam, predominantly constitutive cyclooxygenase (COX-1) inhibitor, given i.p. significantly diminished the i.p. clenbuterol-induced ACTH and corticosterone secretion in control rats and tended to reverse the reduction of ACTH secretion by crowding stress. These results indicate that clenbuterol, a selective β₂-adrenoceptor agonist, is much stronger stimulator of the HPA axis than isoprenaline, a non-selective β-receptor agonist. Social crowding stress reduces to a larger extent the HPA response to β₂-receptor stimulation. Likewise, in the HPA axis stimulation via β₂-adrenoceptors endogenous NO and prostaglandins are significantly involved. Beta₂-adrenoceptor is a dominant functional subtype of β-receptor in the stimulatory and modulatory signals regulating the HPA axis activity under basal and social stress conditions.

Key words: clenbuterol, β₂-adrenergic receptors, stimulation of HPA axis, social stress, NO, PG
INTRODUCTION

Clenbuterol is recognized and used as a selective β₂-adrenergic agonist in both \textit{in vitro} and \textit{in vivo} studies. As a lipophilic compound, clenbuterol easily crosses the blood-brain barrier and may penetrate central brain structures in which β₂-adrenoceptors were found (1). Given peripherally once, or daily over few weeks, as an anabolic agent to animals, it is known to stimulate corticosteroids secretion from adrenal gland (1-3). Chronic administration of clenbuterol affects adrenal morphology and glucocorticoid receptor expression (4). In previous study we found that clenbuterol given i.c.v. strongly increased ACTH and corticosterone secretion 1 h later, suggesting the stimulation of β₂-adrenoceptors in central HPA axis structures (5). Several lines of evidence indicate that the increase in hypothalamic paraventricular nucleus (PVN) monoamines can be linked to the stimulation of the HPA axis. Noradrenaline is known to be crucial for the stimulation of CRH neurons in the PVN which play a major role in the release of ACTH from the anterior pituitary, which in turn leads to increased secretion of glucocorticoids.

Nitric oxide (NO) acts as a signaling molecule in the central nervous system and it participates in the regulation of neuroendocrine function. NO, as one of many participants with other signaling pathways acts at the cellular level via soluble guanylyl cyclase (sGC) which is implicated as the principal physiological receptor for NO in the CNS (6). Nitric oxide is involved in the regulation of monoaminergic neurotransmission (7, 8). Neuronal nitric oxide synthase (NOS) is most highly expressed in cell populations within the hypothalamic PVN (9). A major source of NO at the internal zone of the median eminence might be endothelial in origin rather than neuronal (10). Under \textit{in vivo} conditions both neuronal and endothelial NOS is involved in the NOS-inhibitor induced impairment in ACTH and corticosterone secretion (10, 11).

In freely moving, nonanaesthetized rats nitric oxide is involved in the HPA response to adrenergic and cholinergic receptor stimulation (12-14). In the central stimulation of HPA axis by i.c.v. isoprenaline, a non-selective β-adrenoceptor agonist, endogenous NO exerts a marked stimulatory influence (15). Nitric oxide also participates in the acute ACTH response to physico-emotional stressors in rats at the hypothalamus level (16). Restraint stress evokes a significant increase in nNOS mRNA in the paraventricular hypothalamic nucleus and in medial amygdaloid nucleus which follows the changes in gene expression of nNOS in brain structures related to stress reaction (17).

In the central nervous system prostaglandins (PG) are involved in the regulation of HPA activity in the hypothalamic paraventricular nucleus and medulla, the seat of neurons that project the PVN (18). The stimulatory action of adrenergic agonists on the anterior pituitary corticotroph receptor depends on, and may be modulated by prostaglandins. Our previous studies showed that indomethacin, a non-selective cyclooxygenase inhibitor, administered i.c.v. altered to different extent the responses of HPA axis to the stimulation by adrenergic
agonists that activate different subtypes of adrenergic receptors (19). Prostaglandins can also stimulate steroidogenesis directly in rat adrenal glands (20). Prostaglandins and nitric oxide are important signal transducers in the neurotransmitters and neurohormones regulatory functions of the HPA axis under basal and stress conditions (21). We found that prostaglandins generated by constitutive and inducible cyclooxygenase (COX-1 and COX-2) are not significantly involved in the HPA axis response to i.c.v. isoprenaline a nonselective β-adrenergic receptor agonist (5).

In the brain, the activation of monoamine systems is a major component of the stress response (22, 23). The noradrenergic neurons innervate the hypothalamic PVN and both α- and β-adrenoceptors regulate the secretion of CRH (24). Brain noradrenaline is a major alarm system in stress reactions and alterations in adrenergic receptors are of key importance during adaptation to stress (25). The effect of social stress on the secretory HPA axis response to selective β2-adrenoceptors stimulation has not been elucidated.

The aim of the present experiment was to determine and compare the stimulatory effect of clenbuterol, a selective β2-adrenoceptor agonist, given i.c.v. and i.p. under basal and social crowding stress conditions. We also investigated the involvement of nitric oxide and prostaglandins in the clenbuterol-induced stimulation of the HPA axis under basal and social crowding stress conditions in rats.

MATERIALS AND METHODS

Animals

Experiments were carried out on male Wistar rats weighing 190-220 g. The animals were maintained under standard 12 hour light-dark cycle, lights on at 8 a.m., and temperature 21±2°C, with free access to rodent chow and tap water. Rats were housed 6 per cage for at least 1 week acclimatization period before experimentation. For intracerebroventricular injections, the skulls of rats were prepared one day earlier under light ether anesthesia. The rats remained in their home cages until they were scheduled for treatment. The experiments were performed in accordance with bioethical requirements and were approved by the Local Bioethics Committee.

Experimental procedures

Rats were randomly allocated into required experimental groups: control rats left undisturbed and rats crowded for 3 or 7 days before treatment. All rats from a given cage were assigned to the same experimental group, control 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. Trunk blood samples were collected on ice in 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 -80°C until the time of assay.

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. Control and experimental groups of 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 cage until scheduled for treatment. The stressed rats were crowded in groups of 21 per cage of the same size for 3 or 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.

**Induction of ACTH and corticosterone secretion**

The secretion of ACTH and corticosterone was elicited by i.c.v. or i.p. administration of a selective β₂-adrenergic agonist clenbuterol (10 µg i.c.v. or 0.2 and 0.02 mg/kg i.p.).

General non-selective NOS inhibitor Nω-nitro-L-arginine methyl ester (L-NAME) and neuronal NO synthase (NOS) inhibitor Nω-nitro-L-arginine (L-NNA) were dissolved in sterile physiological saline and injected i.p. 15 min before clenbuterol. Inhibition of the constitutively expressed cyclooxygenase (COX-1) was induced by pretreatment of rats 15 min before clenbuterol with indomethacin 10 µg i.c.v. or 2 mg/kg i.p. or piroxicam (0.02 - 0.2 mg/kg i.p.). Indomethacin a non-selective COX inhibitor and piroxicam, a preferential COX-1 inhibitor, were prepared by sonification in 1% Tween solution and injected i.p. 15 min before clenbuterol. Required doses of the drugs were dissolved in solvent immediately before use and injected i.c.v. in a volume of 10 µl per rat or i.p. in a volume of 2 ml/kg.

**ACTH and corticosterone determinations**

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.

**Chemicals**

Clenbuterol hydrochloride, propranolol hydrochloride, ICI 118 551 hydrochloride, (±)-1-[2,3,(Dihydro-7-methyl-14-inden-4-yl)oxo]-3-[(1-methylethyl)amino]-2-butanol hydrochloride, L-NAME, L-NNA, indomethacin and piroxicam were purchased from Sigma.

**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**

**Effect of β-adrenergic receptor antagonists on the clenbuterol-induced ACTH and corticosterone response**

Clenbuterol, a selective β₂-adrenergic receptor agonist, given either intracebroventricularly (10 µg) or intraperitoneally (0.2 mg/kg), considerably increased ACTH and corticosterone secretion 1 h later. In order to determine the functional β₂-adrenergic selectivity of clenbuterol in central stimulation of HPA axis a selective β₂-adrenergic antagonist ICI 118551 (1 and 10 µg) was
administered i.c.v. 15 min before clenbuterol. This blocker in a dose of 1 µg considerably reduced the clenbuterol-induced ACTH secretion, by 66.3% and corticosterone secretion, by 47.8%. In a higher dose (10 µg) ICI 118551 elicited a weaker reduction in the clenbuterol-induced ACTH, (30.2 %) and did not affect corticosterone secretion (Fig.1A). A significant rise in ACTH and corticosterone secretion induced by i.p. clenbuterol (0.2 mg/kg) was dose-dependently reduced by pretreatment with propranolol (0.1 and 1.0 mg/kg) given by the same route. Propranolol (1.0 mg/kg i.p.) was able to almost totally suppress the clenbuterol-induced ACTH and corticosterone response, by 84.2 and 82.6%, respectively (Fig. 1B).

Clenbuterol-induced ACTH and corticosterone response in rats exposed to crowding stress

In rats crowded for 7 days the stimulatory action of clenbuterol (10 µg) given i.c.v. on ACTH and corticosterone secretion was significantly reduced, by 47 and 30%, respectively. The increase in ACTH secretion induced by clenbuterol (0.2 mg/kg) given i.p. was also strongly reduced (by 55%) in rats crowded for 3 days, whereas corticosterone secretion was not affected (-7%) in these rats, suggesting

\[ \text{ACTH, pg/ml} \]

\[ \text{CORTICOSTERONE, µg/dl} \]

\[ \text{ICI} \quad \text{CLEN} \quad 10 \quad 0.1 \quad 1 \quad 0.2 \quad 1 \quad 0.1 \quad 1 \quad 0.2 \quad 1 \quad \text{mg/kg i.p.} \]

\[ \text{PROP} \quad \text{CLEN} \quad 0.2 \quad 0.2 \quad 0.2 \quad \text{mg/kg i.p.} \]

\[ \text{SOLVENT} \]

\[ \text{++p<0.01 vs. saline control; *p<0.05 and **p<0.01 vs. clenbuterol-treated group.} \]
that corticosterone secretion from adrenal cortex in vivo does not exclusively depend on plasma ACTH levels.

Crowding stress for 3 days did not significantly affect ACTH and corticosterone response induced by a lower dose of clenbuterol (0.02 mg/kg i.p.), which elicited 4 times weaker stimulation of these hormones secretion compared to the secretion after 0.2 mg/kg dose of clenbuterol (Fig. 2).

**Fig. 2.** The effect of crowding stress on the clenbuterol (CLEN)-induced ACTH and corticosterone secretion. Clenbuterol was given i.c.v. or i.p. to rats crowded for 3 or 7 days (21 rats in a cage for 7). Changes are expressed as % of CLEN-induced values in non-stressed rats. **p<0.01 vs. Clenbuterol treated non-stressed group.**

**Fig. 3.** The effect of NOS blockers, L-NAME and L-NNA given 15 min before CLEN on the CLEN-induced ACTH and corticosterone secretion. ++p<0.01 vs. saline control; *p<0.05 and **p<0.01 vs. CLEN-treated group.
Effect of NOS inhibitors on the clenbuterol-induced ACTH and corticosterone secretion

In order to assess the involvement of endogenous NO the in central stimulatory mechanism of HPA axis by i.c.v. clenbuterol, the generation of endogenous NO was inhibited by i.c.v. pretreatment with non-selective NOS inhibitor L-NAME (2 and 10 µg) or preferential neuronal NOS inhibitor L-NNA (2 µg) 15 min prior to clenbuterol. L-NAME (2 µg) significantly inhibited, the clenbuterol-induced increase in ACTH secretion (45.1 %), and corticosterone secretion (33%). In a higher dose (10 µg i.c.v.) L-NAME also significantly diminished the ACTH response to i.c.v. clenbuterol (26.6 %) and slightly decreased the respective corticosterone secretion (6.7 %). Pretreatment with L-NNA (2 µg i.c.v.) markedly, though to a lesser extent than L-NAME, diminished the clenbuterol-elicited ACTH and corticosterone secretion, by 31.5 % and 15.2 %, respectively (Fig. 3).

Indomethacin-induced responses of clenbuterol-elicited ACTH and corticosterone secretion in crowded rats

Indomethacin a non-selective COX inhibitor given to non-stressed rats either i.c.v. (10 µg) or i.p. (2 mg/kg) 15 min before clenbuterol (10 µg i.c.v)
considerably and to similar extent, reduced the clenbuterol-induced ACTH secretion, by 53 and 58 %, and corticosterone secretion by 37.5 and 52 %, respectively. Indomethacin given i.p. elicited moderately stronger inhibitory effects than administered i.c.v. (Fig. 4).

Crowding stress for 7 days significantly impaired the i.c.v. clenbuterol (10 µg)-induced ACTH and corticosterone response, by 47 and 30%, respectively, in comparison with the clenbuterol-induced ACTH and corticosterone response in non-stressed rats. These diminished by prolonged stress hormone responses to clenbuterol were not significantly altered by pretreatment with indomethacin. In crowded rats given i.c.v. indomethacin (10 µg) moderately increased the clenbuterol-induced ACTH response and slightly diminished the corticosterone response. Likewise, indomethacin (2 mg/kg) given i.p. did not significantly alter the clenbuterol-induced responses in crowded rats (Fig. 4).

**Piroxicam-induced responses of clenbuterol-elicited ACTH and corticosterone secretion in crowded rats**

Piroxicam in a lower dose (0.02 mg/kg) given i.p. to non-stressed rats significantly impaired the clenbuterol (0.2 mg/kg i.p.)-induced ACTH secretion, by 38.6 %, and in a higher dose (0.2 mg/kg i.p.) it moderately decreased the clenbuterol-induced ACTH secretion (15.8 %). In either dose piroxicam did not

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**Fig. 5.** Effect of piroxicam (PIROX) on the CLEN-induced ACTH and corticosterone secretion in control and crowded rats. PIROX was injected 15 min before CLEN. ++p<0.01 vs. saline-treated group; **p<0.01 vs. CLEN-treated group. # #p<0.01 vs. nonstressed CLEN-treated group. ^ p<0.05 vs. stressed CLEN-treated group.
significantly alter corticosterone secretion induced by clenbuterol given i.p. This dissociation in the effect of piroxicam on corticosterone secretion in comparison with its influence on ACTH secretion might result from direct action of piroxicam on adrenal cortex in addition to the effect on plasma ACTH levels.

In rats with strong stress-induced desensitization of the ACTH and corticosterone responses to clenbuterol, piroxicam generally reversed these responses.

In a dose of 0.2 mg/kg i.p. piroxicam significantly increased the ACTH response to clenbuterol alone in these rats, by 71.4%. Crowding stress for 3 days did not markedly alter the clenbuterol-induced corticosterone response (-5.4 %) compared with non-stressed rats. Pretreatment with piroxicam (0.02 and 0.2 mg/kg i.p.) reversed the crowding stress-induced decrease responses by 57.1 % and 21.1 %, respectively (Fig. 5).

DISCUSSION

In the present experiment clenbuterol, a selective β₂-adrenoceptor agonist, given i.c.v. appeared to be a much stronger stimulator of HPA axis than isoprenaline, a non selective β-agonist. Although isoprenaline is considered as a combined β₁- and β₂-adrenoceptor agonist, or weaker β₂-receptor agonist, in some reactions β₁-adrenoceptors blockade and β₂-adrenoceptors down regulation elicited similar protective effect against isoprenaline-induced cardiac remodeling process (26). Isoprenaline acts as combined β₁- and β₂-adrenoceptor agonist in different brain structures, including the hypothalamus (27). In our experiment a selective β₂-adrenoceptors inhibitor, compound ICI 118551 (1 µg) given i.c.v. 15 min earlier also considerably reduced the i.c.v. clenbuterol-induced ACTH and corticosterone secretion by 66 % and 54.1 %, respectively.

Clenbuterol (0.2 mg/kg) given i.p. induced much higher increase in plasma ACTH level, up to 1497 pg/ml, than after i.c.v. administration (10 µg), up to 670 pg/ml. The strong clenbuterol-induced increase in ACTH and corticosterone secretion was almost totally reduced (84.2 % and 82.6 %, respectively) by i.p. pretreatment with propranolol (1 mg/kg) a non-selective β-adrenergic receptors blocker. These results suggest that clenbuterol given i.c.v. stimulates β₂-adrenergic receptors in brain structures involved in the regulation of central limb of the HPA axis.

Our present result indicates that social crowding stress desensitizes central mechanisms involved in the clenbuterol-induced ACTH and corticosterone secretion to a larger extent than by isoprenaline in our earlier study (28). This finding suggests that β₂-adrenergic receptors are predominantly involved in the stimulation and desensitization of β₂-adrenergic receptors which mediate HPA responses (29). Stress can desensitize the β-adrenoceptors system rapidly, most probably via receptor internalization and sequestration (30). During stress-induced desensitization, internalization of β₂-adrenergic receptors occur more
frequently though reduced expression of the receptor gene may also be involved (25). In our experiment considerable reduction in the clenbuterol-induced ACTH secretion occurred after 3 days and did not significantly alter after 7 days of crowding. This suggests that subcellular desensitization processes of β2-adrenoceptors persist for a longer time period. Our present and earlier data indicate that β2-adrenoceptors are predominant subtype of central β-receptors that strongly stimulate ACTH secretion under basal conditions and they are more susceptible for desensitization during social crowding stress. The corticosterone response to i.c.v. clenbuterol (10 µg) in crowded rats was diminished to a lesser extent than ACTH response and was not significantly altered when it was stimulated by i.p. administered clenbuterol (0.2 mg/kg). This may reflect the fact that corticosterone secretion induced by i.p. clenburetol depends not only from increased ACTH level (31) but also from a direct and/or indirect stimulation of β-adrenoceptors in the adrenal cortex (32). When clenbuterol in a low dose (0.02 mg/kg i.p.) moderately stimulated ACTH and corticosterone secretion, crowding stress for 3 days resulted in a slight increase in this secretion by (+10.4 %) and (+22 %), respectively. This result indicates that social crowding stress induces desensitization of the evidently higher HPA axis responses after clenbuterol (10 µg i.c.v. or 0.2 mg/kg i.p.) while it sensitizes a weak HPA axis response induced by low dose of clenburterol (0.02 mg/kg i.p.). Chronic stress in rats increased the density of β-adrenoceptors in different brain regions that may be connected with a sensitization of the β2-adrenoceptor-induced increase in HPA response to moderate stimulation by low dose of clenbuterol in the present experiment.

The stimulatory effect of clenbuterol (10 µg i.c.v.) on ACTH and corticosterone secretion under basal conditions was significantly reduced by i.c.v. pretreatment with a non-selective NO synthase blocker, L-NAME (2 and 10 µg). Likewise, pretreatment with neuronal NOS inhibitor, L-NNA (2 µg i.c.v.) significantly decreased the clenbuterol-induced ACTH secretion and moderately diminished corticosterone secretion. NOS is present in hypothalamic paraventricular nuclei which contains most of the CRH neurons that regulate neuroendocrine responses. Microinfusion of NO donor 3-morpholinosydnonimine into the PVN significantly released ACTH (33). Stimuli that affect pituitary hormone release can up-regulate nNOS expression. Activation of the HPA axis by stress or neurotransmitters can be attenuated by pretreatment with the NOS inhibitor L-NAME (34). Although neuronal NOS in the brain does not participate in some immunoendocrinological reactions (35) in our experiment nNOS inhibitor L-NNA (2 µg i.c.v.) resulted in a marked decrease of the clenbuterol-induced ACTH and corticosterone secretion. These decrease was weaker compared to the inhibition evoked by L-NAME, mainly eNOS inhibitor, which suggests that NO synthesized by both eNOS and nNOS participate in the central clenbuterol-induced stimulation of HPA axis activity.

The present results indicate that under basal conditions prostaglandins synthesized by constitutive cyclooxygenase (COX-1) are significantly involved in
the stimulation of ACTH and corticosterone secretion by i.p. clenbuterol since piroxicam, a preferential COX-1 inhibitor, significantly decreased the i.p. clenbuterol-induced ACTH secretion. The stimulation of β-adrenergic receptors increases intracellular cAMP levels and the synthesis of PG in different tissues and cultured coronary endothelial cells (36). The i.c.v. clenbuterol-induced considerable rise in ACTH and corticosterone secretion, was also significantly reduced by COX-1 inhibitor, piroxicam (0.2 µg i.c.v.) by 54 and 34 %, respectively, in our earlier study (5). In rats crowded for 3 days this blocker did not affect ACTH secretion already diminished by stress. The respective changes in corticosterone secretion did not parallel those in ACTH secretion, either in control or in stressed rats. The diminution of sensitivity of HPA axis to prostaglandins in rats exposed to social stress is probably due to desensitization of adenylate cyclase responses to β-adrenergic and PGs stimulation following exposure to stress, since cAMP is the main intracellular second messenger of both β-adrenergic agonists and PGE2 stimulation (37). Prostaglandin E$_2$ is known to induce heterologous desensitization of the β-adrenergic receptor system. Therefore the elevated levels of PGE$_2$ during stress may decrease the efficacy of β-adrenergic agonists in the stimulation of HPA axis observed in the present experiment.

The present results show that clenbuterol, a selective β$_2$-adrenergic receptor agonist, is much stronger stimulator of HPA axis activity than isoprenaline, a nonselective β-adrenoceptor agonist. Social crowding stress reduces considerably the HPA axis response to β$_2$-adrenoceptor stimulation. In the HPA axis stimulation via β$_2$-adrenoceptors endogenous nitric oxide and prostaglandins are significantly involved.

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