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EFFECT OF L-NAME, A SPECIFIC NITRIC OXIDE SYNTHASE INHIBITOR, ON CORTICOTROPIN-RELEASING HORMONE-ELICITED ACTH AND CORTICOSTERONE SECRETION

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This study was designed to determine the role of endogenous nitric oxide (NO) in the corticotropin-releasing hormone (CRH)-induced ACTH and corticosterone secretion, as well as possible involvement of hypothalamic dopamine and noradrenaline in that secretion in conscious rats. CRH given ip stimulated dose-dependently the pituitary-adrenocortical activity measured 1 h later. Dexamethasone (0.2 mg/kg ip) injected 1 h before CRH (1 μg/kg ip) totally abolished the CRH-elicited ACTH and corticosterone secretion, indicating a predominantly pituitary site of CRH-evoked stimulation. L-arginine (120 mg/kg ip) and Nω-nitro-L-arginine methyl ester (L-NAME 5—10 mg/kg ip) did not markedly affect the basal plasma ACTH and corticosterone levels. L-NAME given 15 min before CRH markedly, but not significantly, augmented the CRH-induced ACTH response, and enhanced more potently and significantly the corticosterone response. Pretreatment with L-arginine, a substrate for NOS, slightly diminished the CRH-induced ACTH response and considerably reduced the corticosterone response. L-arginine also significantly reversed the L-NAME-evoked increase in the CRH-induced ACTH and corticosterone secretion. L-NAME did not markedly alter the CRH-induced hypothalamic dopamine and noradrenaline levels, while L-arginine significantly increased noradrenaline level. However, those alterations were not directly correlated with the observed changes in ACTH and corticosterone secretion. These results indicate that in conscious rats NO plays a marked inhibitory role in the CRH-induced ACTH secretion and inhibits more potently corticosterone secretion. Hypothalamic dopamine and noradrenaline do not seem to be directly involved in the observed alterations in ACTH and corticosterone secretion.

Key words: *Corticotropin-releasing hormone, ACTH, corticosterone, nitric oxide, L-NAME, L-arginine.*

INTRODUCTION

Nitric oxide (NO) plays an essential role in modulating many neuroendocrine responses (1). NO is biosynthesized from the guanidino nitrogen of L-arginine by a constitutive or an inducible form of nitric oxide synthase (NOS). It is recently known that constitutive NOS is localized in hypothalamic

neurons which regulate the activity of the hypothalamic-pituitary-adrenal (HPA) axis. Particularly NOS is co-localized with CRH and AVP secreting neurons in the hypothalamic paraventricular nucleus (2, 3). This suggests that NO might play a physiological role in the response of this axis to neuropeptides, neurotransmitters (1, 4—6) and stressful stimuli (7, 8). However, the role of endogenous NO in mediating the release and/or the action of factors involved in regulation of the activity of the HPA axis remains controversial. In rat hypothalamic and anterior pituitary cell cultures *in vitro* a NOS inhibitor blocked the release of interleukin (IL)-1β induced corticotropin-releasing hormone and adrenocorticotropic hormone (ACTH) suggesting that secretion of these neurohormones may be mediated by NO (9). In incubated rat hypothalami the activation of constitutive NOS and the increase of NO release stimulated CRH secretion (10). On the other hand, NO appeared to directly and specifically inhibit the release of CRH from rat hypothalamic explants *in vitro* (11). Nitric oxide mediated IL-1β-induced increase in CRH secretion from perfused hypothalami *in vitro* (12). The blockade of NO activity by NOS antagonists significantly blunted ACTH release in response to noxious stimuli (7) and acute local inflammation (13). Inhibition of NOS activity significantly increased plasma corticosterone secretion in rats, suggesting an inhibitory role of NO in the corticosterone synthesis or secretion (14).

In order to clarify the role of endogenous NO in the CRH-induced stimulation of ACTH and in corticosterone secretion, we investigated the effect of Nω-nitro-L-arginine methyl ester, one of the most widely used potent, competitive inhibitors of all NOS isoforms, and that of L-arginine, a physiological substrate for endogenous NOS. Since NO may also affect the synthesis and release of central catecholamines (15, 16) which are essential factors in regulation the HPA axis (17), hypothalamic levels of dopamine and noradrenaline were also determined to examine if they may be involved in modulation of the HPA axis activity under those circumstances.

MATERIALS AND METHODS

Adult male Wistar rats weighing 200—240 g were used in all experiments. They were housed in groups of 7 in a room temperature of 20±2°C and a diurnal light cycle at least one week prior to experimentation. The rats were given free acces to commercial food and tap water. The animals were randomly assigned to one of the experimental group (6 animals each): the control injected ip with saline, the second group injected with graded doses of CRH, the third group received dexamethasone 1 h before CRH, the fourth group injected with N^G-nitro-L-arginine methyl ester (L-NAME) 15 min before CRH and the fifth group treated with L-arginine+L-NAME 15 min before CRH. CRH, L-NAME, L-arginine and dexamethasone (Sigma) were dissolved in saline immediately before use and injected ip in a volume of 0.2 ml/kg. Control rats were injected with the

same volume of saline. One hour after the last injection, the rats were decapitated immediately after their removal from the cage and their 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 −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 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.

For noradrenaline and dopamine determinations the rats were decapitated at the required time, their brains were quickly removed, placed on glass plates kept on ice and washed with ice-cold saline. The cerebella were descarded and the hypothalami were isolated and stored at −80°C until the further use. For an HPLC assay frozen hypothalami were put into approx. 10 vol. of ice-cold 0.1 M. $HClO_4$ containing 5 mM of ascorbic acid and 25 µg/l of 3, 4 dihydrobenzylamine (internal standard), then they were weighed and homogenized with an Ultra-Turrax homogenizer (10 s at 20000 rpm). The homogenates were centrifuged at 14000 × g and the supernatants were subsequently filtered out through 0.22 µm. RC-58 membranes (BAS MF-1 centrifugal microfilters). The filtrates were injected into the HPLC system. (A BAS-400 liquid chromatograph was used (BAS, USA), equipped with an LC4B/17AT electrochemical detector and 3 µm C_{18} Phase 2 analytical column (100 mm × 3 mm), coupled with 7 µm C_{18} guard column (15 mm × 3 mm). The mobile phase (36 mM citrate-28 mM phosphate buffer pH 3.5, containing 0.77 mM EDTA and 5% methanol) was pumped at 0.9 ml/min through the column thermostatted at 32°C. Separated sample components of dopamine and noradrenaline were detected at an oxidation potential of 0.8 V. All the reagents were of analytical grade (Merck, Germany and Sigma, USA).

The data are presented as mean ± SEM. The statistical significance of differences between groups was estimated by an analysis of variance, followed by individual comparisons with the Duncan test.

RESULTS

Corticotropin releasing hormone given ip in graded doses (0.1—10 µg/kg) resulted 1 h later in gradual increases in corticosterone responses (*Fig. 1*) On a molar basis, the ip CRH-induced corticosterone responses were stronger than those elicited by CRH given intracerebroventricularly (data not shown).

Effect of dexamethasone on the CRH-induced ACTH and corticosterone responses

CRH (1 µg/kg ip) significantly increased the plasma ACTH level from the basal 76.5 ± 18 to 287.5 ± 49 pg/ml, and the corticosterone level from 9.3 ± 0.7 to 26:7 ± 1.9 µg/dl. In order to determine whether the ip CRH-induced pituitary-adrenocortical responses were elicited by stimulation of pituitary

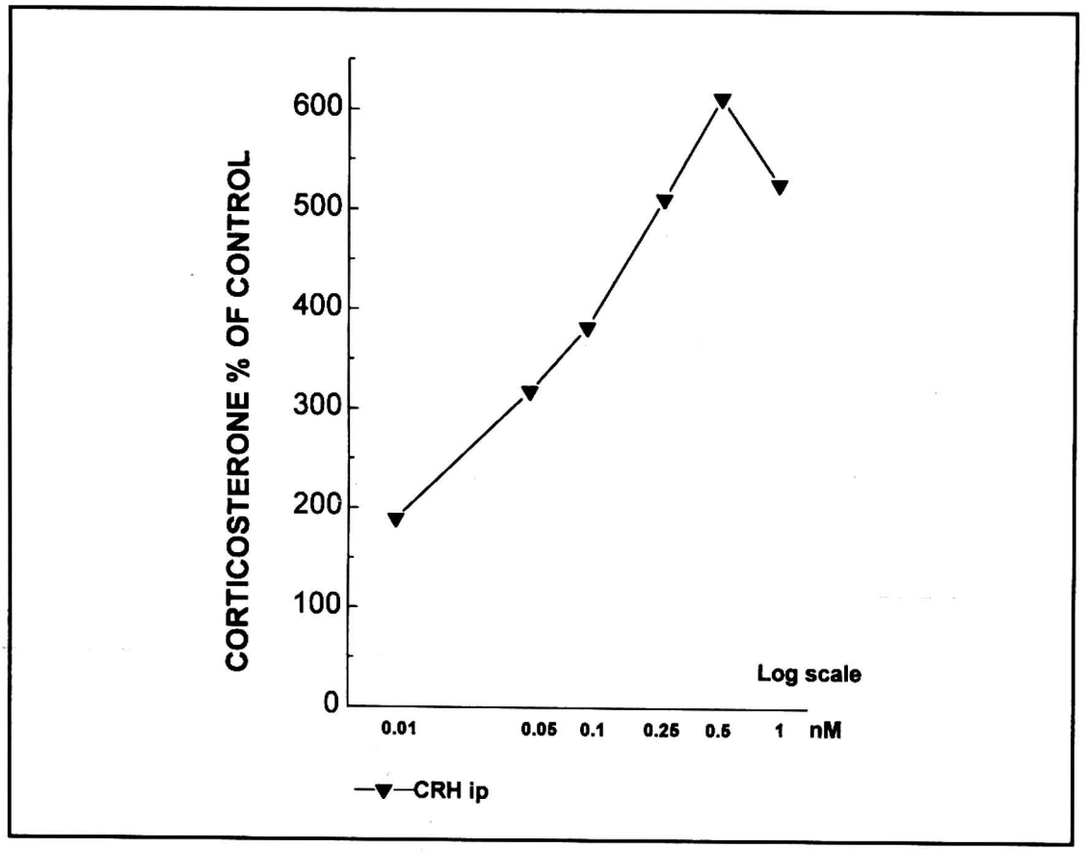

Fig. 1. Corticosterone secretion 1 h after ip administration of corticotropin-releasing hormone (CRH). Log dose-response values of 6 rats.

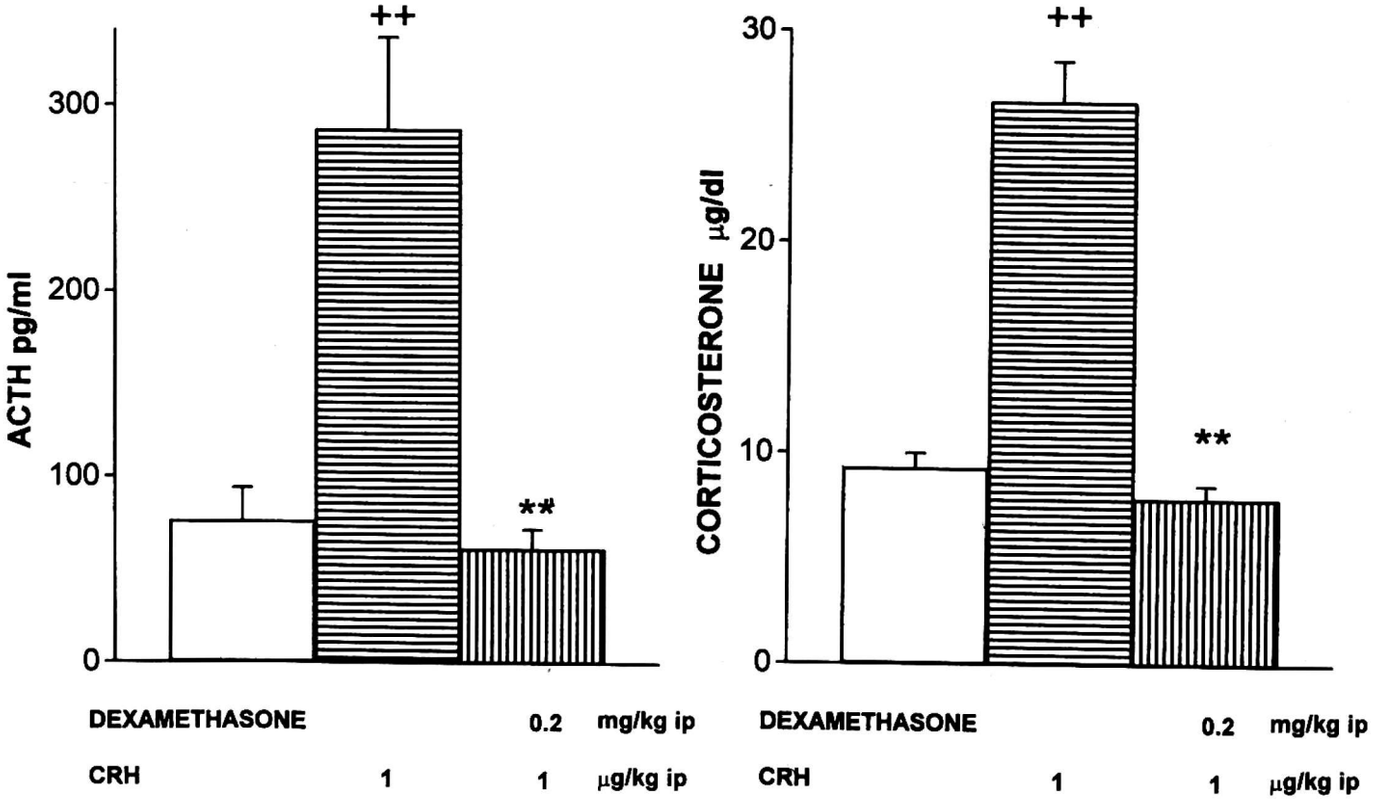

Fig. 2. Effect of dexamethasone on the CRH-induced plasma ACTH and corticosterone levels. Dexamethasone was injected ip 1 h before CRH and 1 h after the last injection the rats were decapitated. In *Fig. 2—4* values represent the mean ± SEM of 6 rats. $^{+}p < 0.05$ and $^{++}p < 0.01$ vs. saline controls, $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. CRH-treated group.

corticotrops, synthetic glucocorticoid dexamethasone was injected ip 1 h prior to CRH. Pretreatment with dexamethasone totally abolished both the ACTH and the corticosterone secretion induced by a subsequent injection of CRH. Dexamethasone (0.2 mg/kg) reduced the CRH-elicited ACTH level to 62 ± 10 pg/ml and the corticosterone level to 8.0 ± 0.6 (*Fig. 2*).

Effect of L-NAME on the CRH-induced ACTH and corticosterone responses

L-NAME (2, 5, 10 mg/kg ip) given alone in doses used in the present experiment did not markedly alter the plasma ACTH and corticosterone levels 1 h after administration (*Fig. 3*). In rats pretreated with these doses of L-NAME the CRH-induced ACTH level 184.7 ± 12.1 pg/ml was gradually raised to 276.9 ± 36.2 pg/ml; however, that maximum increase was not statistically significant. The CRH-elicited corticosterone level, 28.8 ± 1.4 µg/dl, was significantly increased, to 35.7 ± 2.9 µg/dl, by pretreatment with L-NAME, 5 mg/kg (*Fig. 4*).

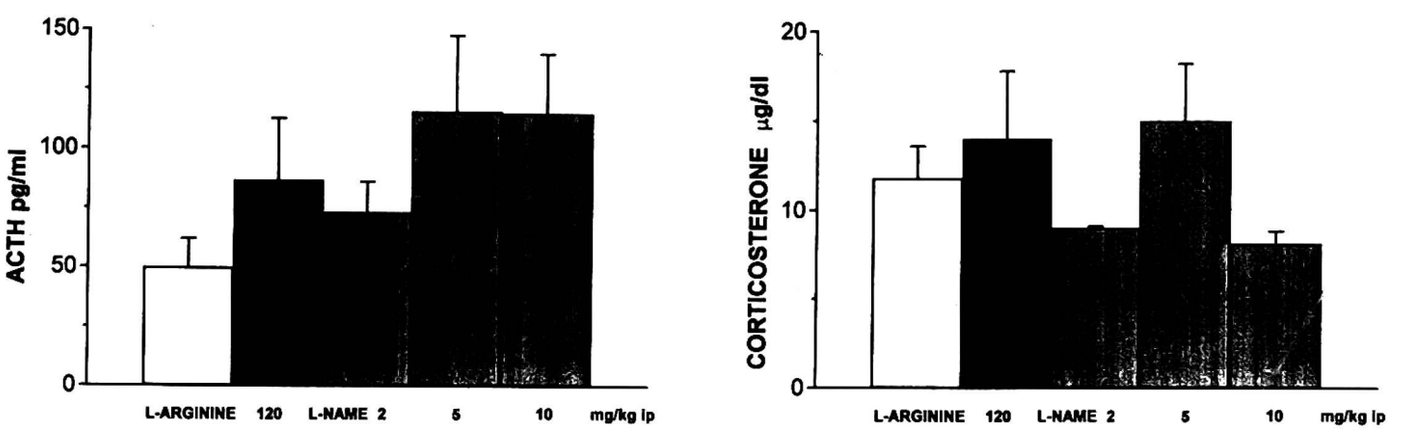

Fig. 3. Effect of L-arginine and L-NAME on basal plasma ACTH and corticosterone levels. See legend to *Fig. 2*.

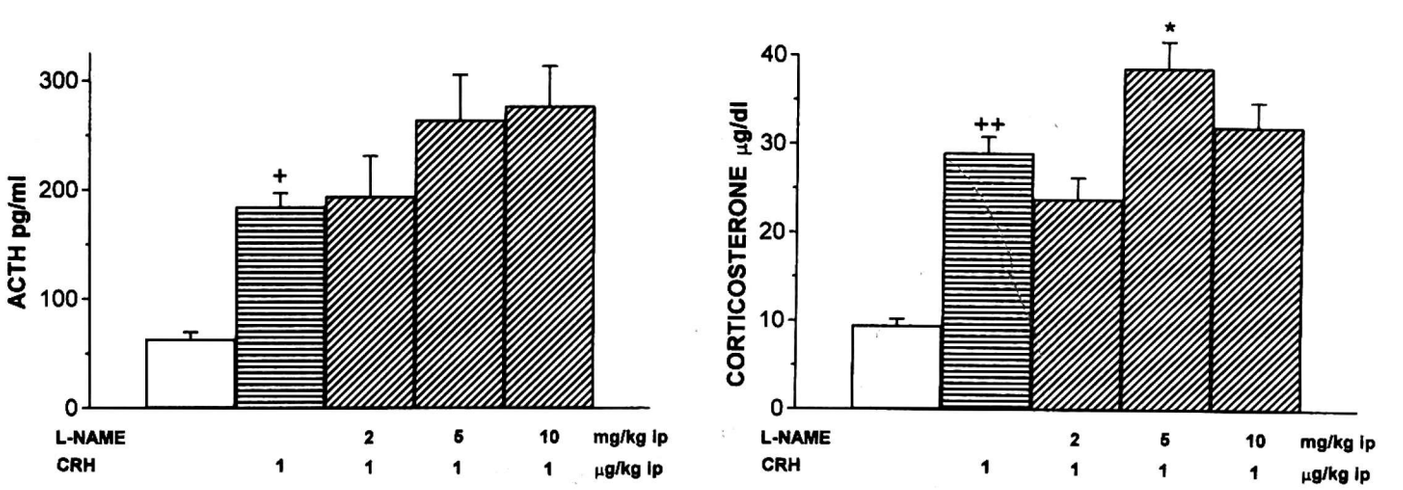

Fig. 4. Effect of L-NAME on CRH-induced plasma ACTH and corticosterone levels. L-NAME was injected ip 15 min before CRH. See legend to *Fig. 2*.

Effect of L-arginine and L-NAME on the CRH-induced ACTH and corticosterone responses

L-arginine (120 mg/kg ip) given alone did not significantly affect the basal plasma ACTH and corticosterone levels (*Fig. 3*). Injected 15 min before CRH L-arginine slightly diminished the CRH-induced ACTH response from 200.5 ± 24.1 to 170.4 ± 46.4 pg/ml but significantly decreased the CRH-elicited corticosterone response, from 28.7 ± 2.9 to 17.9 ± 1.8 µg/dl. In the dose used L-arginine reversed the stimulatory effect of L-NAME on both the CRH-elicited ACTH and corticosterone levels; L-arginine considerably diminished those levels from 299.3 ± 73.1 to 138.5 ± 26.8 and from 39.6 ± 3.9 to 19.1 ± 3.5, respectively (*Fig. 5*).

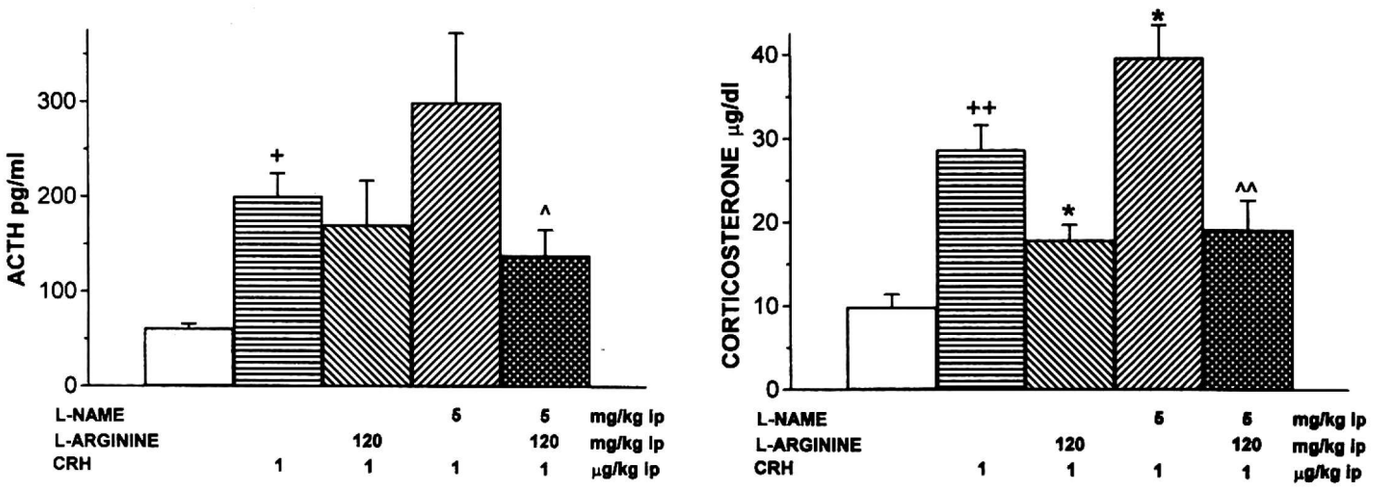

Fig. 5. Effect of L-arginine and L-NAME on CRH-induced plasma ACTH and corticosterone levels. L-arginine and L-NAME separately or together, were given 15 min before CRH. $^{\wedge}p < 0.05$ and $^{\wedge}{}^{\wedge}p < 0.01$ vs. CRH + L-NAME treated group. See legend to *Fig. 2.*

Effect of L-arginine and L-NAME on the CRH-induced hypothalamic catecholamine levels

Corticotropin-releasing hormone (1 µg/kg ip) did not significantly alter control hypothalamic noradrenaline (NA) and dopamine (DA) levels. Pretreatment with L-NAME (5 mg/kg ip) did not measurably affect the CRH-induced hypothalamic NA and DA levels. A higher dose of L-NAME (10 mg/kg ip) increased the CRH-induced NA level from 2436 ± 149 to 2732 ± 220 ng/g and diminished the corresponding DA level from 471 ± 111 to 309 ± 44 ng/g of hypothalamic tissue, however, those changes were not statistically significant. Pretreatment with L-arginine (120 mg/kg ip) significantly increased the CRH-induced hypothalamic NA level, from 2436 ± 149 to 2950 ± 73 ng/g. L-NAME prevented this action of L-arginine. Neither L-arginine nor L-arginine + L-NAME induced any significant effect on the hypothalamic CRH-evoked dopamine levels (*Table 1*).

Table 1. Effect of L-NAME and L-arginine on CRH-induced hypothalamic noradrenaline and dopamine levels.

TREATMENT	NORADRENALINE ng/g wet weight	DOPAMINE ng/g wet weight
Saline control	2541 ± 178	461 ± 115
L-NAME$_{5\,mg/kg}$ + CRH$_{1\,\mu g/kg}$	2458 ± 98	298 ± 14
L-NAME$_{10\,mg/kg}$ + CRH$_{1\,\mu g/kg}$	2732 ± 220	309 ± 34
L-arginine$_{120\,mg/kg}$ + CRH$_{1\,\mu g/kg}$	2950 ± 73 **	334 ± 44
L-arginine$_{120\,mg/kg}$ + L-NAME$_{5\,mg/kg}$ + CRH$_{1\,\mu g/kg}$	2616 ± 190	417 ± 36

L-NAME or L-arginine were injected separately or together ip 15 min before CRH. 1 h after the last injection the rats were decapitated, the brains were rapidly removed, hypothalami isolated and frozen at −70°C. Values represent the mean ± SEM of 6 rats. ** $p < 0.01$ vs. CRH-treated group.

DISCUSSION

The present results show that CRH given systemically dose-dependently increases the secretion of ACTH and corticosterone. Although after systemic administration CRH may directly stimulate corticosterone secretion in hypophysectomized rats or in patients with a proven deficiency in pituitary ACTH without inducing any rise in the level of circulating ACTH, (18, 19) in the presented experiment an increase in the plasma corticosterone level accompanied a parallel rise in ACTH secretion. This indicates that direct activation by CRH of anterior pituitary corticotrops is mainly responsible for release of ACTH and corticosterone secretion. CRH is known to stimulate directly the POMC gene transcription which, in turn, stimulates the ACTH synthesis and release from anterior pituitary corticotrops (20). A substantial penetration by CRH from peripheral circulation of central structures and activation of adrenergic system which stimulates ACTH secretion seems unlikely, since we did not find any marked adrenergic component of the pituitary-adrenocortical activation by ip CRH (21, 22). On a molar basis CRH injected ip was more potent in the pituitary-adrenocortical stimulation (data not shown) than after intracerebroventricular administration. Furthermore, pretreatment with dexamethasone (0.2 mg/kg ip 1 h before CRH) totally abolished the CRH-induced ACTH and corticosterone response in the present experiments. Dexamethasone exerts a negative feed-back control of the anterior pituitary ACTH secretion elicited by various secretagogues by its binding to intracellular steroid receptors and subsequent modulation of protein synthesis (23). Although dexamethasone may inhibit the release of ACTH from

hypothalamic neurons, its effect on the pituitary ACTH secretion is of greater significance in both humans an rats (24, 25). Dexamethasone is known to inhibit CRH-stimulated transcription of the proopiomelanocortin gene in anterior pituitary corticotrops (20).

In the present experiment L-NAME itself did not substantially affect either the plasma ACTH or corticosterone level under basal conditions, which suggests that endogenous NO is not activated in these circumstances in rats. In contrast, inhibition of the endogenous NO synthesis by L-NAME markedly augmented the CRH-induced ACTH and corticosterone secretion. However, the maximum increases in plasma ACTH level observed after doses of 5 and 10 mg/kg of L-NAME, (by 65 and 76%, respectively) were not statistically significant. A larger dose of L-NAME (20 mg/kg) also did not elicit any higher elevation of the CRH-induced ACTH response (data not shown). The maximum and significant increase in the CRH-evoked corticosterone response (by 46.7%) was induced by a dose of 5 mg/kg of L-NAME, while a larger dose (10 mg/kg) resulted in a minor increase (15%). The lack of a parallel increase in ACTH and corticosterone secretion is difficult to explain. Since a partial direct effect of CRH on adrenal steroidogenesis seems unlikely in the present experiments, our results suggest that the observed difference in corticosterone secretion may be releated to a different action of L-NAME in the doses used on adrenal blood flow rather than directly on adrenocortical cells (26). The present results indicate that the effect of L-NAME on the CRH-induced corticosterone response is more potent than on the ACTH response. A similar more potent effect of L-NAME on plasma corticosterone than ACTH level was observed in stressed rats (8).

In the present experiment L-arginine (120 mg/kg ip) moderately diminished the CRH-induced ACTH response and considerably reduced the corticosterone response. L-arginine also reversed the stimulatory effect of L-NAME on the CRH-induced increase in the plasma ACTH and corticosterone levels. These results as well as those obtained with L-NAME also indicate that endogenous NO affects more potently the CRH-induced corticosterone than the ACTH response. A relatively low level of NOS mRNA expression in the anterior pituitary (4) may be responsible for a weaker ACTH response to L-NAME and L-arginine in the present experiment.

We did not find any substantial adrenergic component of the pituitary-adrenocortical stimulation by systemically injected CRH, since neither adrenergic receptors nor central catecholamines markedly affected the CRH-elicited ACTH and corticosterone secretion (21, 22). On the other hand, NO was reported to affect neutransmitters such as catecholamines, which are involved in the control of the HPA activity (27). NO suppressed the release of both dopamine and noradrenaline from the incubated medial basal hypothalamus explants (16). In the present experiment CRH (1 μg/kg ip) did not

alter the hypothalamic noradrenaline or dopamine levels, which confirms our earlier data. Pretreatment with L-NAME (5 or 10 mg/kg ip) also did not markedly affect the catecholamine levels in the hypothalamus, suggesting that the observed concurrent changes in ACTH and corticosterone levels in rats were not substantially connected with alterations in the hypothalamic catecholamine levels. In agreement with the *in vitro* results, L-arginine (120 mg/kg ip) significantly augmented the CRH-induced hypothalamic noradrenaline, but not the dopamine level (16). That increase was accompanied with a moderate or significant decrease in the plasma ACTH and corticosterone levels, respectively, while opossite changes in those hormone levels would rather be expected. Therefore these data indicate that the L-arginine and L-NAME-induced alterations in the hypothalamic catecholamine levels do not correlate with changes observed in the plasma ACTH and corticosterone levels.

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