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# MEDIATION BY NITRIC OXIDE OF THE CARBACHOL-INDUCED CORTICOSTERONE SECRETION IN RATS

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Nitric oxide synthase, an enzyme responsible for nitric oxide (NO) formation has been found in the hypothalamic paraventricular nucleus and median eminence, structures closely associated with regulation of the pituitary activity, and the pituitary gland itself. Nitric oxide modulates the stimulated release of CRH from the rat hypothalamus *in vitro,* which suggests its role in regulating the secretion of ACTH from the pituitary corticotrops and of corticosterone from the adrenal cortex. The purpose of the present study was to elucidate the yet unknown role of endogenous NO in the HPA response to central cholinergic stimulation in conscious rats. Neither L-arginine an NO precursor, nor the NO synthase blockers Nω-nitro-L-arginine methyl ester (L-NAME) and Nω-nitro-L-arginine (L-NNA) caused any consistent changes in the basal serum corticosterone levels. L-arginine, given in higher doses (120—150 mg/kg ip) 15 min prior to icv carbachol (2 μg), markedly diminished the carbachol-induced rise in corticosterone secretion. Systemic pretreatment with the nitric oxide synthase inhibitor L-NAME (5 mg/kg) significantly raised the carbachol-elicited corticosterone response, while addition of L-arginine completely blocked the effect of L-NAME. A similar increase in the carbachol-induced corticosterone response was produced by icv pretreatment with L-NAME (2 μg), indicating a central site of the NO interaction with cholinergic stimulation of the HPA response. L-NAME is a weak inhibitor of neuronal NOS itself, and must first be de-estrified to Nω-nitro-L-arginine to potently inhibit this enzyme. Systemic (10 mg/kg) and icv (1 μg) pretreatment with L-NNA enhanced more effectively the carbachol-induced rise in corticosterone secretion than did pretreatment with L-NAME by either route. These results are the first direct evidence that endogenous NO significantly inhibits the HPA response to central cholinergic, muscarinic receptor stimulation under *in vivo* conditions.

Key words: *nitric oxide, nitric oxide synthase antagonists, central cholinergic stimulation, corticosterone*

## INTRODUCTION

Nitric oxide is regarded as a major and ubiquitous modulator of a variety of physiological reactions. In the central nervous system, this free radical gas acts as a diffusible intracellular signalling molecule (1 – 3). Nitric oxide is synthesized form L-arginine by NO synthase in a NADPH-dependent reaction. In central cholinergic neurons, NOS is colocalized with choline acetyltransferase, and L-NNA the inhibitor of NOS diminishes the release of ACh from the perfused basal forebrain (11, 12). Nitric oxide synthase has recently been identified in cholinergic parasympathetic neurons, and NO is regarded as a cotransmitter at cholinergic terminals (13). Nitric oxide is evidently involved in the cholinergic neurotransmission in multiple brain regions (14). Acetylcholine is known to release CRH from hypothalamic neurons.

The observations that NOS is present in perikarya of the hypothalamus, specifically in the nuclei closely associated with regulation of the pituitary activity (9, 10), as well as in the median eminence and the pituitary itself, suggest that NO might play a physiological role in regulating neuroendocrine functions. Nitric oxide is known to modulate the secretion of hypothalamic and pituitary hormones such as oxitocin, somatostatin, GH, prolactin and LH (11 – 13). Nitric oxide is also involved in modulation of the *in vitro* secretion of CRH from the rat hypothalamus and amygdala (14 – 16) and perfused hypothalami (17), or in the ACTH release from pituitary cell cultures (18). However, in the above *in vitro* experiments NO was found to either mediate the stimulation of CRH release by interleukin-2 (15, 16) or inhibit this stimulation by interleukin 1-β (14, 17).

In the present study we assessed the role of endogenous NO in the HPA activation by central muscarinic receptors in conscious rats under *in vivo* conditions.

## MATERIALS AND METHODS

Adult male Wistar rats (200—230 g) were housed in the animal room, 6 animals per cage, at a room temperature (20—22°C). They were kept under standard feeding and normal day-night cycle lighting conditions. Drugs were dissolved in saline immediately before use and injected i.p. in a volume of 0.2 ml/kg or they were administered into the right lateral cerebral ventricle in a volume of 10 µl to rats whose skulls were prepared 24 h earlier, under light ether anesthesia, for free- hand icv injections. L-arginine was injected ip 15 min before icv administration of carbachol, and the NOS inhibitors Nω-nitro-L-arginine methyl ester (L-NAME) and Nω-nitro-L-arginine (L-NNA) were administered ip or icv 15 min before carbachol. After injections of the drugs, the rats were returned to their cages and decapitated 1 h later. Control animals received simultaneously 0.2 ml or 10 µl of saline and were left undisturbed until decapitation, concurrently with the animals injected with the drugs. One hour after drug administration, the rats were killed by rapid decapitation, and their trunk blood was collected. After centrifugation, aliquots were frozen at −70°C until the assay. The concentration of corticosterone was measured fluorometrically. To avoid corticosterone

fluctuations due to the circadian rhythm, all experiments were performed between 9—11 h, and all decapitations took place between 11—12 h.

*Drugs used*: the arginine derivatives blocking the NO synthase activity, L-NAME and L-NNA, the NO precursor L-arginine, and carbamylcholine hydrochloride (carbachol) were purchased from Sigma.

## *Statistical analysis*

All results are eypressed as means ±SEM. Statistical probabilities were calculated by an analysis of variance, followed by individual comparisons with Duncan's test.

## RESULTS

### *Effect of L-arginine and L-NAME on the carbachol-induced corticosterone secretion*

Carbachol (2 μg), a cholinergic muscarinic receptor agonist administered icv significantly increased the serum corticosterone level, measured 1 h later. Neither L-arginine, a NO precursor, nor the NO synthase blockers L-NAME and L-NNA caused any consistent changes in the basal serum corticosterone levels. L-arginine (120 and 150 mg/kg ip), given 15 min prior to carbachol, markedly reduced, by 37% and 30% ($p. < 0.05$), respectively, the increase in corticosterone secretion evoked by carbachol, (*Table 1*) Blockade of the endogenous NO synthesis by L-NAME (1 and 5 mg/kg), a nitric oxide synthase inhibitor, injected ip 15 min before icv carbachol (2 μg), considerably intensified by 40% and 71%, respectively, the carbachol-elicited increase in the serum

*Table 1.* Effect of L-arginine on the carbachol induced corticosterone secretion

| Treatment | Dose | Corticosterone μg/dl |
|---|---|---|
| Saline | 0.2 ml | 6.0±0.3 |
| Carbachol | 2 μg | 24.2±5.9$^{++}$ |
| L-Arginine + Carbachol | 120 mg/kg +2 μg | 17.4±3.6 |
| | | |
| Saline | 0.2 ml | 9.8±1.6 |
| Carbachol | 2 μg | 36.4±4.2$^{++}$ |
| L-Arginine + Carbachol | 150 mg/kg +2 μg | 28.3±2.5 |

L-arginine was injected ip 15 min prior to icv carbachol. The rats were decapitated 1 h after the last injection. Values represent the means ±SEM of 5—7 animals. $^{++}$ $p. < 0.01$ vs. saline treated group.

corticosterone levels. That increase was totally abolished by addition of L-arginine (120 mg/kg) to L-NAME injection, leaving the carbachol-induced rise in the serum corticosterone level unaffected (*Fig. 1*). Similarly, icv pretreatment with L-NAME in a dose of 2 μg most effectively enhanced, by 64%, the carbachol-induced increase in serum corticosterone levels (*Fig. 2*).

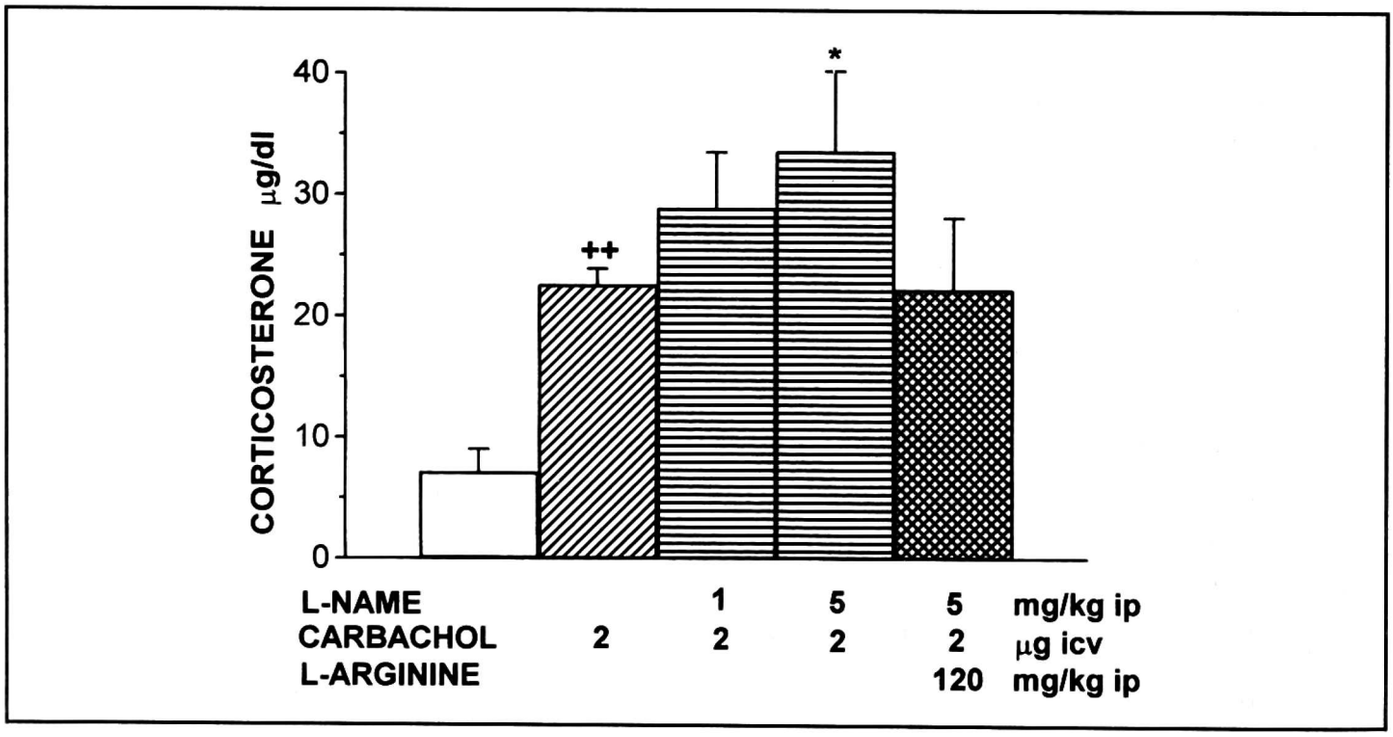


*Fig. 1.* Effect of L-NAME and L-NAME + L-Arginine on carbachol-induced serum corticosterone levels. L-NAME and L-Arginine, separately or together, were injected ip 15 min prior to carbachol. In Fig. 1–4 values represent the mean ±SEM of 6–7 animals. $^{++}$ $p < 0.01$ vs. saline control; * $p < 0.05$ and ** $p < 0.01$ vs. carbachol-treated group.

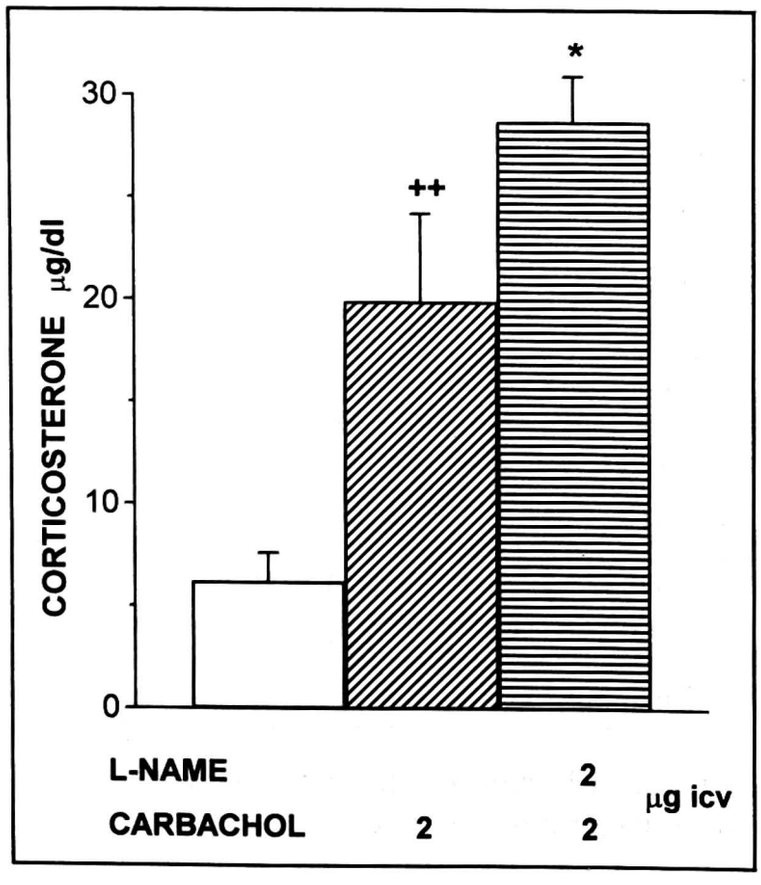


*Fig. 2.* Effect of L-NAME given icv on carbachol-induced corticosterone secretion. See legend to *Fig. 1.*

*Effect of L-NNA on the carbachol-induced corticosterone secretion*

Another NO-synthase blocker, L-NNA (1 and 10 mg/kg), injected ip 15 min prior to icv carbachol (2 μg), elicited much stronger and highly significant increase in the carbachol-induced rise in the serum corticosterone level by 97% and 158%, respectively, in comparison with that produced by L-NAME (*Fig. 3*).

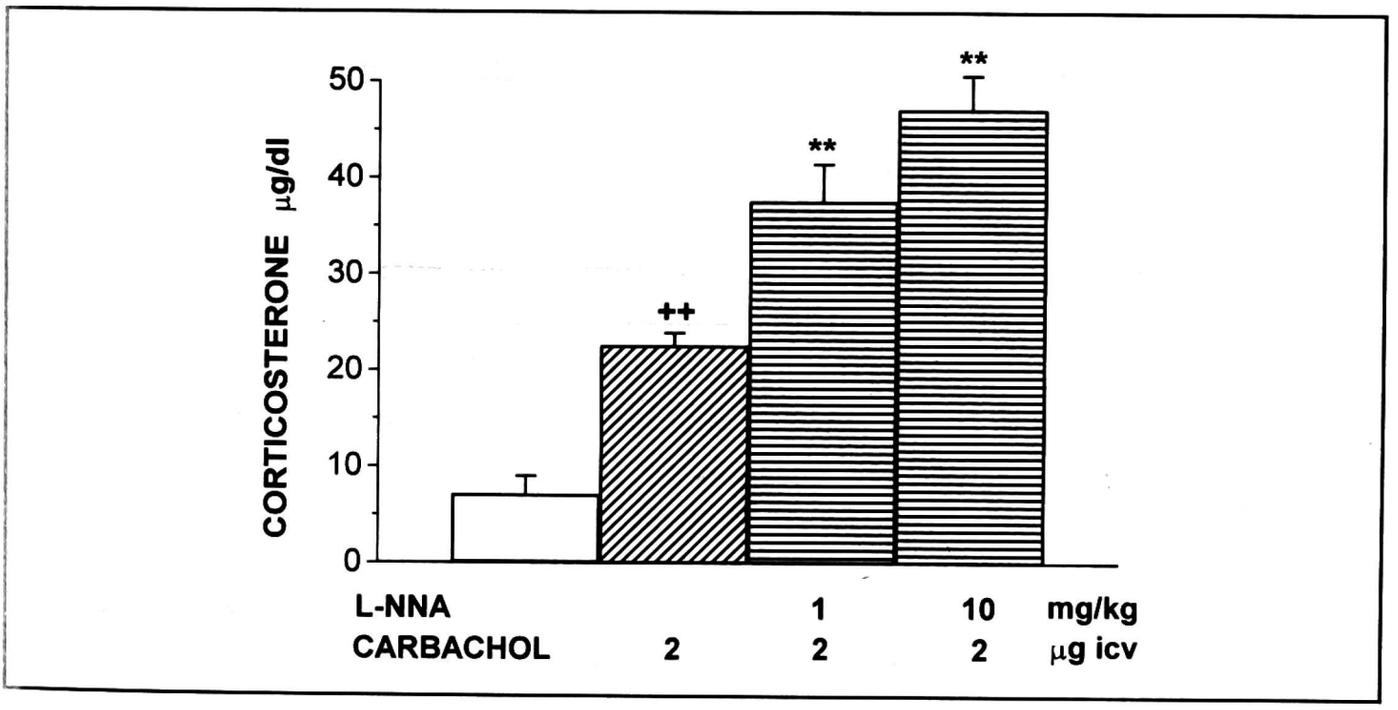


*Fig. 3.* Effect of L-NNA given ip on carbachol-induced corticosterone secretion. See legend to *Fig. 1.*

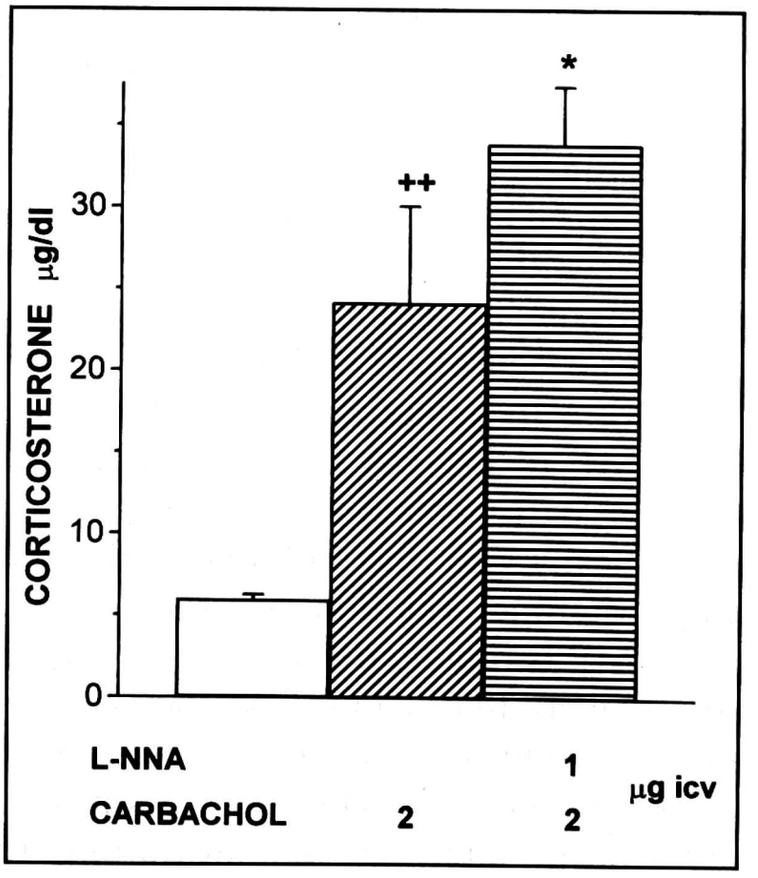


*Fig. 4.* Effect of L-NNA given icv on carbachol-induced serum corticosterone levels. See legend to *Fig. 1.*

Intraventricular pretreatment with L-NNA caused a significant increase in the carbachol-induced corticosterone response which was most pronounced, (by 53%) after a dose of 1 μg and resembled that induced by L-NAME (*Fig. 4*).

## DISCUSSION

We have shown that carbachol, a cholinergic agonist administered icv, activates dose- dependently the HPA axis by stimulating predominantly central muscarinic receptors (19). In the present experiment L-arginine, a NO precursor, did not substantially affect the basal corticosterone level 1 h after its ip administration. Also both the NOS inhibitors L-NAME and L-NNA, in doses used in this study, did not substantially influence the basal corticosterone levels (data not shown). Similarly, neither L-arginine nor L-NAME was able to affect the basal CRH release from rat hypothalamic explants. However, L-arginine significantly inhibited the release of CRH *in vitro* stimulated by nonspecific depolarization with potassium chloride or IL-1β (14). Only when used in high doses, L-NAME was able to induce a sustained increase in the serum corticosterone level in rats (20). The present results clearly show that in conscious rats endogenous NO potently modulates the activity of the HPA axis, elicited by stimulation of central cholinergic muscarinic receptors. In our experiment, pretreatment with L-arginine markedly diminished the corticosterone secretion evoked by icv administration of carbachol. This finding suggests that endogenous NO is an inhibitory mediator of the corticosterone secretion induced by stimulation of central cholinergic receptors. The results obtained with NOS inhibitors support this assumption since these compound have been found to augment the carbachol-induced rise in serum corticosterone levels.

Although L-NAME and L-NNA have similar *in vitro* potencies towards neuronal NOS, in the present experiment L-NNA administered systemically was evidently more effective than L-NAME in increasing the carbachol-induced rise in serum corticosterone levels. L-NAME has a ten fold lower affinity for neuronal NOS than does L-NNA, hence it has first to be de-estrified to L-NNA to potently inhibit nNOS (21—22). A slow rate of deestrification of L-NAME to L-NNA in the periphery, with a half-life of approximately 4 h, may in part explain the lower potency of L-NAME than L-NNA given systemically in the additive effect of the carbachol-induced rise in corticosterone secretion observed in our experiments. On the other hand, L-NAME is rapidly deestrified as soon as it crosses the blood-brain barrier. It seems likely that a lower potency of L-NAME compared to that of L-NNA may be connected with a slower rate of L-NMMA transport across the blood-brain barrier. This assumption is validated by the fact that in the present experiment, after icv administration, the potency of L-NAME does not

markedly differ from that of L-NNA in increasing the carbachol-elicited corticosterone response. Our results strongly suggest that both the NOS blockers, administered systemically or intracerebroventricularly, inhibit endogenous NO synthase which is present in the PVN where the majority of hypothalamic CRH neurons are located. Carbachol given icv can stimulate the HPA axis by activating cholinergic receptors on CRH neurons. When the CRH release *in vitro* is stimulated by depolarization of rat hypothalamic explants or by interleukin-1β (8, 9), local generation of NO may mediate this release. Our data indicate central attenuation of the carbachol-stimulated CRH secretion by NO, since the inhibiton of neuronal NOS by L-NAME and L-NNA consistently and effectively enhances the carbachol-induced corticosterone response.

The present results show for the first time that NO is significantly involved in mediation of the HPA response in conscious rats. They also suggest that the hypothalamus is a site of this interaction. An opposite effect was observed in experiments *in vitro*. Acetylcholine induced CRH release from hypothalamic and amygdala slices, that release being antagonized by L-NNA (15). Also the carbachol-induced secretion of CRH from the mediobasal hypothalamus was inhibited by NMMA, an NOS inhibitor. While *in vitro* NO stimulates the secretion of CRH our *in vivo* data show that endogenous NO inhibits the HPA activity elicited by cholinergic stimulation.

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