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NOTE

Nitric Oxide Plays No Role in ACTH Release Induced by Interleukin-1β, Corticotropin-Releasing Hormone, Arginine Vasopressin and Phorbol Myristate Acetate in Rat Pituitary Cell Cultures

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Abstract. To investigate the role of nitric oxide (NO) in pituitary ACTH secretion , the effect of a nitric oxide synthase (NOS) inhibitor, Nω-Nitro-L-arginine (Nitro-Arg), on ACTH secretion induced by interleukin (IL)-1β, corticotropin releasing hormone (CRH), arginine vasopressin (AVP) and phorbol myristate acetate (PMA) was examined in rat anterior pituitary cell cultures. Nitro-Arg did not affect IL-1β-induced ACTH release in pituitary cell cutures incubated with Dulbecco modified Eagle's medium, Basal Medium Eagle or Krebs Ringer bicarbonate-glucose buffer. It did not affect CRH-, AVP- or PMA-induced ACTH release either. These results suggest that NO does not play an important role in ACTH release at the pituitary level.

Key words: Nitric oxide, ACTH, Interleukin-1, Corticotropin releasing hormone

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NITRIC oxide (NO) is a highly reactive and labile free radical which is synthesized from guanidino nitrogen of L-arginine by NO synthase (NOS) [1]. It was first identified as a mediator of the cytotoxic effect of macrophages and has been shown to be identical to endothelium-derived relaxing factor [2]. NO stimulates guanylate cyclase to accumulate cyclic GMP in the cells [3], and it plays both a cytotoxic and a second messenger role. It is produced not only in the vascular endothelium but also in a various tissues and cells. There are two types of NOS, a constitutive and Ca^{2+}/calmodulin-dependent type NOS (constitutive NOS; cNOS) and an endotoxin/cytokine-inducible NOS (inducible NOS: iNOS). cNOS is expressed not only in the vascular endothelium but in the adrenal gland and neurons within many areas of the rat brain [4–6]. iNOS is expressed in macrophages [7] and vascular smooth muscle [8]. Ohta *et al.* [9] reported that iNOS mRNA was expressed in the cells of a mouse pituitary tumor after exposure to IL-1β. Interleukin-1 (IL-1β), an immune cytokine, has been shown to activate the hypothalamic pituitary adrenal (HPA) axis. IL-1β induces hypothalamic CRH secretion in *in vivo* and *in vitro* experiments [10–12]. It can also stimulate ACTH secretion at the pituitary level [13–15]. It has therefore been assumed that NO may play a role in IL-1β-induced HPA axis stimulation.

However, there has been a controversy among the reports on the role of NO in ACTH secretion. Brunetti *et al.* [16] reported that N^G-Nitro-L-arginine blocked IL-1β-induced ACTH release by rat anterior pituitary cells *in vitro*, suggesting that NO plays a stimulatory role in IL-1β-induced ACTH secretion. On the other hand, other investigators reported an inhibitory role [17, 18] or no role [9] of NO in the ACTH release at the pituitary level. In the present study, we also examined the effect of a NOS inhibitor on IL-1β-induced ACTH release in

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rat anterior pituitary cell cultures. We also examined its effect on ACTH release induced by other ACTH releasing factors such as corticotropin releasing hormone (CRH), arginine vasopressin (AVP) and phorbol myristate acetate (PMA).

Materials and Methods

Preparation of rat anterior pituitary cells

Male Wistar rats, weighing 200–250 g were decapitated. The pituitaries were immediately removed, minced into small pieces and dispersed. The pituitaries were shaken at 37 °C for 20 min with 10 m*l* Hank's-HEPES buffer containing 0.3% collagenase (GIBCO) and 1% bovine serum albumin. Dispersed pituitary cells were washed 3 times with 3 m*l* of sterile Dulbeco modified Eagle's medium (DMEM; GIBCO), containing 10% horse serum, 2.5% fetal calf serum, fungisone, penicillin and strepromycin. The dispersed cells were resuspended in an appropriate volume of serum containing DMEM and were placed in sterile plastic Petri dishes (Falcon, 35 × 10 mm). Each dish contained approximately 1×10^5 cells in 1.5 m*l* medium. The dishes were cultured for 4–7 days in an incubator with 95% air and 5% CO_2 and continuously supplied with water vapor. The culture medium was changed to fresh medium on the fourth day when the experiments were carried out on the seventh day.

Experiments with cultured cells

After 4–7 days of culture, the cells were washed twice with fresh non-serum containing DMEM and then the DMEM containing the test substances was added to the cells. The cells were incubated with either IL-1β (5.5×10^{-9} M), CRH (2.1×10^{-9} M), AVP (10^{-8} M) or PMA (2×10^{-8} M). The concentrations of these materials used were enough to stimulate ACTH release in our previous experiments with pituitary cell cultures. NOS inhibitor, Nω-Nitro-L-arginine (Nitro-Arg, 10^{-5} M and 10^{-4} M) was also added to the cells with or without the above ACTH secretagogues. These concentrations of Nitro-Arg were selected as they suppressed NO synthesis completely *in vitro* [19]. Basal Medium Eagle (BME) and Krebs Ringer bicarbonate glucose buffer (KRBG) were also used when the cells were incubated with IL-1β. When the cells were incubated with IL-1β, incubations were carried out for 5–30 h. Incubations with other ACTH secretagogues were carried out for 4 h. After incubation, medium was collected for ACTH determination. The amount of ACTH in the medium was measured with an ACTH immunoradiometric assay kit (ACTH-II IRMA Kit, Mitsubishi Petro-chemicals, Japan). Recombinant IL-1β was a gift from Du Pont Merck Pharmaceutical Co. (USA). CRH and AVP were purchased from Peptide Institute Inc. (Japan). PMA and Nitro-Arg were purchased from Sigma (USA).

Statistical analysis

Statistical analysis was conducted by Duncan's multiple range test followed by ANOVA.

Results

Nitro-Arg at 10^{-5}–10^{-4}M did not affect baseline ACTH release in pituitary cell cultures (Fig. 1). CRH, PMA and AVP stimulated ACTH release in pituitary cell cultures by 3.6, 4.3 and 2.3 times of the control release, respectively (Figs. 2–3). Nitro-Arg did not affect CRH, PMA or AVP-induced ACTH release in 4 h incubation. Seven h incubation with IL-1β significantly increased ACTH release (1.6 times the control value) in pituitary

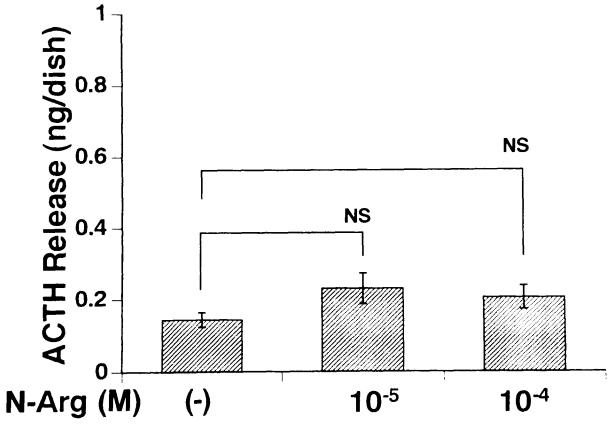

Fig. 1. Effect of Nω-Nitro-L-arginine (N-Arg) on basal ACTH release in rat anterior pituitary cell cultures for 4 h in Dulbecco's modified Eagle Medium (DMEM). Each column and bar represent the mean and SEM, respectively (n=4–5).

cell cultures in DMEM, but Nitro-Arg did not affect IL-1β-induced ACTH release (Fig. 4). IL-1β increased ACTH release slightly but significantly (1.4 times the control value) in 7 h incubation in BME, which contains less l-arginine than DMEM, and Nitro-Arg did not affect IL-1β-induced ACTH release either. IL-1β did not increase ACTH release when the cells were incubated for 5 h in KRBG (Fig. 5), but it increased ACTH release only slightly (1.13 times the control value) in 30 h incubation, and Nitro-Arg did not affect IL-1β-induced ACTH release.

Discussion

Brunetti [16] suggested that NO plays a stimulatory role in IL-1β-induced CRH and ACTH secretion at the levels of the hypothalamus and anterior pituitary, respectively. On the other hand, Costa *et al.* [20] reported that NO directly and specifically inhibited the stimulated release of CRH from rat hypothalamic explants *in vitro*, while leav-

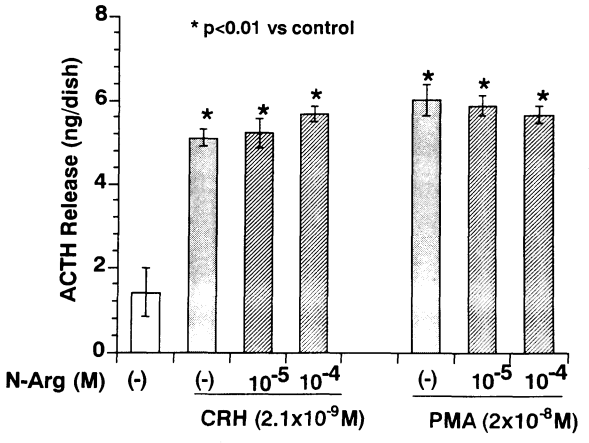

Fig. 2. Effect of N-Arg on CRH- and phorbol myristate acetate (PMA)-induced ACTH release in rat anterior pituitary cell cultures for 4 h in DMEM. Each column and bar represent the mean±SEM, respectively (n=4–5).

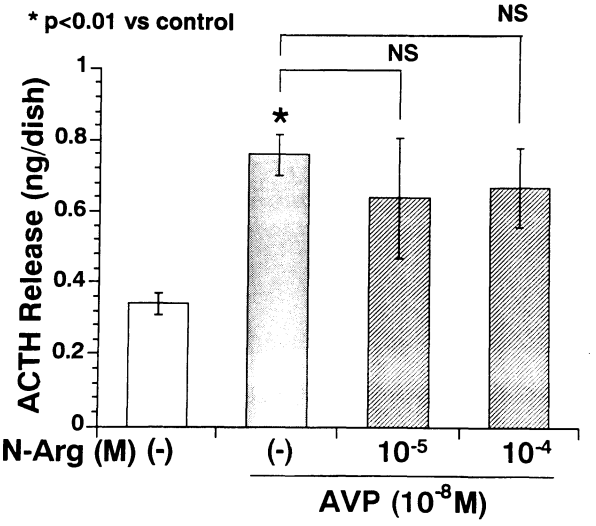

Fig. 3. Effect of N-Arg on AVP-induced ACTH release in rat anterior pituitary cell cultures for 4 h in DMEM. Each column and bar represent the mean±SEM, respectively (n=4–5).

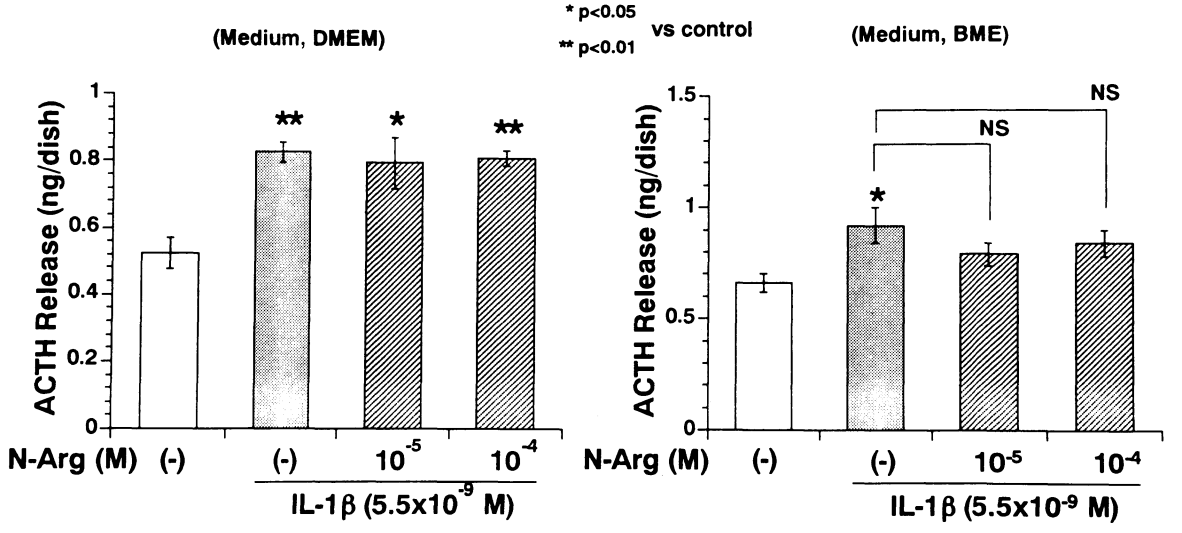

Fig. 4. Effect of N-Arg on IL-1β-induced ACTH release in rat anterior pituitary cell cultures for 7 h in DMEM or Basal Medium Eagle (BME). Each column and bar represent the mean±SEM, respectively (n=4–5).

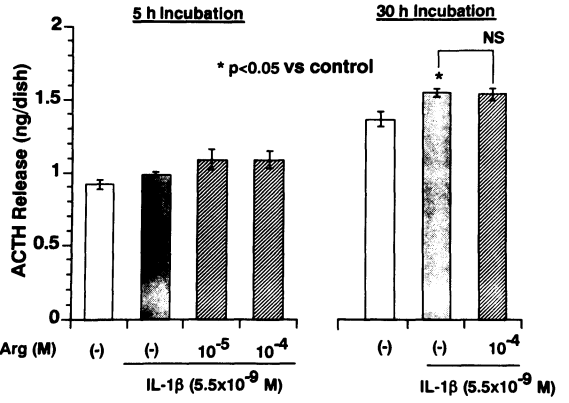

Fig. 5. Effect of N-Arg on IL-1β-induced ACTH release in rat anterior pituitary cell cultures for 5 or 30 h in Krebs Ringer bicarbonate glucose buffer (KRBG). Each column and bar represent the mean±SEM, respectively (n=4–5).

ing basal CRH secretion unaffected. Hulting *et al.* [17] recently reported that NOS was present in monkey corticotrophs and that NOS inhibitor had no clear effect on ACTH secretion, whereas the NO dona nitropruside significantly inhibited CRH-stimulated ACTH release in cultured monkey pituitary cells. Their results suggest that NO plays an inhibitory role in CRH-induced ACTH release at least in the monkey. Very recently, Rivier & Shen [18] reported the results of *in vivo* experiments in which the blockade of NOS activity caused significant extensions of the duration of ACTH releasing action of IL-1β, vasopressin and oxytocin, but it did not significantly alter the stimulatory action of peripherally injected CRH or centrally administered IL-1β. These previous reports suggest that NO exerts an inhibitory influence on the activity of the hypothalamic-pituitary-adrenal (HPA) axis, but it has not been made clear at which level NO exerts its inhibitory effect. The possible levels include the hypothalamus, the median eminence and the anterior pituitary. Rivier & Shen [18] speculated from their data that NO does not act at the level of the hypothalamic paraventricular nucleus or the anterior pituitary, and that the important site of action of NO is the median eminence.

Ohta *et al.* [9] reported that IL-1β stimulated iNOS mRNA expression and NO production in mouse pituitary tumor cells (AtT20/D16). Although NOS inhibitor inhibited IL-1β-induced NO production and cGMP accumulation, it did not block IL-1β-induced ACTH release in cell cultures with DMEM. Our results also show that IL-1β-induced ACTH release was not inhibited by NOS inhibitor, but Nitro-Arg may not completely prevent IL-1β-induced NO production by the pituitary cells in DMEM, as DMEM contains a considerable amount of L-arginine. We therefore also used BME and KRBG buffer as incubation media, as they contain less L-arginine and other amino acids. In these media, IL-1β stimulated less ACTH release than in DMEM. Even in these media Nitro-Arg did not prevent IL-1β-stimulated ACTH release. It therefore seems unlikely that NO plays an important role in IL-1β-induced ACTH secretion at the pituitary level in the rat. Our results also indicate that NO plays no role in CRF-, AVP-, and PMA-induced ACTH release at the pituitary level. It has been suggested that cyclic GMP is not involved in ACTH secretion, suggesting also that NO synthesis is not involved in ACTH release induced by ACTH secretagogues. NO may therefore exert an inhibitory effect on the HPA axis at the median eminence level as Rivier & Shen speculated [18].

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