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Selective Inhibitors of Nitric Oxide Synthase (NOS) Implicate a Constitutive Isoform of NOS in the Regulation of Interleukin-1-Induced ACTH Secretion in Rats

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Nitric oxide synthase (NOS) exists in at least three distinct isoforms: an inducible NOS (NOS II), and two forms which are constitutively expressed—brain NOS (NOS I) and endothelial NOS (NOS III). We have previously shown that the NOS inhibitor, N_ω-nitro-L-arginine methyl ester hydrochloride (L-NAME), markedly potentiates and prolongs the increase in plasma adrenocorticotropin (ACTH) concentrations produced by the intravenous injection of interleukin-1β (IL-1β) in the rat. However, the mechanism of action of L-NAME is unknown. The purpose of the present study was to determine the effects on IL-1β-induced ACTH secretion in the rat, of several NOS inhibitors, whose selectivity for the different NOS isoforms has been well characterized, and which lack the muscarinic receptor antagonist properties that have been reported for L-NAME. Subcutaneous (sc) pretreatment with L-NAME (50–300 μmol/kg) produced the expected pronounced exacerbation of the ACTH response to IL-1β. This effect was mimicked by N^G-nitro-L-arginine, which preferentially inhibits constitutive forms of NOS. In contrast, aminoguanidine, a selective inducible NOS inhibitor at doses up to 3 x 1.8 mmol/kg, was without effect, suggesting that it is a constitutive form of NOS that regulates the ACTH response to IL-1β. Selective inhibition of brain NOS using either 7-nitroindazole (administered intraperitoneally) or L-NAME (administered intracerebroventricularly) did not significantly alter ACTH concentrations after IL-1β. Collectively, these data indicate that NO restrains the ACTH response to IL-1β, and that the NO responsible for this effect is generated by a constitutive, most probably endothelial, isoform of NOS.

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Introduction

Nitric oxide synthase (NOS) is the enzyme that catalyzes the conversion of L-arginine to L-citrulline and the gaseous mediator, nitric oxide (NO). Studies that determined the effects of inhibition of NOS and investigated its localization and regulation have implicated NO in a variety of biological processes, including neuroendocrine secretions (Brunetti, 1994; Snyder and Dawson, 1995). We have shown that the NOS inhibitor, N_ω-nitro-L-arginine methyl ester hydrochloride (L-NAME), potentiates and prolongs the increase in pituitary adrenocorticotropin (ACTH) secretion in response to inflammatory stimuli, such as interleukin-1 (IL-1) (Rivier and Shen, 1994; Rivier, 1995), endotoxin (Rivier and Shen, 1994) and local inflammation (Turnbull and Rivier, 1996). L-NAME has similar effects on ACTH secretion induced by some (vasopressin, oxytocin), but not other (corticotropin-releasing factor [CRF]) hypothalamic ACTH secretagogs (Rivier and Shen, 1994). However, the precise mechanisms by which L-NAME exacerbates ACTH secretion in response to vasopressin and IL-1β appear to differ, with only the L-NAME-enhanced ACTH response to IL-1β being sensitive to adrenergic antagonists (Rivier, 1995). Furthermore, while L-NAME enhances the ACTH response to peripherally administered IL-1β, it has no effect on the response to central IL-1β (Rivier and Shen, 1994) and actually inhibits the elevations in plasma ACTH due to footshock (Rivier, 1994). Collectively, these data clearly indicate specific, and probably multiple, actions of NO in the regulation of the pituitary ACTH secretion.

Given the importance of the magnitude of pituitary-adrenal responses to the development and outcome of inflammatory processes (Chrousos, 1995), we have been particularly interested in the mechanisms by which NO restrains the ACTH response to inflammatory stimuli, such

as IL-1β. The enhancement of IL-1β-induced ACTH secretion by the NOS inhibitor, L-NAME, is unrelated to its effects on blood pressure (Rivier and Shen, 1994), is reversed by competition with L-arginine (Rivier and Shen, 1994), and is at least partially dependent on activation of catecholaminergic mechanisms (Rivier, 1995). NOS is present in rat hypothalamic regions controlling hypophysial secretions (Bredt et al., 1990; Torres et al., 1993; Siaud et al., 1994; Lee et al., 1995), as well as in rat anterior pituitary itself (Ceccatelli et al., 1993). Since IL-1-induced ACTH secretion is dependent on the hypothalamic release of corticotropin-releasing factor (CRF) (Berkenbosch et al., 1987; Sapolsky et al., 1987), several studies have investigated the effects of NOS inhibition on IL-1-induced CRF secretion in vitro (Brunetti et al., 1993; Costa et al., 1993; Sandi and Guaza, 1995). However, results have been contradictory, with some authors reporting enhancing (Costa et al., 1993) and others inhibitory (Brunetti et al., 1993; Sandi and Guaza, 1995) influences.

Several isoforms of NOS have been identified (Forstermann et al., 1995). NOS I is expressed in the central and peripheral nervous systems and is otherwise known as brain or neuronal NOS. NOS II is found in many cell types, such as hepatocytes, macrophages, smooth muscle cells, and glia. Finally, NOS III is synonymous with endothelial NOS. These three isoforms are distinct gene products, differ in terms of their constitutive expression (NOS I and III are constitutively expressed, whereas NOS II is abundantly expressed only after induction by cytokines/endotoxin), are either calcium/calmodulin-dependent (NOS I and III), or -independent (NOS II), and exhibit different kinetic properties (Forstermann et al., 1995). Several NOS inhibitors display selectivity towards the isoform of NOS that they most affect. For example, aminoguanidine selectively inhibits the inducible NOS (II) isoform (Griffiths et al., 1993; Hasan et al., 1993; Misko et al., 1993; Iadecola et al., 1995), whereas N^G-nitro-L-arginine (L-NNA), a poor inhibitor of NOS II (Furfine et al., 1993), potently inhibits both constitutively expressed NOS isoforms (I and III) (Dwyer et al., 1991; Furfine et al., 1993; Mayer et al., 1993). Indazole derivatives (e.g., 7-nitro-indazole, 7-NI), on the other hand, inhibit brain NOS (NOS I) without affecting blood pressure (i.e., a NOS III mechanism) (Babbedge et al., 1993; Moore et al., 1993a, 1993b, 1994; MacKenzie et al., 1994). Our previous studies (Rivier and Shen, 1994; Rivier, 1995) showed that the NOS inhibitor L-NAME, exacerbated ACTH secretion due to cytokines, but a recent report (Buxton et al., 1993) demonstrating that L-NAME, and other alkyl esters of arginine are muscarinic receptor antagonists, questions whether the effects we observed were indeed a result of the specific inhibition of NOS. The present experiments were undertaken to compare the effects of a number of NOS inhibitors, L-NNA, aminoguanidine and 7-NI, which are not alkyl esters of arginine, and whose selectivity for the NOS isoforms is well characterized.

Results

L-NNA and L-NAME, but not Aminoguanidine, Enhance the ACTH Response to IL-1β

Plasma ACTH concentrations in animals treated (sc) 3 h earlier with DMSO were 7.3 ± 0.9 pg/mL, and remained relatively constant after the iv injection of only vehicle. Neither aminoguanidine, L-NAME, nor L-NNA (each dissolved in DMSO) had a significant effect on plasma ACTH concentrations 3–5 h after injection of each NOS inhibitor alone (Fig. 1). IL-1β produced a small (peak 135 ± 24 pg/mL), and transient (peak at 15 min), increase in plasma ACTH in animals pretreated with only DMSO. A single injection (sc) of either L-NAME or L-NNA 3 h earlier, dose-dependently potentiated the ACTH response to IL-1β, producing both higher peak concentrations (DMSO, 135 ± 24 pg/mL; 300 μmol/kg L-NAME, 218 ± 41 pg/mL; 300 μmol/kg L-NNA, 341 ± 68 pg/mL), and a more prolonged ACTH response. In contrast, a single injection of aminoguanidine at doses as high as 1.8 mmol/kg, had no effect on the ACTH response (peak 113 ± 15 pg/mL) to IL-1β (Fig. 1).

In further experiments, L-NAME (300 μmol/kg), L-NNA (300 μmol/kg), or aminoguanidine (1.8 mmol/kg) were administered (sc) at –24 h, -14 h, and –3 h before IL-1β (see Fig. 2). In these experiments, L-NNA, but not L-NAME or aminoguanadine, produced a small, but statistically significant elevation of basal plasma ACTH concentrations (DMSO, 19 ± 7 pg/mL; L-NNA, 50 ± 4 pg/mL; P = 0.002, Scheffé's multiple comparison test). Both L-NAME and L-NNA (each 3 × 300 μmol/kg) produced a dramatic exaggeration of the peak ACTH concentrations (at 15 min DMSO, 194 ± 45 pg/mL; L-NAME, 505 ± 81 pg/mL; L-NNA, 539 ± 122 pg/mL) and prolonged the ACTH response to IL-1β. However, aminoguanidine (3 × 1.8 mmol/kg) had no effect on IL-1β-induced ACTH secretion (peak, 173 ± 53 pg/mL, Fig. 2).

7-NI Does Not Enhance the ACTH Response to IL-1β

Neither L-NAME (100 μmol/kg, dissolved in peanut oil) nor 7-NI (either 100 or 300 μmol/kg) when administered ip 3 h before, affected basal ACTH secretion (Fig. 3). IL-1β induced a similar increase in plasma ACTH concentration as described in earlier experiments. While L-NAME (100 μmol/kg) produced a marked exacerbation of the ACTH response to IL-1β, 7-NI at either 100 or 300 μmol/kg was completely without effect (Fig. 3). A higher dose of 7-NI (550 μmol/kg) produced elevation of basal plasma ACTH concentrations, but did not produce an exacerbation of the ACTH response to IL-1β (data not shown).

The Intracerebroventricular (icv) Injection of L-NAME Does Not Enhance the ACTH Response to IL-1β

The absence of a potentiation of the ACTH response to IL-1β by 7-NI suggested that brain NOS (NOS I) was not important in the regulation of ACTH secretion in response to this stimulus. To test this hypothesis further, we inves-

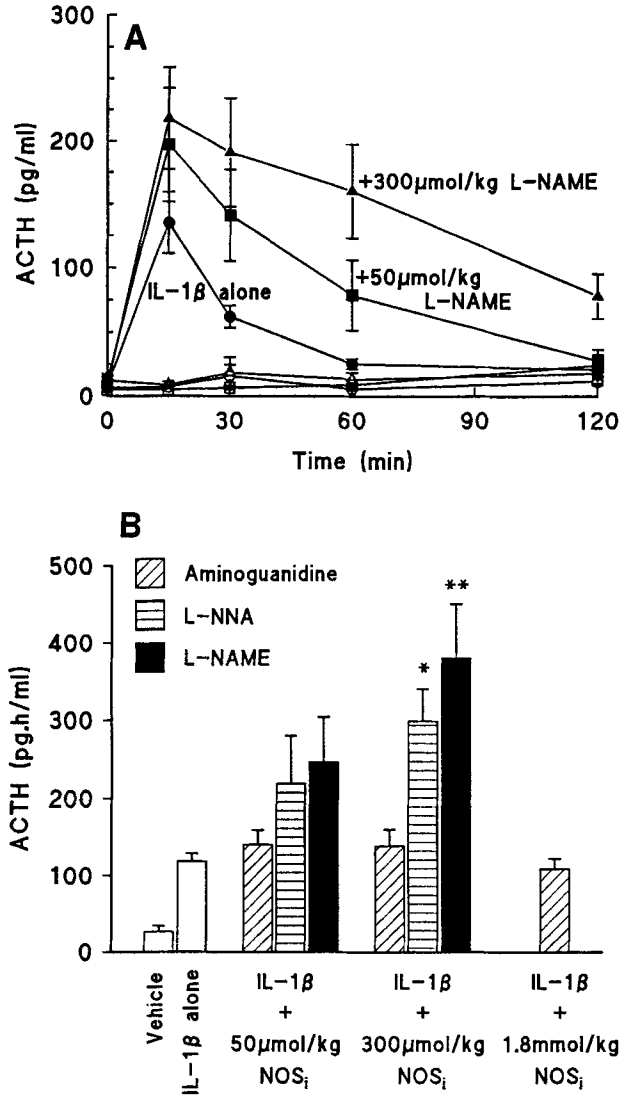

Fig. 1. (A) The effect of L-NAME on IL-1β-induced elevations in plasma ACTH concentrations in the rat. L-NAME, (0 [circles], 50 [squares] or 300 [triangles] μmol/kg) was administered (sc) 3 h before the iv injection of either vehicle (open symbols) or IL-1β (100 ng/kg, closed symbols). All drugs were dissolved in DMSO. **(B)** Comparison of the effects of acute administration of aminoguanidine, L-NNA or L-NAME on IL-1β-induced elevations in plasma ACTH. Data are presented as the integration of plasma ACTH concentrations measured 0, 15, 30, 60, and 120 min after IL-1β injection. *p < 0.05, **p < 0.01 vs IL-1β alone (Dunnett multiple comparison test following ANOVA [$F_{7,43}$ = 5.20, P = 0.0002]). n = 5–7 animals per group.

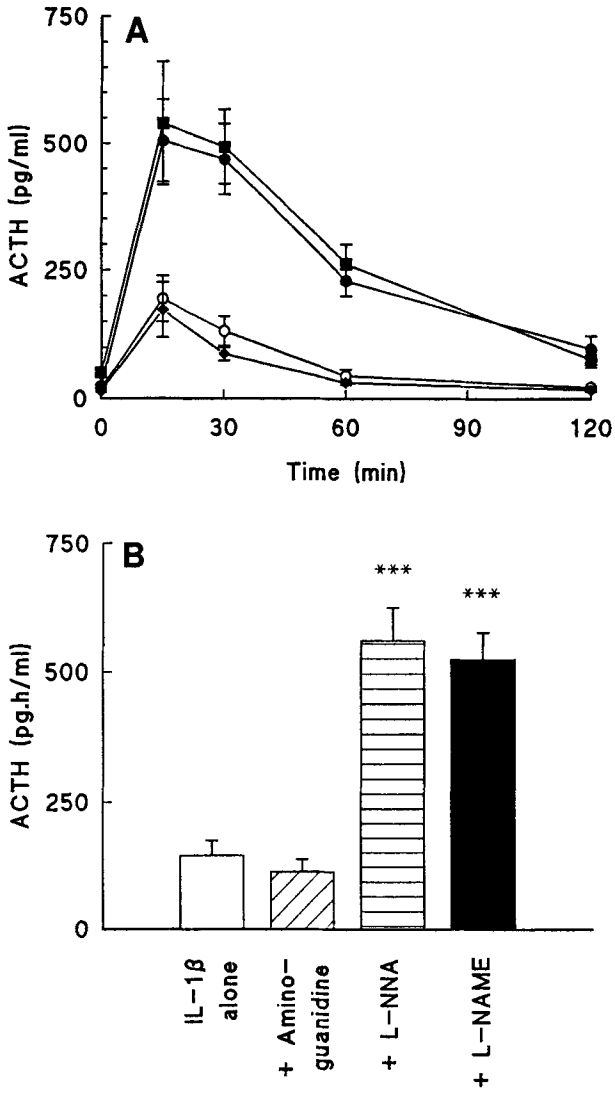

Fig. 2. (A) The effect of chronic administration of L-NAME (closed circles), L-NNA (closed squares) or aminoguanidine (closed diamonds) on IL-1β-induced elevations in plasma ACTH concentrations in the rat (vehicle [DMSO] pretreated group injected with IL-1β represented with open circles). NOS inhibitors were administered sc at doses of either 300 μmol/kg (L-NAME and L-NNA) or 1.8 mmol/kg (aminoguanidine) on three separate occasions: at 24, 14, and 3 h before IL-1β (100 ng/kg, iv). **(B)** Integrated plasma ACTH concentrations over the 120 min after IL-1β. ***p < 0.001 vs IL- 1β alone (Dunnett multiple comparison test following ANOVA [$F_{3,16}$ = 24.43, p < 0.0001]). n = 5–6 animals per group.

tigated the effects of L-NAME (0.15 and 0.75 μmol/kg, dissolved in physiological 0.9% saline) injected directly into the brain (icv) on the ACTH response to IL-1β. Pretreatment with L-NAME icv did not mimic the effects of L-NAME or L-NNA given systemically, indeed the ACTH response to IL-1β in animals treated with the highest dose icv (0.75 μmol/kg) was 50% smaller than in animals treated with IL-1β alone, though this effect did not achieve statistical significance (Fig. 4).

Discussion

Previous studies have shown that the intravenous administration of the NOS inhibitor, L-NAME, potentiates the ACTH response to IL-1β (Rivier and Shen, 1994; Rivier, 1995). The present experiments indicate that this effect is unlikely to be a result of the reported antagonist actions of L-NAME at muscarinic receptors (Buxton et al., 1993), since similar effects are observed using the nonalkyl-ester

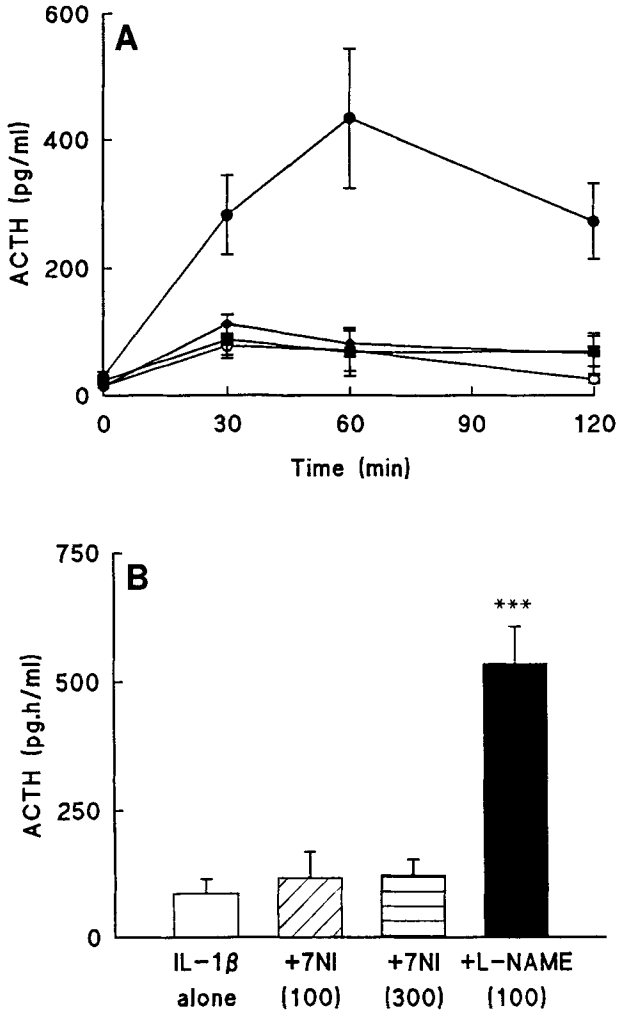

Fig. 3. **(A)** The effect of 7-NI (100 μmol/kg [closed squares] and 300 μmol/kg [closed diamonds]) and L-NAME (100 μmol/kg [closed circles]) administered ip at –3 h on the ACTH response to IL-1β. Vehicle- (peanut oil) treated groups are represented by open circles. **(B)** Integrated plasma ACTH concentrations over the 120 min after IL-1β. ***$p < 0.001$ vs IL-1β alone (Dunnett multiple comparison test following ANOVA [$F_{3,17} = 21.24$, $p < 0.0001$]). n = 5–6 animals per group.

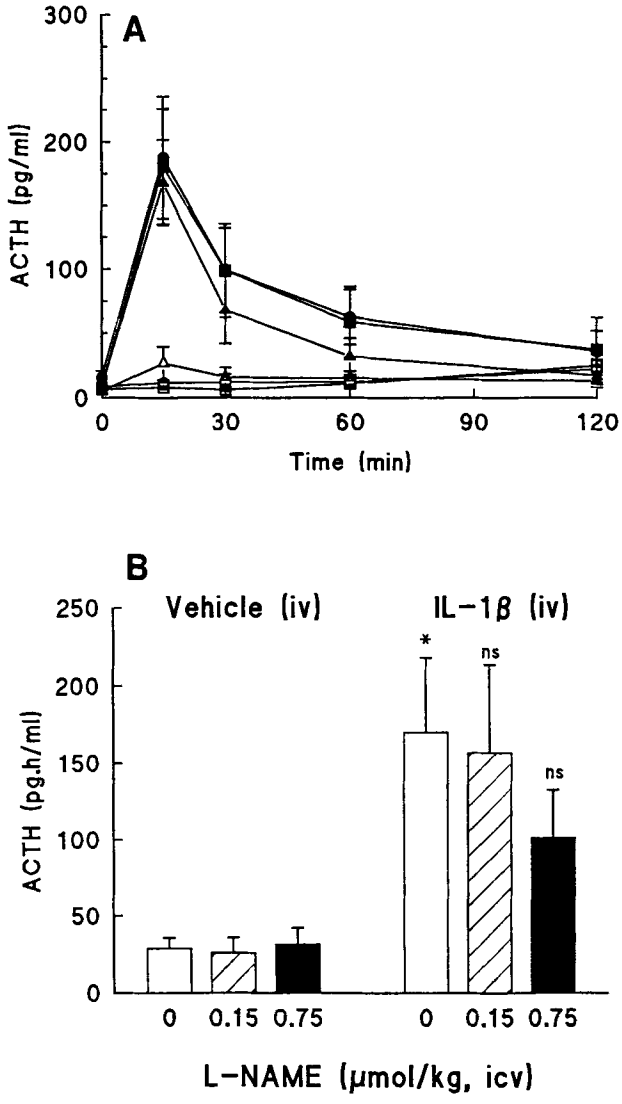

Fig. 4. **(A)** The effect of L-NAME (icv) on IL-1β-induced elevations in plasma ACTH concentrations in the rat. L-NAME, (0 [circles], 0.15 [triangles] or 0.75 [squares] μmol/kg) was administered (icv) 1 h before the iv injection of either vehicle (0.9% saline; open symbols) or IL-1β (100 ng/kg, closed symbols). **(B)** The effect of L-NAME (icv) on IL-1β-induced elevations in plasma ACTH. Data are presented as the integration of plasma ACTH concentrations measured 0, 15, 30, 60, and 120 min after IL-1β injection. *$p < 0.05$ vs vehicle (iv)/0 μmol/kg L-NAME (icv); ns not significant vs IL-1β (iv)/0 μmol/kg L-NAME (icv) (Scheffé's multiple comparison test following ANOVA [$F_{5,32} = 3.23$, $p = 0.01$]). n = 5–7 animals per group.

form of this inhibitor, L-NNA. It seems reasonable to conclude, therefore, that the effects of these inhibitors on the ACTH response to IL-1β are produced specifically by inhibition of NO formation.

Cytokines such as IL-1 induce the expression of NOS II (Moncada et al., 1991). However, the present experiments indicate that NOS II does not play a major role in the regulation of ACTH secretion due to IL-1β in the rat. Firstly, the effects of NOS inhibition are observed within 15 min of IL-1β injection, a time-frame unlikely to be sufficient for the induction of NOS II protein. Secondly, the pronounced exacerbation of ACTH secretion due to IL-1β produced by L-NAME and L-NNA, but not by aminoguanidine, implicates a constitutive isoform of the enzyme in this effect of NOS inhibition (*see* Table 1 for summary). Aminoguanidine is a relatively potent inhibitor of endotoxin- or cytokine-induced NO generation in a number of cell types (e.g., insulinoma cells [Hasan et al., 1993], smooth muscle cells [Griffiths et al., 1993], macrophages [Misko et al., 1993]), but has little or no effect on NO produced by endothelial NOS (Griffiths et al., 1993; Hasan et al., 1993; Misko et al., 1993). This apparent selectivity for the inducible isoform of NOS (NOS II) has also been demonstrated in vivo, and similar dosing regimes (100 mg/kg [i.e., 0.74 mmol/kg] twice daily) to

Table 1
Effect of Selective Inhibition of NOS Isoforms on the Increase in Plasma ACTH Concentrations Due to Intravenous (iv) IL-1β

NOS inhibitor	NOS isoform most sensitive to inhibitor	Refs.	Effect on IL-1β-induced ACTH secretion
Amino-guanidine	II (inducible)	(Griffiths et al., 1993; Hasan et al., 1993; Misko et al., 1993; Iadecola et al., 1995)	none
Nitro-arginine (L-NNA)	I (brain) and III (endothelial)	(Dwyer et al., 1991; Furfine et al., 1993; Mayer et al., 1993)	enhancement
7-nitroindazole (7-NI)	I (brain)	(Babbedge et al., 1993; Moore et al., 1993a; 1993b; MacKenzie et al., 1994; Mayer et al., 1994)	none

those employed in the present study (0.05–1.8 mmol/kg) have been shown to dramatically inhibit inducible, but not constitutive, NOS activity (Iadecola et al., 1995). L-NNA, on the other hand, is more potent at inhibiting constitutive rather than inducible NOS (Dwyer et al., 1991; Furfine et al., 1993; Mayer et al., 1993). Together, these data lead us to conclude that a constitutive isoform of NOS (either NOS I or NOS III) generates the NO that restrains the ACTH response to IL-1.

To determine whether brain NOS (NOS I) is important in the regulation of ACTH secretion induced by IL-1, we tested the effects of 7-NI. Experiments in both rats and mice indicate that 7-NI selectively inhibits brain NOS (Moore et al., 1993a; 1993b). A recent study (MacKenzie et al., 1994) demonstrated that an ip dose of 30 mg/kg (250 μmol/kg) of 7-NI produces prolonged (> 3 h) and pronounced (>60%) inhibition of brain NOS. In the present work, doses of 7-NI as high as 300 μmol/kg were without effect on the ACTH response to IL-1β. Furthermore, L-NAME injected directly into the cerebroventricles, at doses that inhibit NOS activity within subcortical structures by 50–100% for several hours (Salter et al., 1995), did not mimic the effects of L-NAME or L-NNA given systemically. These experiments strongly suggest that brain NOS is not an important regulator of L-1β-induced ACTH secretion. This would imply that the observed effects of constitutive NOS inhibitors are due primarily to effects on endothelial NOS (NOS III). However, it is important to note that while L-NAME produces a marked hypertension, this effect occurs in both L-1β and vehicle-treated rats. In contrast, the dramatic exacerbation of ACTH secretion is observed only in the former, indicating that the effects of inhibition of NOS III on systemic blood pressure do not account for their effects on ACTH secretion in response to IL-1β. Whether effects of NOS III inhibitors on local blood flow, in particular in the hypophysial portal vasculature (Ceccatelli et al., 1992), contribute to this neuroendocrine effect, remains to be determined.

In summary, the present data demonstrate that NO restrains the ACTH response to the systemic injection of IL-1. A constitutive, rather than inducible, isoform of NOS is implicated in this effect. That selective inhibition of brain NOS is without effect on the elevations in plasma ACTH concentrations due to IL-1β, leads us to conclude that endothelial NOS plays a significant role in the regulation of IL-1-induced ACTH secretion.

Materials and Methods

Animals and Surgical Procedures

Male Sprague-Dawley rats (170–240 g BW) were purchased from Harlan Sprague Dawley Laboratories (Indianapolis, IN), and housed in animal facilities adjacent to experimental rooms. They were maintained on a 12-h light, 12-h dark cycle (lights on at 0600h) and provided rat chow and water ad libitum. All procedures described were approved by The Salk Institute Animal Use and Care Committee.

Rats were equipped with intravenous (iv) catheters, 48 h before experimentation, as described previously (Turnbull and Rivier, 1996). In one series of experiments, animals received injections directly into the cerebroventricles via indwelling guide cannulae, which were implanted 7–9 d before iv cannulation. Briefly, rats were anesthetized with an SC injection of ketamine (100 mg/kg)/acepromazine (4 mg/kg)/xylazine (10 mg/kg) and mounted on a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). With the incisor bar set at –3.3 mm, a guide cannula (cut to 5 mm length; Plastics One, Roanoke, VA) was implanted at coordinates: lateral (L) 1.4 mm; anterior-posterior (A-P) 0.4 mm; dorso-ventral (D-V) 3.5 mm, and secured with three screws and dental cement. On the day of the experiment, an injection cannula (protruding 0.5 mm beyond the tip of the guide cannula) was connected at least 2 h before the commencement of the experiment. Intracerebroventricular (icv) injections (5 μL) were administered over a period of 60 s. After completion of the experiment, animals were sacrificed, and 5 μL indian ink injected via the icv assembly. Only data from animals that showed a distribution of ink throughout the ventricular system (i.e., third, fourth, and lateral ventricles and cerebral aqueduct) were included in subsequent analyses.

Drugs

Recombinant human interleukin- 1β (rhIL- 1β) was a kind gift from Dr. Tony Troutt (Immunex, Seattle, WA). IL-1β was administered iv (total volume 0.5 mL) at a dose of 100 ng/kg. Control animals received an equivalent volume of 0.9% w/v sterile saline containing 0.1% w/v bovine serum albumbin.

The NOS inhibitors, 7-nitro-indazole (7-NI) and L-NNA, were purchased from Calbiochem (San Diego, CA) and aminoguanidine and L-NAME were obtained from Sigma Chemical Co. (St. Louis, MO). These inhibitors display differing solubility characteristics, with only aminoguanidine and L-NAME being relatively soluble in physiological 0.9 % saline. However, in experiments where different NOS inhibitors were compared, the same vehicle was used for each inhibitor. L-NNA was dissolved in dimethylsulfoxide (DMSO, Sigma) and 7-NI was dissolved in domestic peanut oil.

ACTH Assay

Plasma ACTH concentrations were determined by a two-site immunoradiometric assay (IRMA, Allegro, Nichols Institute, San Juan Capistrano, CA) using 50 µL of plasma. All samples from the same experiment were assayed in a single IRMA. Assay sensitivity was 5 pg/mL, and coefficients of variation at 37 and 344 pg/mL were 2.8 and 5.1% within assays and 10.5 and 6.2% between assays.

Data Presentation and Statistical Analysis

Each experiment was performed at least twice, with similar results obtained on each occasion. The data are presented as the mean ± SEM of single representative experiments. Statistical analyses were performed on ACTH concentrations integrated over the time-course of the experiment (area under the curve, pg.h/mL) using one-way ANOVA, and Scheffé's or Dunnett's multiple comparison test as posthoc, as appropriate. The numbers of rats employed in each experimental group (n) is indicated in the figure legends. A two-tailed probability of less than 5% (i.e., $p < 0.05$) was considered statistically significant.

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