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Influence of Carbon Monoxide, and its Interaction with Nitric Oxide, on the Adrenocorticotropin Hormone Response of the Normal Rat to a Physico-Emotional Stress

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Abstract

We determined whether the gas carbon monoxide (CO) altered the adrenocorticotropin hormone (ACTH) response to mild inescapable electrofootshocks, and whether it interacted with nitric oxide (NO). Peripheral injection of the NO synthase (NOS) inhibitor Nwnitro-L-arginine-methylester (L-NAME), a compound which readily crosses the blood–brain barrier, produced the expected blunting of the ACTH response to the shocks. This effect was mimicked by other arginine analogues such as L-nitroarginine (L-NNA) and N^G-methyl-L-arginine (NMMA). The subcutaneous (s.c.) administration of the heme oxygenase (HO) blockers tin mesoporphyrin (SnMP) or tin protoporphyrin (SnPP) significantly decreased brain HO levels, indicating that both compounds had penetrated the brain. Blood pressure showed a modest increase in response to SnMP, and no change after SnPP. SnMP and SnPP both decreased shock-induced ACTH release, though the magnitude of this effect was slightly less than that of L-NAME. The influence of SnPP was further augmented in rats with concomitant blockade of NO formation, which suggests that both NO and CO are necessary for the full response of this axis to electrofootshocks. Finally, the ability of SnPP to significantly blunt the expression of the mRNA for the immediate early gene NGFI-B in the paraventricular nucleus (PVN) of rats exposed to shocks, indicates that the influence of CO was exerted on hypothalamic neuronal activity. Collectively, our results show that NO and CO exert a stimulatory effect on the HPA axis response to mild electrofootshocks, and that at least part of this influence takes place on hypothalamic neurons and/or their afferents.

The gases nitric oxide (NO) and carbon monoxide (CO) are now considered to be potential neurotransmitters in the mammalian brain (1–6). Physiological roles for NO in the CNS are suggested by the discrete and highly conserved distribution of the enzyme responsible for NO formation, NO synthase (NOS), in subpopulations of central neurones of all mammalian brains examined so far. The localization of NOS in hypothalamic structures known to control the activity of the hypothalamic-pituitary-adrenal (HPA) axis (7–11), had suggested in particular that NO might regulate adrenocorticotropin hormone (ACTH) secretion (12–14). NOS catalyses the oxidation of one of the guanidino nitrogen atoms of L-arginine to yield NO and the by-product, L-citrulline. Investigation of the physiological significance of the L-arginine/NO pathway has relied in particular on manipulation of NOS using guanidino-substituted analogues of L-arginine that inhibit the conversion of L-arginine to NO (15, 16). We have previously shown that the NO inhibitor Nwnitro-L-arginine-methylester (L-NAME) significantly inhibited ACTH secretion in rats exposed to physical or emotional stressors (17). These results suggest that NO exerts a stimulatory influence on the brain circuitries that are important for the activation of the HPA axis during these types of stressors, a hypothesis in agreement with some (18, 19), but not all (20) results obtained with isolated hypothalamic fragments. In the present work, we extend our previous findings to demonstrate that other arginine analogues such as L-nitroarginine (L-NNA) and N^G-methyl-L-arginine (L-NMMA), which mostly inactivate the constitutive (endothelial and neuronal) forms of NOS (15, 21), similarly blunt the

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ACTH response to electrofootshocks. We thought that this was important in view of the controversy regarding the possibility that L-NAME might also act on muscarinic receptors (22), and was necessary to validate the use of L-NAME in our subsequent studies focused on interactions between NO and CO.

CO is formed by the action of the enzyme heme oxygenase (HO) (5, 6), a microsomal enzyme that catalyses the oxidative cleavage of the α-meso carbon bridge of heme b (Fe-protoporphyrin IX). This reaction results in the formation of biliverdin, free iron and CO. Like NO, CO has been proposed as a modulator of the HPA axis. HO-2, the isoform that is primarily present in the brain (23–25), is found in parts of the endocrine hypothalamus that are relevant for ACTH secretion (26, 27), and its levels are influenced by corticosterone (28, 29). Studies investigating the role of this gas on hormones of the HPA axis have so far been carried out in isolated tissues, and have yielded contradictory results. In one report, cultured hypothalamic cells exposed to CO or to the heme analogue haematin were shown to increase their basal corticotropin-releasing factor (CRF) secretion while the synthetic metalloporphyrin zinc protoporphyrin (ZnPP), which was used as an HO inhibitor, decreased CRF release (30). In another report hemin, also an heme analogue, was found to inhibit KCl-stimulated CRF release, while ZnPP was without effect (31). These seemingly discordant data are reminiscent of those found with regard to NO, which as discussed above, indicate either stimulatory (18, 19) or inhibitory (20) effects on CRF secretion by isolated hypothalamic tissues. An additional problem has recently emerged that complicates the interpretation of studies aimed at investigating the role played by CO; namely the fact that researchers have used HO inhibitors that were subsequently found to also alter NOS activity (32). Indeed when four metalloporphyrin inhibitors of HO were used to assess CO production and the induction of long-term potentiation (LTP), only those that also inhibited NOS in the hippocampus interfered with LTP while specific inhibitors of HO formation such as tin mesoporphyrin (SnMP) did not (33). These data suggest that some previous results investigating the influence of CO on components of the HPA axis may need to be reexamined, and that *in-vivo* studies are also warranted to better understand the role played by this gas in the intact animal.

The purpose of the present work was to determine the effects of blockade of endogenous CO formation on the plasma ACTH and hypothalamic neuronal responses to mild inescapable electrofootshocks, and to compare this influence with that of NO. Interference with NO formation was induced by injecting L-NAME, and decrease in CO formation was induced by SnMP or tin protoporphyrin (SnPP), which act as potent competitive inhibitors of HO (33–37). L-NAME crosses the blood–brain barrier [see, for example, (38–40)] and blocks the activity of all three NOS isoforms, i.e. neuronal, endothelial and inducible (41). When we started our experiments SnPP, but not SnMP, was reported by some investigators to also penetrate into the brain (42) and effectively disrupt HO-2 protein formation and activity, though controversy remained regarding this point (43, 44). As in our study, the intracerebroventricular (i.c.v.) injection of metalloporphyrins at doses reported to be necessary to block HO activity, induced significant behavioural changes such as marked agitation and even convulsions, we decided to administer these compounds systemically, i.e. subcutanously (s.c.). In order to determine whether these treatments produced the desired decrease in brain CO formation, we measured HO levels. In view of the paucity of published data on the use of metalloporphyrins on various physiological responses, and of the controversy regarding the contribution of changes in blood pressure to the HPA axis response following blockade of either NO or CO formation, we also measured changes in blood pressure after s.c. injection of L-NAME, SnMP or SnPP. Finally, because of reports of functional interactions between CO and NO (45, 46), and because of the possibility that these gases might have opposite effects on the HPA axis(see above), we examined the combined influence of SnPP and L-NAME.

Results

Comparison among the effects of L-NAME, L-NNA and L-NMMA on the ACTH response to electrofootshocks

Rats were injected with the vehicle, L-NAME, L-NNA or L-NMMA (50 mg/kg, s.c.) 3 or 6 h before being exposed to mild electrofootshocks, or left undisturbed. Results obtained when the inhibitors were administered 3 or 6 h before the shocks were similar so only the 3 h data are presented. All three arginine analogues induced a small but significant ($P<0.05$) increase in baseline ACTH levels (corresponding to, on average, an increase from 21 ± 4 to 44 ± 6 pg/mL at each time point) (Fig. 1). Though not shown on the figure,

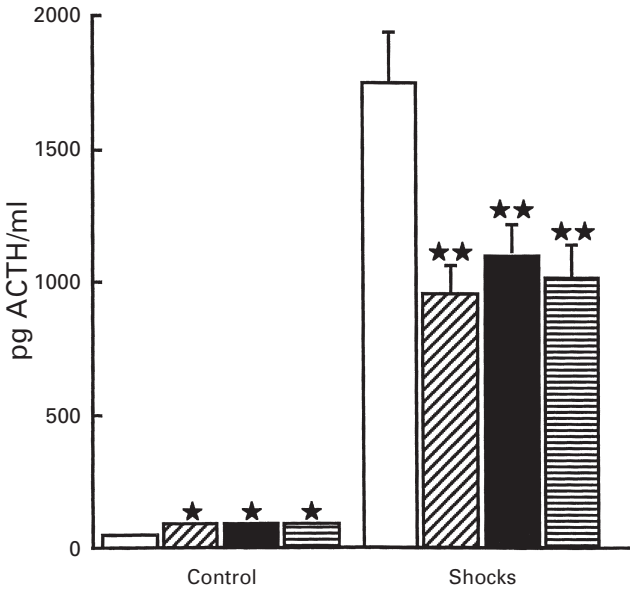

FIG. 1. Effects of the s.c. injection of the vehicle, L-NAME, L-NNA or L-NMMA on the ACTH response to mild electrofootshocks. The vehicle or the inhibitors were injected s.c. at 50 mg/kg 3 h before the shocks. Cumulative ACTH release was measured by adding hormone concentrations at the 10 and 30 min time points of shock exposure. Each bar represents the mean ± SEM of seven rats. *, $P<0.05$ and **, $P<0.01$ *versus* vehicle. □, Vehicle; ▨, L-NAME; ■, L-NNA; ▤, L-NMMA.

this resulted in a significant elevation of baseline corticosterone levels from 18±4 to 45±8 ng/mL. L-name produced the expected significant ($P<0.01$) blunting of the ACTH response, measured over the 30-min shock period. Pretreatment with L-NNA or L-NMMA induced comparable effects.

Effects of L-NAME, SnMP or SnPP on blood pressure

In view of the present controversy regarding the ability of metalloporphyrins to alter blood pressure, we thought that it was important to measure this parameter in our paradigm. We had previously shown that the intravenous (i.v.) injection of L-NAME (30 mg/kg) produced a significant and very rapid (within 5 min) increase in blood pressure (47), a finding that agreed with previous reports [quoted in (47)]. As the present work relied on the s.c. injection of a different dose of this arginine derivative, we determined the influence of this particular treatment, and compared it to the influence of SnMP and SnPP. As shown in Fig. 2, we confirmed the ability of L-NAME (50 mg/kg, s.c.) to significantly ($P<0.01$) elevate blood pressure. While SnPP (60 mg/kg) did not significantly alter this parameter, there was a modest effect of SnMP which was not statistically significant at any individual point, although a significant overall effect was present ($P<0.05$).

Measurement of whole brain HO activity following systemic injection of L-NAME, SnMP or SnPP

In view of the still unresolved controversy regarding the ability of systemically injected HO inhibitors to decrease activity levels of the enzyme in the brain (see Introduction), we measured HO levels in the whole brain. As indicated in Table 1, SnMP and SnPP, but not L-NAME, significantly ($P<0.01$) decreased HO levels 3 or 6 h later. Interestingly, despite reports that SnPP more readily penetrates the brain than SnMP, we consistently observed that both compounds significantly lowered HO levels. These results provide compelling evidence that both SnMP and SnPP are capable of inhibiting CO production following systemic administration.

TABLE 1. Heme Oxygenase Levels in Whole Brain Extracts.

Treatments	HO levels (pmol/mg protein/min)
3 h injections:	
Vehicle	21.1±3.4
L-NAME	21.4±3.8
SnPP	10.2±1.6**
SnMP	3.7±1.5**
6 h injections:	
Vehicle	13.8±2.6
L-NAME	15.3±2.4
SnPP	4.2±1.4*
SnMP	0.5±0.2*

The inhibitors were injected s.c. at 50–60 mg/kg. Each point represents the mean±SEM of 5–8 rats. *, $P<0.01$ from vehicle.

Effects of SnMP or SnPP on the ACTH response to electrofootshocks

The aim of these experiments was to determine whether blockade of HO activity altered the ACTH response to shocks. Rats were injected with the vehicle, SnMP or SnPP (60 mg/kg, s.c.). Shocks significantly increased plasma ACTH levels, and both SnMP and SnPP significantly ($P<0.05$–0.01) blunted this response regardless of whether they were administered 3 or 6 h earlier (Fig. 3).

Effect of SnPP, alone or in combination with L-NAME, on the ACTH response to electrofootshocks

These experiments investigated the presence of a possible interaction between blockade of NO and CO formation. The inhibitors were injected at 50–60 mg/kg, s.c., 3 h before the shocks. L-name alone or with SnPP produced a small, though significant ($P<0.01$) increase in basal ACTH levels, while SnPP did not (Fig. 4). We observed the expected rise in plasma ACTH levels following exposure to the shocks, a response that was significantly ($P<0.01$ and 0.05, respectively) decreased by L-NAME and SnPP. Combined treatment with both compounds produced an effect that was

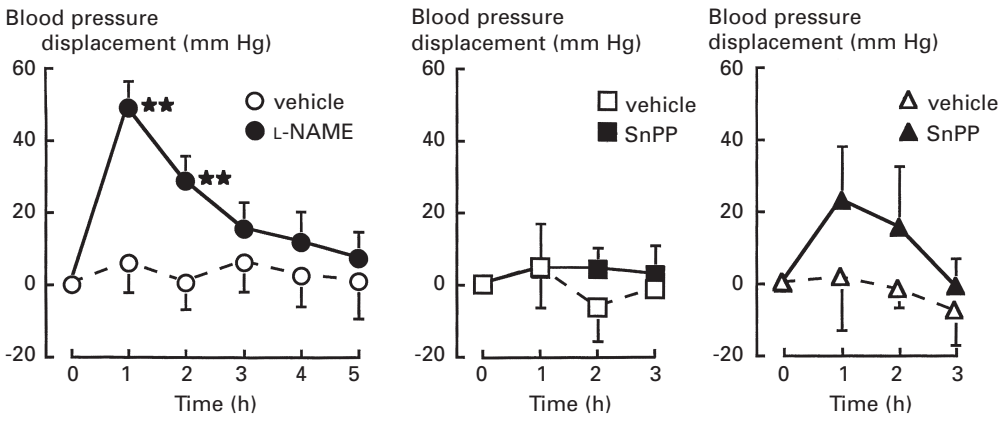

FIG. 2. Effects of L-NAME (50 mg/kg, s.c.), SnMP (60 mg/kg, s.c.) or SnPP (60 mg/kg, s.c.) on blood pressure. Each point represents the mean±SEM of 4–8 animals. **, $P<0.01$ *versus* the corresponding time point in vehicle-injected rats.

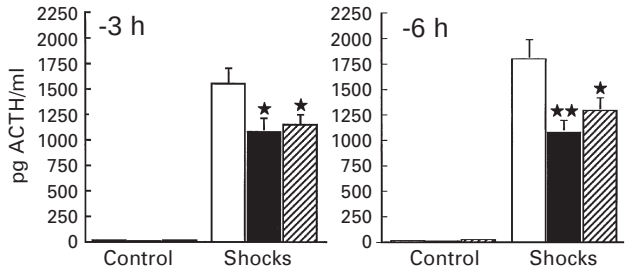

FIG. 3. Comparison between the effects of SnMP or SnPP (60 mg/kg, s.c.) administered 3 or 6 h before the shocks. Cumulative ACTH release was measured by adding hormone concentrations at the 10 and 30 min time points of shock exposure. Each bar represents the mean ± SEM of seven rats. *, $P<0.05$ and **, $P<0.01$ *versus* vehicle. □, Vehicle; ■, SnMP; ▨, SnPP.

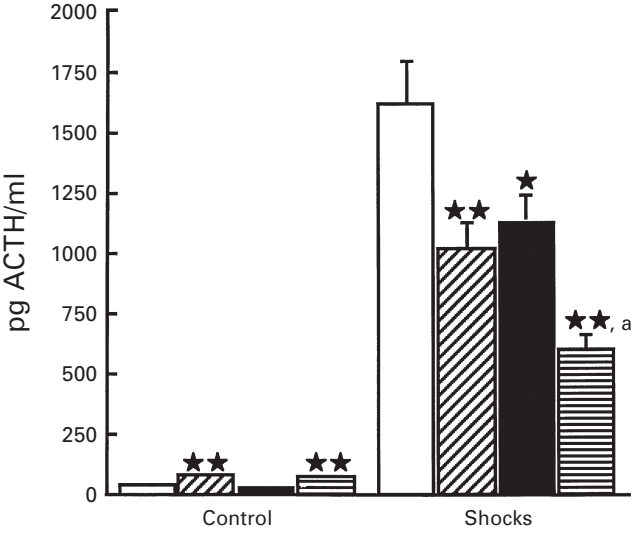

FIG. 4. Single or combined effects of L-NAME (50 mg/kg) and SnPP (60 mg/kg) on the ACTH response to mild electrofootshocks. The vehicle or the inhibitors were injected s.c. 3 h before the shocks. Cumulative ACTH release was measured by adding hormone concentrations at the 10 and 30 min time points of shock exposure. Each bar represents the mean ± SEM of seven rats. *, $P<0.05$ and **, $P<0.01$ *versus* vehicle; a, $P<0.01$ *versus* L-NAME or SnPP. □, Vehicle; ▨, L-NAME; ■, SnPP; ▤, L-NAME + SnPP.

significantly larger ($P<0.01$) than that observed with either compound injected alone.

Hypothalamic neuronal activity following exposure to electrofootshocks in the presence or absence of SnMP or SnPP

These experiments were designed to test the hypothesis that the ability of SnMP and SnPP to decrease the ACTH response to shocks was due at least in part to a blunted hypothalamic neuronal activation. Experiments using SnMP and SnPP yielded comparable results, and only those focused on SnPP are illustrated here. Transcripts for NGFI-B were barely detectable in control rats or those injected with SnPP alone, but increased significantly ($P<0.01$) following shocks (Fig. 5 and Table 2). As expected, this change was primarily observed in the parvocellular division of the paraventricular nucleus (pPVN), with a smaller response measured in the magnocellular division of this nucleus (mPVN). Prior administration of SnPP significantly ($P<0.01$) blunted the shock-induced up-regulation of NGFI-B mRNA levels in both sections of the PVN. Primary transcripts (hnRNA levels) for CRF, which were restricted to the pPVN, showed the same pattern (Fig. 6).

TABLE 2. Statistical Analysis of the Data Presented in Fig. 5.

Treatment	pPVN	mPVN
Vehicle	2.4 ± 0.3	2.8 ± 0.3
SnPP	2.0 ± 0.4	2.2 ± 0.2
Shocks	34.7 ± 4.1*	15.4 ± 1.7*
SnPP + shocks	22.9 ± 3.6*†	9.0 ± 1.3*†

Data are expressed in terms of O.D. (arbitrary units) for NGFI-B mRNA levels. Each point represents the mean ± SEM of 5 rats. *, $P<0.01$ *versus* vehicle; †, $P<0.01$ *versus* shocks alone.

Effect of SnPP, alone or in combination with L-NAME, on the ACTH response to CRF or VP

The ACTH response to mild electrofootshocks depends on endogenous CRF and arginine vasopressin (VP) (17, 48, 49). We therefore thought that it was important to investigate the influence of NO and/or CO on the ability of these peptides to release ACTH. As we had previously reported (47), pretreatment with L-NAME (50 mg/kg, s.c., −3 h) significantly augmented the ACTH response to VP (0.4 μg/kg), while only marginally and not significantly altering the effect of CRF (0.5 μg/kg) (Fig. 7). Blockade of CO formation by SnPP (60 mg/kg) did not modify the effect of the peptides alone, nor did it alter the effect of L-NAME on VP-induced ACTH release. Similar results were obtained in rats injected with higher doses of CRF (1 or 4 μg/kg) or VP (1 μg/kg) (data not shown).

Discussion

We confirm here our previous findings (17) that blockade of NO formation with L-NAME blunts the ACTH response to mild electrofootshocks. On the basis of these results, we had proposed that in the whole animal, endogenous NO exerted a stimulatory influence on the circuitries responsible for the activation of the HPA axis by this stressor. It has sometimes been argued that L-NAME might act by influencing muscarinic receptors (22). However, the present finding that the effect of L-NAME could be duplicated by other arginine analogues that do not exhibit this property (22), as well as our recent report that 7-nitroindazole, a more specific inhibitor of neuronal NOS (50, 51), also blunts shock-induced ACTH release (52), argues against this possibility. Another mechanism that has been invoked to explain the neuroendocrine effects of blockade of NO formation is that this induces significant rises in blood pressure that might result in altered blood circulation in the brain (53–55). However, our recent finding that the i.c.v. injection of the NO donor SIN-1 significantly increases NGFI-B mRNA and CRF hnRNA levels in the PVN (56), the ability of L-NAME to interfere

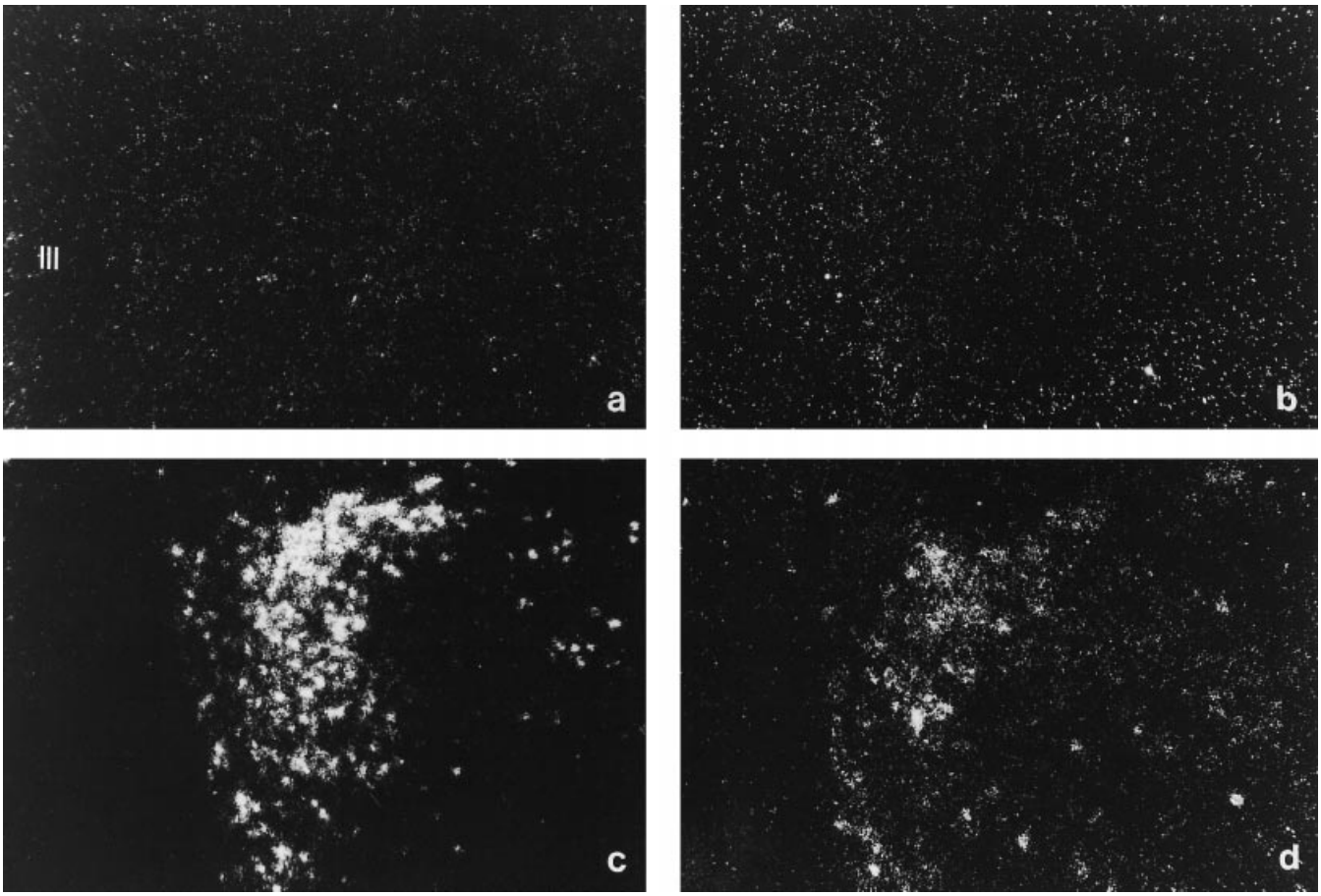

FIG. 5. Dark-field photomicrographs illustrating the interaction between shocks and SnPP on NGFI-B mRNA levels in the PVN. Brains were obtained 30 min after the beginning of the stress. SnPP (60 mg/kg) was injected s.c. 3 h earlier. Magnification, 340 ×. (A) vehicle; (B) SnPP; (C) shocks; (D) SnPP + shocks. III, 3rd ventricle. The statistical analysis of the data is presented in Table 2.

with the increase in PVN neuronal activation induced by shocks (57), as well as the present observation that the BP effects of L-NAME had subsided by the time that the rats were tested for ACTH response, support the concept of a specific stimulatory influence of NO on hypothalamic neurons important for the HPA axis. Another mechanism that we considered potentially important in explaining the inhibitory effect of L-NAME in rats exposed to electrofootshocks is the presence of increased corticosteroid feedback. This possibility was suggested by the ability of L-NAME to slightly, but significantly, elevate baseline corticosterone concentrations. We believe that this possibility is also unlikely because, at least in our hands, acute (within 4 h) increases in corticosterone release (as opposed to exogenously injected dexamethasone) do not significantly alter the ability of footshocks to release ACTH (C. Rivier, unpublished observations). Also, as mentioned before, specific blockade of neuronal NOS, which does not increase adrenal steroid concentrations, similarly blunts shock-induced ACTH secretion. Finally, some investigators have raised the possibility that the decreased ACTH response to shocks observed in rats pre-treated with L-NAME might be explained by their decreased wakefulness (58, 59). However, according to these reports, reduced wakefulness only lasts 1 h, while the inhibitory influence of L-NAME is observed whether this reagent is administered 5 min or 3 h prior to the shocks (52). Therefore we believe that the influence of endogenous NO on the ACTH response to shocks represents a specific effect mediated at least in part through a stimulatory effect of this gas on neurons of the PVN of the hypothalamus and/or their afferents.

An interesting, but so far unresolved, paradox lies in the fact that even though L-NAME decreases the ACTH response to shocks (17), it not only slightly increases basal ACTH levels, but also significantly potentiates the effect of VP on corticotroph activity (47). The rise in baseline ACTH concentrations is usually modest and may be related to the increase in blood pressure. As the magnitude of this hormonal change is small, reconciling it with the ability of blockade of NO formation to significantly decrease the HPA axis response to shocks, is not necessarily difficult. The interactions between NO and VP during shocks are more difficult to understand. Because of the extreme rapidity of the influence of L-NAME on the pituitary response to VP, the hypothesis that a putative inhibition of hypothalamic VP release by L-NAME might upregulate receptors for this peptide can probably be ruled out. As, in our hands, endogenous VP mediates the HPA axis response to shocks (17), our present results suggest that despite the dramatic enhancement of the ACTH response to

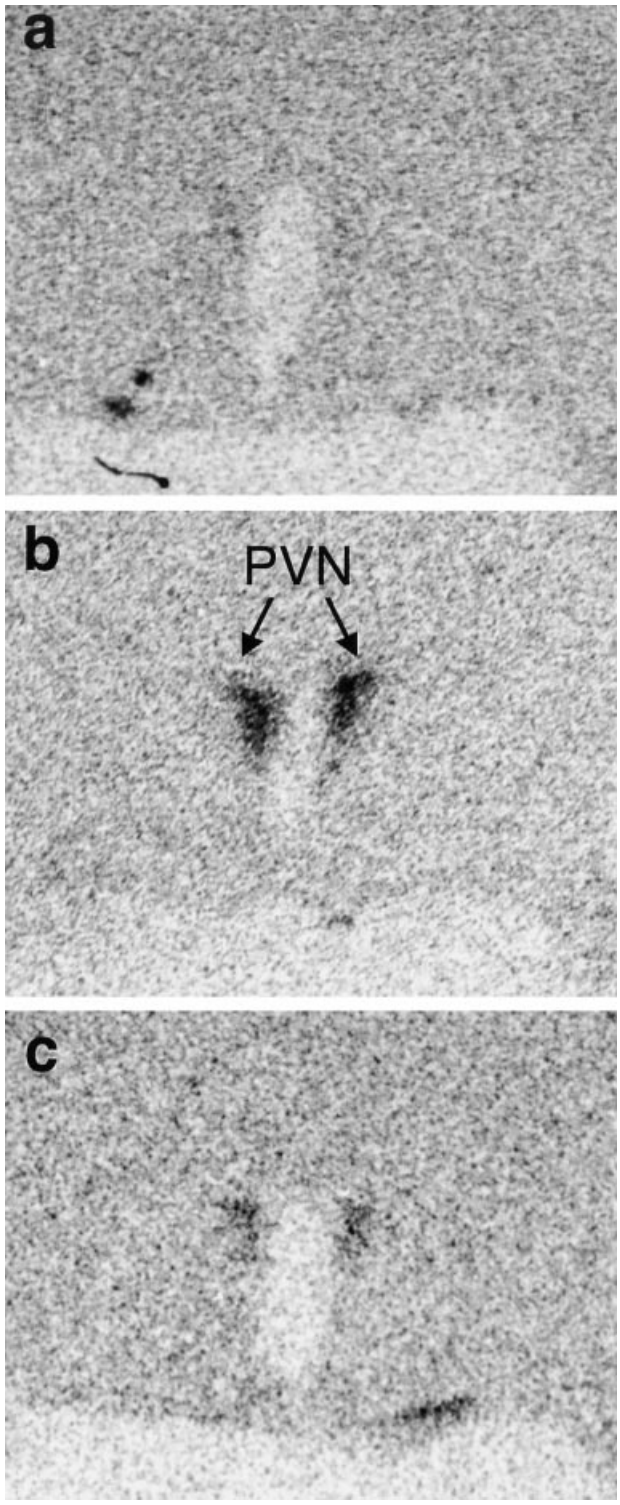

FIG. 6. Autoradiographic photos displaying brain sections hybridized with a [^{33}P]-riboprobe for CRF hnRNA on X-ray film. Brains were obtained 30 min after the beginning of the shocks. SnPP (60 mg/kg) was injected s.c. 3 h earlier. Magnification, 30×. (A) vehicle or SnPP; (B) shocks; (C) SnPP + shocks.

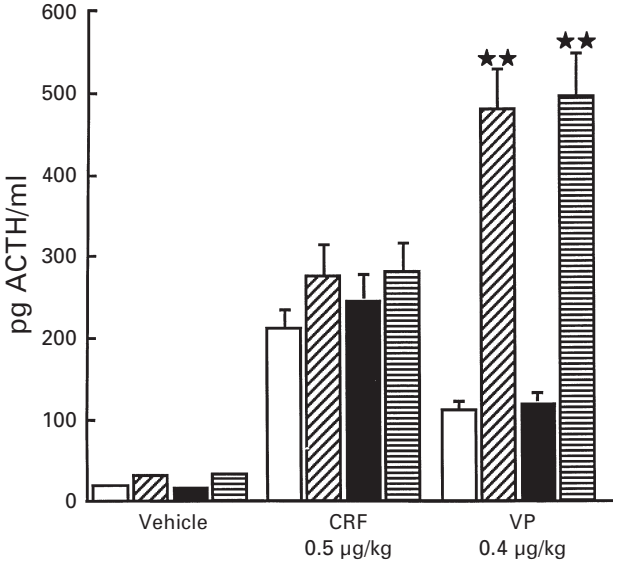

FIG. 7. Single or combined effect of L-NAME (50 mg/kg) and SnPP (60 mg/kg) on the ACTH response to either CRF (0.5 µg/kg, i.v.) or VP (0.4 µg/kg, i.v.). ACTH hormone levels were measured 10 min after administration of the vehicle, CRF or VP. The vehicle, L-NAME, SnPP, or L-NAME plus SnPP were administered s.c. 3 h before the peptides. Each bar represents the mean ± SEM of five rats. **, $P<0.01$ *versus* vehicle. □, Vehicle; ▨, L-NAME; ■, SnPP; ▤, L-NAME.

VP in the presence of L-NAME, the ability of this compound to inhibit the response of hypothalamic neurons to shocks predominates. What we do not know, however, is the magnitude of pituitary sensitization to VP by L-NAME at peptide concentrations present during exposure to shocks. Measurement of increases in plasma VP levels during this stressor, and studies of the effect of L-NAME on the ACTH response to these specific peptide concentrations, might help determine whether this interaction is significant. Our own experience with experiments in which NO formation was blocked, coupled with the information available in the published literature, makes it amply obvious that our understanding of the mechanisms through which this gas modulates the activity of the HPA axis is still in its infancy. Indeed there are still a number of apparently contradictory data that remain difficult to reconcile, as well as many provocative issues that need resolving.

We also report here that like NO, the influence of endogenous CO on the brain pathways responsible for ACTH released by exposure to electrofootshocks appears to be a stimulatory one. To our knowledge, this represents the first demonstration of this effect in the whole animal. While the evidence provided by HO inhibitors remains indirect, the fact that SnPP and SnMP significantly blunted the appearance of CRF transcripts in the PVN of shocked rats, supports the hypothesis that CO stimulates the neuronal activity of the endocrine hypothalamus. As mentioned in the Introduction, controversy remained regarding the ability of SnMP and SnPP to cross the blood–brain barrier following systemic administration. We show here that at the doses and time regimens we used, both compounds significantly decreased HO activity levels in the brain. We examined the whole brain rather than only the

PVN. However, we are currently conducting studies to determine if these changes take place specifically in the PVN. Interestingly, the decrease in the ACTH response to shocks was usually comparable in rats injected with SnMP or SnPP, even though brain HO levels were more dramatically lowered by SnMP.

Though they share some biological activities such as their ability to bind to the iron in heme (46, 60, 61), CO and NO may function differently, as suggested by significant differences in their localization (6, 62), and by their relative abilities to activate guanydyl cyclase (61, 63). In addition, CO is reported to inhibit NOS activity in primary culture of rat cerebellar granule cells by preventing NO-induced cGMP formation (45). We therefore thought it of interest to determine potential interactions between these two gases on the HPA axis. If, as reported in cerebellar cultures, CO interfered with NO production, SnPP, by increasing concentrations of this latter gas, should have increased ACTH levels in shocked rats. The opposite was true. In addition, we show here that the combined effect of removing NO and CO appears additive. At present, we still know too little of the anatomical circuitry in which these gases play a role, to allow us to delineate the sites of their action on neuroendocrine functions, and to determine the basis for their potential interaction. Nevertheless, our results support the concept that the two gases exert similar, not opposite, effects on the HPA axis response to shocks.

One mechanism through which CO and NO inhibitors might alter the HPA axis response to shocks, is through their ability to modify the stimulatory effect of ACTH secretagogues such as CRF and/or VP. We had reported that L-NAME did not significantly alter CRF-induced ACTH release, while potentiating the effect of VP, and had proposed that this mechanism might play a role in the enhanced ACTH response to systemic cytokine treatment (17). The present work confirmed that blockade of endogenous NO formation increased the stimulatory effect of VP on ACTH release. In contrast, blockade of CO formation with SnPP or SnMP did not significantly alter either the ability of CRF or VP to stimulate ACTH secretion, or modify the influence of L-NAME on VP-induced ACTH release. These observations lend support to the hypothesis that the ability of CO to blunt the ACTH response to electrofootshocks is not primarily exerted through changes in pituitary responsiveness. In unpublished preliminary experiments, we also observed that the administration of SnMP produced only a modest effect on the HPA axis response to blood-borne cytokines [ACTH response (pg/mL) measured 15 and 30 min after IL-1β (100 ng/kg): IL-1β alone, 374 ± 55 and 326 ± 38; SnMP + IL-1β, 257 ± 35 and 273 ± 35]. This is in marked contrast to the influence exerted by NO, which we consistently find to be very inhibitory in this paradigm (12, 47). It thus appears that the two gases markedly differ in their ability to influence various portions of the HPA axis, with the influence of CO seemingly restricted to the PVN while that of NO may include a direct pituitary effect as well as a possible presynaptic influence within the median eminence (47).

In conclusion we have shown that in intact rats, both NO and CO are necessary for the full HPA axis response to electrofootshocks. We propose that this effect is exerted on PVN neuronal activity, either directly or indirectly via their afferents, and that at least with regard to CO, this influence is not exerted through changes in pituitary responsiveness to its trophic signals.

Materials and methods

Animals

Adult male Sprague-Dawley rats (ca. 250–270 g) were kept under standard light (12 h lights : 12 h darkness, lights on at 0630) and feeding regimens. Forty-eight to 72 h before the experiment, the animals underwent aseptic insertion of a right jugular venous catheter under rapid (< 3 min) halothane anaesthesia (13). These catheters were used to inject treatments and withdraw blood. Blood samples (0.3 mL each time) were collected for a maximum of four times/rat and immediately replaced with an equivalent volume of apyrogenic saline, a procedure which in our hand does not alter baseline activity of the HPA axis. Exposure to mild electrofootshocks (1 mA, 1 s duration, two shocks/min) was carried out as previously described (17). Each experiment was done at least twice. All procedures were approved by the Salk Institute Animal Care and Use Committee.

Reagents

L-NAME, L-NNA and L-NMMA, purchased from Sigma Corp. (St. Louis, MO, USA), were diluted in saline and injected s.c. at 50 mg/kg in a 0.2 mL volume. These doses are larger than those shown to block > 95% of brain NOS activity following systemic administration (15, 41, 64, 65). Preliminary studies also indicated that increasing the doses up to 120 mg/kg did not produce a larger effect, which suggested the efficacy of the concentration we chose. To specifically block HO, we administered SnMP or SnPP (33, 36, 37, 42), purchased from Porphyrin Products (Logan, UT, USA). The HO inhibitors were dissolved in DMSO, sonicated under red light (66) and injected s.c. in a volume of 0.2–0.4 mL. Dose–response curves were generated in preliminary studies and indicated that doses of 40 and 60 mg/kg produced a maximum effect in the shock paradigm, with higher doses (up to 120 mg/kg) not being more effective. We therefore chose doses of 50–60 mg/kg, which are larger than those reported to block brain HO activity (42, 67). Preliminary experiments were also conducted to determine the time course of action of SnPP. Comparable hormonal results were obtained when SnPP was administered once (3 or 6 h before the experiment) or twice (15 h as well as 3 h before the experiment). Consequently, subsequent experiments were conducted with a single injection, which was done 3 or 6 h before the experiment. In all studies, control rats received the vehicle corresponding to the reagent tested in a particular experiment. Non-quantitative, though attentive, observation of the animals carried out during measurement of blood pressure, failed to indicate obvious stress-related behavioural responses to any of the inhibitors tested. Finally, the potential influence of NO and CO inhibitors on the pituitary response to its trophic signals was determined by injecting human/rat CRF or VP into animals previously administered L-NAME or SnPP. These peptides were synthesized by solid phase methodology (68), provided by Dr Jean Rivier (The Salk Institute, San Diego, CA, USA) and diluted in 0.04 M phosphate buffer, pH 7.4, containing 0.1% BSA and 0.01% ascorbic acid. They were injected i.v. in a 0.5 mL volume.

Blood pressure measurement

Blood pressure was measured via the tail cuff method using a Programmed Electro-Sphygmomanometer (PE-300) (69). All rats were habituated to the apparatus and procedures for at least four 15-min sessions. On the test day, two baseline sessions (averaged) were followed by a drug injection, then by a session each hour for up to 5 h. Each session was of 15 min duration in which eight blood pressure measurements (averaged) were observed at 1.5 min intervals. Data are expressed as displacement (mmHg) from the pre-drug baseline measure.

Adrenocorticotropin hormone assay

Plasma ACTH levels were determined by a commercially available two-site immunoradiometric assay (Allegro kit, Nichols Institute, San Juan Capistrano, CA, USA), which has been validated for the measurement of rat ACTH (47).

Heme oxygenase assay

Measurement of HO activity levels were done in whole brains from a previously published protocol (70) with minor modifications. This assay relies

on the quantification of bilirubin production as an index of CO formation, and has an intra- and interassay coefficient of variation of $<5\%$ and 11%, respectively. The tissues are homogenized in buffer (10 mM tris-HCl, 250 mM sucrose, pH 7.5), centrifuged at $10{,}000 \times g$ for 20 min at 4 °C, the supernatants are centrifuged at $100{,}000 \times g$ for 1 h at 4 °C, and the pellets are resuspended in buffer (20 mM tris-HCl, 0.1 mM EDTA, 20% glycerol, 0.4% triton X-100). For each sample, a reaction mixture is made: 50 μL resuspended sample, 400 μL assay buffer (0.1 M potassium phosphate monobasic, 1 mM EDTA, pH 7.4), 10 μL biliverdin reductase (isolated supernatant following the centrifuge steps, from kidney tissue), 6 μL of 1 mM heme solution (6.52 mg hemin (Sigma), 250 μL 0.1 N NaOH, 100 mg tris-base, 132 mg bovine serum albumin, 9.5 mL water, 200 μL 0.1 N HCl), and 2 μL cytochrome P450 reductase (Sigma) solution (0.04 units). The reaction mixture (210 μL) is added to a test tube and reference tube, and 21 μL of either 2.75 mM NADPH (Sigma) or assay buffer is added to the test and reference tubes, respectively. The tubes are incubated at 37 °C for 8–30 min, placed into an ice-bath to terminate the reaction, then scanned on a spectrophotometer using the reference tube as the blank. The change in optical density of the test tube at the peak (530 nm) compared to the trough (465 nm) is divided by the extinction coefficient of bilirubin (40/mM/cm), and values are expressed in pmol/mg protein/min. Protein quantification of each sample is by the Bradford method using a Bio-Rad protein assay kit (Aldrich Laboratories, Milwaukee, IL, USA).

cRNA probe synthesis and preparation

The pGEM3 plasmid containing an EcoR I fragment of CRF intron (provided by Dr S. Watson) was linearized with Hind III. The pBluescript SK-1 vector containing rat NGFI-B cDNA (provided by Dr J. Milbrandt) was linearized with BamH I. Radioactive cRNA copies were synthesized by incubation of 250 ng linearized plasmid in 6 mM $MgCl_2$, 36 mM Tris (pH 7.5), 2 mM spermidine, 8 mM dithiothreitol, 25 mM ATP/GTP/CTP, 5 mM unlabelled UTP, [α-^{35}S or ^{33}P]UTP, 1 U RNasin (Promega, Madison, WI, USA) and T7 (for CRF introns) or T3 (for NGFI-B) for 60 min at 37 °C. Unincorporated nucleotides were removed using Quick-Spin columns (Boehringer Mannheim, Indianapolis, IN, USA). A sense probe was used as a control for non-specific signal in some adjacent sections for *in-situ* hybridization.

In-situ *hybridization histochemistry*

Four to six rats/group were deeply anaesthetized with chloral hydrate, a drug that does not increase immediate early genes/peptides RNA levels, or ACTH concentrations. The animals were then perfused transcardially with saline followed by 4% paraformaldehyde/0.1 M borate buffer, pH 9.5. The brains were removed and postfixed in 4% paraformaldehyde for 4–5 days, then placed overnight in 10% sucrose/4% paraformaldehyde/0.1 M borate buffer. They were cut into 30 μm coronal slices obtained at 120 μm intervals throughout the hypothalamus, and stored at −20 °C in a cryoprotectant solution (50% 0.1 M phosphate-buffered saline, 30% ethylene glycol and 20% glycerol) until histochemical analysis.

Hybridization histochemical localization of each transcript was carried out using ^{35}S- or ^{33}P-labelled cRNA probes. Cytoplasmic RNA concentrations were measured for NGFI-B, and nuclear levels of RNA were measured for CRF. Protocols for riboprobe synthesis, hybridization and autoradiographic localization of mRNA signals were adapted from Simmons *et al.* (71). All solutions were treated with diethylpyrocarbonate (Dep. C) and sterilized in order to prevent RNA degradation. Sections mounted onto gelatin- and poly L-lysine-coated slides were desiccated under vacuum overnight, fixed in 4% paraformaldehyde for 30 min and digested by proteinase K (10 μg/mL in 50 mM tris HCl, pH 7.5, and 5 mM EDTA, at 37 °C for 25 min). Thereafter, brain sections were rinsed in sterile Dep. C water followed by a solution of 0.1 M triethanolamine (TEA, pH 8.0), acetylated in 0.25% acetic anhydride in 0.1 M TEA and dehydrated through graded concentrations of alcohol (50, 70, 95 and 100%). After vacuum drying for a minimum of 2 h, 90 μL hybridization mixture (10^7 cpm/mL) was spotted on each slide, sealed under a coverslip, and incubated at 60 °C overnight in a slide warmer. Coverslips were then removed and the slides rinsed in 4 × SSC at room temperature. Sections were digested by RNase A (20 μg/mL, 37 °C, 30 min), rinsed in descending concentrations of SSC (2 ×, 1 ×, 0.5 × SSC), washed in 0.1 × SSC for 30 min at 65 °C (1 × SSC: 0.15 M NaCl, 15 mM trisodium citrate buffer, pH 7.0) and dehydrated through graded concentrations of alcohol. After being dried under the vacuum, sections were exposed at 4 °C to X-ray film (Kodak, Rochester, NY, USA) for 15–72 h, defatted in xylene, and dipped in NTB2 nuclear emulsion (Kodak; diluted 1 : 1 with distilled water). Slides were exposed for 6–42 days, developed in D19 developer (Kodak) for 3.5 min at 15 °C and fixed in rapid fixer (Kodak) for 6 min. Thereafter, tissues were rinsed in running distilled water, counterstained with thionin (0.25%), dehydrated through graded concentrations of alcohol, cleared in xylene and cover slipped with DPX.

Quantitative analysis of in-situ *hybridization results*

Semiquantitative densitometric analysis of hybridization signals for RNAs of interest was carried out in nuclear emulsion-dipped slides. Brain paste standards containing serial dilutions of ^{35}S-UTP, used for quantification of mRNA signal, were prepared concurrently to ensure that optical density was found within the linear range of the standard curve (72). In addition, analyses with emulsion-coated slides were carried out with 2–3 different exposure times in order to confirm that signals were not saturated. Densitometric analyses of autoradiographic signals were done over the confines of cells within the PVN using a Leitz optical system coupled to a Macintosh II computer and Image software (version 1.61, W Rasband, NIH). Dark-field measurements for the parvo-and magnocellular divisions of the PVN (pPVN and mPVN) were obtained separately. While it is possible to exclude magnocellular neurones scattered in the parvicellular PVN region (73), we did not use this method. It thus remains possible that counts throughout the medial parvicellular PVN included some magnocellular neurones. Grey level measurements were taken under dark-field illumination of hybridized sections over the medial pPVN, as defined by redirected sampling from the corresponding Nissl stained sections under bright-field images. Data were expressed in gray scale values of 1–256. All grey level measurements were corrected for background. Signals were measured in both sides of the brain, and mean values for all animals(4–6/group) were determined for each rats in 3–4 sections throughout the PVN. While image analysis was performed similarly for all probes, we could not analyse emulsion-coated sections for CRF hnRNA because of the long exposure time required to detect these signals.

Statistical analysis

Statistical analysis was performed by one-and two-way factorial ANOVA, Tukeys *post-hoc* test, and/or Dunn-Sidak procedure for contrasting means. Estimating cumulative release by measuring the area of the curve above baseline is usually considered inappropriate for open-ended curves. We therefore used the following formula instead:

$$\text{cumulative ACTH levels} =$$

$$a/x + 1/x(b-a/2) + b/x + 1/x\ (c-b/2) + c/y + 1/y(d-c/2)$$

where a–d represent the time points of the bleeds and x and y represent the fraction of total time encompassed by the interval between bleeds. Baselines were not subtracted for the graphs shown in the present work, but separate statistical analysis that included them indicated comparable results (not shown).

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