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Short communication

Endotoxin stimulates an endogenous pathway regulating corticotropin-releasing hormone and vasopressin release involving the generation of nitric oxide and carbon monoxide

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Abstract

Although the administration of endotoxin in vivo activates the neuroendocrine stress axis in the process of crosstalk between the immune and endocrine axes, the direct application of endotoxin to the hypothalamus in vitro does not stimulate the release of the hypothalamic peptides controlling the hypothalamo–pituitary–adrenal (HPA) axis, corticotropin-releasing hormone (CRH) and vasopressin. The hypothesis has therefore been tested that endotoxin may also activate inhibitory pathways, specifically those involving the generation of nitric oxide (NO) and carbon monoxide (CO). Studies were performed on the isolated rat hypothalamus using endotoxin in the presence or absence of inhibitors of heme oxygenase (which generates CO) and nitric oxide synthase, and ferrous hemoglobin. Endotoxin alone decreased both CRH and vasopressin secretion from the hypothalamus. However, when applied together with a nitric oxide synthase inhibitor, the inhibitory effect on CRH was lost. Conversely, co-administration with heme oxygenase inhibitors transformed the inhibition of vasopressin to stimulation, while having no effect on the inhibition of CRH. Ferrous hemoglobin reversed the inhibition of vasopressin, but did not lead to stimulation. It is therefore concluded that endotoxin may stimulate endogenous pathways that lead to the generation of NO, which in turn inhibits CRH. In addition, it generates CO, which modulates the release of vasopressin. These gases are thus potential counter-regulatory controls to the activation of the HPA.

Keywords: Endotoxin; LPS; Vasopressin; CRH; Carbon monoxide; Nitric oxide

1. Introduction

The defense of an organism in the face of hostile microbial attack involves the coordinated display of a panoply of specific and non-specific immune mechanisms. Such defenses include inflammatory mediators that determine the type and timing of the influx of immune cells. However, such inflammatory mediators can also cause tissue damage, and the inflammatory focus needs to be contained in both time and space. It has been suggested that this is the primary role of glucocorticoids, which are activated by numerous cytokines such as interleukin-1 (IL-1) (Munck et al., 1984; Grossman, 1991). Lipopolysaccharide (LPS) has been shown to increase corticotropin (ACTH) and corticosterone in the rat (Rettori et al., 1994; Mekauche et al., 1996), principally via the generation of hypothalamic factors such as corticotropin-releasing hormone (CRH) and vasopressin involved in the regulation of the hypothalamo–pituitary–adrenal (HPA) axis (Berkenbosch et al., 1987; Sapolsky et al., 1987; Suda et al., 1990; Navarra et al., 1991; Yasin et al., 1994). In addition, endotoxin may activate peripheral autonomic nerves which, in turn, relay the information to the central nervous system (Tilders et al., 1994). However, there is evidence that at high doses endotoxin may act directly on the hypothalamus to stimulate IL-1 production and secretion (Hagan et al., 1993; Hillhouse and Mosley, 1993; Mirtella et al., 1994; Rettori et al., 1994). In spite of this, we and others have been unable to show an increase of

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either CRH or vasopressin after the direct application of endotoxin to acute hypothalamic explants (Spinedi et al., 1992; Pozzoli et al., 1994a). We therefore hypothesized that the failure of endotoxin to directly increase CRH and vasopressin from the hypothalamus may be due to simultaneous activation of endogenous pathways that lead to the generation of substances that inhibit both CRH and vasopressin. In particular, the gaseous neurotransmitters nitric oxide (NO), which is generated by nitric oxide synthase (NOS), and carbon monoxide (CO), which is generated by heme oxygenase (HO), have been shown to modulate both CRH and vasopressin release, and both enzymes are located in the hypothalamic paraventricular and supraoptic nuclei (Grossman et al., 1994; Vincent et al., 1994). We have therefore speculated that generation of these gases might obscure a direct stimulatory effect of LPS on CRH and vasopressin release, and have tested this by measuring CRH and vasopressin released from acute rat hypothalamic explants.

2. Materials and methods

2.1. Tissue preparation

The experiments were performed using male Wistar rats according to previously described protocols for the acute removal and incubation of hypothalamic explants (Tsagarakis et al., 1988; Yasin et al., 1993a). The hypothalamic blocks obtained after dissection were bisected longitudinally through the mid-sagittal plane, and the two hypothalamic halves were incubated in the same vial. After preincubation for 80 min, the tissue was incubated in fresh medium for a 20-min control period (period B1): this was followed by aspiration and replacement with fresh medium for one or more 20 min incubation periods, in either medium alone (control basal groups) or medium containing graded concentrations of test substances (test groups; period B2), the total length of each experiment being 2 h. Samples obtained from the incubations were stored at -20°C until assay for vasopressin or CRH immunoreactivity.

2.2. Experimental protocol

In the first series of experiments, the effect of LPS at a concentration of 10 ng/ml, 100 ng/ml, 1 μg/ml and 10 μg/ml on basal vasopressin release was studied. On the basis of these results, further experiments were performed on selected doses of LPS, namely 1 μg/ml and 10 μg/ml. The effect of the NOS inhibitor, N^G-monomethyl-L-arginine (L-NMMA) at a concentration of 10^{-3} M and 10^{-4} M, both alone and in the presence or absence of LPS 1 μg/ml, was investigated. The effect of the HO inhibitors zinc–protoporphyrin–IX (ZnPP9, 10^{-7} M) and the more specific Sn–mesoporphyrin–IX (SnMP9, 10^{-6} M; Boni et al., 1993) in the presence or absence of LPS (1 μg/ml and 10 μg/ml) on vasopressin release, was also investigated. Finally, the effect of ferrous hemoglobin A_0 10^{-5} M and 10^{-6} M, a scavenger of both NO and CO, was investigated in the presence or absence of LPS 1 μg/ml and 10 μg/ml.

In a separate series of studies, the effect of similar drugs and drug combinations was performed with the measurement of CRH: insufficient medium was available for the assay of both vasopressin and CRH in the same study. The experiments were broadly comparable to those carried out for vasopressin, but the NO antagonist L-NO-Arg was used in place of L-NMMA to confirm the results.

2.3. Hormone assays and drugs

The concentrations of vasopressin and CRH in the incubation medium was measured by radioimmunoassay as previously described (Tsagarakis et al., 1988; Yasin et al., 1993a). All drugs used in this study were freshly prepared and diluted immediately before their addition to the incubation vials. LPS (lipopolysaccharide from *Escherichia coli*, serotype O26:B26) and hemoglobin (ferrous hemoglobin A_0) were obtained from Sigma (Poole, Dorset, UK). LPS was initially diluted in phosphate-buffered saline and then to the desired concentration in fresh EBSS. N^G-monomethyl-L-arginine monoacetate salt (L-NMMA) was obtained from Calbiochem-Novabiochem (La Jolla, USA), L-NO-Arg from Bachem Feinchemikalien AG (CH4416 Bubendorf, Switzerland). Zinc–protoporphyrin–IX (ZnPP9) was obtained from Research Biochemicals International (Natoick, MA, USA) and was dissolved initially in 0.1 M NaOH and then to the desired concentration in the incubation medium. Sn–mesoporphyrin–IX was obtained from Porphyrin Products (Logan, UT, USA) and was dissolved initially in slightly basic aqueous solution, and then to the desired concentration in fresh EBSS.

2.4. Statistical analysis

Data are expressed as ratios of hormone released during experimental to the preceding control incubations. The data were analysed by an overall one-way analysis of variance to test if there was any significant drug effect, followed by Student's *t*-test with Dunnett's correction for multiple comparisons where appropriate.

3. Results

Vasopressin release was unaffected by the addition of LPs at a concentration of 10 ng/ml or 100 ng/ml, but decreased significantly after the addition of LPS at a concentration of 1 μg/ml and 10 μg/ml (both $P < 0.01$ compared to control). When hypothalami were incubated with LPS 1 μg/ml in the presence of the NOS inhibitor,

L-NMMA (10^{-3} M or 10^{-4} M), the level of vasopressin was not significantly different from either basal or LPS-inhibited conditions (Fig. 1B). However, when hypothalami were incubated with LPS 1 μg/ml in the presence of ZnPP9 10^{-7} M, vasopressin release was significantly *stimulated* when compared to the control incubation ($P < 0.001$; Fig. 1A). Co-incubation with both ZnPP9 (10^{-7} M) and L-NMMA (10^{-3} M) in the presence of LPS (1 μg/ml) demonstrated significant stimulation of vasopressin release ($P < 0.001$ compared to control), which was comparable to that seen when L-NMMA was not added (Fig. 1B). Vasopressin release also increased significantly when hypothalami were incubated with an alternative more specific inhibitor of heme oxygenase, SnMP9 10^{-6} M, in addition to LPS 1 μg/ml ($P < 0.05$; Fig. 1C). Using LPS 10 μg/ml, SnMP9 10^{-6} M reversed the LPS-induced suppression but did not reveal stimulation. When hypothalami were incubated with LPS 1 μg/ml in the presence of ferrous hemoglobin A_0 10^{-6} M or 10^{-5} M, vasopressin release was similar to that observed under control conditions (Fig. 1D). Essentially, similar results were seen with LPS 10 μg/ml (data not shown). None of the antagonists used, nor ferrous hemoglobin, affected the basal release of vasopressin.

For CRH, LPS 1 ng/ml caused no change in the basal release of CRH, but this was significantly inhibited by incubation in the presence of LPS 1 μg/ml ($P < 0.05$). When L-NMMA 10^{-6} M or 10^{-3} M was added to LPS 1 μg/ml, the release of CRH was similar to basal secretion, and significantly different to the incubation involving LPS alone ($P < 0.05$; Fig. 2A). However, no *stimulation* of CRH release was seen at either dose of L-NMMA. Simi-

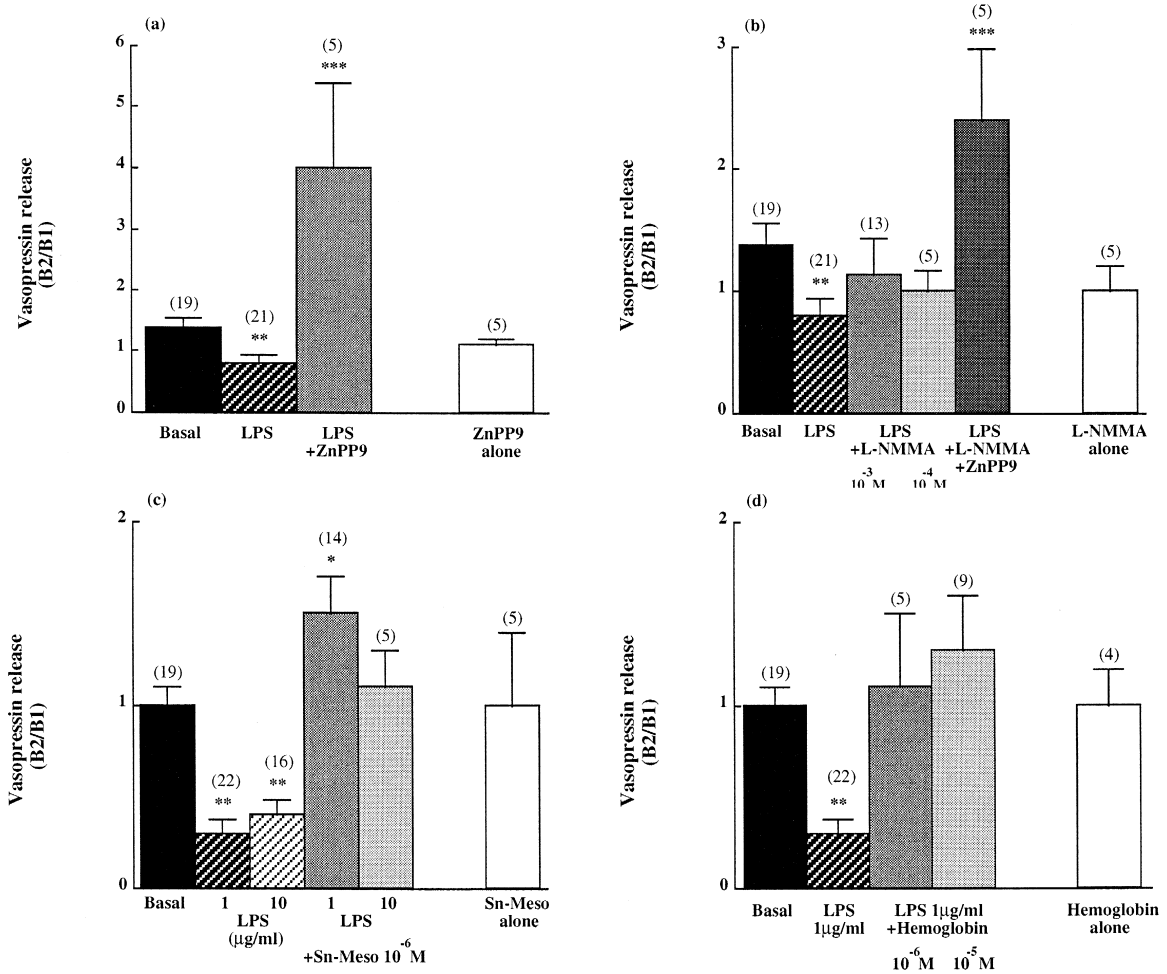

Fig. 1. (A) The effect of LPS (1 μg/ml), ZnPP9 (10^{-7} M) alone and ZnPP9 (10^{-7} M) in the presence of LPS (1 μg/ml) on vasopressin release. Bars represent means ± S.E.M. and numbers in parentheses indicate number of hypothalami. $**=p<0.01$, $***p<0.001$ as compared to control. (B) The effect of LPS (1 μg/ml), L-NMMA (10^{-3} M) alone, LPS (1 μg/ml) in the presence of L-NMMA (10^{-3} M and 10^{-4} M) and LPS (1 μg/ml) in the presence of both L-NMMA 10^{-3} M and ZnPP9 10^{-7} M on vasopressin release. Bars represent means ± S.E.M. and numbers in parentheses indicate number of hypothalami. $**=p<0.01$, $***=p<0.001$ as compared to control. (C) The effect of LPS (1 μg/ml and 10 μg/ml) in the absence and presence of Sn–mesoporhyrin–IX (Sn–Meso) 10^{-6} M on vasopressin release. Bars represent means ± S.E.M., and numbers in parentheses indicate number of hypothalami. $**p<0.01$, $*p<0.05$ as compared to control. (D) The effect of LPS (1 μg/ml) in the absence and presence of ferrous hemoglobin A_0 (Hb) 10^{-5} M and 10^{-6} M on vasopressin release. Bars represent means ± S.E.M. and numbers in parentheses indicate number of hypothalami. $**p<0.01$ as compared to control.

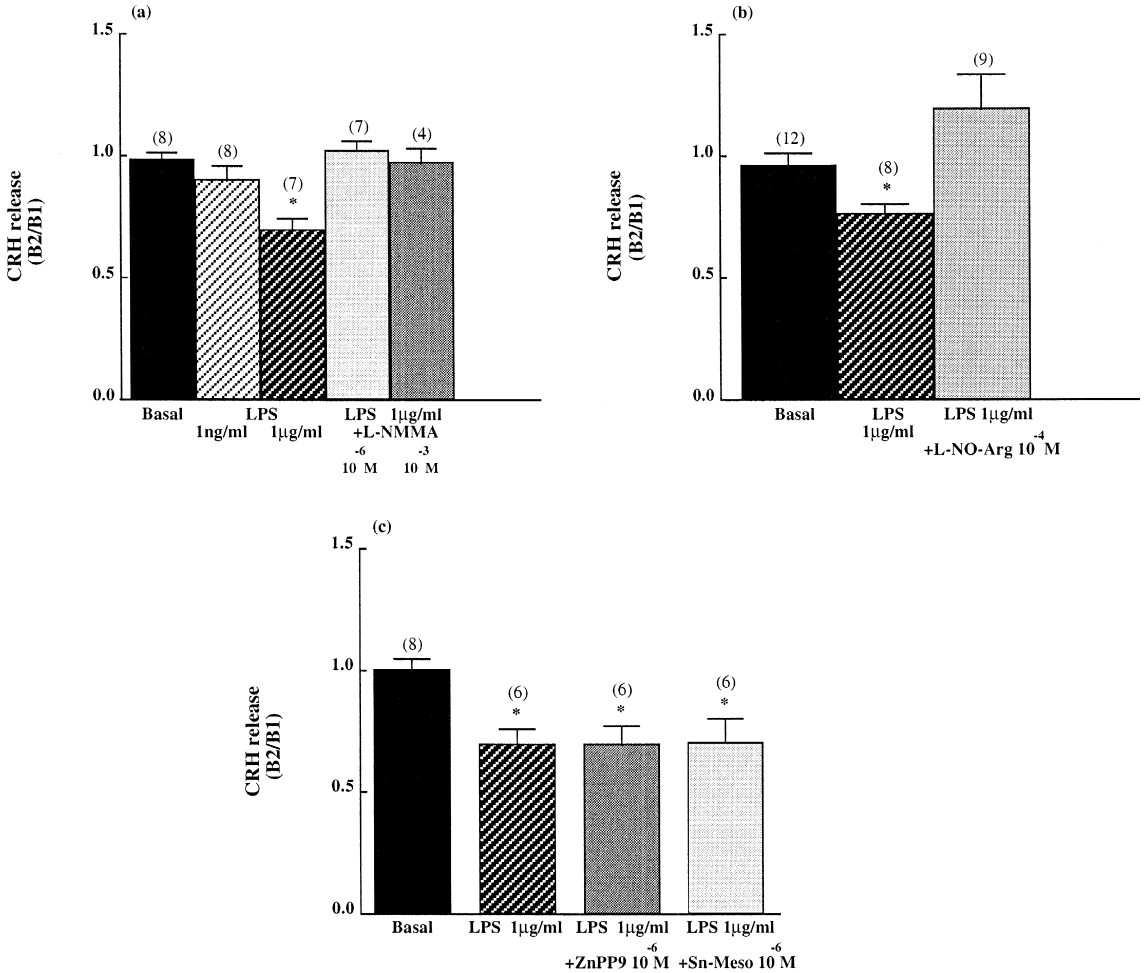

Fig. 2. (A) The effect of LPS (1 ng/ml and 1 μg/ml) and L-NMMA (10^{-6} M and 10^{-3} M) in the presence of LPS (1 μg/ml) on basal CRH release. Bars represent means ± S.E.M., and numbers in parentheses indicate number of hypothalami. $* = p < 0.05$ as compared to control. (B) The effect of LPS (1 μg/ml) in the absence and presence of L-NO-Arg (10^{-4} M) on basal CRH release. Bars represent means ± S.E.M. and numbers in parentheses indicate number of hypothalami. $* = p < 0.05$ as compared to control. (C) The effect of LPS (1 μg/ml) and LPS (1 μg/ml) in the presence of either ZnPP9 (10^{-6} M) or Sn–mesoporphyrin–IX (Sn–Meso 10^{-6} M) on basal CRH release. Bars represent means ± S.E.M. and numbers in parentheses indicate number of hypothalami. $* p < 0.05$ as compared to control.

larly, co-incubation with L-NO-Arg 10^{-4} M and LPS 1 μg/ml reversed the inhibition shown with LPS alone (Fig. 2B). Contrary to the results found for vasopressin, neither ZnPP9 (10^{-6} M) nor SnMP9 (10^{-6} M) had any effect on the inhibition of CRH shown by LPS 1 μg/ml (Fig. 2C).

4. Discussion

The present data suggest that LPS actually inhibits rather than stimulates the acute release of both vasopressin and CRH from rat hypothalamic explants. In the case of CRH, this inhibition can be reversed by agents which inhibit the generation of NO, returning CRH release to basal levels; the data for vasopressin are less clearcut, and do not definitely incriminate NO in LPS-induced inhibition. By contrast, agents that block CO production revealed a stimulatory role for LPS in the release of vasopressin, but not for CRH. Taken together, the results are consistent with the acute effects of endotoxin on neuroendocrine function being masked by concurrent activation of endogenous pathways leading to the generation of NO and CO.

There are a number of reports on the putative effects of NO generation on CRH and vasopressin in vitro which are not entirely consistent (Brunetti et al., 1993; Costa et al., 1993; Karanth et al., 1993), but the response of the HPA to inflammatory challenges in vivo are most consistent with NO being activated in this situation to restrain the responsiveness of the HPA (Rivier and Shen, 1994; Rivier, 1995). The situation with regard to vasopressin is less controversial, there being a consensus that NO is an important inhibitory regulator (Summy-Long et al., 1993; Yasin et al., 1993b; Kadowaki et al., 1994). The current results now demonstrate that there may be acute and direct effects of endotoxin to activate NOS, and hence suppress CRH and possibly vasopressin release. These results may also be compared to the effects of LPS in vivo, whereby the

inducible form of NOS, iNOS, is upregulated in the hypothalamus after some delay in response to intra-peritoneal injection (Wong et al., 1996; Jacobs et al., 1997). However, using NO antagonists we were unable to show any *stimulatory* effect of LPS on either vasopressin or CRH release: it would appear that NO generation is responsible for the acute inhibitory modulation of CRH by LPS, while the situation regarding vasopressin is currently not definable.

Carbon monoxide may also act as a physiological regulator (Mansouri and Perry, 1982, 1984; Brune and Ullrich, 1987; Vedernikov et al., 1989; Utz and Ullrich, 1991; Verma et al., 1993; Zhuo et al., 1993). Carbon monoxide is formed endogenously by the action of heme oxygenase (Tehnunen et al., 1969), and has been shown to inhibit vasopressin (Mancuso et al., 1997a) and oxytocin (Kostoglou-Athanassiou et al., 1996) release, although its effects on CRH appear to vary according to the precise experimental conditions (Parkes et al., 1994; Pozzoli et al., 1994b). However, we have been able to demonstrate that the HO substrate, hemin, is most effective at suppressing vasopressin release at a concentration at which CO generation is maximal (Mancuso et al., 1997a). Our present results now show that LPS can directly activate HO, such that specific inhibition of its generation allows a stimulatory effect of LPS on vasopressin to become evident. Furthermore, we were unable to demonstrate stimulation of either vasopressin or CRH using specific NO antagonists such as L-NMMA or L-NO-Arg. This adds support to our conclusion that the acute stimulation of vasopressin release by LPS in the presence of the porphyrin antagonists is due to blockade of CO production. Thus, our results are broadly compatible with the notion that LPS activates both NOS and HO to generate NO and CO, the latter in particular suppressing its direct stimulatory effect on vasopressin.

The second messengers for both NO and CO may be guanylate cyclase, but this is not necessarily the case, and others have been suggested, including a cytochrome *P*450-dependent oxygenase for CO in the fetal ductus arteriosus (Coceani, 1994). Activation of a common second messenger would explain why there was no additional effect when NO and CO generation were simultaneously blocked, although it is then difficult to understand the specificity of the effect for each neuropeptide, especially as the generating enzymes for these gaseous neurotransmitters are extensively co-stored in the hypothalamus (Ewing and Maines, 1992) and diffuse in a non-synaptic manner. It is possible that the separate locations of CRH and vasopressin-containing neurons, plus the very different half-lives of the two gases, determine their differential effects on the two neuropeptides. Alternatively, recent data incriminating guanylate cyclase as the second messenger for NO and cyclo-oxygenase for CO may help explain these discordant results (Mancuso et al., 1997b, 1998).

We have previously been unable to demonstrate induction of either constitutive or inducible isoforms of HO mRNA in the rat hypothalamus using either high dose or low dose endotoxin, in a model system where the induction of inducible nitric oxide synthase was readily demonstrable (Jacobs et al., 1997). This suggests that while endotoxin directly and acutely may increase HO activity, in the longer term, induction of message only occurs for the enzymes generating NO rather than CO. This temporal dislocation may, in turn, indicate that the roles of these two gases, which we have found in our system to have broadly similar effects on CRH, vasopressin and oxytocin, may be distributed in time such that CO mediates an early and NO a late modulatory role on vasopressin release. Whether this translates to the in vivo situation, and indeed whether this is relevant to vasopressin as a modulator of the HPA axis, clearly requires further study. Nevertheless, our data support the concept that these counter-regulatory pathways are an important component of the organism's susceptibility to endotoxic shock.

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