Acute and Subacute Effects of Endotoxin on Hypothalamic Gaseous Neuromodulators

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ABSTRACT: Although two-way communication between the hypothalamus and the immune system is now well established, particularly for the hypothalamo–pituitary–adrenal axis, the role of the gaseous neurotransmitters nitric oxide (NO) and carbon monoxide (CO) is much less well understood in terms of hypothalamic function. These agents are an important part of the peripheral inflammatory response; and their synthetic enzymes, NO synthase (NOS) and heme oxygenase (HO), respectively, have been localized to the hypothalamic PVN and SON. The induced generation of both NO and CO leads to the suppression of CRH and vasopressin, the major stimulators of the HPA. Thus, the addition of hemin to hypothalamic explants is maximally active at 1 μM in attenuating the release of CRH and vasopressin, and this dose is also most effective in generating biliverdin and associated CO. CO generation is also able to stimulate cyclooxygenase to produce prostaglandin E_2, an established intermediary in the cytokine-stimulated activation of the HPA. Finally, inducible NOS mRNA is specifically induced in the hypothalalmus in response to endotoxin, in parallel to interleukin-1. These data provide increasing evidence in favor of NO and CO as counterregulatory agents in the HPA response to immune activation.

GASES IN THE HYPOTHALAMUS

Nitric oxide (NO) is now well established as a major endogenous vasorelaxant and is also involved in mediating nonimmunological antimicrobial activity via a variety of cell types, most particularly those of the macrophage lineage.[1] The enzyme involved in the synthesis of NO, nitric oxide synthase (NOS), has been described in

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two major isoforms, which are products of two separate genes: type II NOS or "inducible" NOS (iNOS) is the major inducible form and is synthesized by macrophages in response to a variety of inflammatory stimuli and is relatively independent of the calcium milieu, whereas type III NOS (eNOS) is the principal endothelial-derived isoform that is constitutively present in the endothelium and whose activity is highly dependent on prevailing calcium concentrations.[2] In addition, a specific neuronal isoform, type I or nNOS, has also been described as an independent gene product that is functionally most closely related to the endothelial enzyme; nNOS has been implicated in neuronal signaling, specifically in the peripheral autonomic nervous system and in long-term potentiation in central pathways.[3]

NO has also been shown to be involved in modulating neuroendocrine function, with administration of NO donors and NOS antagonists *in vivo* causing changes in pituitary–gonadal,[4,5] pituitary–adrenal,[6,7] and neurohypophyseal axes.[8,9] With regard to the latter, NO antagonism has been shown to enhance the responsiveness of the hypothalamo–pituitary–adrenal (HPA) axis to endotoxin administration, suggesting that endotoxin activated an inhibitory NOS pathway.[6,7] Furthermore, NO donors have been reported to attenuate the induced release of CRH[10] and vasopressin[11] *in vitro*, while nNOS has been shown by both immunocytochemistry and *in situ* hybridization to be concentrated in the hypothalamic paraventricular (PVN) and supraoptic nuclei (SON),[12,13] specifically in both neurohypophyseal[14] and corticotropin-releasing hormone (CRH)-containing neurons.[15] These data suggest that hypothalamic NOS is an important inhibitory modulator of HPA responsiveness to inflammatory stress. However, studies on changes in hypothalamic NOS enzyme or messenger RNA levels in response to endotoxin have been few, and frequently conflicting.[16]

Another gaseous neurotransmitter, carbon monoxide (CO), has also been implicated in neuroimmune regulation: generation of CO by smooth muscle cells has been shown to be vasodilatory,[17] and its production within an inflammatory milieu may serve to limit the inflammatory response.[18] CO is synthesized by two isoforms of heme oxygenase (HO), an inducible HO_1, and a constitutive HO_2, both of which have been localized to the PVN and SON.[19,20] There is recent evidence that the generation of CO inhibits the secretion of CRH[21] and oxytocin,[22] and stimulates the release of gonadotrophin-releasing hormone,[23] paralleling results for NO, and there appears to be interdependence of the NO/NOS and CO/HO pathways in the central nervous system (CNS).[24] It is likely that both NO and CO act through the same pathways, binding metalloproteins such as guanylate cyclase which are allosterically modified to increase, for example, cyclic GMP. However, the involvement of NO and CO in the HPA response to endotoxin has not been systematically investigated. We therefore studied the possible involvement of these gaseous neuromodulators on the HPA by looking at the effects of (i) *in vivo* endotoxin administration on hypothalamic messenger RNA levels for the major NOS and HO transcripts, and (ii) of *in vitro* endotoxin administration on vasopressin release in the presence of specific NO and CO enzyme inhibitors.

EXPERIMENTAL DESIGN

In Vivo

Male Sprague-Dawley rats (200–250 g body weight) were injected intraperitoneally with endotoxin (*E. coli* 055 B5) dissolved in sterile normal saline (250

μg/100 g) to give a final volume of 0.5 ml. Two control groups were used, animals either receiving 0.5 ml sterile normal saline or no injection at all. The animals were killed by decapitation 1, 3, 8, or 24 hours after endotoxin administration ($n = 4$ per group). Immediately after decapitation the brain was dissected, and the liver was removed; the hypothalamus and liver tissue was snap frozen, and total RNA extracted and quantified.[25] A 5-μg sample of total RNA was reverse transcribed using the method described by Chew *et al.*,[26] and then subjected to PCR amplification using β actin as internal control and primers for either neuronal NOS, inducible NOS, heme oxygenase$_1$, heme oxygenase$_2$, or interleukin-1β. A 10-μl sample of the final reaction product was run on a 2% agarose gel containing ethidium bromide; and after electrophoresis the gels were viewed using UV light on a transilluminator, and Polaroid photographs were taken as a record of the results. Densitometry of the resulting bands gave absorbance readings for each of the fluorescent product bands. Results were expressed as a ratio of the resultant optical densities for the specific gene to β actin.

Individual comparisons were made using Student's *t*-tests, with Dunnett's correction for multiple comparisons, where appropriate.

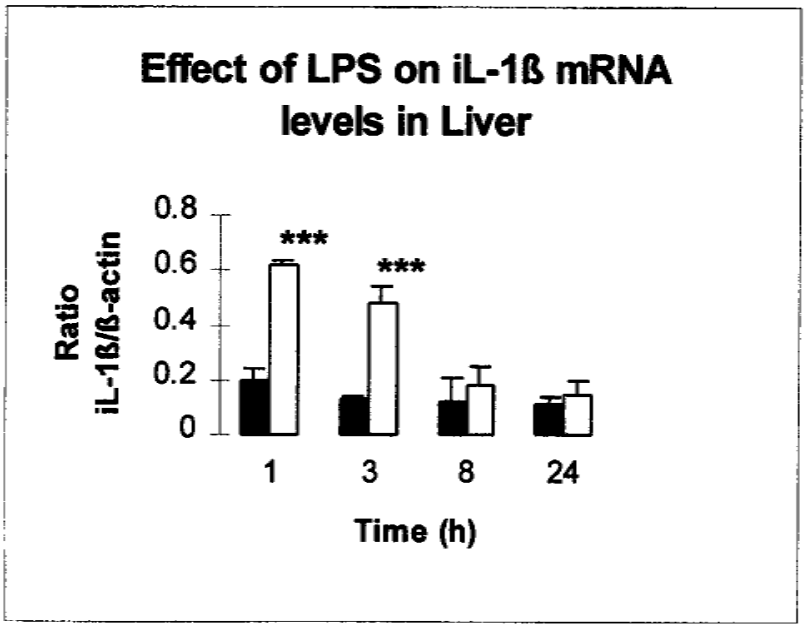
In Vitro

We used a previously validated acute hypothalamic explant model to investigate the acute effects of endotoxin (*E. coli* 055 B5) on the release of vasopressin and CRH. Male Wistar rats (200–250 g) were killed at 09.00–10.00 hours, and their hypothalami rapidly removed. After 60 minutes of stabilization in Earle's supplemented balanced salt solution, they were incubated for 20-minute periods in the presence or absence of a variety of drug solutions. The ratio of hormone release in the presence of a given drug or drug combination (B2) was related to that in the preceding incubation in medium alone (B1) to compute the B2/B1 ratio, and compared by means of Student's *t*-test (with Dunnett's correction for multiple comparisons, where appropriate) to a parallel incubation in the absence of additives. Initial studies showed that LPS caused interference in the CRH RIA, and only data on vasopressin will be shown here.

RESULTS OF *IN VIVO* STUDIES

The liver tissue removed from rats receiving LPS showed a rapid and significant increase in the level of IL-1β when compared to control animals (FIG. 1**A**). This remained significantly raised for 3 hours ($p < 0.001$) and was seen to return to control levels at 8 hours. A similar pattern was seen in the hypothalamus (FIG. 1**B**) with peak values of mRNA at 1 hour ($p < 0.001$); however, these remained high at 8 hours, returning to control levels at 24 hours.

There was no significant effect of LPS on hypothalamic nNOS compared to the control injection at any time point studied. By contrast, administration of LPS showed a marked effect on iNOS expression in both rat liver (FIG. 2**A**) and hypothalamus (FIG. 2**B**.) The hepatic tissue showed low basal levels of iNOS, which began rising 1 hour after LPS and were greatly enhanced at 3 hours ($p < 0.001$). By 8 hours the response had returned to control levels and remained at this level at 24 hours. The hypothalamus showed a similar trend but at a reduced level of induction. At 1 hour a small increase was shown, which by 3 hours had become



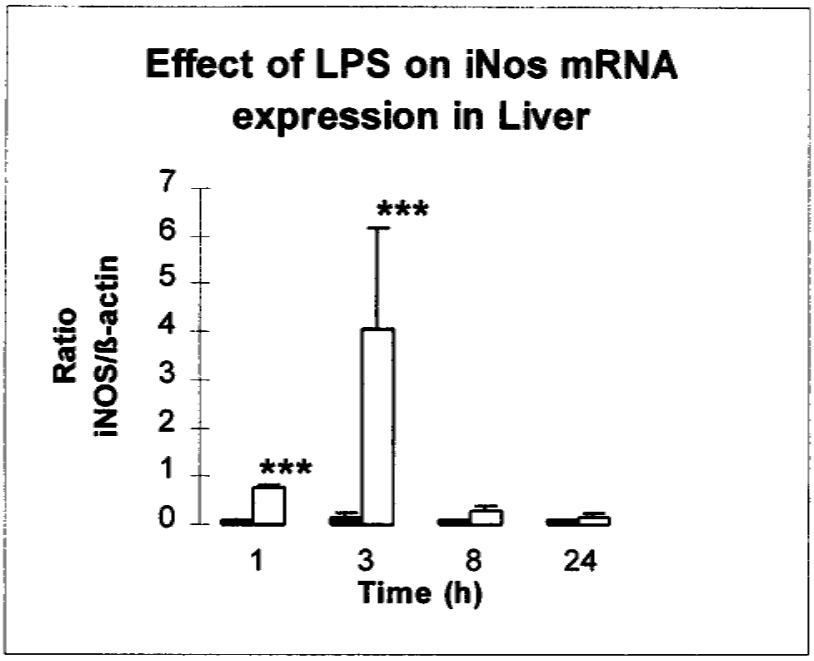
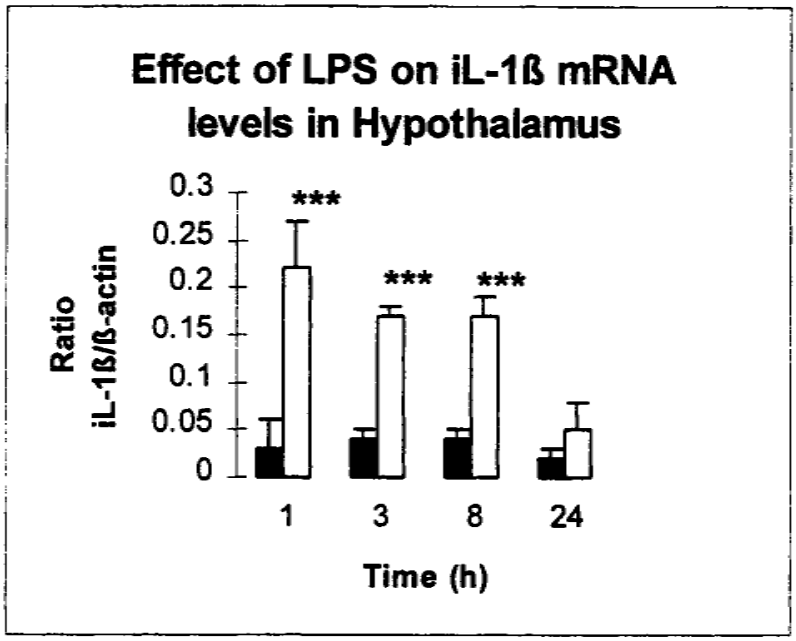


FIGURE 1. A *(upper panel)*: The effect of i.p. LPS on levels of interleukin-1β mRNA levels in rat liver. **B** *(lower panel)*: The effect of i.p. LPS on levels of iNOS mRNA levels in rat liver. *** $p < 0.001$ compared to control.



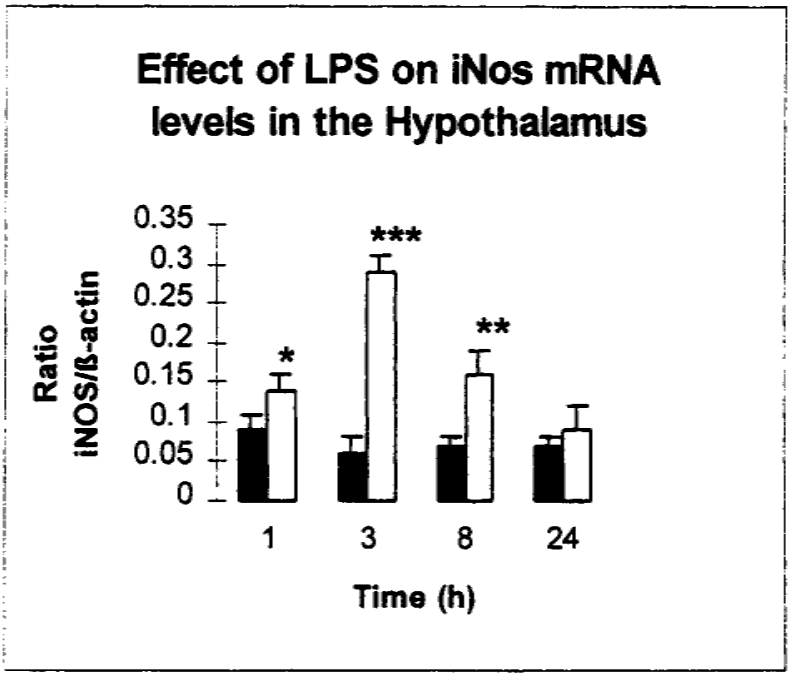


FIGURE 2. A *(upper panel)*: The effect of i.p. LPS on levels of interleukin-1β mRNA levels in rat hypothalamus. **B** *(lower panel)*: The effect of i.p. LPS on iNOS mRNA levels in rat hypothalamus. $^{*}p < 0.05$; $^{**}p < 0.005$; $^{***}p < 0.001$ compared to control.

statistically significant ($p < 0.001$). Unlike the liver, this remained significantly elevated at 8 hours, but had returned to control levels by 24 hours.

Both HO_1 and HO_2 were identified under basal conditions in both liver and hypothalamic tissue. A small but statistically significant increase in HO_1 was seen in liver tissue 1 hour after LPS, but from 3 hours onwards there was no significant difference between control and LPS-injected rats. LPS had no effect on either hypothalamic HO_1 or HO_2 at any time point.

RESULTS OF *IN VITRO* STUDIES

Endotoxin caused significant inhibition of the release of vasopressin at doses of 1 and 10 μg/ml (FIG. 3). Co-incubation of the explants with endotoxin and the NO synthase inhibitor, L-NMMA, showed inhibition of vasopressin that was of a similar magnitude (FIG. 4). However, co-incubation with the heme oxygenase antagonist, zinc prototoporphyrin-9 (ZnPP-9), reversed this inhibition and replaced it with significant stimulation (FIG. 5). Similarly, co-incubation with the heme oxygenase antagonist tin mesoporphyrin (SnMP), said to be more specific for heme oxygenase than ZnPP-9, also converted endotoxin-induced inhibition to stimulation (data not shown). Finally, addition of ferrous hemoglobin to endotoxin led to loss of inhibition, although no significant stimulation was noted (data not shown). None of these agents had any effect when added alone.

DISCUSSION

In the liver, endotoxin-treated rats showed an early and marked rise in IL-1β mRNA that gradually returned to control levels by 24 hours. Similarly, changes in iNOS and, to a lesser extent, HO_1 mRNA were seen in the same animals. These results are concordant with extensive previous studies demonstrating activation of these inducible agents during endotoxemia as part of the acute-phase response.[27,28] Although the cells of origin of these transcripts cannot be addressed by these studies, previous work has suggested that these arise predominantly from hepatic Kupffer cells, which are a component of the reticulo-endothelial system and act as sessile macrophages/monocytes.

We were particularly interested in exploring changes in the hypothalamus, as this has been thought to be the major locus for the acute stimulation of the HPA in response to endotoxin. Previous studies had shown that endotoxin activates the HPA through a predominantly central—hypothalamic—action, as does IL-1β. As IL-1β has been shown to directly stimulate the release of both CRH[29] and vasopressin[30] and IL-1β has been demonstrated within the hypothalamus,[31] it has been suggested that the effects of endotoxin administration may at least in part be mediated by activation of hypothalamic IL-1β.[32] Changes in hypothalamic IL-1β peptide in response to LPS have been demonstrated,[33,34] although alterations in IL-1β mRNA in response to endotoxin have been more difficult to demonstrate. Higgins and Olschowka[35] were able to show that intracerebral injection of endotoxin could increase IL-1β expression in surrounding tissue,

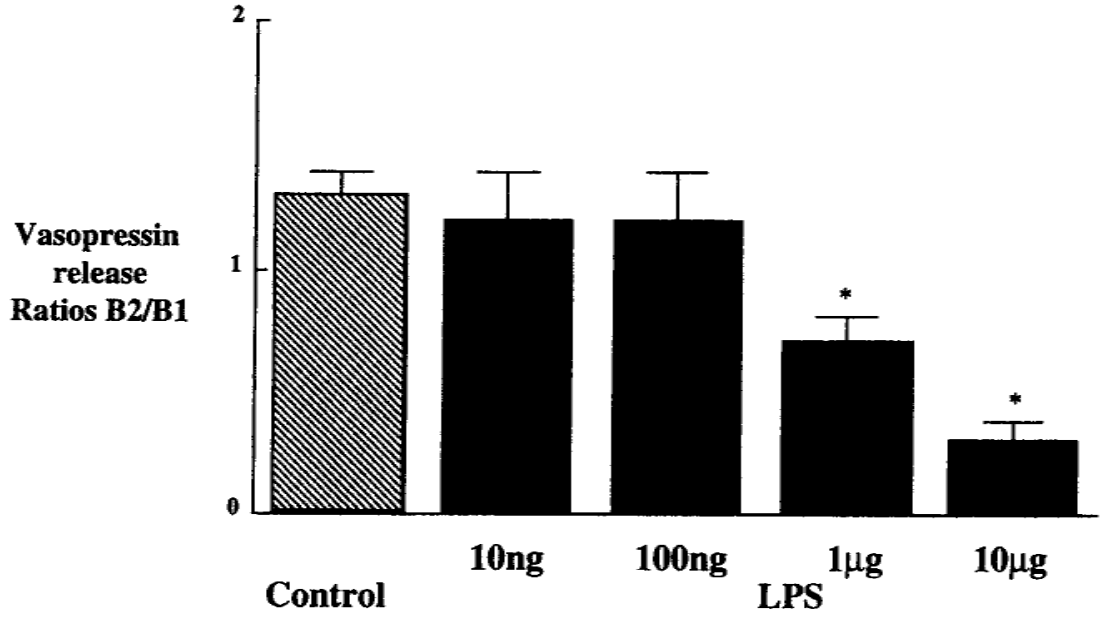


FIGURE 3. Dose–response curve for the inhibition of vasopressin release from the rat hypothalamus *in vitro*. $^*p < 0.05$ compared to control.

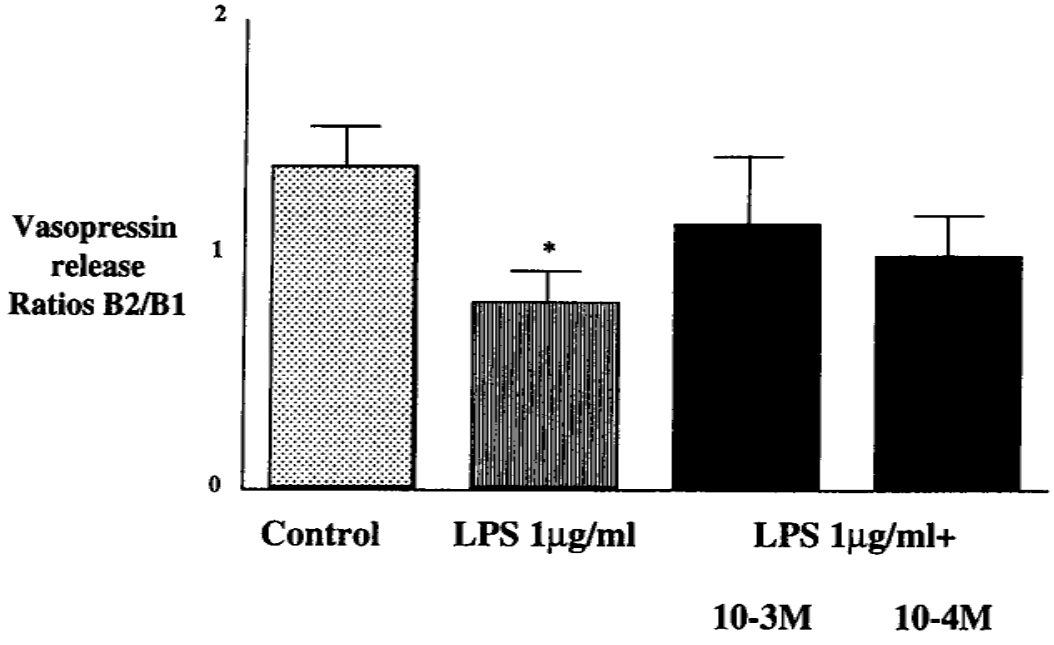


FIGURE 4. The addition of the NOS inhibitor, L-NMMA *(black bars)*, showed little effect on the inhibition of vasopressin release by LPS 1 μg/ml from the rat hypothalamus *in vitro*. $^*p < 0.05$ compared to control.

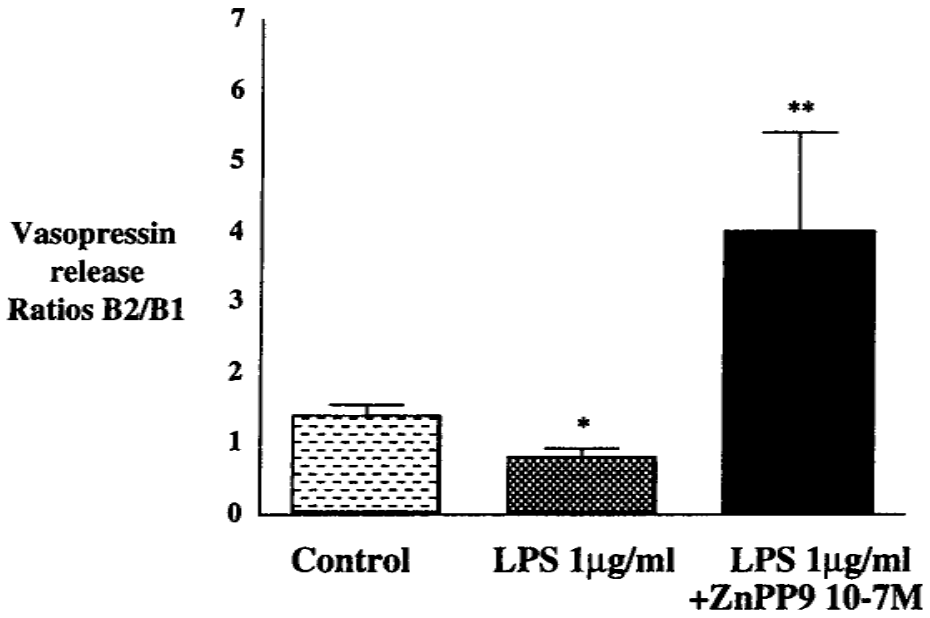


FIGURE 5. The addition of the heme oxygenase inhibitor, ZnPP-9, transformed the suppression of vasopressin release by LPS 1 μg/ml into significant stimulation. Similar changes were noted with the heme oxygenase inhibitor, SnMP. $^{*}p < 0.05$; $^{**}p < 0.01$ compared to control.

and subsequently IL-1 induction in the hypothalamus was demonstrated 60 minutes after LPS 2 mg/kg i.p. by RT-PCR.[36] More recently, the kinetics of the early phase of IL-1 induction in mouse hypothalamus was demonstrated in the three hours following i.p. LPS using a radio-labeled RT-PCR technique.[37] Our results are compatible with these reports and extend the time domain to 24 hours post-injection. It may be noted that repeated injections of endotoxin will increase hypothalamic CRH,[38] and thus, while not directly implicating IL-1β in this process, our data are consistent with the notion that hypothalamic IL-1β may mediate neuroendocrine responses to endotoxin. Interleukin-1 receptor RNA has been located in the rat hypothalamus, including the PVN, although at this site it appears to be localized to endothelial rather than neuronal or glial cells.[39] It is therefore possible that secreted IL-1 first activates endothelium and then that intermediary regulators, such as eicosanoids including prostaglandin E_2,[40] are involved in the transfer of the signal from endothelium to CRH- and vasopressin-containing neurons.

Previous studies have not generally been successful in demonstrating changes in NOS enzyme protein or activity in response to peripheral inflammation.[41–43] Neuronal NOS mRNA is now known to be "inducible" under certain circumstances, and in one recent study appeared to transiently increase within the hypothalamus after a relatively low dose of LPS.[44] The current data clearly demonstrated a rapid and very marked elevation in iNOS transcripts, but with no significant change in nNOS mRNA at any time over the 24 hours assessed. Inducible NOS has promoter elements that confer LPS inducibility,[45] and these results are in keeping with the very recent demonstration of iNOS induction by

an approximately 10 times higher dose of endotoxin as revealed by *in situ* hybridization.[16] It is possible that we missed a comparatively slight early change in nNOS message levels as previously suggested by *in situ* hybridization, but an alternative possibility is that the very significant increase in iNOS mRNA, and the assumed considerable generation of NO, may have led to downregulation of the nNOS isoform.[46] In the light of these positive findings, it is unclear why increases in neither iNOS activity nor enzyme have previously been reported in endotoxin-treated rats. Mitchell *et al.* found no change in NOS enzyme activity in whole rat brain following LPS 5 mg/kg i.p.,[41] whereas a careful immunocytochemical study was equally unsuccessful in demonstrating other than a minimal increase in iNOS staining in the hypothalamus after application of LPS 2.5 mg/kg intravenously.[43] It is possible that there is post-transcriptional regulation of NOS mRNA such that the enzyme is induced but its translation inhibited, but there is no evidence for this mechanism regulating iNOS at other sites, where the dominant control of enzyme activity is via enzyme induction. An alternative possibility is that the enzyme induction occurs in blood-borne monocytes marginating through endothelial capillaries in our tissue samples (see also below), but then this should also have occurred in the tissue extracted by Mitchell *et al.* Furthermore, induction of iNOS *within* hypothalamic cells has been reported, albeit using a much higher dose of LPS.[16] Thus, the reasons for these discrepancies remain difficult to explain at the current time.

HO_1 is a heat-shock protein which is present in tissues at very low concentrations under basal conditions, but can still be identified in the human brain in the absence of stimulation.[19,20,24] However, its promoter sequence contains a potential binding site for a heat-shock transcription factor,[47] and under appropriate stimulation can be markedly induced by endotoxin. Our results confirm that intraperitoneal endotoxin can induce HO_1 in the liver, although the change in level is not as pronounced as that seen for iNOS or IL-1β. HO_2, by contrast, is not classically inducible, and indeed appears to lack the promoter regulatory elements characteristic of heat-shock proteins. In this study also, while HO_2 transcripts were clearly identified in rat liver, no consistent response to endotoxin was noted. In the CNS, specifically the hypothalamus and cerebellum, mRNA for both HO_1 and HO_2 was identified under basal conditions. While early studies had suggested that only HO_2 was significantly present in the absence of stimulation,[19] more recent work has identified both isoforms, although the distribution of HO_2 appears to be more widespread.[20] Furthermore, our data showed that endotoxin administration was unassociated with any change in the CNS of either HO_1 or HO_2. It was especially notable that HO_1 remained impervious to endotoxin, particularly in the light of the changes seen in iNOS and IL-1β since NO itself has been shown to induce HO_1 expression,[24] while to some extent attenuating its own induction.[46] It should also be noted that the absolute levels of the HO enzymes were quite variable in the CNS, such that small changes may have eluded detection.

The cell(s) of origin of the mRNA detected, particularly for those which altered in response to endotoxaemia, cannot be identified by the present study. Neuronal NOS, and both isoforms of HO, have been identified in neurons, but according to the current study the transcripts for these enzymes are not affected by our regimen of endotoxin administration. Previous work has located IL-1β in both glial cells and neurons, the glial element predominantly, if not wholly, being microglial.[32,37] Inducible NOS has also been induced in glial cells, although a recent elegant study has shown specific induction in cultured cerebellar neurons.[48] It

may also be noted that Wong *et al.* found induction in neurons, glia and endothelium, although using rather higher doses than we used. As a freely diffusible gas, NO may cause changes in a sphere approximately 1000 μm in diameter, and thus the precise site of release may not be critical so long as it remains within this geographically limited sphere of influence. The pathophysiological significance of these findings is uncertain but intriguing. As noted above, the regulation of iNOS activity is principally by means of transcription, and it is therefore probable that a change in mRNA is reflected in an alteration in NO secretion. This suggests that the degree of systemic inflammation induced in the current model will generate NO within the hypothalamus, which may have neuroendocrine and/or cytocidal activity. There is disagreement in the published studies concerning the direction of CRH modulation following NO generation in rat hypothalamic explants; most studies have demonstrated stimulation,[49–51] but the single report of inhibition is compatible with the published *in vivo* data.[6,7] This would in turn suggest that the induction of iNOS down-regulates responsiveness in the HPA. The iNOS system appears to be particularly important in defense against protozoal parasites such as *Leishmania*, and in bacteraemic states may be actually prejudicial to survival.[52] While this is principally due to the peripheral activity of iNOS, it is clearly possible that at least part of this pathogenic response is due to central attenuation of HPA responsiveness.

While we and others have shown that endotoxin can induce the acute release of interleukin-1 from the hypothalamus, and that IL-1 is a secretagogue for both CRH and vasopressin, the inability of endotoxin to stimulate the release of either CRH or vasopressin release *in vitro* has been puzzling. We had originally postulated that there may be induction of NO by endotoxin that counterregulated or even overcompensated for any stimulation, but the lack of response to L-NMMA in our *in vitro* study suggests that this is unlikely. By contrast, the acute explant studies apparently demonstrated the involvement of an inhibitory pathway to vasopressin release in response to endotoxin which could be blocked by porphyrin analogues which antagonise heme oxygenase. Until recently, ZnPP-9 was the most frequently used agent in exploring the putative involvement of CO in a number of processes, although its specificity has been challenged. In particular, it has been suggested that many of the effects of ZnPP-9 might be secondary to antagonism of NO synthase.[53] Our results, showing a lack of any marked effect of L-NMMA, and similar responses to SnMP (which is not thought to affect NOS[53]), support the notion that endotoxin actually *stimulates* vasopressin release at the same time it activates an inhibitory pathway involving heme oxygenase. While heme oxygenase can catalyse a number of reactions, our previous results suggest that the modulation of vasopressin release by heme oxygenase intermediates is dependent on the generation of CO.[54] Previous work by ourselves and others has demonstrated pharmacological effects of CO manipulation on neuroendocrine function, but to our knowledge this is the first demonstration that endogenous CO may play a pathophysiological role.

In summary, we suggest that endotoxin will directly and acutely activate hypothalamic CO to counter-regulate other stimulatory pathways to vasopressin release, while in the longer term it will, directly or indirectly, increase the expression of inducible NOS in the hypothalamus. We speculate that hypothalamic gaseous neuromodulators are an important component of the HPA response to endotoxin challenge, and that they serve to attenuate this responsiveness and hence to act as central pro-inflammatory agents.

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