Inducible nitric oxide synthase gene expression in the brain during systemic inflammation

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Inducible nitric oxide synthase (iNOS) is a transcriptionally regulated enzyme that synthesizes nitric oxide from L-arginine[1] that has a key role in the pathophysiology of systemic inflammation and sepsis. Transgenic animals with a null mutation for the iNOS gene are resistant to hypotension and death caused by *Escherichia coli* lipopolysaccharide (LPS)[2,3]. The regulation of peripheral iNOS has been well studied in sepsis, but little is known about iNOS regulation in the brain during systemic inflammation or sepsis. We show that at baseline there is no detectable iNOS gene expression in the brain, but a detailed neuroanatomical study reveals that early in the course of systemic inflammation there is a profound induction of iNOS messenger RNA in vascular, glial and neuronal structures of the rat brain, accompanied by the production of nitric oxide (NO) metabolites in brain parenchyma and cerebrospinal fluid (CSF). We propose that the spillover of nitrite into the CSF has the potential to be a diagnostic marker for systemic inflammation and sepsis. Pharmacological interventions aimed at regulating iNOS function in the brain might represent a new treatment strategy in sepsis. Brain iNOS may be relevant to the pathophysiology, diagnosis and treatment of systemic inflammation and sepsis.

Nitric oxide is a labile free radical gas that conveys biological information in a manner differing from all other classical transmitters. NO is not released at the synaptic level, but diffuses from cells; it does not bind reversibly to specific receptors, but forms covalent bonds with enzymes; it is not inactivated by reuptake or enzymatic degradation, but rather by diffusion from targets and by forming linkages to anions or scavenger proteins[4].

Different forms of NOS have been identified and are encoded by separate genes located in separate chromosomes[5]. A constitutive pathway consists of a reservoir of calcium-dependent NOS, activation of which does not require new enzyme protein synthesis[6]. The classic constitutive NOS (cNOS and neuronal (nNOS)) pathway is localized in the brain. Glutamate binding to *N*-methyl-D-aspartate (NMDA) receptors increases the levels of intracellular Ca^{2+}, which in turn activates nNOS via calmodulin for the mediation of rapid events, such as neurotransmission[7]. The calcium-independent inducible pathway is transcriptionally regulated and therefore requires new NOS mRNA for its activity[1]. After induction, iNOS is active for a period of 4 to 24 hours and synthesizes NO in nanomolar concentrations, more than 100 times those produced by cNOS (ref. 8). Inducible NOS (iNOS) operates in inflammatory cells[9]. NO both inactivates key bacterial enzymes and is converted to cytotoxic metabolites such as nitrite[4]; NO derived from inflammatory cells is also thought to mediate the altered vascular permeability that occurs during inflammation[10].

In the central nervous system, NO is best known as an important modulator of glutamate release[11,12]; NO metabolites such as peroxynitrite are exceedingly neurotoxic[13], and NO-mediated glutamate release is thought to play a role in neurodegenerative processes such as Alzheimer's disease and neuro-AIDS (ref. 7). NO present in inflammatory cells modulates local cytotoxicity, edema formation and leukocyte traffic and is thought to be involved in the pathophysiology of inflammatory disorders such as ulcerative colitis[14–16]. Finally, NO is thought to play a key role in the mortality caused by sepsis. High-dose LPS administration causes endotoxemia and sepsis, which may be lethal[17]. Inhibitors of NOS, the rate-limiting enzyme that generates NO from the amino acid L-arginine, can reverse or prevent the hypotension induced in animals by LPS, hemorrhage and anaphylactic shock. Moreover, Wei *et al.*[2] and MacMicking *et al.*[3] showed that null mutant iNOS mice ($iNOS^{-/-}$) are resistant to the hypotension and death caused by LPS. Those data further established that iNOS has a crucial role in LPS-induced death.

The pathophysiology of systemic inflammation and sepsis involves the central nervous system (CNS), affecting sleep, temperature regulation, behavior and neuroendocrine function, as well as several peripheral systems, causing gastrointestinal, renal and cardiovascular alterations[18–20]. The peripheral components of the pathophysiology of sepsis have been well established; however, the neuroanatomical localization of iNOS mRNA during sepsis has not been yet accomplished. The purpose of the present report was to explore the hypothesis that during pathogenic states such as systemic inflammation and sepsis, iNOS gene expression is induced in the brain, leading to increases in citrulline in brain parenchyma and to the spillover of nitrites in CSF. Demonstration that iNOS was expressed as a consequence of experimentally induced sepsis also would raise the possibility that the measurement of metabolites of NO in the CSF could serve as a marker for sepsis and as a means of monitoring its course and that antagonists of iNOS in the brain might potentially influence the natural history of the generalized inflammatory response syndrome and sepsis.

We report here that the intraperitoneal injection of LPS, which induced clear manifestations of systemic inflammation, such as piloerection, mild febrile shaking and lethargy, produced a marked increase in the levels of expression of the gene encoding for iNOS in several regions of the rat brain. We examined the entire rat brain and pituitary gland, which were dissected in one block and sectioned coronally every 1.0 mm. Our *in situ* hybridization histochemistry experiments showed that whereas at baseline there was no expression of the iNOS gene in the rat brain, LPS-induced sepsis caused a pattern of iNOS gene expression that included not only glial and vascular localization, but also neuronal localization (Fig. 1, 2 and 3). Meninges (Fig. 2, *a* and *d*), choroid plexus, median eminence (Fig. 2, *b* and *e*) and

subfornical organ are vascular-rich areas that showed marked iNOS gene expression during sepsis (Fig. 1). Two neuronal hypothalamic nuclei showed strikingly high induction of iNOS mRNA: the paraventricular (PVN) nucleus (Fig. 1*n*, 2, *c* and *f*, and 3*a*) and the arcuate nucleus (Fig. 2, *b* and *e*, and 3*b*), indicating that, contrary to current thinking, neurons can express inducible as well as constitutive NOS. iNOS mRNA levels were also markedly induced in both endocrine glands that are situated in close proximity to the brain, the pituitary (Fig. 1*j*) and the pineal (Fig. 1*k*).

We found that the pronounced induction of iNOS mRNA in the brain and meninges was reflected by increased levels of NO metabolites in brain parenchyma and in CSF. In medial basal hypothalamus (MBH) the levels of citrulline, a by-product of NO metabolism, increased from 95,654 ± 4,557 (mean ± s.e.m.) c.p.m./MBH in controls to 122,239 ± 4,575 c.p.m./MBH in LPS-treated animals (Student's *t*-test, unpaired: $P < 0.01$). There was spillover of nitrite (NO_2^-), a stable metabolite of NO, into CSF during sepsis. We measured nitrite levels in the CSF: at baseline nitrite levels were not detectable in CSF (<1.0 μM); 6 hours after LPS administration, at the peak of iNOS mRNA induction in the brain, CSF nitrite levels were 17.8 ± 3.9 μM in LPS-treated animals (Student's *t*-test, unpaired: $P < 0.002$). It is likely that in states of sepsis the choroid plexus and meninges, areas we showed to have a profound induction of iNOS mRNA, release NO into the CSF.

Inducible nitric oxide synthase is transcriptionally regulated[1]. Increases in iNOS mRNA levels are therefore indicative of increased NO levels. That is reflected by our finding of increased citrulline levels in brain parenchyma and by the spillover of nitrite into the CSF during sepsis.

The marked increase in the levels of iNOS gene expression in the brain and indices of activation of NO activity in the central nervous system (CNS) following LPS administration have potential pathophysiologic, diagnostic and therapeutic implications. From a pathophysiologic perspective, it is known that an acute encephalopathy[21,22] and persistent deterioration in cognitive function[23] can occur as a consequence of sepsis. Activation of iNOS in widespread areas of the brain could potentially lead to glutamate neurotoxicity with acute and long-lasting consequences. On the other hand, because NO neurons themselves are resistant to NO and NMDA-induced neurotoxicity[24], our findings of iNOS mRNA induction in the PVN and arcuate nuclei of the hypothalamus suggest that these key neuronal nuclei may be relatively less vulnerable during systemic inflammation.

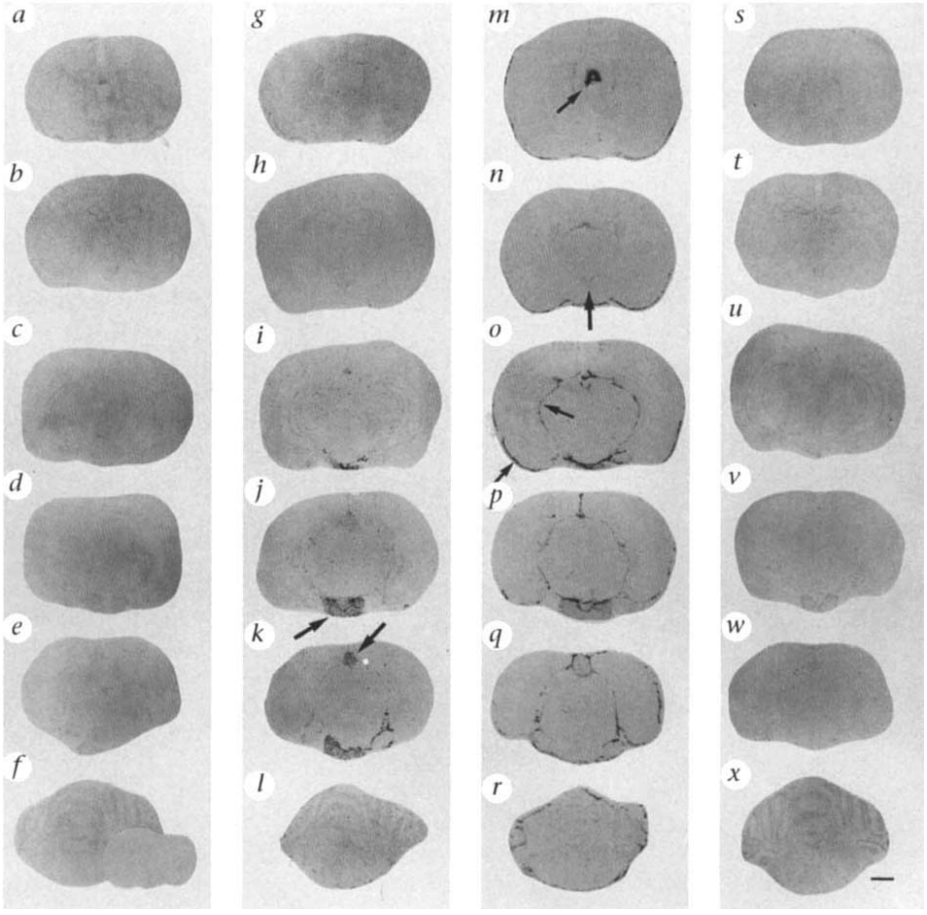

Fig. 1 Localization of iNOS mRNA in the rat brain by *in situ* hybridization histochemistry after treatment with LPS. A series of film autoradiographs is arranged from rostral to caudal, showing the regional distribution of brain regions positively hybridized using a radiolabeled antisense probe generated from a cDNA encoding the rat iNOS mRNA. Control brain slices are shown in the first column: *a* to *f* represent the hybridization of iNOS antisense riboprobe in the brain of control rats; note the lack of positively hybridized cells throughout the brain; in *f* the small image on the right shows a control hybridization using a sense probe. Two hours after a single LPS injection i.p. (5.0 mg/animal), the induction of iNOS throughout the rat brain is shown in the second column (*g* to *l*). There is a remarkable induction of iNOS in the pituitary gland (arrow in *j*) and in the pineal gland (arrow in *k*). iNOS induction also occurred in the choroid plexus and perivascular regions. Six hours after a single LPS injection, the induction of iNOS throughout the rat brain is shown in the third column (*m* to *r*). There is a strong induction of iNOS in the subfornical organ (arrow in *m*), in the paraventricular (PVN) nucleus of the hypothalamus (arrow in *n*), in the choroid plexus, in the meninges surrounding the brain (left arrow in *p*). Then, 24 h after a single LPS injection the levels of iNOS mRNA throughout the rat brain are considerably decreased, but have not returned to baseline (fourth column, *s* to *x*). Scale bar, 0.4 mm for the right-hand image in *f*; scale bar, 1.6 mm in all other images.

In the light of the substantial presence of iNOS mRNA in the PVN and arcuate nuclei after peripheral LPS administration, it is likely that NO plays an important role in the neuroendocrine response to sepsis, especially in hypothalamic–pituitary–adrenal axis activation. It has been postulated that NO mediates the effects of neurotransmitters such as norepinephrine on corticotropin-releasing hormone (CRH) via activation of cyclooxygenase and prostaglandin-E_2 release[25–27]. CRH-mediated pituitary–adrenal activation during inflammatory stress has been shown to play an important physiologic role in the counterregulation of the inflam-

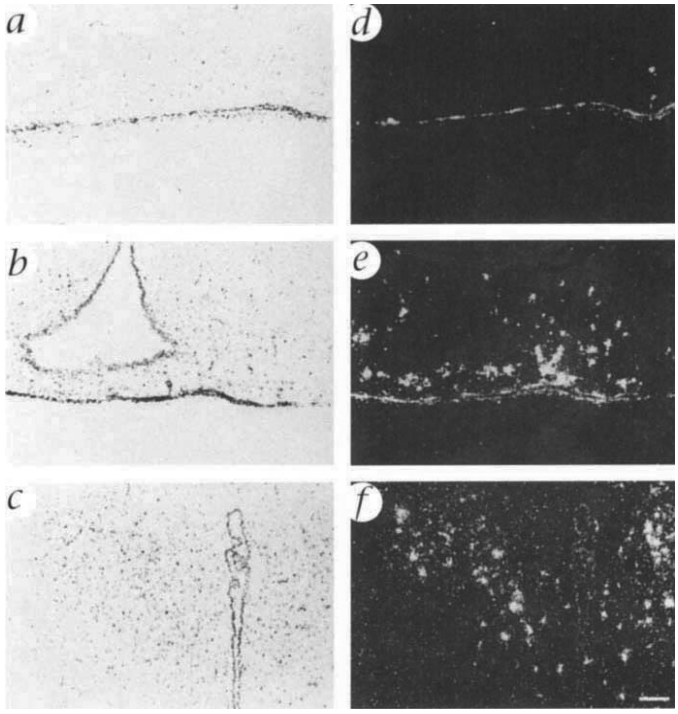

Fig. 2 Low-power magnification images showing the localization of iNOS in brain regions by *in situ* hybridization histochemistry 6 h after LPS treatment. The bright-field photomicrograph images shown in the first column (*a* to *c*) correspond to the brain regions shown in the second column (*d* to *f*) in dark-field photomicrographs. *a* and *d*, the meninges; *b* and *e*, a detailed image of the median eminence and arcuate nucleus of the hypothalamus; *c* and *f*, the PVN. Among the neurosecretory structures in the hypothalamus, signals were observed predominantly in the PVN and arcuate nuclei, and median eminence. Note the preponderance of positively hybridized cells within the nuclei in the PVN and the arcuate nucleus compared with the areas surrounding the nuclei. White dots in the dark-field images represent silver grains overlying iNOS mRNA; note the remarkable concentration of white dots in the meninges, median eminence, arcuate nucleus and PVN. Scale bar, 240 μm.

matory response and interruption of this negative feedback loop has pathologic consequences[28]. The integrity of this feedback loop in sepsis should be investigated; moreover, the use of NO antagonists in sepsis may disrupt this loop and its potential salutary effects on the outcome of systemic inflammation and sepsis.

A lumbar puncture is a standard diagnostic procedure in the investigation of any case of suspected sepsis of unknown source. The presence of NO metabolites in CSF shortly after LPS injection may be a definitive indicator of the fact that an inflammatory state has reached the stage of the systemic inflammatory response syndrome. CSF levels of nitrite may therefore be an early marker of systemic inflammation and sepsis. Despite current treatment, sepsis has a high mortality rate of up to 51% (ref. 29). The diagnosis of sepsis is a subject of great clinical interest, because therapeutic interventions offer more promise if initiated early. It is particularly important to clinically detect iNOS induction within the first hours of the onset of sepsis. We showed that within the first hours of systemic inflammation there is a profound increase in the expression of the gene encoding for iNOS in the brain, associated with the spillover of nitrite into the CSF. The hypothesis that CSF nitrite levels might be an early marker of sepsis should therefore be tested in humans.

As $iNOS^{-/-}$ mice are protected from death after high-dose LPS administration[2,3], the inhibition of iNOS may represent a new treatment strategy for sepsis; however, the use of NOS antagonists in the treatment of sepsis can be problematic in the light of their potential capacity to induce a state of irreversible vasoconstriction[4]. If central iNOS is playing a role in the vasodilatation of the septic state, the use of specific, centrally acting iNOS antagonists may ameliorate hypotension without resulting in profound vasoconstriction. The search for such agents might represent a new therapeutic strategy for the systemic inflammatory response syndrome and sepsis. Work in this area should, however, proceed with caution because some of the potential roles of iNOS in the brain during sepsis, such as activation of the hypothalamic–pituitary–adrenal axis by NO-induced increases in the levels of CRH in the PVN (ref. 25), may be beneficial during systemic inflammation and sepsis. Future studies should examine the contributions of NO-neuroendocrine interactions to the outcome of sepsis.

Methods

Animals. Studies were carried out in accordance with animal protocols approved by the National Institutes of Health. Virus-free and pathogen-free male Sprague-Dawley (200–250 g, Harlan, Indianapolis, Indiana) rats were housed in a light-controlled (12 h on/12 h off) and temperature-controlled environment, with food and water *ad libitum*. Animals were treated with 5.0 mg of *Escherichia coli* LPS (055:B5, Sigma Chemical Co.), prepared in 0.5 ml saline and administered intraperitoneally (i.p.). LPS administration i.p. at this dosage level induces cytokine-mediated systemic inflammation in rodents, modeling a systemic inflammatory response syndrome[17]. Control groups received 0.5 ml saline i.p. Different groups of animals ($n = 6$/group) were studied 0, 2, 6 or 24 h after injection of LPS, or saline (control groups). Injections were timed so that the animals were killed between 10:00 and 10:30 a.m., thus avoiding the possibility of circadian variations in the outcome measures. To prevent the confounding effects of stress on gene expression, animals were removed from their home cages by an animal handler not involved in the decapitation process and were decapitated within 45 s of individual removal from home cages.

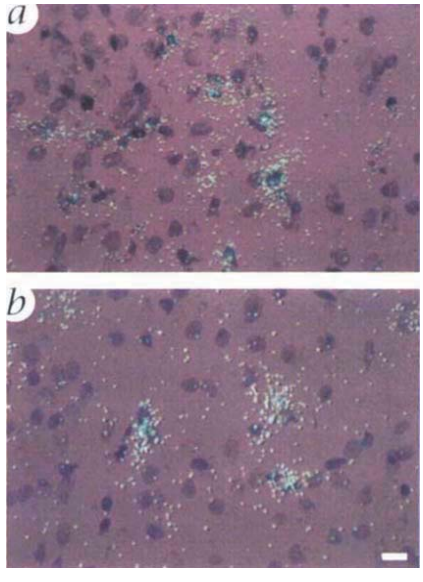

Fig. 3 High-magnification bright-field color photomicrographs of iNOS mRNA in hypothalamic nuclei. After treatment with LPS, cells strongly hybridized with iNOS mRNA probe were seen in the PVN (*a*) and in the arcuate nuclei (*b*) of the hypothalamus. Bright, light blue dots over the cells represent silver grains overlying iNOS mRNA. Scale bar, 15 μm.

***In situ* hybridization histochemistry.** Brains were rapidly removed and stored at –70 °C before processing for *in situ* hybridization histochemistry. A ribonucleotide probe, directed against the rat iNOS sequence, was generated from the rat iNOS cDNA (generously provided by D. Feinstein, the New York Hospital-Cornell Medical Center, New York, New York)[30]. Transcription of antisense and sense probes was carried out using the Riboprobe System (Promega Biotech, Madison, Wisconsin) in the presence of [α-^{35}S]UTP (sp. act. 1000–1500 Ci/mmol, New England Nuclear).

Sectioning, fixing, *in situ* hybridization histochemistry, autoradiography and anatomical localization of the probe at the cellular level were performed as described by Wong *et al.*[31,32]. Approximately 5 × 10^5 c.p.m. of radiolabeled probe was used per 50 µl hybridization buffer. To test the specificity of both the antisense probe and the hybridization method, controls were generated by using a labeled sense probe, or excess cold probe (100×). Hybridization and post-hybridization treatments were concomitantly carried out on antisense and control sections. Sections were exposed to X-ray film (Hyperfilm B max, Amersham Corp., Arlington Heights, Illinois) for 10–15 days. The films were developed in D19 (Eastman Kodak Co., Rochester, New York) for 5 min at 20 °C. Anatomical localization of the probe at the cellular level was performed by dipping the slides into NTB-2 nuclear track emulsion (Eastman Kodak Co.) dilutes 1:1 with distilled water, developed after 8 weeks of exposure and counterstained with cresyl violet. The localization of grains was studied with light microscopy (Leitz, Wetzlar, Germany). Excess cold (100×) and sense probe were used to control specificity of the probe and the hybridization method.

Measurement of citrulline in medial basal hypothalamus. As arginine is converted into NO and equimolar quantities of citrulline, and the NO disappears rapidly from the tissue, whereas citrulline remains, the formation of labeled citrulline from [^{14}C]arginine is a convenient measure of NOS activity in the tissue at the end of the incubation. The tissue is homogenized and incubated with [^{14}C]arginine and cofactors that cause the maximum production of NO and citrulline. Citrulline is separated from arginine by column chromatography and counted in a liquid scintillation counter[33].

Measurement of nitrate levels in cerebrospinal fluid. The CSF concentration of nitrite (NO_2^-), a metabolite of NO, in the CSF was quantified colorimetrically using the Griess reaction[34]. Griess reagent (100 µl, Promega Biotech) were added to 50 µl of CSF samples in duplicate (CSF samples from two rats were pooled together in each well). Absorbance at 540 nm (A_{540}) was measured using a microplate reader. NO_2^- concentrations were calculated by comparing the A_{540} of standard solutions of sodium nitrite prepared in artificial CSF (ESA, Bedford, Massachusetts).

Acknowledgments

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1. Xie, Q.-W. *et al.* Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. *Science* **256**, 225–228 (1992).
2. Wei, X.-Q. *et al.* Altered immune responses in mice lacking inducible nitric oxide synthase. *Nature* **375**, 408–411 (1995).
3. MacMicking, J.D. *et al.* Altered response to bacterial infection and endotoxic shock in mice lacking inducible nitric oxide synthase. *Cell* **81**, 641–650 (1995).
4. Moncada, S. & Higgs, A. The L-arginine-nitric oxide pathway. *N. Engl. J. Med.* **329**, 2002–2012 (1993).
5. Nathan, C. & Xie, Q.-W. Nitric oxide synthases: Roles, tolls, and controls. *Cell* **78**, 915–918 (1994).
6. Bredt, D.S. & Snyder, S.H. Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. *Proc. Natl. Acad. Sci. USA* **87**, 682–685 (1990).
7. Dawson, T.M. & Snyder, S.H. Gases as biological messengers: Nitric oxide and carbon monoxide in the brain. *J. Neurosci.* **14**, 5147–59 (1994).
8. Kuo, P.C. & Schroeder, R.A. The emerging multifaceted roles of nitric oxide. *Ann. Surg.* **221**, 220–235 (1995).
9. Iyengar, R., Stuehr, D.J. & Marletta, M.A. Macrophage synthesis of nitrite, nitrate, and N-nitrosamines: Precursors and role of the respiratory burst. *Proc. Natl. Acad. Sci. USA* **84**, 6369–6373 (1987).
10. Ialenti, A., Ianaro, A., Moncada, S. & Di Rosa, M. Modulation of acute inflammation by endogenous nitric oxide. *Eur. J. Pharmacol.* **211**, 177–182 (1992).
11. Bredt, D.S. & Snyder, S.H. Nitric oxide mediates glutamate-linked enhancement of cGMP levels in the cerebellum. *Proc. Natl. Acad. Sci. USA* **86**, 9030–3 (1989).
12. Dawson, V.L., Dawson, T.M., London, E.D. Bredt, D.S. & Snyder, S.H. Nitric oxide mediates glutamate neurotoxicity in primary cortical cultures. *Proc. Natl. Acad. Sci. USA* **88**, 6368–6371 (1991).
13. Beckman, J.S., Beckman, T.W., Chen, J., Marshall, P.A. & Freeman, B.A. Apparent hydroxyl radical production by peroxynitrite: Implications for endothelial injury from nitric oxide and superoxide. *Proc. Natl. Acad. Sci. USA* **87**, 1620–1624 (1990).
14. Middleton, S.J., Shorthouse, M. & Hunter, J.O. Increased nitric oxide synthesis in ulcerative colitis. *Lancet* **341**, 465–466 (1993).
15. Boughton, S.N. *et al.* Nitric oxide synthase activity in ulcerative colitis and Crohn's disease. *Lancet* **342**, 338–340 (1993).
16. Lundberg, J.O., Hellstrom, P.M., Lundberg, J.M. & Alving, K. Greatly increased luminal nitric oxide in ulcerative colitis. *Lancet* **344**, 1673–1674 (1994).
17. Li, P. *et al.* Mice deficient in IL-1β converting enzyme are defective in production of mature IL-1β and resistant to endotoxic shock. *Cell* **80**, 401–411 (1995).
18. Dennhardt, R., Gramm, H.J., Meinhold, K. & Voigt, K. Patterns of endocrine secretion during sepsis. *Prog. Clin. Biol. Res.* **308**, 751–756 (1989).
19. Bone, R.C. Toward an epidemiology and natural history of SIRS (systemic inflammatory response syndrome). *JAMA* **268**, 3452–3455 (1992).
20. Dinarello, C.A., Gelfand, J.A. & Wolff, S.M. Anticytokine strategies in the treatment of the systemic inflammatory response syndrome. *JAMA* **269**, 1829–1835 (1993).
21. Young, G.B., Bolton, C.F., Archibald, Y.M., Austin, T.W. & Wells, G.A. The electroencephalogram in sepsis-associated encephalopathy. *J. Clin. Neurophysiol.* **9**, 145–152 (1992).
22. Young, G.B., Bolton, C.F., Austin, T.W., Archibald, Y.M., Gonder, J. & Wells, G.A. The encephalopathy associated with septic illness. *Clin. Invest. Med.* **13**, 297–304 (1990).
23. Rossor, M.N. Alzheimer's disease. in *Oxford Textbook of Medicine,* vol. 3 (eds. Weatherall, D.J., Ledingham, J.G.C. & Warell, D.A.) 3971–3974 (Oxford Univ. Press, Oxford, 1996).
24. Dawson, V.L., Dawson, T.M., Bartley, D.A., Uhl, G.R. & Snyder, S.H. Mechanisms of nitric oxide-mediated neurotoxicity in primary brain cultures. *J. Neurosci.* **13**, 2651–2661 (1993).
25. Costa, A., Trainer P., Besser, M. & Grossman, A. Nitric oxide modulates the release of corticotropin-releasing hormone from the rat hypothalamus in vitro. *Brain Res.* **605**, 187–192 (1993).
26. Rivier, C. & Shen, G.H. In the rat, endogenous nitric oxide modulates the response of the hypothalamic-pituitary-adrenal axis to interleukin-1 beta, vasopressin, and oxytocin. *J. Neurosci.* **14**, 1985–1993 (1994).
27. Sandi, C. & Guaza, C. Evidence for a role of nitric oxide in the corticotropin-releasing factor release induced by interleukin-1 beta. *Eur. J. Pharmacol.* **274**, 17–23 (1995).
28. Sternberg, E.M. *et al.* A central nervous system defect in biosynthesis of corticotropin-releasing hormone is associated with susceptibility to streptococcal cell wall-induced arthritis in Lewis rats. *Proc. Natl. Acad. Sci. USA* **86**, 4771–4775 (1989).
29. Barriere, S.L. & Lowry, S.F. An overview of mortality risk prediction in sepsis. *Crit. Care Med.* **23**, 376–393 (1995).
30. Galea, E., Reis, D.J. & Feinstein, D.L. Cloning and expression of inducible nitric oxide synthase from rat astrocytes. *J. Neurosci. Res.* **37**, 406–414 (1994).
31. Wong, M.-L., Gold, P.W. & Licinio, J. *In situ* hybridization techniques for the localization of interleukin-1 and interleukin-1 receptor antagonist mRNA in brain. *Methods Neurosci.* **16**, 81–99 (1993).
32. Licinio, J., Bongiorno, P., Gold, P.W. & Wong, M.-L. The gene encoding for the novel transacting factor propiomelanocortin corticotropin-releasing hormone responsive element binding protein 1 (PCRH-REB-1) is constitutively expressed in rat pituitary and in discrete brain regions containing CRH or CRH receptors: Pathophysiological implications. *Endocrinology* **136**, 4709–4712 (1995).
33. Canteros, G. *et al.* Nitric oxide synthase (NOS) content of hypothalamic explants: Increased by norepinephrine and inactivated by NO and cyclic GMP. *Proc. Natl. Acad. Sci. USA* (in the press).
34. Green, L.C. *et al.* Analysis of nitrate, nitrite, and ^{15}N nitrate in biological fluids. *Anal. Biochem.* **126**, 131–138 (1982).