Comparisons of the Effects of Anesthesia and Stress on Release of Tumor Necrosis Factor–α, Leptin, and Nitric Oxide in Adult Male Rats

CLAUDIO A. MASTRONARDI, WEN H. YU, AND SAMUEL M. MCCANN[1]

Pennington Biomedical Research Center, Louisiana State University, 6400 Perkins Road, Baton Rouge, Louisiana 70808–4124

Bacterial lipopolysaccharide (LPS) stimulates massive release of tumor necrosis factor-alpha (TNF-α) together with nitric oxide (NO) and a lessor release of leptin. We hypothesized that other types of stress such as that of surgery might also release these cytokines and NO. Adult male rats were anesthetized with ketamine/acepromazine/xylazine anesthesia (90 + 2 + 6 mg/ml, respectively) and an external jugular catheter was inserted for removal of blood samples (0.6 ml) at various times postoperatively. Plasma TNF-α was almost undetectable in decapitated rats and was near zero immediately following the placement of the jugular catheter (time zero [t0]). As the rats awakened from anesthesia, there was a rise in TNF-α at 30 min that peaked at 2 hr with a 400-fold increase and then precipitously declined 40-fold to a level still greater than zero at 3 hr. At 6 hr on the following morning, TNF-α values were near zero, but following connection of tubing and withdrawal of the initial blood sample, there was a 100-fold increase 1 hr later, followed by a decline over the next 3 hr. In contrast, plasma [NO_3/NO_2] from decapitated rats was 117 μM. Values at t0 were decreased and plummeted 4-fold within 30 min, then rose slightly in the ensuing 3 hr. At 6 hr on the next day [NO_3/NO_2] values were lower than at t0 and declined gradually during the next 4 hr. Leptin gradually declined from pre-operative concentrations, reaching a minimum at 3 hr and its concentration was unaffected by the bleeding stress of the second day. We conclude that release of TNF-α, [NO_3/NO_2], and leptin are neurally controlled since plasma levels of all three declined as a result of anesthesia. TNF-α secretion was remarkably stress responsive, whereas NO release appeared to be suppressed by the combined operative and bleeding stress, and leptin was stress unresponsive. [Exp Biol Med Vol. 226(4):296–300, 2001]

Key words: plasma leptin; TNF-α; [NO_3/NO_2]; surgical and bleeding stress

This research was supported by the National Institutes of Health (grant no. MH51853).

[1] To whom requests for reprints should be addressed at Pennington Biomedical Research Center, Louisiana State University, 6400 Perkins Road, Baton Rouge, LA 70808–4124. E-mail: mccannsm@pbrc.edu

Received June 21, 2000.
Accepted November 10, 2000.

0037-9727/01/2264-0296$15.00

Different types of stress produce alterations in the immune, endocrine, and nervous systems. Rather than acting independently, these systems are closely interrelated through their messenger molecules: cytokines, hormones, neurotransmitters, and nitric oxide (NO) (1), constituting a complex network that allows the organism to respond as a whole to noxious stimuli and changes of the environment in order to preserve homeostasis.

In the inflammatory stress induced by peripheral or central administration of lipopolysaccharide (LPS) in rodents, there are a number of changes in the immune system such as stimulation of release of acute phase proteins, induction of NO synthase (NOS) activity and synthesis, and increased release of cytokines (2–7). Tumor necrosis factor-alpha (TNF-α) and interleukin-1 released from immune cells reach the central nervous system (CNS) and increase release of corticotrophin-releasing hormone (CRH) that activates the pituitary-adrenal axis (7). LPS also alters the release of other pituitary hormones in rats, increasing prolactin release and decreasing growth hormone and luteinizing hormone release (8). There are a variety of changes in the CNS caused by LPS as well, many of which are probably mediated by LPS-induced cytokines such as alterations of central and peripheral catecholamine (9, 10) levels and alteration of neurotransmitter release in different areas of the brain (11). These result in induction of fever and loss of appetite, libido, and somnolence.

In previous research we have determined that an intravenous injection of LPS in conscious, freely moving rats bearing indwelling jugular catheters increased the release of TNF-α almost 50-fold within 30 min (12) and also increased leptin release 2-fold over baseline 2 hr after injection (13, 14). Similar results were found in *in vivo* experiments in mice (15).

Since the dramatic release of TNF-α that began almost immediately after the intravenous injection of LPS was much greater quantitatively than the equally rapid release of adrenocorticotrophic hormone (ACTH) (8, 12), we concluded that TNF-α was a very important stress hormone that might be released in response to other stresses such as sur-

gery. Consequently, to further investigate the possible relationship between TNF-α, leptin, and NO during surgical stress, we decided to measure plasma concentrations of leptin, TNF-α, nitrates, and nitrites [NO_3/NO_2], as an index of NO production before, during, and after placing external jugular catheters in anesthetized (ketamine/acepromazine/xylazine) male rats.

Materials and Methods

Animals. Two groups of adult male rats (347 ± 7.5 g, large) and (500 ± 9 g, larger) of the Holtzman strain (Harlan, Sprague Dawley, Inc.) were employed. After arrival the animals were allowed to acclimate for 2 weeks. They were housed two per cage in a room with controlled temperature (23°–25°C) and lighting (lights on from 0700–1900 hr). A standard pellet diet and tap water were available *ad libitum*. Procedures involving animals were approved by the Pennington Biomedical Research Center Institutional Animal Care and Use Committee, protocol 97.

Decapitated Animals. A total of 10 rats were decapitated (large, $n = 4$) and (larger, $n = 6$) by means of a guillotine and trunk blood was collected in sterile 15-ml capacity plastic tubes containing 6 μl of heparin 5000 IU/ml.

Repeated Blood Sampling. Twelve rats (large, $n = 4$) and (larger, $n = 8$) were brought to the laboratory from the vivarium and were housed individually on the morning of the experiment. After resting for 1 hr they were anesthetized by an intraperitoneal injection of 0.1 ml/100 g body wt. of ketamine/acepromazine/xylazine anesthesia (90 + 2 + 6 mg/ml, respectively). After the rats were completely anesthetized, a skin incision was made and a silastic catheter was introduced into the right external jugular vein and advanced to the right atrium according to the technique of Harms and Ojeda (16). Immediately after the operation, polyethylene tubing was connected to the silastic catheter and 0.6 ml of blood was withdrawn and replaced with an equal volume of 0.9% NaCl containing 500 IU/ml of heparin (saline). Thereafter, blood samples were collected at 30, 120, and 180 min during the first day and replaced with equal volumes of saline. After the 180-min sample was withdrawn, the rats were disconnected from the polyethylene tubing. Water and the pellet diet were available after this time until the following morning. Then, the animals were reconnected to the polyethylene tubing at 0600 hr and a 0.6-ml blood sample was withdrawn and replaced with saline as before. Thereafter, blood samples were collected at 60, 120, and 240 min and replaced with equal volumes of saline. Blood was centrifuged (700*g*) for 15 min and the plasma was stored at −70°C until assays for determination of TNF-α, leptin, and [NO_3/NO_2] were performed.

Plasma [NO_3/NO_2] Measurement. [NO_3/NO_2] was determined using a kit purchased from Cayman Chemical Co. (Ann Arbor, MI). The optical density was determined at 540 nm using a microplate reader (Bio-Rad, model 550).

Plasma Leptin Determination. Leptin was measured by radioimmunoassay (RIA) using rat leptin RIA kits purchased from Linko Research, Inc. (St. Charles, MO).

Plasma TNF-α Measurement. TNF-α was determined by the sandwich enzyme immunoassay technique using a rat TNF-α kit bought from Quantikine M, R&D Systems (Minneapolis, MN). The optical density was determined at 450 nm with a wavelength correction set at 540 nm using a microplate reader (Bio-Rad, model 550).

Statistical Analysis. Since there was a strong, significant correlation of all parameters with body weight, data were normalized by body weight and expressed as mean ± SEM. Statistical differences between two means were calculated by Student's *t* test when the data passed the normality and equal variance test. When the data failed to pass this test a nonparametric test was performed, namely the Mann Whitney U-test.

Results

Plasma Concentrations of Leptin, [NO_3/NO_2], and TNF-α Before Operation. Ten animals were decapitated at the time the operation was performed in the other rats and trunk blood was collected. Plasma leptin concentrations were significantly ($P < 0.01$) greater in the group of larger than in the large rats (Fig. 1). Plasma [NO_3/NO_2] was similarly related to the weight of the rats (Fig. 2). Plasma TNF-α levels were below the sensitivity of the assay; i.e. less than 12.5 pg/ml.

Plasma Concentrations of Leptin, [NO_3/NO_2], and TNF-α After Operation. In the first day, immediately following the placement of the jugular catheter (time zero [t0]) at which time the rats were fully anesthetized, plasma TNF-α was near zero (Fig. 3). Within 30 min there was a rise ($P = 0.01$) in TNF-α that peaked at 2 hr with a 400-fold increase ($P < 0.0003$) and then precipitously declined 40-fold to a level still greater than zero at 3 hr ($P < 0.001$). On the second day, at 6 hr TNF-α values were near zero, but following connection of tubing and withdrawal of the initial blood sample, there was a 100-fold increase ($P < 0.001$) 1 hr later followed by a decline over the next 3 hr.

In contrast, [NO_3/NO_2] was almost three times higher in decapitated animals than in the anesthetized, operated rats at [t0] of the first day ($P < 0.001$; Fig. 4). Values declined 4-fold ($P < 0.001$) within 30 min (Fig. 5) and remained at that concentration until 2 hr. By 3 hr there was a slight increase, (PNS versus 0.5 and 2 hr, respectively), but values were still significantly lower than at [t0] ($P < 0.05$). Plasma [NO_3/NO_2] at [t0] of the second day was similar to that at [t0] of the first day, but it was still significantly lower than preoperative values determined in decapitated rats ($P < 0.001$; Fig. 4). Thereafter, values started to decline, reaching a minimum at 4 hr ($P = 0.01$ versus [t0] of the second day and $P < 0.01$ versus [t0] of the first day (Fig. 5).

There were no significant differences between plasma leptin values in decapitated rats and anesthetized, operated animals at [t0] of the first day. After operation, plasma leptin concentration declined, reaching a minimum at 3 hr

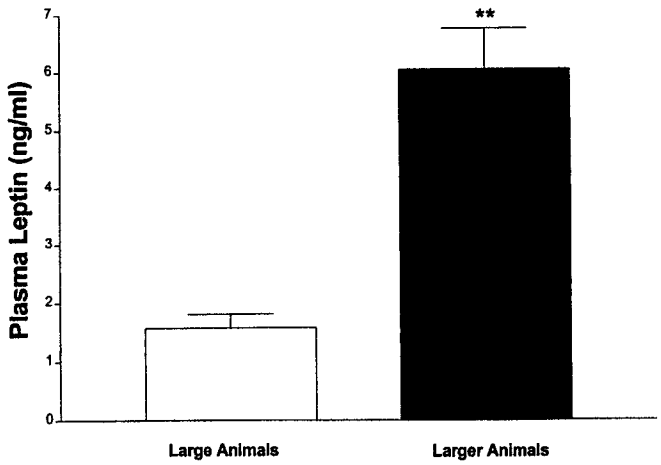

Figure 1. Plasma leptin concentration in rats of different body weight. Large rats (347 ± 7.5 grams; $n = 4$) and larger rats (500 ± 9, $n = 8$). The height of the bar indicates the mean; vertical lines above the mean represents 1 SEM. $^{**}P < 0.01$ versus small rats.

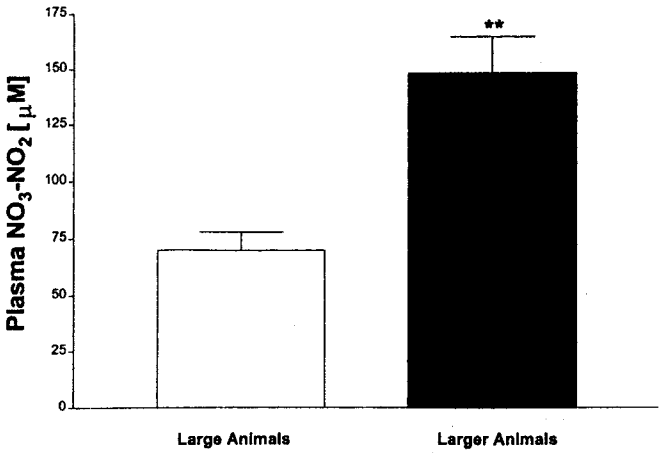

Figure 2. Plasma [NO_3/NO_2] concentration of the same rats as in Figure 1 of different body weights. $^{**}P < 0.01$ versus large rats.

($P < 0.01$; Fig. 6). On the second day, plasma leptin was higher than the minimum determined the day before ($P < 0.01$) and was not significantly different from the preoperative concentration. There were no significant variations of plasma leptin concentration during the second day.

Discussion

Since the stress of injection of LPS induced a release of the cytokines TNF-α and leptin, and NO, we hypothesized that these compounds might also be released during stress as epitomized by surgery. The surgery was performed under anesthesia with ketamine/acepromasine/xylazine. Anesthesia rapidly decreased plasma [NO_3/NO_2] since the values of [NO_3/NO_2] were highly significantly reduced below those obtained in decapitated animals by the time the jugular catheter had been placed, at which time the rats were still completely anesthetized. The practically undetectable levels of TNF-α, on the other hand, were not altered at this time. Since plasma TNF-α rapidly increased as the rats awakened, it appears that anesthesia blocked the initial release of TNF-α induced by surgical stress. Plasma leptin values gradually declined post-operatively and were highly significantly lowered by 3 hr post-operatively. In a subsequent experiment we also showed that simply anesthetizing the animals lowered leptin concentrations similarly. Therefore, the decline in leptin levels following surgery was not caused by the stress of surgery, but was caused by anesthesia. We believe that the release of leptin and NO is under neural control since plasma concentrations of both substances decreased following initiation of anesthesia. The decrease in [NO_3/NO_2] was much more rapid and of greater magnitude than that of leptin, suggesting that the inhibition of NO release was of greater magnitude than that of leptin.

We believe that ketamine is the responsible element of this combination of ketamine/ acepromazine/xylazine, since it alone at this dose induces anesthesia. Ketamine, the prin-

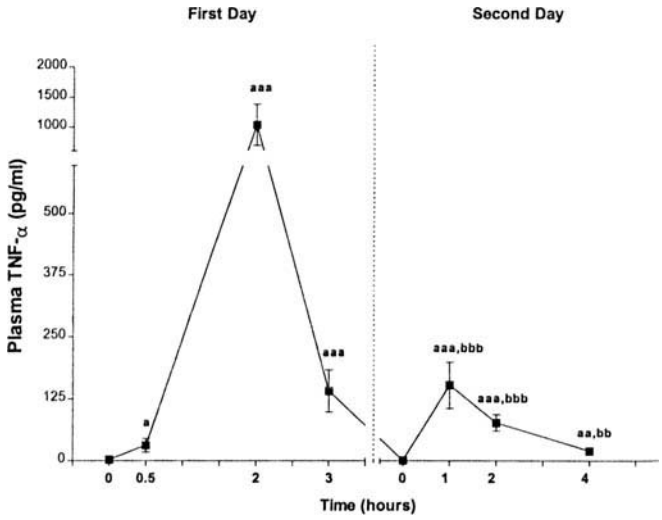

Figure 3. Plasma TNF-α after operation. Individual values were normalized by body weight and expressed as pg/ml/450 g. [t0] of first day was between 0800 and 11:30 hr and [t0] of the second day was between 0600 and 6:30 hr. $^{a}P < 0.05$, $^{aa}P < 0.01$, and $^{aaa}P < 0.001$ versus [t0] of the first day, $^{bb}P < 0.01$ and $^{bbb}P < 0.001$ versus [t0] of the second day.

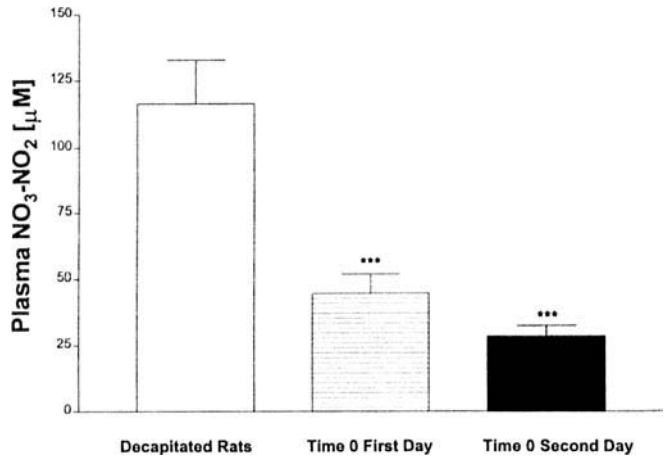

Figure 4. Comparison of plasma [NO_3/NO_2] between decapitated animals and [t0] of first and second day. $^{***}P < 0.001$ versus decapitated animals.

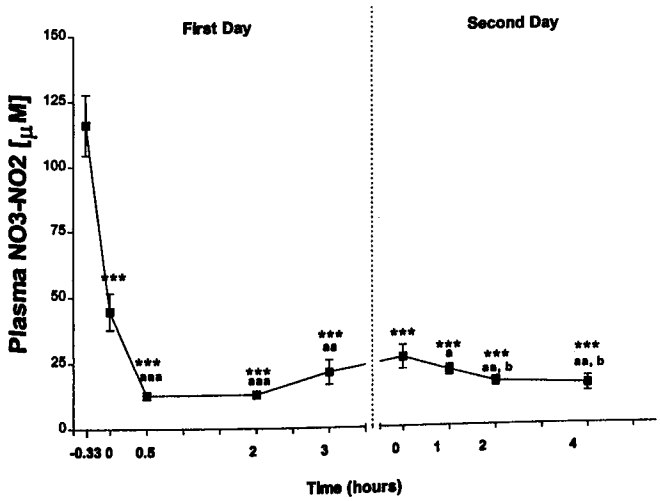

Figure 5. Plasma [NO_3/NO_2] after operation. Individual values were normalized by body weight and expressed as pg/ml/450 g. $^{a}P < 0.05$, $^{aa}P < 0.01$ versus [t0] first day, and $^{aaa}P < 0.001$ versus [t0] of the first day, $^{b}P < 0.05$ versus [t0] second day. $^{***}P < 0.001$ versus decapitated animals.

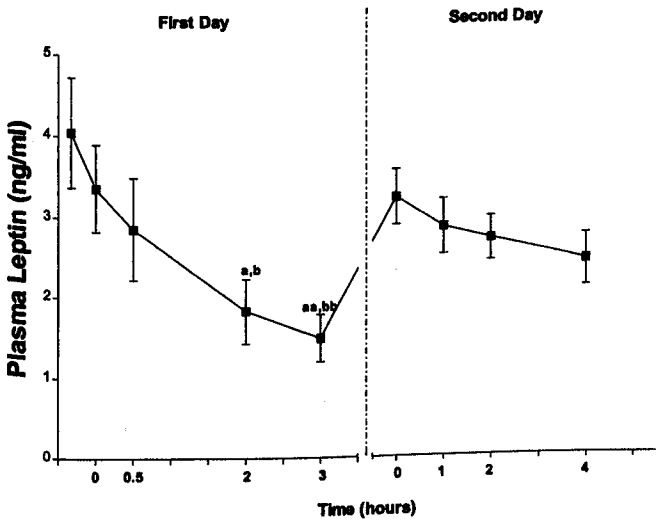

Figure 6. Plasma leptin after operation. Individual values were normalized by body weight and expressed as ng/ml/450 g. $^{a}P < 0.05$ and $^{aa}P < 0.01$ versus [t0] of the first day, $^{b}P < 0.05$ and $^{bb}P < 0.01$ versus [t0] of the second day.

cipal compound of the anesthetic used, is a noncompetitive inhibitor of N-methyl-D-aspartate (NMDA) receptors (17). NMDA receptors are widely distributed throughout the brain and spinal cord (18). Glutamic acid, the natural agonist of NMDA receptors, increases NO production (19). The rapid reduction of plasma [NO_3/NO_2] after induction of anesthesia suggests that glutamic acid-induced NO production may be an important contributor to plasma [NO_3/NO_2]. Plasma [NO_3/NO_2] at 6 hr of the second day was still significantly lower than its pre-operative concentration, suggesting that the combination of surgical stress and bleeding also reduced NO production. Plasma [NO_3/NO_2] fell significantly during the removal of blood samples on the second morning, reaching a minimum after 4 hr. The total volume of blood removed and replaced by saline during the 2 days of the experiment was 4.8 ml, which represented approximately 12% of the blood volume of the rats. We hypothesize that the blood loss caused a compensatory increased sympathetic and decreased parasympathetic tone to the vascular system to maintain blood pressure. The decreased cholinergic input to the vascular endothelium would cause decreased activation of endothelial(e) NOS, resulting in decreased release of NO into the blood stream and consequent decreased plasma NO_3/NO_2 (20, 21).

In vivo studies in humans have shown that ^{14}C-ketamine reaches the brain very rapidly and is distributed in high concentration to the striatum, thalamic nuclei, and cortical regions (22) and presumably also to the hypothalamus and brain stem where it may block CNS control of TNF-α, [NO_3/NO_2], and leptin release. Since glutamic acid is the principal excitatory transmitter in the brain and it is present in roughly one-third of the synapses within the brain, we hypothesize that the elimination of the excitatory effects of glutamic acid by ketamine may be the mechanism responsible for ketamine-induced anesthesia. We plan to determine if the results would be similar with other anesthetics such as Nembutal or ether.

Surgical stress dramatically increased TNF-α release and so did the inflammatory stress induced by LPS, where the rapid rise in TNF-α far exceeded the magnitude of the increase in plasma ACTH that occurred from similar doses (8), showing clearly that TNF-α is an important stress hormone. Initiation of blood sampling on the morning of the second day also initiated a rapid increase in TNF-α within 30 min, albeit of lesser magnitude than that which followed the surgery. Therefore, it appears that TNF-α is the most stress responsive hormone yet discovered.

The time elapsed to produce these rapid releases of TNF-α is insufficient for the release to be accounted for by *de novo* synthesis of the cytokine. Instead, it must be released from stores somewhere in the body. Indeed, it has recently been shown that TNF-α is expressed in the anterior pituitary gland in the folliculostellate cells and also in hormone-producing cells (23). We have detected TNF-α in extracts of the liver where it may be stored in Kupffer cells that are modified macrophages (Mastronardi CA, Yu WH, McCann SM, unpublished data). We hypothesize that stresses of many types will cause a release of TNF-α by activation of the hypothalamus. In fact, the pathway may be very similar to that which brings about the release of CRH and vasopressin from stress, namely, afferent input to the brain stem followed by activation of noradrenergic neurons in the brain stem that project to the hypothalamus and activate TNF-α release from the anterior pituitary gland. Indeed, the release of CRH or vasopressin into the hypophyseal portal vessels may stimulate TNF-α release. Alternatively, release of norepinephrine or epinephrine into those vessels may be responsible (24). Activation of the peripheral sympathetic nervous system by afferent input to the brain stem may induce TNF-α release from immune cells in liver, spleen, and lymph nodes.

In contrast to the dramatic effect of stress on TNF-α release, there was no clear effect of stress on the release leptin into the circulation, either from the surgical stress of the first day or the bleeding stress of the first or second day. In the case of the other stress that we have studied, namely, that of LPS, again there was a dramatic increase in TNF-α, but in this case there was also a gradual increase in leptin release, which continued linearly for 2 hr, whereas the TNF-α response terminated rapidly. Thus, we conclude that leptin can respond to stress, but the sensitivity is much less than that of TNF-α. Conversely, NO release declined rapidly while the rats were anesthetized, recovered slightly, and finally reached a minimum at the end of the experiment, probably related to the hemorrhage-induced decline in NO production either by inhibition of nitroxidergic terminals ending on blood vessels (25) and/or by decreased stimulation of eNOS by other hemorrhage-induced mechanisms provoking a reduction in NO release (26). This response is diametrically opposite to the marked release of NO brought about by induction of inducible NOS by LPS (2, 3).

1. Besedovsky HO, Del Rey A. Immune-neuroendocrine circuits: Integrative role of cytokines. Front Neuroendocrinol **13:**61–94, 1992.
2. Wong ML, Rettori V, Al-Shekhlee A, Bongiorno PB, Canteros G, McCann SM, Gold PW, Licinio J. Inducible nitric oxide synthase gene expression in the brain during systemic inflammation. Nat Med **5:**581–584, 1996.
3. McCann SM, Kimura M, Karanth S, Yu WH, Rettori V. Role of nitric oxide in the neuroendocrine responses to cytokines. Ann N Y Acad Sci **840:**174–184, 1998.
4. Gottschall PE, Komaki G, Arimura A. Increased circulating interleukin-1 and interleukin-6 after intracerebroventricular injection of lipopolysaccharide. Neuroendocrinology **6:**935–938, 1992.
5. Turnbull AV, Lee S, Rivier C. Mechanisms of hypothalamic-pituitary-adrenal axis stimulation by immune signals in the adult rat. Ann N Y Acad Sci **840:**434–443, 1998.
6. Fattori E, Cappelletti M, Costa P, Sellitto C, Cantoni L, Carelli M, Faggioni R, Fantuzzi G, Ghezzi P, Poli V. Defective inflammatory response in interleukin 6-deficient mice. J Exp Med **180:**1243–1250, 1994.
7. Kakizaki Y, Watanobe H, Kohsaka A, Suda T. Temporal profiles of interleukin-1β, interleukin-6, and tumor necrosis factor-α in the plasma and hypothalamic paraventricular nucleus after intravenous or intraperitoneal administration of lipopolysaccharide in the rat: Estimation by push-pull perfusion. Endocr J **46:**487–496, 1999.
8. Rettori V, Dees WL, Hiney JK, Lyson K, McCann SM. An interleukin-1-alpha-like neuronal system in the preoptic-hypothalamic region and its induction by bacterial lipopolysaccharide in concentrations which alter pituitary hormone release. Neuroimmunomodulation **1:**251–258, 1994.
9. Wang YS, White TD. The bacterial endotoxin lipopolysaccharide causes rapid inappropriate excitation in rat cortex. J Neurochem **72:**652–660, 1999.
10. Song DK, Im YB, Jung JS, Suh HW, Huh SO, Park SW, Wie MB, Kim YH. Differential involvement of central and peripheral norepinephrine in the central lipopolysaccharide-induced interleukin-6 responses in mice. J Neurochem **72:**1625–1633, 1999.
11. MohanKumar SM, MohanKumar PS, Quadri SK. Lipopolysaccharide-induced changes in monoamines in specific areas of the brain: Blockade by interleukin-1 receptor antagonist. Brain Res **824:**232–237, 1999.
12. McCann SM, Mastronardi CA, Yu WH. Lipopolysaccharide (LPS)-induced TNF-α release is controlled by the central nervous system. The Endocrine Society 82nd Annual Meeting, Toronto, Canada, June 21–24, 2000.
13. Mastronardi CA, Yu WH, McCann SM. Lipopolysaccharide (LPS)-induced leptin release is neurally controlled. The Endocrine Society 82nd Annual Meeting, Toronto, Canada, June 21–24, 2000.
14. Mastronardi CA, Yu WH, McCann SM. Lipopolysaccharide-induced leptin release is not mediated by nitric oxide, but is blocked by dexamethasone. Neuroimmunomodulation **8:**91–97, 2000.
15. Finck BN, Kelley KW, Dantzer R, Johnson RW. *In vivo* and *in vitro* evidence for the involvement of tumor necrosis factor-α in the induction of leptin by lipopolysaccharide. Endocrinology **139:**2278–2283, 1998.
16. Harms PC, Ojeda SR. A rapid and simple procedures for chronic cannulation of the rat jugular vein. J Appl Physiol **36:**391–392, 1974.
17. Hargreaves RJ, Hill RG, Iversen LL. Neuroprotective NMDA antagonists: The controversy over their potential for adverse effects on cortical neuronal morphology. Acta Neurochir Suppl (Wien) **60:**15–19, 1994.
18. Bhargava HN, Kumar S. Sensitization to the locomotor stimulant effect of cocaine modifies the binding of [3H]MK-801 to brain regions and spinal cord of the mouse. Gen Pharmacol **32:**359–363, 1999.
19. Rettori V, Kamat A, McCann SM. Nitric oxide mediates the stimulation of luteinizing-hormone releasing hormone release induced by glutamic acid *in vitro*. Brain Res Bull **33:**501–503, 1994.
20. Furchgott RF, Carvalho MH, Khan MT, Matsunaga K Evidence for endothelium-dependent vasodilation of resistance vessels by acetylcholine. Blood Vessels **24:**145–149, 1987.
21. Ignarro LJ, Buga GM, Wood KS, Byrns RE, Chaudhuri G Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. Proc Natl Acad Sci U S A **84:**9265–9269, 1987.
22. Kumlien E, Hartvig P, Valind S, Oye I, Tedroff J, Langstrom B. NMDA-receptor activity visualized with (S)-[N-methyl-11C]ketamine and positron emission tomography in patients with medial temporal lobe epilepsy. Epilepsia **40:**30–37, 1999.
23. Theas S, Pisera D, Duvilanski B, De Laurentiis A, Pampillo M, Lasaga M, Seilicovich A. Estrogens modulate the inhibitory effect of tumor necrosis factor-alpha on anterior pituitary cell proliferation and prolactin release. Endocrine **12:**249–255, 2000.
24. McCann, SM, Franci CR, Anselmo-Franci JA, Karanth S and Rettori V. Role of the hypothalamic pituitary adrenal axis in control of the response to stress and infection. Brazilian J Med Biol Res **224:**152–158, 2000.
25. Toda N, Toda M, Ayajiki K, Okamura T. Cholinergic nerve function in monkey ciliary arteries innervated by nitroxidergic nerve. Am J Physiol **274:**H1582–H1589, 1998.
26. Conner EM, Aiko S, Fernandez M, Battarbee HD, Gray L, Grisham MB. Duration of the hemodynamic effects of N(G)-nitro-L-arginine methyl ester *in vivo*. Nitric Oxide **2:**85–93, 2000.