Experimental Gerontology, Vol. 33, Nos. 7/8, pp. 813–826, 1998

0531-5565/98 $19.00 + .00

ELSEVIER

PII S0531-5565(98)00050-3

THE NITRIC OXIDE HYPOTHESIS OF AGING

S.M. McCann,[1] J. Licinio,[2] M.-L. Wong,[2] W.H. Yu,[1] S. Karanth[1] and V. Rettorri[3]

[1]Pennington Biomedical Research Center (LSU), 6400 Perkins Road, Baton Rouge, Louisiana 70808-4124; [2]Clinical Neuroendocrinology Branch, National Institute of Mental Health, NIH, Bldg. 10, Rm. 3S231, 10 Center Dr MSC 1284, Bethesda, Maryland 20892-1284, and [3]Centro de Estudios Farmacologicos y Botanicos, Consejo Nacional de Investigaciones Cientificas y Tecnicas (CEFYBO-CONICET), Serrano 665, 1414 Buenos Aires, Argentina

Abstract—Nitric oxide (NO), generated by endothelial (e) NO synthase (NOS) and neuronal (n) NOS, plays a ubiquitous role in the body in controlling the function of almost every, if not every, organ system. Bacterial and viral products, such as bacterial lipopolysaccharide (LPS), induce inducible (i) NOS synthesis that produces massive amounts of NO toxic to the invading viruses and bacteria, but also host cells by inactivation of enzymes leading to cell death. The actions of all forms of NOS are mediated not only by the free radical oxidant properties of this soluble gas, but also by its activation of guanylate cyclase (GC), leading to the production of cyclic guanosine monophosphate (cGMP) that mediates many of its physiological actions. In addition, NO activates cyclooxygenase and lipoxygenase, leading to the production of physiologically relevant quantities of prostaglandin E_2 (PGE_2) and leukotrienes. In the case of iNOS, the massive release of NO, PGE_2, and leukotrienes produces toxic effects. Systemic injection of LPS causes induction of interleukin (IL)-1β mRNA followed by IL-β synthesis that induces iNOS mRNA with a latency of two and four hours, respectively, in the anterior pituitary and pineal glands, meninges, and choroid plexus, regions outside the blood–brain barrier, and shortly thereafter, in hypothalamic regions, such as the temperature-regulating centers, paraventricular nucleus containing releasing and inhibiting hormone neurons, and the arcuate nucleus, a region containing these neurons and axons bound for the median eminence. We are currently determining if LPS similarly activates cytokine and iNOS production in the cardiovascular system and the gonads. Our hypothesis is that recurrent infections over the life span play a significant role in producing aging changes in all systems outside the blood–brain barrier via release of toxic quantities of NO. NO may be a major factor in the development of coronary heart disease (CHD). Considerable evidence has accrued indicating a role for infections in the induction of CHD and, indeed, patients treated with a tetracycline derivative had 10 times less complications of CHD than their controls. Stress, inflammation, and infection have all been shown to cause induction of iNOS in rats, and it is likely that this triad of events is very important in progression of coronary arteriosclerosis leading to coronary occlusion. Aging of the anterior pituitary and pineal with resultant decreased secretion of pituitary hormones and the pineal hormone, melatonin, respectively, may be caused by NO. The induction of iNOS in the temperature-regulating centers by infections may cause the decreased febrile response in the

Correspondence to: S.M. McCann. Tel: (225) 763-3042; Fax: (225) 763-3030; E-mail: mccannsm@mhs.pbrc.edu

aged by loss of thermosensitive neurons. iNOS induction in the paraventricular nucleus may cause the decreased nocturnal secretion of growth hormone (GH) and prolactin that occurs with age, and its induction in the arcuate nucleus may destroy luteinizing hormone-releasing hormone (LHRH) neurons, thereby leading to decreased release of gonadotropins. Recurrent infections may play a role in aging of other parts of the brain, because there are increased numbers of astrocytes expressing IL-1β throughout the brain in aged patients. IL-1 and products of NO activity accumulate around the plaques of Alzheimer's, and may play a role in the progression of the disease. Early onset Parkinsonism following flu encephalitis during World War I was possibly due to induction of iNOS in cells adjacent to substantia nigra dopaminergic neurons leading to death of these cells, which, coupled with ordinary aging fall out, led to Parkinsonism. The central nervous system (CNS) pathology in AIDS patients bears striking resemblance to aging changes, and may also be largely caused by the action of iNOS. Antioxidants, such as melatonin, vitamin C, and vitamin E, probably play an important acute and chronic role in reducing or eliminating the oxidant damage produced by NO.

Key Words: nitric oxide synthase, cyclic GMP, cyclooxygenase, bacterial lipopolysaccharide, cytokines, hypothalamus, brain, pituitary gland, pineal gland, degenerative diseases, inflammation, infection, stress

INTRODUCTION

THE CAUSES OF AGING are undoubtedly multifactorial. One of the most prominent current theories of aging is the free radical theory. According to this theory, free radicals generated through mitochondrial metabolism can act to cause abnormal function and cell death. Various toxins in the environment can injure mitochondrial enzymes, leading to increased generation of free radicals that, over the life span, would eventually play a major part in aging (Kirkwood and Kowald, 1997).

At the Third International Symposium on the Neurobiology and Neuroendocrinology of Aging, I (McCann, 1997) presented evidence to suggest that excessive production of the free radical, nitric oxide (NO), in the central nervous system (CNS) and its related glands, such as the pineal and anterior pituitary, may be the most important factor in aging of these structures. Evidence for this hypothesis has been accruing rapidly. Because of the fact that the synthesis of inducible NO synthase (iNOS) following injection of bacterial lipopolysaccharide (LPS) in the rat was much greater outside the blood–brain barrier (Wong *et al*., 1996), for example, in the anterior pituitary and pineal gland, than inside this barrier, it occurred to us that NO might play a role in aging of every organ system of the body. The evidence for this concept is particularly well developed to explain the pathogenesis of coronary arteriosclerosis.

NO has been found to play an ubiquitous role in the control of physiological functions throughout the body. The toxic effects of the soluble gas occur following infection or inflammation that cause the production of iNOS, which floods the tissue with toxic concentrations of NO. Here, we will first briefly review the methods of formation of NO in the body and its physiological role in the organs where we believe it also plays an important role in aging, and then present the evidence that NO is largely responsible for the aging process.

Production of NO in the body

Three isoforms of NO synthase (NOS) occur in the body (McCann and Rettori, 1996; McDonald and Murad, 1996). The first of these is iNOS. LPS, or cytokines [interleukin (IL)-1, IL-2, IL-6, tumor necrosis factor (TNF)] act via receptors on the cell surface of immune cells,

particularly macrophages and other cells, such as endothelial cells, to activate DNA-directed mRNA synthesis that induces synthesis of iNOS. A single injection of LPS leads to release of massive amounts of NO, beginning within a few hours, peaking at 18 h, and then declining by 24 h. This enzyme is active as soon as it is induced, because it contains within itself calcium and calmodulin, which are required for activation of all isoforms of the enzyme. Like all forms of NOS, iNOS converts arginine and equimolar molecules of oxygen in the presence of various cofactors, such as NADPH and tetrahydrobiopterin, into NO and equimolar amounts of citrulline. The NO decays in solution with a half time of 5 to 10 s, whereas citrulline remains in the cell and can even be recycled into arginine to provide further substrate for conversion by NOS into NO and citrulline. There is also a transport mechanism that carries arginine into the cell to provide substrate.

NO is a soluble gas and diffuses to neighboring cells and bacteria and viruses. The high concentration formed after the induction of iNOS interferes with metabolism, leading to death of viral and bacterial invaders and also host cells. NO blocks cellular enzymes required in metabolism and also activates soluble guanylate cyclase (sGC), a soluble enzyme present in the cytoplasm of cells. The activation occurs via interaction of NO with the Fe^{2+} in the heme portion of the molecule, thereby altering its conformation and activating it. This causes conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP), which mediates many of the physiological actions of NO in mammalian cells. NO also activates cyclooxygenase-generating prostaglandins and lipoxygenase-forming leucotrienes that are also toxic in high concentrations.

Endothelial (eNOS) is a constitutive enzyme present in vascular endothelium (Bredt *et al*., 1990). Cholinergic stimulation by parasympathetic innervation of the vessels activates the enzyme by increasing the intracellular free calcium concentration [Ca^{++}], which combines with calmodulin. This interacts with eNOS, activating it. The NO produced diffuses to overlying smooth muscle and activates sGC. The cGMP released reduces intracellular [Ca^{++}], thereby relaxing the vascular smooth muscle.

A third isoform of the enzyme neural (n) NOS is a constitutive enzyme, like eNOS, present in many neurons in the central and autonomic nervous systems (Bredt *et al*., 1990). It is present in high concentrations in the cerebellum, cerebral cortex, and hippocampus, and in very high concentrations in the hypothalamus, particularly in the paraventricular and supraoptic neurons. The axons of these neurons project to the median eminence and neural lobe of the pituitary gland where very large amounts of enzyme are present. This form of the enzyme, like eNOS, requires activation by synaptic input, causing elevation of intracellular [Ca^{++}], which combines with calmodulin, which activates the enzyme leading to the production of NO.

Role of NO in the CNS

The first evidence that NO played a role in the CNS was derived from experiments using cerebellar or hippocampal explants (Bredt *et al*., 1990). These showed that NO plays an important role in cerebellar function via activation of sGC and induction of cGMP formation. However, because of the complexity of function in the cerebellum, it is difficult to say what particular physiologic functions of the cerebellum are altered by NO. Garthwaite *et al*., (1988) showed that NO may be important in induction of long-term potentiation in the hippocampus, which is thought to play a role in memory (Bredt *et al*., 1990; Murad and McDonald, 1996; McCann and Rettori, 1996, for further references). Palmer *et al*. (1987) showed by mass spectroscopy that the substance was indeed NO. Earlier work had previously pointed clearly to

NO; however, it is possible that the major product is not NO itself, but nitroso compounds formed by combination of NO with various other compounds. Indeed, slow release of NO from nitroso compounds may account for a longer duration of action of NO than can be explained on the basis of the free radical itself, which, as indicated above, decays within seconds in solution.

Role of NO in hypothalamic function

It has been clearly established that NO plays a key role in controlling the physiological release of a number of hypothalamic peptides and classical neurotransmitters. It has been shown to control the release of corticotrophin-releasing hormone (CRH) by the CRH neurons in the PVN (Karanth *et al*., 1993). The stimulatory effect is mediated by cholinergic neurons that act on muscarinic receptors to stimulate the release of NO from nNOS-containing neurons, termed NOergic neurons, in the paraventricular nucleus. This NO diffuses to the CRH-containing neurons, and activates the release of CRH. The mechanism involves stimulation of release of prostaglandin E_2 (PGE_2) and leukotrienes in the CRH neurons via activation of cyclooxygenase and lipoxygenase. In the case of the cyclooxygenase (COX), it is clear that NO activates constitutive COX (COX-1) by interaction of the free radical with the heme group of the enzyme, altering its conformation. Lipoxygenase, also contains Fe^{2+}, but a heme group has yet to be demonstrated. Nonetheless, this enzyme is definitely activated because sodium nitroprusside (NP), which releases NO, increases conversion of ^{14}C arachidonic acid to leucotrienes (Rettori *et al*., 1992).

NO is thought to act physiologically, mainly by activation of sGC (McDonald and Murad, 1996). Indeed, NP increases the release of cGMP from hypothalamic explants (Canteros *et al*., 1995). We hypothesize that NO releases CRH and other releasing hormones via the activation of GC, which releases cGMP, which in turn, increases intracellular [Ca^{++}], leading to activation of phospholipase-A2. This converts membrane phospholipids into arachidonate, which is then converted to PGE_2 and leukotrienes by the activated cyclooxygenase and lipoxygenase, respectively (6). The result is activation of adenylate cyclase, conversion of ATP to cAMP, which activates protein kinase A, causing extrusion of CRH granules. The CRH then enters the hypophyseal portal vessels, reaches the anterior lobe of the pituitary, and causes release of adrenocorticotropic hormone (ACTH) (Karanth *et al*., 1993).

Role of NO in luteinizing hormone-releasing hormone (LHRH) release

Release of LH is pulsatile and controlled by pulsatile release of LHRH into hypophysial portal vessels that deliver it to the sinusoids of the anterior pituitary where it acts on the gonadotropes to promote the release of LH. LHRH also increases follicle-stimulating hormone (FSH) release. Although NO controls LH release, we have found no evidence for a role for NO in control of FSH release, and postulate that the putative FSH-releasing hormone (FSHRH) is not under NO control (Rettori *et al*., 1993).

On the basis of in vitro studies, we postulate that the pathway of activation of LHRH release is as follows: either direct activation of noradrenergic terminals in the region of the arcuate-median eminence region or their activation by glutamergic neurons, via *N*-methyl-D-aspartic (NMDA) receptors, causes the release of norepinephrine, which acts by α_1 adrenergic receptors on the NOergic neurons to cause the release of NO (Kamat *et al*., 1995). This diffuses to the LHRH terminals intermingled with NOergic neurons nearby in the median eminence-arcuate region, stimulating the release of LHRH by the identical mechanism described above for CRH—namely by activation of sGC, COX-1 and lipoxygenase—leading to the release of LHRH

secretory granules into the hypophyseal portal vessels (Canteros *et al*., 1995). The intermingling of NOergic and LHRH terminals in this region has been demonstrated (Canteros *et al*., 1995).

NO controls LHRH release that mediates mating behavior in both male and female rats by activating brain stem neurons controlling lordosis in the female and penile erection in the male rat. The role of NO in mediating LHRH release controlling both of these activities has been demonstrated in vivo (Mani *et al*., 1994).

We have recently demonstrated that oxytocin stimulates the release of LHRH via NO. The low concentrations required (10^{-8}–10^{-10}M) probably fall within the physiological range in view of the high concentrations of oxytocin in the median eminence in juxtaposition to LHRH terminals (Rettori *et al*., 1997). There is an ultrashort-loop negative feedback by which NO released from NOergic neurons feeds back to inhibit the release of oxytocin.

Not only does NO stimulate the release of many hypothalamic peptides, but it also stimulates the release of the inhibitory neurotransmitter, gamma amino-butyric acid (GABA) (Seilicovich *et al*., 1995a). This blocks the response of the LHRH terminal to NO. Therefore, GABA mediates a feed-forward negative feedback to inhibit pulsatile LHRH release. Furthermore, NO also inhibits the release of both norepinephrine and dopamine from the medial basal hypothalamus, constituting another negative feedback of pulsatile LHRH release by feeding back on the terminals of the noreadrenergic and dopamineric neurons to inhibit the release of both of these transmitters, one of which, and probably both of which, stimulate the release of NO that drives LHRH release (Seilicovich *et al*., 1995b).

Interestingly, 30 min after addition of norepinephrine or NMDA, both stimulators of LHRH release, there is an increase in content of NOS in the hypothalamus measured by the citrulline method (Canteros *et al*., 1995). This method is an index of the quantity of NOS in the tissue because the labeled arginine is added to the homogenate and the labeled citrulline formed is measured. That this represents de novo synthesis of NOS is indicated by the fact that this effect is blocked by the inhibitor of DNA-directed RNA synthesis, actinomycin (Rettori *et al*., unpublished).

Thus, pulsatile LHRH release is mediated by noradrenergic neuronal terminals that activate NOergic neurons. The NO synthesized diffuses to the LHRH neurons and activates LHRH release. The pulses of LHRH are terminated by NO-induced release of GABA that blocks the response to the LHRH neuron to NO and NO-induced inhibition of further norepinephrine and dopamine release.

Role of NO in control of other hypothalamic peptides and neurotransmitters

Growth hormone (GH) release is also pulsatile, but the greatest release occurs during early sleep at night in humans. In vivo studies have shown that the inhibitor of NO synthase, N^G-monomehtyl-L-arginine (NMMA), can block pulsatile release of GH in the rat (Rettori *et al*., 1994a). Furthermore, NO can also stimulate somatostatin release and its messenger RNA levels in the paraventricular nucleus by activation of sGC on the basis of in vitro studies utilizing explants of the paraventricular region (Aguila, 1994). We hypothesize that the pulsatile release of GH that occurs under normal conditions is brought about principally by NO stimulation of GH-releasing hormone (GHRH) release. At the same time, somatostatin release is probably inhibited. In the interpulse interval when GHRH release is absent, somatostatin release mediated by NO increases. IL-1 not only inhibits GHRH release, but also stimulates somatostatin release, thereby inhibiting GH release during infections. The IL-induced prolactin release is also mediated by NO (Rettori *et al*., 1994b) probably by NO stimulation of prolactin-releasing

peptides, such as oxytocin (Rettori *et al*., 1997) and by inhibition of the release of dopamine, a potent prolactin release inhibiting hormone, into the hypophyseal portal vessels (Duvilanski *et al*., 1995).

All of these results indicate that under physiological conditions, NO plays a fundamental part in the control of neurotransmitter and neuropeptide release in the hypothalamus. Therefore, these areas where NO is released physiologically will be subjected to low levels of pulsatile NO throughout the life of the individual. It is not clear whether such levels can be toxic, but they may be. No studies have been done to determine whether long-term exposure to low levels of NO is damaging to neurons and/or glia. Interestingly, Schultz *et al*. (1996) reported neurofibrillary degeneration in nerve fibers in the arcuate nucleus of aged men but not women. Possibly, pulsatile release of NO driving LHRH release over the life span may have caused this degeneration. Because aged women failed to show the degeneration, there appears to be a sex difference, probably mediated by sex hormones such as estrogen, which has been shown to alter NOS concentrations.

The role of NO in control of anterior pituitary function

Neural NOS has been localized in anterior pituitary cells. At least two cell types contain the enzyme; one of these, the folliculostellate cells (FS), which are modified macrophages, are known to secrete IL-6 and other cytokines. The other type is the gonadotropes that secrete LH. The in vitro secretion rates of most pituitary hormones are low because of withdrawal of hypothalamic stimulation, but the secretion of prolactin is greatly enhanced because of withdrawal of hypothalamic inhibition by dopamine. In the case of prolactin, its secretion can be increased by inhibiting NOS with N^G-monomethyl-L-arginine (NMMA), a competitive inhibitor of NOS or nitroarginine methyl ester (NAME), another inhibitor of the enzyme. On the other hand, NP that spontaneously releases NO, lowers prolactin release. The prolactin-inhibiting action of dopamine, the principle prolactin inhibiting hormone, appears to be mediated via NO, because the action of dopamine to lower prolactin release was blocked by inhibition of NOS. We hypothesize that DA released into portal vessels reaches the anterior lobe, where it acts on D_2 receptors in folliculostellate cells and or gonadotropes to activate sGC increasing cGMP, which then suppresses secretion of prolactin from the lactotrophs. Indeed, cGMP decreased prolactin secretion (Duvilanski *et al*., 1995).

Adenosine is secreted by the FS cells, and is the most powerful stimulant of prolactin secretion from anterior pituitaries in vitro yet identified, increasing release at concentrations of 10^{-10}–10^{-5} M with maximal release of three times basal at 10^{-8} M. The action appears to be mediated by an autocrine activation of adenosine 1 receptors on the surface of the FS cells, which activates inhibitory G proteins (Gi) that lower intracellular [Ca^{++}], thereby inhibiting nNOS within the FS cells that decreases NO production. The reduced paracrine NO inhibition of the lactotropes increases prolactin release (Yu *et al*., 1998).

In contrast to prolactin, the release of which is inhibited by the hypothalamus, the release of the gonadotropins, LH, and FSH is stimulated by the hypothalamic peptide, LHRH, which stimulates LH and to a lesser extent FSH and by FSHR factor (FSHRF) (lamprey III LHRH or a closely related peptide), which preferentially stimulates FSH release. The mechanism is by stimulation of LHRH and FSHRF receptors, respectively, leading to increased [Ca^{++}], activation of nNOS in the gonadotropes with resultant generation of cGMP, which in turn, activates PKG, leading to extrusion of gonadotropin secretory granules (Yu *et al*., 1997).

Gonadotropin secretion is pulsatile. Pulses can consist of the simultaneous release of FSH and

LH, brought about by prior simultaneous release of FSHRF and LHRH, or selective pulses of FSH or LH brought about by prior release of the respective RH. The relative abundance of the pulses of each type is governed by sex hormones. Thus, on the basis of the research done so far, it appears that the pituitary gland is exposed to NO throughout normal life. Again, whether or not these concentrations could be toxic is not clear.

The effect of infection on cytokine and NO formation in brain, pituitary and pineal glands

Recent work indicates that CNS infection is a powerful inducer of cytokine production in glia and neurons of the brain, which causes induction of iNOS and production of potentially toxic quantities of NO. Injection of bacterial LPS induces the pattern of pituitary hormone secretion that characterizes infection. Intravenous (IV) LPS induces a dose-related release of ACTH and prolactin, a transient release of GH followed by profound inhibition, decreased secretion of thyrotropin-releasing hormone (TSH), and inhibition of LH, and to a lesser extent FSH, release in rat. This pattern is caused by effects of LPS directly on the brain because following IV injection of an intermediate dose of LPS, this pattern of pituitary hormone response occurred, and also there was an induction of IL-1α immunoreactive neurons in the preoptic-hypothalamic region. These cells were shown to be neurons by the fact that double staining revealed the presence of neuronspecific enolase. The neurons are present in saline-injected control animals, but increased in number by a factor of 2 within two hours following injection of LPS. They were located in a region that also contains the thermosensitive neurons. They may be the cells that are stimulated to induce fever following injection of LPS. They have short axons that did not clearly project to the areas containing the various hypothalamic releasing and inhibiting hormones, but they could also be involved in the stimulation or inhibition of their release, which occurs following infection (Rettori *et al*., 1994c).

This study led to further research in which we determined the effect of IP injection of a moderate dose of LPS on the induction of Il-1β and iNOS mRNA in the brain, pituitary, and pineal gland. The results were very exciting, because an induction of IL-1β and iNOS mRNA occurred with the same time course as found in the periphery following injection of LPS, namely, clear induction of iNOS mRNA within two hours, reaching a peak in two or six hours, followed by a decline to near basal levels at the next measurement by 24 h after the single injection of LPS. The induction of both mRNAs occurred in the meninges, the choroid plexus, the circumventricular organs—such as the subfornical organ and median eminence—in the ependymal cells lining the ventricular system, and very surprisingly, in parvocellular neurons of the PVN and arcuate nucleus (AN), areas of particular interest because they contain the hypothalamic releasing and inhibiting hormone and also other neurotransmitters controlled by NO (Wong *et al*., 1996).

The greatest induction occurred in the anterior lobe of the pituitary, where the iNOS mRNA was increased at two hours by a factor of 45, and the pineal, where the activity was increased by a factor of 7 at six hours, whereas the increase in the PVN was fivefold. At six hours, the medial basal hypothalamus was found to have an increased content of NOS measured in vitro, and the collected cerebrospinal fluid (CSF) had increased concentrations of the NO metabolite, nitrate. These results indicate that the increase in iNOS mRNA was followed by de novo synthesis of iNOS that liberated NO into the tissue and also into the CSF. Presumably, LPS was bound to its receptors in the circumventricular organs and in the choroid plexus. These receptors, as in macrophages, activated DNA-directed IL-1β mRNA synthesis, which in turn, caused the synthesis of IL-1β. IL-1β then activated iNOS mRNA and synthesis.

How can neurons in the AN and PVN be activated as they are inside the blood–brain barrier? In the case of the AN, the neurons may have axons that project to the median eminence. These neurons may have LPS receptors on their cell surface, which then induce IL-β mRNA and IL-β synthesis. This may then induce iNOS mRNA. Alternatively, LPS acting on its receptors may simultaneously induce IL-β mRNA and iNOS mRNA.

Active transport mechanisms for IL-1 and other cytokines (Banks and Kastin, 1995), and perhaps LPS, are present in the choroid plexus. The cells of the choroid plexus on the basis of our results must have LPS receptors on them. LPS must stimulate IL-1β and iNOS mRNA followed by synthesis of IL-1β and iNOS in the choroid plexus. LPS and IL-1β are then transported into the CSF. LPS is carried by CSF flow to the third ventricle, where it either crosses the ependyma or acts on terminals of PVN neurons in the ependyma to induce IL-1β and iNOS mRNA.

These results raise the possibility that even moderate infection, without direct CNS involvement, can increase iNOS levels and lead to production of toxic levels of NO. Therefore, it is possible that repeated infections over the life span could lead to brain damage in areas where there is large induction of iNOS in neurons, such as the PVN—the site of the cell bodies of most of the releasing and inhibiting hormone neurons—and the AN-median eminence region, which is also the site of production of GHRH, many neurotransmitters, and the site of passage of axons of many of the releasing hormone neurons, such as LHRH neurons, which project to the median eminence. There may also be damage to glial elements, meninges, and to the choroid plexus over the lifespan. The induction of IL-1α neurons in the temperature-regulating regions of the preoptic area should also be followed by induction of iNOS. Exposure to high levels of NO in this region may kill thermosensitive neurons and thus be responsible for the decreased febrile response to infection in the elderly. Measurement of iNOS activity in aged male rats (greater than two years of age) revealed a significant increase in NOS activity in comparison with that in young adults, which provides the first experimental support for this concept (Rettori, unpublished data).

The greatest increase in iNOS mRNA following LPS injection occurred in the anterior pituitary gland. Therefore, the likelihood of damage to the cells in this gland during infection is great. This, coupled with the damage to the releasing hormone neurons, could account for aging changes in secretion of pituitary hormones. For example, GH and prolactin are released largely at night, and nocturnal GH release is known to be impaired with age.

The massive induction of iNOS in the pineal could very well contribute to the gradual reduction in function of this gland associated with decreased nocturnal melatonin levels and finally even calcification of the gland, which occurs with aging (Pierpaoli, *et al*., 1997). Melatonin is an antioxidant, and has been shown to reduce oxidative damage produced by brain ischemia and reperfusion (Przedborski *et al*., 1996). There is evidence that exogenous melatonin increases the life span of mice (Pierpaoli, *et al*., 1997). Therefore, NO-induced pineal "aging" may play a role in aging.

Role of NO in other neurodegenerative diseases

There is already considerable evidence that NO plays a role in neuronal cell death, which brings on Parkinsonism by loss of the neurons of the nigrostriatal dopaminergic system. Indeed, the toxin, 1-methyl-4phenyl-1234-tetrahydropyradine (NPTP) induces a Parkinsonism-like syndrome. It is transported into the substantia nigra dopaminergic neurons by the dopamine transporter. It then interferes with mitochondrial metabolism, leading to the production of

oxygen free radicals. Apparently, the basal production of NO can then be sufficient to cause toxicity via its diffusion into the dopaminergic neurons and combination with superoxide to generate peroxynitrite, a much more potent free radical than either superoxide or NO itself (Przedborski *et al*., 1996). Another probable mechanism for toxicity of NO in all sites is by combination with the heme groups, in various enzymes, thereby inactivating them and blocking cellular respiration leading to cell death (McDonald and Murad, 1996; Przedborski *et al*., 1996).

These findings provide an explanation for the high incidence of early onset Parkinsonism in many people who served in World War I and developed influenza. There was a major epidemic of influenza with encephalitis, which presumably led to generation of large amounts of NO in the region of the substantia nigra that then caused loss of dopaminergic neurons and eventual development of Parkinsonism many years before it would have appeared as a result of normal aging. The appearance of Parkinsonism with age is probably related to the quite rapid decline, beginning at age 45, in dopaminergic neurons in this region even in normal individuals (Knoll, 1997), which may also be caused by enhanced NO generation during infections.

In Alzheimer's, an important neurodegenerative disease, plaques form consisting of amyloid. Surrounding these plaques are many abnormal astrocytes, which, by immunocytochemistry, contain IL-1 (Griffin, 1998). The IL-1 should cause the induction of iNOS and production of NO, which may be a large factor in neuronal cell death in the vicinity of the plaques in this condition. Indeed, prostanoids presumably formed by action of NO accumulate adjacent to these plaques.

Even in normally aging brain, there is an increased incidence of these abnormal astrocytes (Griffin, 1998). Their production of IL-1 and NO could be partly responsible for the general neuronal cell loss that occurs with aging. NO is probably also involved in producing cell death around any area of inflammation in the CNS, for example, in multiple sclerosis, or after brain trauma (Griffin, 1998).

In Huntington's chorea, there is a mutation of the Huntington protein that is associated with the selective loss of basal ganglion neurons that characterize this disease. Recently, a brain-specific protein that is associated with Huntington has been identified, and has been termed Huntington-associated protein (HAP-1). The location of this protein with neurons containing nNOS mRNA, with dramatic enrichment in both the pseudopedunculopontine nuclei, the accessory olfactory bulb, and the supraoptic nucleus of the hypothalamus, with colocalization of HAP-1 and nNOS in some of these neurons, suggests that, here again, NOS could generate sufficient NO to produce the neuronal cell loss responsible for Huntington's disease (Xiao-Jiang *et al*., 1996).

The role of NO in coronary atherosclerosis

Because we had found such profound induction of iNOS in the anterior pituitary and pineal glands, areas outside the blood–brain barrier, it occurred to us that LPS would probably induce similar changes in all organs outside the blood–brain barrier, a prime example being the coronary arteries. Indeed, we have initiated studies to determine if there is induction of IL-1β and iNOS mRNA in the endothelium of the vascular system.

A great deal of evidence has accrued, suggesting the possibility that chronic infections may have a relationship with coronary heart disease (CHD) (Danesh *et al*., 1997). In the 1970s, experimental infection of germ-free chickens with avian herpes virus induced pathologic changes resembling those in human CHD (Fabricant, 1978). There have been many studies showing the presence of high titers of antibodies against various organisms in patients with

CHD. Although there is always some question about such studies, the incidence is such as to make it appear very likely that antibodies against *Helicobacter pylori*, *Chlamydia pneumonia*, *Cytomegalovirus*, or other herpes viruses are very common in these patients. There is even an association with severe dental carries (Danesh *et al*., 1997).

Stimulated by these reports, there have now been two reports of treatment of patients with CHD with tetracycline derivatives (Gurfinkel *et al*., 1997; Gupta, *et al*., 1997). In both studies further complications of CHD were significantly reduced in the treated groups. In one study, treatment reduced the complications 10-fold (Garfinkel, 1997).

Tetracyclines have now been studied in chondral cell cultures from patients with osteoarthritis and in cell cultures from animals with experimentally produced arthritis. They have been shown to have chondro-protective effects (Amin *et al*., 1996). NO is spontaneously released from human cartilage affected by osteo- or rheumatoid arthritis in quantities sufficient to cause cartilage damage. In a recent report, tetracyclines have been shown to reduce the expression and function of human osteoarthritis-effected NOS (iNOS) (Amin *et al*., 1996). It appears that in addition to the antibacterial action of these drugs, tetracyclines inhibit the expression of NOS, leading to reduction in the toxic consequences of production of NO. It is likely that these compounds will be beneficial in the treatment of osteoarthritis, as well as CHD. They will also probably be of therapeutic value in rheumatoid arthritis and cardiomyopathy, both thought to be autoimmune diseases caused largely by excess NO.

The current theory of CHD is that it is induced by an elevation of plasma cholesterol above the normal limit of 200 mg%. However, if one looks at the incidence of CHD vs. the concentration of plasma cholesterol, one finds that as cholesterol passes the 200 mg% concentration, there is only a very slight increase in the incidence of the disease as one reaches 250 mg% and the slope of the incidence begins to rise between 250 and 300 and rises quite rapidly as one approaches 400 mg%. There are many cases of CHD in patients with perfectly normal cholesterol. Indeed, increased LDL cholesterol has been considered particularly ominous, whereas HDL cholesterol has been thought to be protective. However, in many cases, CHD develops and has its downward progression in the presence of normal cholesterol and other lipids.

In a recently studied case, a 72-year-old male, the patient had gradually rising cholesterol values from 200 mg% at the age of 40 to 220 at the age of 60, but had no symptoms of CHD. In late November, 1996, 72 h after return from a trip to England, he contracted influenza, followed by pneumonia, with a fever of 103.5°F and a pulse of 120. The pneumonia was probably bacterial, because it was responsive to ampicillin.

This severe infection was followed by the development of angina pectoris within five weeks, which progressed to the point that he finally sought medical attention five months later. At this time, he had advanced CHD. His weight was normal. His cholesterol was 240 mg%, with a slightly elevated LDL and low HDL, but normal triglycerides. Angiography revealed extensive disease that was judged unsuitable for either balloon angioplasty or coronary bypass surgery, and he was placed on a cholesterol synthesis inhibitor that normalized his total cholesterol, LDL, and HDL within one month. The angina gradually improved over the next five months.

At that point in mid-November 1997, the subject took a trip to Europe and the Middle East. Beginning in Frankfurt, Germany, then to Cairo, Egypt, Luxor, and by Nile Steamer to Aswan, back to Cairo, then to Israel and back to Germany. He developed a bad cold, necessitating antibiotic treatment just before departure. He reactivated his osteoarthritis while tramping through the ruins of Egypt. The cold recurred one week after it had ceased, and there was additional activation of the arthritis by walking the streets of Jerusalem. Additional stress was

occasioned by the marked time changes, alternate cold vs. hot, dry weather, and the stress of the middle Eastern situation at that point in time. However, his angina was still improving. In Germany, it was cold and damp, and his cold and arthritis were active. On return to this country, there was an extraordinary exacerbation of the osteoarthritis followed by a rapid downhill progression of his angina pectoris. Because of the success with zinthromycin in reducing CHD complications, he was treated with this drug. The treatment was followed by marked amelioration of his arthritis, but the angina continued to worsen, and he developed angina during sleep, unrelieved by nitroglycerin.

Finally, he was admitted to the hospital. Angiography demonstrated that his coronaries had deteriorated greatly since first examined, even in the presence of perfectly normal lipids: cholesterol of 170 mg%, normal HDL and LDL, and low triglycerides. Fortunately, there was no evidence of myocardial infarction, and he survived and is recovering from quadruple bypass surgery (McCann, unpublished data, 1998).

DISCUSSION

The data presented above indicate that there are many areas in the brain where there is regular periodic physiological release of NO throughout the life span. This probably occurs in the hippocampus, cerebellum and, in particular, in the hypothalamus, in which NO controls most of the hypothalamic peptidergic neurons such as CRH, LHRH, GHRH, somatostatin, oxytocin, and vasopressin, and also activates the release of GABA and inhibits that of norepinephrine and dopamine. It is not definite that this physiological release could ever reach levels that would produce neuronal cell damage; however, Schultz *et al*. (1996) have recently reported that in men, but not women, over 75, there were neurodegenerative changes in the arcuate-median eminence region associated with an increase in neurofibrillary protein. This is the site of the interaction between NO and LHRH neurons.

The fact that injection of moderate amounts of LPS to mimic the effect of bacterial infection induces increased numbers of IL-1α immunoreactive neurons in the region of the thermosensitive neurons in the preoptic hypothalamic region, plus increased IL-β mRNA and iNOS mRNA in the PVN, AN, median eminence, choroid plexus, meninges, and in massive amounts in the anterior pituitary and pineal with consequent release of NO, suggests that toxic amounts of NO could exist in these regions during moderate infections, even though there is no direct involvement of the brain.

Destruction of neurons in the temperature regulating centers and in the paraventricular and arcuate median-eminence region following multiple infections over the life span may be responsible for the reduced febrile response and pituitary hormone secretion in response to infection, respectively. Presumably, LPS acts on receptors in the choroid plexus and after its transport into the CSF on neuronal terminals in the wall of the third ventricle, which induce IL-1 and iNOS mRNA with resultant production of NO at their cell bodies in the PVN. Repeated bouts of infection over the life span of the individual, even without direct CNS involvement, could lead to neuronal cell loss in the hippocampus, because NO is important in memory formation. Cerebellar and cortical dysfunction could also ensue.

The evidence that NO is involved in a number of neurodegenerative diseases, among them Huntington's disease and Parkinsonism, is already impressive. In chronic CNS infections such as AIDS, there would certainly be even greater responses in the aforementioned areas, plus increased iNOS in glia producing large amounts of NO. Indeed, CNS AIDS has led to

Alzheimer-like changes in the brain (Griffin, 1988). Therefore, NO may cause much of the neuropathologic changes in CNS AIDS.

Even more impressive is the induction of iNOS in the pituitary following LPS, which with age could alter the responses of the pituitary to infection. The dramatic induction of iNOS mRNA in the pineal should lead to high concentrations of NO that could result in the death of pineal cells and reduction of melatonin secretion, leading to impaired resistance to free radicals that are normally scavenged by melatonin and acceleration of aging.

In humans, it is already well known, as indicated before, that infections with release of bacterial or viral products, such as LPS, causes the induction of cytokines, which are released and travel through the blood stream. LPS and the released cytokines combine with their receptors on the coronary artery endothelial cells. They induce iNOS in the endothelial cells and in macrophages that might be adherent to or resident within the vessel. The result would be production of 1,000 times more NO than would be released by eNOS. NO would oxidize LDL and cause the production of prostaglandins, leucotrienes that are damaging to the vessel. NO itself would have toxic effects to bring about cell injury and death. There would be generation and enlargement of the atherosclerotic plaques producing a rapid, downhill course of the coronary disease. It is also known, as indicated, that inflammation, as for example in severe osteoarthritis (Amin *et al*., 1996), as in the patient in question, causes the induction of cytokines that would circulate to the coronaries and also induce iNOS.

Finally, in rats, it has been shown that stress itself, even without tissue damage, can cause the induction of nNOS in the same areas that have been studied in the case of iNOS induction by LPS (Kishomoto *et al*., 1996), and therefore, presumably also in the vascular system, although this has not yet been studied. Whether or not psychological or physical stress can cause induction of NOS in the coronary endothelium of humans has not been determined, but it is a well-known fact that stress predisposes to CHD and myocardial infarction. In fact, executives who fired their employees were twice as prone to have a heart attack around that time as on ordinary days. Even if stress-induced heart attacks are not directly caused by NO, they may be caused by increased vasoconstriction associated with the stress-induced withdrawal of NOergic vasodilator tone or augmented adrenergic vasoconstrictor tone. Further studies are needed to determine which of these possibilities is correct in this case; however, the evidence is rapidly mounting that the final mediator of the effects of inflammation and infection on the coronaries is the massive amounts of NO released by iNOS that cause a rapid progression of CHD, leading to the development of angina pectoris and finally myocardial infarction. It appears that the triad of stress, infection, and inflammation are the main factors that precipitate rapid deterioration of the coronaries mediated in large part by NO. NO can cause CHD even in the presence of a normal lipid profile.

Other organ systems and therapeutic implications

Space does not allow development of this hypothesis in other organ systems, but quite clearly it is probable that the aging effects of sunlight on the skin are also mediated by the inflammatory response and production of massive amounts of NO. Similarly, infections could mediate aging changes in the gonads, digestive system, and every other organ of the body. The reduction in incidence of infectious disease via public health and sanitation measures, from immunization and from their successful treatment with chemo- and antibiotic therapy, may account for the increased longevity in developed countries by reducing exposure to toxic concentrations of NO.

In conclusion, although much work needs to be done, it is already known that treatment of

patients with antioxidants, vitamin C and E, which would reduce the toxic effects of NO, is of value in patients with CHD. This is probably the mechanism of their protective effects against CHD. Melatonin, as indicated above, is a naturally occurring antioxidant that has been shown to increase the life span of mice. Finally, compounds that inhibit the production of NO directly, such as inhibitors of NOS or agents that inhibit the production of NOS, such as corticoids, the tetracyclines, and α-MSH may prove useful in slowing the aging process. Aspirin blocks cyclooxygenase I, thereby reducing production and toxicity of prostanoids produced by NO, accounting for its protective effect in CHD. New cyclooxygenase II inhibitors should be even more effective than aspirin. It may even be beneficial to decrease the production of NO in infections by the use of inhibitors of NOS, such as NAME, or if they can be developed, specifically of iNOS.

Acknowledgments—This work was supported by NIH Grants DK43900 and MH51853. We would like to thank Judy Scott and Jill Lucius for their excellent secretarial assistance.

REFERENCES

AGUILA, M.C. Growth hormone-releasing factor increases somatostatin release and mRNA levels in the rat periventricular nucleus via nitric oxide by activation of guanylate cyclase. *Proc. Natl. Acad. Sci. USA* **91,** 782–786, 1994.

AMIN, A.R., ATTUR, M.G., THAKKER, G.D., PATEL, P.D., VYAS, P.R., PATEL, R.N., PATEL, I.R., and ABRAMSON, S.B. A novel mechanism of action of tetracyclines: Effects on nitric oxide synthases. *Proc. Natl. Acad. Sci. USA* **93,** 14014–14019, 1996.

BANKS, W.A., KASTIN, A.J., and BROADWELL, R.D. Passage of cytokines across the blood–brain barrier. *Neuroimmunomodulation* **2,** 241–248, 1995.

BREDT, D.S., HWANG, P.M., and SNYDER, S.H. Localization of nitric oxide synthase I indicating a neural role for nitric oxide. *Nature* **347,** 768–770, 1990.

CANTEROS, G., RETTORI, V., FRANCHI, A., GENARO, A., CEBRAL, E., FALETTI, A., GIMENO, M., and McCann, S.M. Ethanol inhibits luteinizing hormone-releasing hormone (LHRH) secretion by blocking the response of LHRH neuronal terminals to nitric oxide. *Proc. Natl. Acad. Sci. USA* **92,** 3416–3420, 1995.

DANESH, J., COLLINS, R., and PETO, R. Chronic infections and coronary heart disease: Is there a link? *Lancet* **350,** 430–436, 1997.

DUVILANSKI, B.H., ZAMBRUNO, C., SEILICOVICH, A., PISERA, D., LASAGA, M., DEL, C., DIAZ, M., BELOVA, N., RETTORI, V., and McCann, S.M. Role of nitric oxide in control of prolactin release by the adenohypophysis. *Proc. Natl. Acad. Sci. USA* **92,** 170–174, 1995.

FABRICANT, C.G., FABRICANT, J., LITRENTA, M.M. and MINICK, C.R. Virus-induced atherosclerosis. *J. Exp. Med.* **148,** 335–340, 1978.

GARTHWAITE, J., CHARLES, S.L., and CHESS-WILLIAMS, R. Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intercellular messenger in the brain. *Nature* **336,** 385–388, 1988.

GRIFFIN, S. *Neuroimmunomodulation Symposium*. Am. Assoc. Immunol. Mtg., San Francisco, April, 1998.

GUPTA, S., LEATHAM, E.W., CARRINGTON, D., MENDALL, M.A., KASKI, J.C., and CAMM, A.J. Elevated *Chlamydia pneumoniae* antibodies, cardiovascular events, and azithromycin in the male survivors of myocardial infarction. *Circulation* **96,** 404–407, 1997.

GURFINKEL, E., BOZOVICH, G., DAROCA, A., BECK, E., and MAUTNER, B., for the ROXIS Study Group. Randomised trial of roxithromycin in non-Q-wave coronary syndromes: ROXIS pilot study. *Lancet* **350,** 404–407, 1997.

KAMAT, A., YU, W.H., RETTORI, V., and McCann, S.M. Glutamic acid stimulated luteinizing-hormone releasing hormone release is mediated by alpha adrenergic stimulation of nitric oxide release. *Brain Res. Bull.* **37,** 233–235, 1995.

KARANTH, S., LYSON, K., and McCann, S.M. Role of nitric oxide in interleukin 2-induced corticotropin-releasing factor release from incubated hypothalami. *Proc. Natl. Acad. Sci. USA* **90,** 3383–3387, 1993.

KIRKWOOD, T.B.L. and KOWALD, A. Network theory of aging. *Exp. Gerontol.* **32,** 395–399, 1997.

KISHIMOTO, J., TSUCHIYA, T., EMSON, P.C., and NAKAYAMA, Y. Immobilization-induced stress activates neuronal nitric oxide synthase (nNOS) mRNA and protein in hypothalamic–pituitary–adrenal axis in rats. *Brain Res.* **720,** 159–171, 1996.

KNOLL, J. Sexual performance and longevity. *Exp. Gerontol.* **32,** 539–552, 1997.

MANI, S.K., ALLEN, J.M.C., RETTORI, V., O'MALLEY, B.W., CLARK, J.H., and McCann, S.M. Nitric oxide mediates sexual behavior in female rats by stimulating LHRH release. *Proc. Natl. Acad. Sci. USA* **91,** 6468–6472, 1994.

McCANN, S.M. The nitric oxide hypothesis of brain aging. *Exp. Gerontol.* **32,** 431–440, 1997.

McCANN, S.M. and RETTORI, V. The role of nitric oxide in reproduction. *Proc. Soc. Exp. Biol. Med.* **211,** 7–15, 1996.

McDONALD, L.J. and MURAD, F. Nitric oxide and cyclic GMP signaling. *Proc. Soc. Exp. Biol. Med.* **211,** 1–6, 1996.

PALMER, R.M.J., FERRIGE, A.G., and MONCADA, S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* **327,** 524–526, 1987.

PIERPAOLI, W., BULIAN, D., DALL'ARA, A., MARCHETTI, B., GALLO, F., MORALE, M.C., TIROLO, C., and TESTA, N. Circadian melatonin and young-to-old pineal grafting postpone aging and maintain juvenile conditions of reproductive functions in mice and rats. *Exp. Gerontol.* **32,** 587–602, 1997.

PRZEDBORSKI, S., JACKSON-LEWIS, V., YOKOYAMA, R., SHIBATA, T., DAWSON, V.L., and DAWSON, T.M. Role of neuronal NO in 1-methyl-4-phenyl-1,2, 3, 4-tetrahydopyridine (MPTP)-induced dopaminergic toxicity. *Proc. Natl. Acad. Sci. USA* **93,** 4565–4571, 1996.

RETTORI, V., GIMENO, M., LYSON, K., and McCann, S.M. Nitric oxide mediates norepinephrine-induced prostaglandin E_2 release from the hypothalamus. *Proc. Natl. Acad. Sci. USA* **89,** 11543–11546, 1992.

RETTORI, V., BELOVA, N., DEES, W.L., NYBERG, C.L., GIMENO, M., and McCann, S.M. Role of nitric oxide in the control of luteinizing hormone-releasing hormone release in vivo and in vitro. *Proc. Natl. Acad. Sci. USA* **90,** 10130–10134, 1993.

RETTORI, V., BELOVA, N., GIMENO, M., and McCann, S.M. Inhibition of nitric oxide synthase in the hypothalamus blocks the increase in plasma prolactin induced by intraventricular injection of interleukin-1α in the rat. *Neuroimmunomodulation* **1,** 116–120, 1994a.

RETTORI, V., BELOVA, N., YU, W.H., GIMENO, M., and McCann, S.M. Role of nitric oxide in control of growth hormone release in the rat. *Neuroimmunomodulation* **1,** 195–200, 1994b.

RETTORI, V., CANTEROS, G., RENOSO, R., GIMENO, M., and McCann, S.M. Oxytocin stimulates the release of luteinizing hormone-releasing hormone from medial basal hypothalamic explants by releasing nitric oxide. *Proc. Natl. Acad. Sci. USA* **94,** 2741–2744, 1997.

RETTORI, V., DEES, W.L., HINEY, J.K., LYSON, K., and McCann, S.M. An interleukin-1α-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, 1994c.

SCHULTZ, BRAAK, H., and BRAAK, E. A sex difference in neurodegeneration of the human hypothalamus. Abstract presented at the 3rd International Symposium on Neurobiology and Neuroendocrinology of Aging, Bregnez, Austria, 1997.

SEILICOVICH, A., DUVILANSKI, B.H., PISERA, D., THIES, S., GIMENO, M., RETTORI, V., and McCann, S.M. Nitric oxide inhibits hypothalamic luteinizing hormone-releasing hormone release by releasing γ-aminobutyric acid. *Proc. Natl. Acad. Sci. USA* **92,** 3421–3424, 1995a.

SEILICOVICH, A., LASAGA, M., BEFUMO, M., DUVILANSKI, B.H., DEL, C., DIAS, M., RETTORI, V., and McCann, S.M. Nitric oxide inhibits the release of norepinephrine and dopamine from the medial basal hypothalamus of the rat. *Proc. Natl. Acad. Sci. USA* **92,** 11299–11302, 1995b.

WONG, M.L., RETTORI, V., AL-SHEKHELEE, A., BONGIORNO, P.B., CANTEROS, G., McCANN, S.M., GOLD, P.W., and LICINIO, J. Inducible nitric oxide synthase gene expression in the brain during systemic inflammation. *Nat. Med.* **2,** 581–584, 1996.

XIAO-JIANG, L., SHARP, A.H., SHI-HUA, L., DAWSON, T.M., SNYDER, S.H., and ROSS, C.A. Huntington-associated protein (HAPI): Discrete neuronal localizations in the brain resemble those of neuronal nitric oxide synthase. *Proc. Natl. Acad. Sci. USA* **93,** 4839–4844, 1996.

YU, W.H., KIMURA, M., WALCZEWSKA, A., PORTER, J.C., and McCANN, S.M. Adenosine acts by A_1 receptors to stimulate release of prolactin from anterior-pituitaries in vitro. *Proc. Natl. Acad. Sci. USA* **95,** 7795–7798, 1998.

YU, W.H., WALCZEWSKA, A., KARANTH, S., and McCANN, S.M. Nitric oxide mediates leptin-induced luteinizing hormone-releasing hormone (LHRH) and LHRH and leptin-induced LH release from the pituitary gland. *Endocrinology* **138,** 5055–5058, 1997.