Role of nitric oxide in the regulation of cardiovascular autonomic control

Saqib CHOWDHARY and Jonathan N. TOWNEND
University of Birmingham Department of Cardiovascular Medicine, Queen Elizabeth Hospital, Birmingham B15 2TH, U.K.

ABSTRACT

Alteration in function of the cardiac autonomic nervous system has proved to be a powerful predictor of cardiac death or serious arrhythmia in patients with cardiac disease, yet little is known about the mechanisms by which this system is regulated. Recent evidence suggests that the gaseous molecule nitric oxide (NO) may act as an important mediator in this pathway. Histochemical staining techniques have identified neuronal populations that contain NO synthase within medullary cardio-regulatory sites and their peripheral autonomic pathways. Drugs that modulate the NO pathway (administered both systemically and into the central nervous system) cause changes in pre- and post-ganglionic sympathetic nerve activity that imply that NO serves to inhibit central sympathetic outflow. There is also evidence that NO may attenuate cardiovascular end-organ responses to sympathetic stimulation. Studies suggest that NO modulates cardiac vagal control, increasing the activity of central vagal motoneurons and, more contentiously, contributing to the bradycardic effects of vagal stimulation. NO also modulates so-called ‘indirect’ vagal inhibition of sympathetic cardiac responses. Additionally, central attenuation of baroreflex-mediated vagal control has been described. There is relatively little information available on the importance of NO in the regulation of human cardiovascular autonomic control. Further well-controlled studies are required.

BACKGROUND

Importance of cardiac autonomic control

Abnormalities of sympathetic and parasympathetic cardiac control are present in cardiovascular disease states, such as cardiac failure [1–4] and myocardial infarction [5]. Recent large studies have demonstrated that, in both conditions, the degree of autonomic dysfunction is a strong and independent indicator of prognosis [6,7]. This has been widely interpreted as evidence that autonomic dysfunction in cardiovascular disease is not merely a reflection of cardiac dysfunction, but directly and adversely influences the clinical course of the disease. The mechanisms by which impaired cardiac autonomic control may exert adverse effects are not fully understood, but an increased vulnerability to ventricular fibrillation appears likely. Animal evidence shows that high levels of sympathetic activity increase the vulnerability of ischaemic myocardium to lethal arrhythmias [8], while high cardiac vagal tone exerts a protective anti-arrhythmic effect [8,9]. Despite the increasing evidence for the importance of abnormalities of cardiac autonomic function in heart disease, the mechanisms by which it is controlled remain poorly understood. Recent studies suggest that endogenous nitric oxide (NO) may play an important role in the control of this regulatory system.

NO and the nervous system

Although recognized primarily as a mediator of the endothelial control of vascular smooth muscle, NO is an...
important mechanism of intercellular signalling in numerous tissues, including the nervous system. NO acts via the second messenger cGMP. It is synthesized from its precursor l-arginine by the enzyme NO synthase (NOS), which occurs in three distinct isoforms. These consist of the constitutive calcium-dependent enzymes eNOS (originally identified in vascular endothelium, but also present in platelets, myocardium and endocardium) and nNOS (present in neuronal tissue), and the calcium-independent, cytokine-inducible isoform iNOS (expressed in macrophages and other tissues during immune stimulation) (for reviews, see [10,11]). Localization of neuronal populations that possess nNOS has been achieved by histochemical staining using NADPH-diaphorase and immunohistochemistry [12]. NOS activity has been demonstrated in central and peripheral sites throughout the cardiac autonomic nervous system, including the receptors and effectors of the baroreflex pathway (Table 1).

The initial evidence for a physiological role for NO in nervous transmission came from the in vitro demonstration of NO release in response to N-methyl-D-aspartate receptor stimulation in rat cerebellar cells by glutamate [32]. Neuronal NO may also be released directly by nerve endings into the vascular adventitia, resulting in the relaxation of smooth muscle. Such ‘nitrergic’ nerves (previously termed non-adrenergic, non-cholinergic) may act on vascular smooth muscle within the cerebral circulation [27,33,34], the temporal and mesenteric arteries [35–37], as well as the corpus cavernosum [38]. Although some investigators have documented NOS-immunoreactive fibres in peripheral arteries [28], this has not been a uniform finding [27], and the role of nitrergic nerves in the control of systemic arterial tone remains uncertain. However, evidence is accumulating in support of a role for NO in the modulation of both sympathetic and parasympathetic control of the cardiovascular system.

Table 1  Distribution of NOS within the cardiac autonomic nervous system

<table>
<thead>
<tr>
<th>Autonomic site</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Central</td>
<td></td>
</tr>
<tr>
<td>Nucleus tractus solitarius (NTS)</td>
<td>[13–17]</td>
</tr>
<tr>
<td>Rostral ventrolateral medulla (RVLM)</td>
<td>[14,17,18]</td>
</tr>
<tr>
<td>Caudal ventrolateral medulla</td>
<td>[14,17]</td>
</tr>
<tr>
<td>Dorsal motor nucleus of the vagus</td>
<td>[15,17,19,20]</td>
</tr>
<tr>
<td>Nucleus ambiguous</td>
<td>[13,15–17]</td>
</tr>
<tr>
<td>Peripheral</td>
<td></td>
</tr>
<tr>
<td>Carotid sinus and body</td>
<td>[21,22]</td>
</tr>
<tr>
<td>Cardiac vagal neurons</td>
<td>[17,23]</td>
</tr>
<tr>
<td>Pre-ganglionic sympathetic neurons</td>
<td>[24,25]</td>
</tr>
<tr>
<td>Sympathetic ganglia</td>
<td>[25,26]</td>
</tr>
<tr>
<td>Innervation of sino-atrial and atrio-ventricular nodes</td>
<td>[23]</td>
</tr>
<tr>
<td>Perivascular nerves</td>
<td>[26–28]</td>
</tr>
<tr>
<td>Intrinsic cardiac neurons</td>
<td>[29–31]</td>
</tr>
</tbody>
</table>

**NO AND SYMPATHETIC CARDIOVASCULAR CONTROL**

Several approaches have been employed in order to determine the role of NO in the control of sympathetic nervous activity. These can be categorized as follows. (1) Determination of the effects of systemic inhibition of NOS on measures of sympathetic nervous activity. (2) Investigation of the effects of NO within the central nervous system (CNS) on sympathetic nervous activity. (3) Investigation of the effect of NO on the reflex control of sympathetic nerve activity. (4) Determination of the effects of NO on the cardiac and vascular responses to sympathetic stimulation. The evidence arising from each series of studies will be discussed in turn.

**Determination of the effects of systemic inhibition of NOS on measures of sympathetic nervous activity**

l-Arginine analogues, such as Nω-monomethyl-l-arginine (l-NMMA), Nω-nitro-l-arginine (l-NNA) and its methyl ester (l-NAME), are non-isomeric-specific inhibitors of NOS which have proved to be valuable tools in the investigation of the physiological roles of NO. These agents differ in their relative potencies and specificities for the different isoforms of NOS. l-NNA shows some selectivity for nNOS, for which it is an order of magnitude more potent than l-NMMA; l-NAME and l-NNA show reduced potency for iNOS compared with the constitutive isoforms, whereas l-NMMA shows equal potency for all isoforms. Recently non-amino-acid compounds such as 7-nitroindazole have been developed that selectively inhibit nNOS (for review, see [39]).

Non-specific inhibition of NOS by the systemic administration of l-arginine analogues results in a vasoconstrictor response and an increase in mean arterial blood pressure in both animals [40–45] and man [46–51]. This has been explained by the removal of tonic vasodilatation resulting from endothelial NO production [10]. However, the pressor response to systemic NOS inhibition appears to result partly from activation of the sympathetic nervous system, suggesting that neuronal NO may exert a tonic inhibitory influence on sympathetic nervous activity. The evidence in support of this can be summarized as follows.

1. Acute pharmacological ganglion blockade caused a significantly greater drop in blood pressure during chronic NOS inhibition with l-NAME than in control animals [45,52,53]. Acute β-blockade also caused greater falls in heart rate and blood pressure in the l-NAME-treated animals than in controls [45].
induced by acute L-NAME administration in baroreceptor-denervated cats was reversed by suppression of central sympathetic outflow effected through cooling of the rostral ventrolateral medulla (RVLM) [54]. (3) The hypertensive response to chronic L-NAME ingestion was greatly attenuated in previously sympathectomized rats, while the vascular responses to endothelial NO-mediated stimuli remained intact [52]. (4) Directly recorded renal sympathetic nerve activity (RSNA) showed a biphasic response to systemic L-NMMA in anaesthetized rats [55]. There was an initial decrease in RSNA (ascribed to baroreflex-mediated sympathetic inhibition), followed by a sustained increase, despite the persistent rise in blood pressure. Surgical baroreceptor deafferentation potentiated the increase in blood pressure, abolishing the initial reduction and enhancing the late increase in RSNA. Both effects were abolished by cervical spinal transection. Pretreatment with L-arginine late increase in RSNA. Both effects were abolished by ure, abolishing the initial reduction and enhancing the deafferentation potentiated the increase in blood pressure. Surgical baroreceptor deafferentation potentiated the increase in blood pressure, abolishing the initial reduction and enhancing the late increase in RSNA. Both effects were abolished by cervical spinal transection. Pretreatment with L-arginine prevented the late increase in RSNA, suggesting that the potentiating effects of L-NMMA on sympathetic activity were due specifically to inhibition of NO synthesis. (5) In anaesthetized rabbits, stimulation of endogenous NO synthesis with intravenous L-arginine caused a decrease in cerebral sympathetic nerve activity and RSNA, despite a fall in blood pressure [56]. (6) Directly recorded cardiac sympathetic nerve activity showed a 15% increase on intravenous L-NMMA treatment in baroreceptor-denervated rabbits, an effect that was reversed by subsequent L-arginine administration [57].

There are contradictory findings, including a lack of change in RSNA in response to L-NNA or selective nNOS inhibition in conscious intact and sinoaortic-denervated rabbits [58,59], that cannot be readily explained by species or methodological differences. However, the weight of evidence does appear to suggest a significant inhibitory role for NO in the control of sympathetic nerve activity.

**Investigation of the effects of NO within the CNS on sympathetic nervous activity**

Evidence for a central site for inhibition of sympathetic activity by NO has come from studies of the effects of administering drugs that act via the NO pathway directly into the CNS. In anaesthetized rats, intracerebroventricular injection of L-NAME produced increases in blood pressure and heart rate that were blocked by atenolol, but not by atropine [60]. Rises in blood pressure and RSNA also resulted from intracerebroventricular [61] and intracisternal [62,63] administration of NOS inhibitors in rabbits and rats, an effect that was abolished by cervical spinal cord transection [62]. Similarly, stimulation of NO synthesis within the CNS by intracerebroventricular injection of L-arginine caused reductions in arterial pressure and in directly recorded abdominal sympathetic nerve activity in rats [64].

Studies employing the microinjection of agents that affect the NO pathway into specific medullary nuclei have allowed localization of possible CNS sites of nitrergic modulation of sympathetic activity. The nucleus tractus solitarius (NTS) is the first relay site for baroreceptor afferent fibres. In anaesthetized rabbits, the administration of L-NMMA into the NTS resulted in increases in arterial pressure and RSNA in both baroreceptor-intact [65,66] and baroreceptor-denervated [65] animals. Conversely, stimulation of endogenous NO activity by direct injection of L-arginine into the NTS caused a decrease in both arterial pressure and sympathetic activity [66]. The RVLM is the final site of sympathetic processing in the brainstem, and also appears to contain neurons susceptible to modulation by NO. Here, injection of L-NNA caused increases in blood pressure and preganglionic sympathetic nerve activity in baroreceptor-denervated cats [67], as well as in RSNA in baroreceptor-intact rats [66]. Injection of L-arginine and the NO donor sodium nitroprusside into the RVLM produced decreases in mean arterial pressure and RSNA in the rat [66], and also in the cat [68]. This last study also showed that injection of NO donors into the caudal ventrolateral medulla caused an increase in RSNA, suggesting that NO attenuates activity at this inhibitory site [68] (Figure 1). Since the effects of NO on these two medullary sites would result in antagonistic influences on sympathetic nerve activity, this role of NO generation as a mechanism constraining central sympathetic outflow would seem to be explained by a dominant action at the RVLM.

In summary, the findings following central administration of modulators of the NO pathway, either within the cerebral ventricles or into specific medullary sites of autonomic processing, consistently support the concept of tonic restraint of central sympathetic outflow by NO. This role of NO generation as a mechanism constraining a rise in sympathetic nerve activity is indirectly supported by the demonstration of an increase in NO-producing neurons within the brain following experimentally induced stress. This was observed at a number of sites of autonomic processing within the CNS of conscious rats, including the NTS and the ventrolateral medulla [69].

**Investigation of the effects of NO on the reflex control of sympathetic nerve activity**

While studies have demonstrated the ability of NO to modulate basal sympathetic activity, nitrergic modulation of the baroreflex control of sympathetic activity remains controversial. There is evidence that NO attenuates baroreceptor–RSNA gain in conscious animals. Liu et al. [58] found that the gain of the baroreceptor–RSNA reflex was increased by acute systemic NOS inhibition in the conscious rabbit. Similar findings were obtained in conscious rats [70]. A central effect was suggested by Matsumura et al. [61], who showed that the admin-
Intracardiac neurons (ICNs) are formed from efferent post-ganglionic autonomic and afferent cardiac fibres, but also consist of local circuit neurons. These NOS-containing neurons exhibit increased activity with NO donors, predominantly resulting in augmentation of cardiac responses (although a small subpopulation are inhibitory) [30]. The overall effect on baroreceptor–RSNA gain of NO has been reported as being inhibitory or neutral. The functional effect of NO on vagal baroreceptor–heart-rate gain has been reported as being inhibitory.

Administration of NOS inhibitors directly into the cerebral ventricles of conscious rabbits resulted in an increase in baroreceptor–RSNA gain, while NO donors caused a fall. However, Liu et al. [58] felt that central NO was unlikely to modulate this reflex, as there was no significant change in RSNA responses to aortic afferent nerve stimulation in anaesthetized rabbits. Although this part of their work is open to criticism with respect to the possible confounding effects of anaesthesia, further studies have shown that selective blockade of nNOS is
without effect on baroreflex control of RSNA in either conscious or anaesthetized rabbits [59]. Other experiments in anaesthetized rabbits also revealed no evidence for significant modulation of the baroreflex control of either pre-ganglionic (cervical) or post-ganglionic (renal) sympathetic nerve activity by endogenous NO [56].

Such inconsistencies can only partly be explained by differences between conscious and anaesthetized animal models, systemic versus central NO modulation, methodological differences in assessment of baroreflex gain, or species variations. However, recent evidence does appear to indicate a role for NO in more complex dynamic indices of the baroreflex control of sympathetic activity. Hironaga et al. [63] showed that sustained pressure applied to the vasoconstrictor effects of sympathetic nerves. In the intact rat heart, NOS inhibitors in the vagotomized rabbit [76] and ferret [77] indicates that neurally produced NO may also contribute significantly to this effect in vivo. The mechanisms by which NO modulates adrenergic control are not fully understood. It has been shown that NO inhibits the voltage-sensitive L-type calcium channel, thereby reducing the inward calcium current (\(I_{\text{ca}}\)) that occurs in response to adrenergic stimulation [78–80]. This reduces the duration of the cardiac action potential and the availability of ‘trigger calcium’, which is central to excitation–contraction coupling.

A further mechanism of NO-mediated inhibitory regulation of cardiac sympathetic responses may be presynaptic inhibition of noradrenaline release from sympathetic nerves. In the intact rat heart, NOS inhibitors enhanced, while NO donors reduced, cardiac noradrenaline release during sympathetic nerve stimulation [31]. The investigators concluded that neuronal NO was responsible for this effect, as the influence of L-NAME on evoked noradrenaline release was not inhibited by chemical removal of the endothelium. Further, using Western blotting and immunofluorescence, they demonstrated numerous nNOS-negative intrinsic neurons in the rat heart (for further refs., see Table 1). Regulation of cardiac noradrenaline release has also been documented in anaesthetized autonomically denervated dogs, in which levels of cardiac noradrenaline spillover evoked by sympathetic stimulation were decreased by intra-pericardial L-arginine [81].

Taken together, the in vitro and in vivo evidence strongly suggests that cardiac responses to adrenergic stimulation, both inotropic and chronotropic, are under an inhibitory nitrergic influence. The mechanisms mediating this effect are likely to include modulation of \(I_{\text{ca}}\) and presynaptic inhibition of noradrenaline release at cardiac sympathetic nerve endings.

Vascular tissue
Inhibition by NO of peripheral vasoconstrictor responses to sympathetic nerve activity has been documented, and may occur via both pre- and post-synaptic mechanisms. In vitro, NOS inhibition enhanced vasoconstrictor responses to both noradrenaline and sympathetic nerve stimulation [82], while NO resulted in attenuation of the constrictor effects of sympathetic
nerve stimulation [83]. However, Vo et al. [82] found that the responses to a variety of vasoconstrictors were also inhibited by l-NNA, and suggested that increased endothelial NO synthesis (due to increased shear stress [84]) during vasoconstriction was acting non-specifically as a functional antagonist by opposing the effect of adrenergic vasoconstriction.

A specific post-junctional interaction between NO and noradrenaline was suggested by the finding that l-NAME enhanced vasoconstrictor responses to noradrenaline to a much greater extent than its effect on the responses to angiotensin II [54]. Similarly, in isolated human saphenous vein rings, the contractile responses to noradrenaline were augmented during both NOS inhibition and endothelial denudation, whereas the response to potassium was not affected. Thus the authors suggested that NO produced by endothelial cells inhibits sympathetic contractile responses, ‘probably by acting at a post-junctional level’ [85].

In addition to this post-synaptic action, Greenberg et al. [86,87] found evidence for an additional presynaptic inhibitory influence of NO. Exogenous NO reduced the efflux of radiolabelled noradrenaline in canine pulmonary vessels [86] and mesenteric artery [87] during transmural nerve stimulation.

Available evidence therefore indicates that NO inhibits peripheral vascular responses to sympathetic vasoconstriction not only by acting directly as a vasodilator, but also by inhibiting sympathetic noradrenergic transmission at the pre- and post-junctional levels.

**NO AND PARASYMPATHETIC CARDIAC CONTROL**

In comparison with its modulation of the sympathetic nervous system, the influence of NO on cardiac vagal control has received little attention. In particular, there is a paucity of data on the effects of NO on central vagal activity. No direct recordings of vagal efferent activity have been made during central or systemic administration of agents that affect the NO pathway. Much of the information regarding central vagal modulation has to be inferred from the effects of NO on the baroreflex control of heart rate. This is possible because the immediate bradycardia (< 10 s) that occurs in response to baroreceptor loading is vagally mediated [88]. For this reason we shall consider data on the effect of NO upon the baroreceptor–heart-rate reflex together with modulation of the cardiac vagus.

More comprehensive study has been made of the influence of NO upon cardiac responses to vagal efferent activity. Here NO appears to modulate both the effects of muscarinic activation (‘direct’ vagal effect) and the muscarinic antagonism of sympathetic cardiac control (‘indirect’ vagal effect).

**Afferent baroreceptor activity**

The effect of NO on directly recorded baroreceptor afferent activity was investigated in the isolated rabbit carotid sinus at constant pressure by Matsuda et al. [89]. Dose-dependent inhibition of carotid sinus nerve firing at fixed pressure, and a shift to the right in the pressure–activity curve, were observed on injection of either a saturated solution of NO or the NO donor S-nitroscysteine. There was no evidence of vascular relaxation, suggesting a direct effect on neuronal excitability. Others have also shown significant attenuation of carotid sinus nerve activity with high doses of NO donors, but found no effect when using doses presumed to supply NO at physiological concentrations [90]. In addition, neither scavenging of endogenous NO by haemoglobin nor NOS inhibition by l-NAME altered baroreceptor activity in the experiments of Matsuda et al. [89]. Thus the physiological role of endogenous NO in modulating baroreceptor firing remains in doubt, although a possible role is suggested in disease states accompanied by high levels of NO production.

**Central modulation of vagal activity and the baroreceptor–heart-rate reflex**

The sensitivity or ‘gain’ of the baroreceptor–heart-rate reflex can be quantified by measuring the increase in R–R interval (interval between successive R waves on an ECG) per unit rise in arterial pressure. Acute administration of NOS inhibitors appears to increase the gain of the baroreceptor–heart-rate reflex. In conscious rats, intravenous administration of l-NAME resulted in an increase in the gain of the baroreceptor–heart-rate reflex, an effect that was reversed by infusion of sodium nitroprusside [91], implying that endogenous NO attenuates reflex vagal cardiac control. In a detailed study of the effects of NO on the baroreceptor control of heart rate, Liu et al. [58] showed that, in the conscious rabbit, baroreceptor–heart-rate gain was also increased by l-NNA, and that this effect was reversed by l-arginine. Atropine, but not metoprolol, prevented this increase in baroreflex sensitivity, suggesting a predominantly vagal mechanism. A central rather than peripheral effect was thought to be the mode of action, because whereas l-NNA resulted in an increased bradycardic response to afferent (aortic nerve) stimulation, there was no effect on the heart-rate response to vagal efferent stimulation. The authors concluded that NO produced within the CNS exerts an important influence on the arterial baroreflex control of heart rate, reducing the gain of the reflex. The same group recently demonstrated that non-pressor administration of the selective nNOS inhibitor 7-nitroindazole also enhanced the baroreflex control of heart rate [59], suggesting that this action is mediated by nNOS. A central site of modulation was confirmed recently by the demonstration that intracerebroven-
tricular injection of L-NAME caused an increase in baroreceptor–heart-rate gain, whereas administration of NO donors blunted the reflex [61].

There is little evidence that dissents from this position. Although Jimbo et al. [56] showed no change in baroreceptor–heart-rate gain with acute infusions of l-arginine or L-NMMA in anaesthetized rabbits, this result may have been influenced by the anaesthetic agent. Unlike the effects of acute NOS inhibition, the chronic oral administration of L-NAME to rats appeared to reduce baroreceptor–heart-rate gain in the majority of studies [33,92–94]. However, these results are difficult to interpret because of the inhibitory effect of chronic hypertension on baroreceptor–heart-rate gain.

The precise site within the CNS that mediates the inhibitory action of NO on the baroreflex remains unclear. The NTS receives primary baroreceptor afferent fibres and contains neurons with NOS activity (for refs., see Table 1). If the NO-mediated inhibition of the baroreflex occurred within the NTS, it might be expected that NO would show inhibitory influences on neuronal activity there. However, this does not appear to be so. The neurotransmitter L-glutamate causes NO release [32], and when applied to the NTS it mimics the effects of baroreflex activation. Microinjection of L-NMMA into the NTS of rats inhibited the decrease in blood pressure caused by L-glutamate, suggesting that the release of NO by L-glutamate stimulates, rather than inhibits, baroreflex processing within the NTS [95]. Single-unit recording of NTS neurons showed that their activity was reduced by systemic injection of L-NAME at constant blood pressure [96], whereas L-arginine and sodium nitroprusside increased the neuronal discharge rate [97]. Hence it appears that NO increases NTS neuronal activity in response to a given level of baroreceptor afferent stimulation, making it an unlikely site for nitricergic inhibition of the baroreflex. NO also appears to enhance, rather than inhibit, the activity of neurons within the dorsal motor nucleus of the vagus. This has been demonstrated by directly recorded increases in the motoneuron firing rate caused by NO donors and L-arginine in vitro [19]. However, it should be noted that the dorsal motor nucleus of the vagus contains cardiac vagal motoneurons with C-fibre axons, and that activity in such neurons has been shown to be insensitive to modulation by baroreceptor afferents, providing a slow tonic, rather than reflex-mediated, influence on heart rate [98]. Baroreflex-sensitive vagal motoneurons show rapid phasic activity via B-fibre axons and are located primarily within the nucleus ambiguus (for a review, see [98]). The action of NO at this site has not been investigated, but may be critical for understanding the influence of NO on central vagal outflow to the heart. It is possible that NO may have discrepant effects on cardiac vagal outflow, inhibiting baroreflex control (B-fibre-mediated), but increasing tonic activity (C-fibre-mediated). This might explain the apparent inconsistency of NO increasing neuronal activity within the NTS and the dorsal motor nucleus of the vagus, but reducing baroreceptor–heart-rate reflex gain.

Peripheral vagus

Direct vagal effects

Muscarinic receptor stimulation has long been shown to elevate cGMP levels in heart muscle [99]. The possibility that NO may be an intermediary in the generation of this cGMP response is supported by the finding that the bradycardia resulting from muscarinic stimulation is in part dependent upon the production of NO. In vitro, both L-NMMA and Methylene Blue blocked the negative chronotropic effect of muscarinic stimulation in spontaneously beating neonatal rat myocytes [72]. This suggests that a constitutive isoform of NOS is present in cardiac myocytes, modifying their response to muscarinic receptor stimulation. The same group has shown that the endothelial isoform, eNOS, is indeed constitutively expressed in cardiac myocytes at the transcript and protein levels [100].

In vivo studies have also yielded support for a physiological mediation of muscarinic transmission by NO. Conlon et al. [101] showed that, in the anaesthetized, β-blocked ferret, L-NAME significantly attenuated the bradycardic response to efferent vagus nerve stimulation. Species variations may exist with respect to this effect of endogenous NO, as similar well conducted experiments in the rabbit showed no modulation of the chronotropic response to efferent vagal stimulation during infusion of L-NNA or a specific nNOS inhibitor [58,76]. However, enhancement of the bradycardic response to vagal stimulation in rabbits has been demonstrated with exogenous NO donors [102]. The use of L-NAME in the experiments on ferrets may be criticized in the light of a report that alkyl esters such as L-NAME (although not nitroarginine compounds such as L-NMMA or L-NNA) exhibit activity as competitive antagonists at muscarinic receptors, an effect not reversed by L-arginine [103]. However, Conlon et al. [101] showed that L-arginine inhibited the effects of L-NAME on heart rate during vagal stimulation, making it unlikely that the effects were due to direct muscarinic antagonism.

Thus in vitro and in vivo evidence suggests that NO augments the chronotropic response to parasympathetic stimulation in at least some mammalian species.

Indirect vagal effects

An important component of the action of the parasympathetic nervous system on the heart is thought to be inhibition of β-adrenergic responses; this has been termed ‘indirect’ cardiac vagal activity or ‘accentuated antagonism’. Until recently, this interaction of sympathetic and parasympathetic pathways was thought to be due to
Figure 2  Possible mechanisms of NO-dependent indirect vagal control of the L-type calcium channel

Regulation of $I_{Ca}$ directly influences contractility and heart rate via its control of cardiac action potential duration and the availability of trigger calcium in cardiac myocytes and sino-atrial node cells. Sympathetic augmentation of this current is effectuated through cAMP-dependent phosphorylation of the L-type calcium channel ($L$-Ca\(^{2+}\) channel). Muscarinic receptor (MR) stimulation is linked to this control system via an inhibitory G-protein ($G_i$), decreasing adenylate cyclase activity. Muscarinic agonist binding may also stimulate NO-dependent cGMP production. A cGMP-stimulated cAMP phosphodiesterase (PDE) [109] may then induce hydrolysis of cAMP. Activation of an endogenous cGMP-dependent protein kinase (PKG) is another possible pathway, leading to direct inhibition of $I_{Ca}$ [though with a requirement for coexistent cAMP-dependent protein kinase (PKA) activity] [110]. Additional abbreviations: ACh, acetylcholine; NA, noradrenaline; $\beta$, $\beta$-adrenergic receptor; $G_s$, stimulatory G-protein. + and — denote stimulation and inhibition respectively.

Inhibition of adenylate cyclase via an inhibitory G-protein activated by muscarinic agonist binding. There is now evidence that this action may also be mediated by the NO/cGMP pathway. In adult rat ventricular myocytes, inhibition of the NO/cGMP pathway using either Methylene Blue or L-NMMA abolished the muscarinic attenuation of isoprenaline-induced increases in contractility [100]. However, in frog ventricular myocytes, L-NMMA did not affect the acetylcholine-induced attenuation of isoprenaline contractile responses [104]. One explanation for this discrepancy may lie in the report that NOS is absent from frog ventricular myocytes, although it is present in the endocardial endothelium [104]. Indeed, in intact frog hearts, L-NMMA did reduce the inhibitory effect of acetylcholine on heart rate during adrenergic stimulation [105]. NO may also facilitate indirect vagal action by augmenting the rate of decrease of the heart rate in response to vagal stimulation during adrenergic drive [106].

There is also in vivo evidence for a role for NO in effecting indirect vagal activity. In closed-chest dogs, intracoronary L-NMMA attenuated the inhibitory action of vagal stimulation on the inotropic responses to dobutamine [107]. In a further study, L-NMMA was infused directly into the sino-atrial and atrio-ventricular nodal arteries in dogs, and the effects on sinus discharge rate and atrio-ventricular node conduction time were determined. L-NMMA inhibited both the effects of vagal stimulation alone (direct activity) and the vagal inhibition of sympathetically mediated effects (indirect activity) [108].

The mechanism by which NO generation influences indirect cardiac vagal activity is not fully defined, but may be due to cGMP-mediated inhibition of $I_{Ca}$ via a number of possible pathways (Figure 2). This is supported by in vitro studies on isolated rabbit sino-atrial node cells showing that NOS inhibitors prevented cholinergic inhibition of the sympathetically augmented $I_{Ca}$ [111,112]. Further evidence for a role for L-NMMA in the modulation by NO of indirect vagal control of heart rate was provided in studies on isolated guinea-pig atria. L-NMMA slowed the rate of heart-rate decrease with vagal stimulation, an effect duplicated by nifedipine, an inhibitor of the L-type calcium channel [106]. In isolated ventricular myocytes, L-NMMA abolished the cholinergic attenuation of adrenergically augmented $I_{Ca}$ [79,100], suggesting that this mechanism may influence indirect vagal control of contractile function as well as the chronotropic response.

Early studies on human tissue have been equivocal. In atrial myocytes, inhibition of cGMP production by Methylene Blue antagonized the inhibitory effect of acetylcholine on isoprenaline-stimulated $I_{Ca}$. However, L-NMMA did not reproduce this effect [113]. In human papillary muscle strips, neither Methylene Blue nor L-NMMA inhibited the effect of carbachol on adrenergically stimulated contractility [114].

© 1999 The Biochemical Society and the Medical Research Society
In conclusion, while the data in relation to endogenous NO as a mediator of the direct cardiac response to muscarinic stimulation are supportive but inconsistent, there is strong evidence for a role for NO in the mediation of indirect vagal control. The precise mechanism of this action remains undefined, although, as with the modulation of cardiac sympathetic responses, indirect inhibition by NO of $I_{ca}$ appears to be important.

**HUMAN IN VIVO STUDIES**

Data on the effects of NO on the human cardiac autonomic nervous system are comparatively sparse. Hansen et al. [48] undertook microelectrode recording of post-ganglionic sympathetic nerve activity in the common peroneal nerve and showed that a high-dose L-NMMA infusion associated with a significant rise in blood pressure resulted in a 61% fall in sympathetic nerve activity. They concluded that this decrease in sympathetic nerve activity was due to baroreceptor activation, as it was not significantly different from that obtained during a similar pressor response to phenylephrine. Furthermore, no change in sympathetic nerve activity was observed when the pressor response, and therefore baroreceptor activation, was prevented using a combination of phentolamine and lower body negative pressure. While recent work initially appeared to confirm these results, studies using lower doses of 1-NMMA, producing minimal baroreflex loading, did suggest significant modulation of sympathetic nerve activity by NO in comparison with equipressor doses of phenylephrine [50,51]. These findings are consistent with the animal data, and suggest that NO may indeed act to ‘buffer’ sympathetic nerve activity in humans.

The autonomic responses to NOS inhibition have also been assessed using measures of heart rate and blood pressure variability. The increase in arterial pressure and fall in heart rate during 1-NMMA infusion were accompanied by a rise in high-frequency power (a measure of vagal activity), but no change in baroreflex sensitivity [49]. These results are difficult to interpret, as no appropriate controls for alterations in blood pressure were studied.

Numerous investigators have studied the effects of modulating the NO pathway in disease states in humans, but the important question of the part played by nitrergic modulation of the autonomic nervous system has been relatively overlooked. In light of the in vitro evidence of nitrergic inhibition of $\beta$-adrenergic inotropic responses, Hare et al. [115,116] studied the effects of NO on $\beta$-adrenergic contractile responses in humans. Intracoronary infusion of 1-NMMA had no effect on baseline measures of left ventricular contractility. However, it significantly potentiated the contractile response to dobutamine in patients with heart failure, although not in subjects with normal cardiac function [116]. The reasons for this discrepancy have not been elucidated, but one possibility would seem to be that levels of intrinsic NO production within healthy hearts are not sufficient to inhibit $\beta$-adrenergic signalling. Alternatively, modification of other elements of the cellular $\beta$-adrenergic transduction pathway in heart failure may render the system more sensitive to the effects of NO inhibition.

There are many pathological states that are associated with abnormalities of the generation or function of NO, such as hypertension, heart failure, diabetes, hypercholesterolaemia, atheroma and septic shock (for reviews, see [11,117]). Many of these conditions are also associated with autonomic dysfunction. Heart failure is characterized by endothelial dysfunction manifested by reduced responses to endothelial-dependent vasodilators. However, there is evidence that, rather than this condition being characterized by low NO production, NO generation may even be enhanced [118,119]. If NO serves to buffer sympathetic activity while enhancing cardiac vagal control in patients with cardiac failure, it may be postulated that modulation of cardiac autonomic control by NO in this condition, characterized by sympathetic overactivity [1–3] and vagal withdrawal [4], represents another failed counter-regulatory mechanism. Hepatic failure is associated with high levels of NO generation, thought to be due to endotoxin release and iNOS induction; it is also characterized by sympathetic overactivity. The effect of administration of L-arginine on heart-rate variability has been studied in patients with liver cirrhosis. Confusingly, indices of both parasympathetic and sympathetic modulation of heart rate were increased, but the study was uncontrolled and the results are therefore difficult to interpret [120]. In patients with end-stage renal disease, elevated markers of endogenous NO production were inversely correlated with cardiac sympathetic activity determined by spectral analysis of heart rate [121]. The possible causal relationship between alterations in NO pathway activity and abnormalities of autonomic function in these disorders remains an area requiring further detailed study.

**CONCLUSIONS**

NO is a ubiquitous intracellular messenger in many mammalian systems, and the cardiovascular autonomic nervous system has proven to be no exception. There is strong histochemical evidence for the presence of NOS throughout cardiac autonomic neurons. Elucidating the functional effects of NO has proved to be a more difficult task, particularly when trying to address the clinically important question of the overall modulation of each limb of the system. The fundamental difficulty in extrapolating the results of studies on isolated systems in animal models to the intact physiological state is par-
particularly true for NO. The labile nature of NO implies a tightly localized functional effect, allowing contradictory actions in its regulation of the same system at different sites. Further difficulty arises in that characteristics of autonomic control that are unaffected by constitutively generated levels of NO may be influenced by much higher levels seen during iNOS induction in disease states. Overall, a picture of NO as a sympathetic and a vagotonic agent does emerge from the animal data.

It is difficult to draw conclusions from the few existing human studies with regard to the effects of NO on cardiovascular autonomic control, due to a lack of well-controlled protocols. If indeed NO increases vagal and decreases sympathetic influence on cardiovascular control in humans, manipulation of the NO/cGMP system may be a therapeutic option in disease states such as congestive cardiac failure and myocardial infarction, where mortality and the risk of arrhythmic events may be directly influenced by autonomic dysfunction. In this respect it is intriguing to note that ischaemic dog hearts perfused with L-arginine in vivo were less susceptible to ventricular arrhythmias induced by sympathetic stimulation [81].

Finally, evidence reviewed in this article shows that exogenous NO modulates activity at many key sites in the arterial baroreflex arc, ranging from the receptors, through sites of central neuronal integration, to the effectors themselves. Animal data suggest that NO blunts the baroreceptor–heart-rate reflex. Whether this finding also applies to human physiology remains unanswered, but much of the current data on baroreflex sensitivity in humans has been obtained using the NO donors sodium nitroprusside and glyceryl trinitrate to unload the reflex. These results must now be called into question. There is a clear requirement for further well-controlled clinical studies to elucidate the possible role played by NO in human cardiovascular autonomic control in both health and disease.

ACKNOWLEDGMENTS

We gratefully acknowledge the helpful advice of Professor J. H. Coote in the preparation of this manuscript. Both authors are funded by the British Heart Foundation.

REFERENCES

44 Cunha, R. S., Cabral, A. M. and Vasquez, E. C. (1993) Evidence that the autonomic nervous system plays a major role in the l-NAME-induced hypertension in conscious rats. Am. J. Hypertens. 6, 806–809


Sears, C. E., Choate, J. K. and Paterson, D. J. (1998) Inhibition of nitric oxide synthase slows heart rate recovery from cholinergic activation. J. Appl. Physiol. 84, 1596–1603


