JCI The Journal of Clinical Investigation

Interaction between neuronal nitric oxide synthase and inhibitory G protein activity in heart rate regulation in conscious mice.

P Jumrussirikul, … , J M Hare, R D Berger

J Clin Invest. 1998;102(7):1279-1285. https://doi.org/10.1172/JCI2843.

Research Article

Nitric oxide (NO) synthesized within mammalian sinoatrial cells has been shown to participate in cholinergic control of heart rate (HR). However, it is not known whether NO synthesized within neurons plays a role in HR regulation. HR dynamics were measured in 24 wild-type (WT) mice and 24 mice in which the gene for neuronal NO synthase (nNOS) was absent (nNOS-/- mice). Mean HR and HR variability were compared in subsets of these animals at baseline, after parasympathetic blockade with atropine (0.5 mg/kg i.p.), after beta-adrenergic blockade with propranolol (1 mg/kg i.p.), and after combined autonomic blockade. Other animals underwent pressor challenge with phenylephrine (3 mg/kg i.p.) after beta-adrenergic blockade to test for a baroreflex-mediated cardioinhibitory response. The latter experiments were then repeated after inactivation of inhibitory G proteins with pertussis toxin (PTX) (30 microgram/kg i.p.). At baseline, nNOS-/- mice had higher mean HR (711+/-8 vs. 650+/-8 bpm, P = 0.0004) and lower HR variance (424+/-70 vs. 1,112+/-174 bpm2, P = 0.001) compared with WT mice. In nNOS-/- mice, atropine administration led to a much smaller change in mean HR (-2+/-9 vs. 49+/-5 bpm, P = 0.0008) and in HR variance (64+/-24 vs. -903+/-295 bpm2, P = 0.02) than in WT mice. In contrast, propranolol administration and combined autonomic blockade led to similar changes in mean HR between the two groups. […]

Interaction Between Neuronal Nitric Oxide Synthase and Inhibitory G Protein Activity in Heart Rate Regulation in Conscious Mice

Pitayadet Jumrussirikul, Jay Dinerman, Ted M. Dawson, Valina L. Dawson, Ulf Ekelund, Dimitrios Georgakopoulos, Lawrence P. Schramm, Hugh Calkins, Solomon H. Snyder, Joshua M. Hare, and Ronald D. Berger

Department of Medicine, Department of Neurology, Department of Neuroscience, Department of Physiology, and Department of Biomedical Engineering, Johns Hopkins University, Baltimore, Maryland 21287

Abstract

Nitric oxide (NO) synthesized within mammalian sinoatrial cells has been shown to participate in cholinergic control of heart rate (HR). However, it is not known whether NO synthesized within neurons plays a role in HR regulation. HR dynamics were measured in 24 wild-type (WT) mice and 24 mice in which the gene for neuronal NO synthase (nNOS) was absent ($nNOS^{-/-}$ mice). Mean HR and HR variability were compared in subsets of these animals at baseline, after parasympathetic blockade with atropine (0.5 mg/kg i.p.), after β-adrenergic blockade with propranolol (1 mg/kg i.p.), and after combined autonomic blockade. Other animals underwent pressor challenge with phenylephrine (3 mg/kg i.p.) after β-adrenergic blockade to test for a baroreflex-mediated cardioinhibitory response. The latter experiments were then repeated after inactivation of inhibitory G proteins with pertussis toxin (PTX) (30 μg/kg i.p.). At baseline, $nNOS^{-/-}$ mice had higher mean HR (711±8 vs. 650±8 bpm, $P = 0.0004$) and lower HR variance (424±70 vs. 1,112±174 bpm^2, $P = 0.001$) compared with WT mice. In $nNOS^{-/-}$ mice, atropine administration led to a much smaller change in mean HR (−2±9 vs. 49±5 bpm, $P = 0.0008$) and in HR variance (64±24 vs. −903±295 bpm^2, $P = 0.02$) than in WT mice. In contrast, propranolol administration and combined autonomic blockade led to similar changes in mean HR between the two groups. After β-adrenergic blockade, phenylephrine injection elicited a fall in mean HR and rise in HR variance in WT mice that was partially attenuated after treatment with PTX. The response to pressor challenge in $nNOS^{-/-}$ mice before PTX administration was similar to that in WT mice. However, PTX-treated $nNOS^{-/-}$ mice had a dramatically attenuated response to phenylephrine. These findings suggest that the absence of nNOS activity leads to reduced baseline parasympathetic tone, but does not prevent baroreflex-mediated cardioinhibition unless inhibitory G proteins are also inactivated. Thus, neuronally derived NO and cardiac inhibitory G protein activity serve as parallel pathways to mediate autonomic slowing of heart rate in the mouse. (*J. Clin. Invest.* 1998. 102:1279–1285.) Key words: nitric oxide • G protein • autonomic nervous system • parasympathetic • baroreflex

Introduction

Nitric oxide (NO)[1] has been implicated in autonomic regulation of various aspects of cardiovascular function. NO is recognized as a major endothelium-derived relaxing factor (1), and has been shown to participate in parasympathetic coronary vasodilation (2, 3) and in inhibition of sympathetic peripheral and coronary vasoconstriction (4, 5). NO has been shown to modulate myocardial contractility in response to both cholinergic (6, 7) and β-adrenergic (8) stimulation. There is also evidence that NO plays an obligatory role in cholinergic modulation of automaticity in isolated myocytes (9, 10) and that it participates in vagal control of heart rate (HR) in intact mammals (11, 12).

NO is known to stimulate soluble guanylyl cyclase to produce cyclic GMP (13, 14), a molecule that acts as a second messenger in autonomic signaling in the heart (15, 16). However, it remains unclear whether NO plays a role in preganglionic mediation of autonomic activity. NO is a ubiquitous molecule, and its autonomic influences may relate to a multitude of diverse effects within the central nervous system, myocardium, and vasculature (17). Therefore, it is difficult to establish the role of NO in mediating autonomic reflexes in the intact organism based on extrapolation from in vitro studies. To determine the effects of neuronally derived NO on autonomic HR regulation, we compared HR dynamics in wild-type (WT) mice with those in mice in which the gene for neuronal NO synthase (nNOS) had been disrupted (18). This model enabled investigation of the specific role of neuronally produced NO in HR regulation in an intact conscious mammal.

Methods

Animal preparation. 24 WT and 24 nNOS knockout ($nNOS^{-/-}$) mice, weighing 20–25 g and aged 10–12 wk, were studied. $nNOS^{-/-}$ mice were obtained from a breeding colony established at Johns Hopkins University using animals previously produced by homologous recom-

Address correspondence to Ronald D. Berger, M.D., Ph.D., Carnegie 592, Johns Hopkins Hospital, 600 N. Wolfe St., Baltimore, MD 21287. Phone: 410-614-2751; FAX: 410-614-1345; E-mail: ron@tachy.cdisc.jhu.edu

Received for publication 20 January 1998 and accepted in revised form 21 August 1998.

J. Clin. Invest.

0021-9738/98/10/1279/07 $2.00
Volume 102, Number 7, October 1998, 1279–1285
http://www.jci.org

1. *Abbreviations used in this paper:* G_i, inhibitory G protein; HR, heart rate; nNOS, neuronal NO synthase; $nNOS^{-/-}$, homozygous disruption of nNOS gene; NO, nitric oxide; PTX, pertussis toxin; WT, wild-type.

bination (18). Because the nNOS$^{-/-}$ mutation was initially made on a 129SvEv agouti and C57B6 background, we used both 129SvEv and C57B6 WT mice as controls.

In 20 WT and 18 nNOS$^{-/-}$ mice, an electrocardiographic transmitter (Data Sciences Int., St. Paul, MN) with subcutaneous leads was implanted in the peritoneal cavity using aseptic technique under anesthesia with phenobarbital 1 mg i.p. Mice were allowed to recover for 5–10 d before undergoing experimental protocols. All animals were maintained in accordance with the guidelines of the Animal Care and Use Committee at Johns Hopkins and the Guide for the Care and Use of Laboratory Animals (Department of Health and Human Services publication No. NIH 83-23, revised 1985).

Protocol 1: Baseline HR dynamics and autonomic blockade. In 16 WT and 12 nNOS$^{-/-}$ mice instrumented with electrocardiographic transmitters, ECG recordings (see below) were obtained in the fully conscious drug-free (baseline) state, while the animals were unrestrained in their cages. Among these mice, five of the WT and six nNOS$^{-/-}$ mice received atropine 0. 5 mg/kg i.p. ECG recordings were then repeated after a 10-min equilibration phase. After acquisition of baseline ECG recordings on a different day, 14 WT and 11 nNOS$^{-/-}$ mice received propranolol 1 mg/kg i.p. ECG recordings were again obtained after a 10-min waiting phase. In five WT and four nNOS$^{-/-}$ mice, atropine 0.5 mg/kg i.p. was then injected to achieve combined β-adrenergic and muscarinic cholinergic blockade, after which ECG recordings were obtained once again. The dosages of atropine and propranolol we used are similar to those used by other workers in mice (19, 20) and rabbits (21), although we did not specifically test the completeness of autonomic blockade with agonist challenge. We chose a waiting period of 10 min for equilibration because changes in HR occurred within 5–10 min of intraperitoneal injection of these pharmacologic agents, after which little additional change in HR was observed.

Protocol 2: Baroreflex HR modulation. The inhibitory limb of the HR baroreflex was studied in four WT and six nNOS$^{-/-}$ instrumented mice in the fully conscious state. These 10 animals were different from those used in the first protocol. After collecting baseline ECG recordings, animals were given propranolol 1 mg/kg i.p., and ECG recordings were repeated 10 min later. Phenylephrine 3 mg/kg i.p. was then administered, and ECG recordings were obtained after 1 min. The next day, pertussis toxin (PTX) 30 μg/kg i.p. was administered to inactivate inhibitory guanine nucleotide proteins (G_i and G_o) in cardiac tissue (22). 3 d later, the protocol of propranolol and phenylephrine injections was repeated.

Protocol 3: Acute blood pressure and baroreceptor sensitivity. We did not attempt to measure blood pressure in the chronically instrumented animals in protocols 1 and 2. However, in another four WT and six nNOS$^{-/-}$ mice, baseline blood pressure and baroreceptor sensitivity were measured acutely under anesthesia. Anesthesia was initiated with methoxyflurane inhalation followed by injection of urethane 750 mg/kg i.p., with additional injections titrated so that the animal remained unresponsive to tail pinch by forceps, assessed by changes in HR and blood pressure. The animals were intubated with a blunted 19-gauge needle via tracheotomy and were ventilated with a custom-designed constant pressure ventilator with 100% oxygen at 120 breaths/min. The carotid artery was cannulated and instrumented with a Millar micromanometer, positioned to achieve a stable pressure waveform. Systolic blood pressure and HR were determined from the micromanometer signal, averaged over 5-min measurements. Phenylephrine 3 mg/kg i.p. was then administered and the peak change in systolic blood pressure and HR was recorded. The baroreceptor sensitivity was then computed as the ratio of change in HR to change in systolic blood pressure.

ECG recording and analysis. In the chronically instrumented animals (protocols 1 and 2), ECG signals were recorded using a telemetry receiver (Data Sciences Int.) connected to an analog-to-digital converter (Biopac Systems, Inc., Santa Barbara, CA) interfaced with a 486-based computer. Signals were obtained in 5-min epochs and were recorded with 12-bit precision at a sampling rate of 1,000 Hz onto magnetic media for off-line analysis. Whenever possible, two consecutive 5-min data epochs were obtained at each ECG recording point in the experimental protocols.

The digitized ECG records were transferred to a workstation (SUN Microsystems, Inc., Mountain View, CA) for analysis. R-waves were found by automatic peak detection, and each 5-min RR interval series was resampled as described by Berger et al. (23) to provide a series of evenly spaced HR samples. Artifacts in the resulting HR series caused by mouse movement were removed by linear spline technique (24). Records consisting of $> 10\%$ artifact were excluded from further analysis. The mean and variance in HR were then computed for each epoch. When two usable epochs were available for a given point in the data collection, the HR means and variances for the two epochs were averaged.

Statistical analysis. Data are expressed as mean±standard error. Except where noted, tests of drug effect were made by comparison with the most recent prior baseline state. Comparisons between data points within a group were made with the paired two-tailed Student's *t* test, and comparisons between groups were made with the unpaired two-tailed Student's *t* test. Statistical significance was accepted at the level of $P < 0.05$.

Results

Baseline HR dynamics. Results from the first protocol showed that compared with WT mice, nNOS$^{-/-}$ mice had a significantly higher mean HR (711±8 vs. 650±8 bpm, $P = 0.0004$) and lower HR variance (424±70 vs. 1,112±174 bpm^2, $P = 0.001$). These data are shown in Fig. 1.

Autonomic blockade. The effects of parasympathetic, sympathetic, and combined autonomic blockade on mean HR and HR variability are shown in Fig. 2. Compared with the effect in WT mice, in nNOS$^{-/-}$ mice atropine administration led to a much smaller change in mean HR (−2±9 vs. 49±5 bpm, $P = 0.0008$) and in HR variance (64±24 vs. −903±295 bpm^2, $P = 0.02$). By contrast, propranolol administration led to similar changes in mean HR (−84±11 vs. −84±18 bpm, P = NS) in the two groups, although the change in HR variance was greater in WT mice (46±95 vs. −462±231 bpm^2, $P = 0.02$). Combined autonomic blockade also caused a similar fall from baseline in mean HR between the two groups (−92±30 vs. −63±22 bpm, P = NS), but it elicited a greater fall in HR variance in the WT mice (−226±132 vs. −1,259±352 bpm^2, $P = 0.02$).

Baroreflex pressor response. Protocol 2 was designed to enable investigation of the baroreceptor-mediated HR response to a pressor challenge. Since muscarinic-cholinergic effects are dependent on G_i and G_o activity, we examined the HR response to phenylephrine in WT and nNOS$^{-/-}$ mice both in the setting of intact G protein activity and after G_i and G_o inactivation by administration of PTX. The pressor challenge was performed after β-adrenergic blockade so that any observed change in HR mean or variability would be due to activation of the inhibitory limb of the baroreflex and not the result of sympathetic withdrawal.

Fig. 3 shows the response in WT and nNOS$^{-/-}$ mice to injections of phenylephrine, before and after treatment with PTX. Changes in HR mean and variance resulting from phenylephrine injection were measured relative to the β-adrenergic blocked state. In WT mice, phenylephrine injection elicited a fall in mean HR, which was partially attenuated after PTX treatment (−299±36 vs. −196±35 bpm, $P = 0.04$), and a rise in HR variance that was similar before and after G protein inactivation (1,654±461 vs. 1,452±564 bpm^2, P = NS). Phenylephrine elicited a response in nNOS$^{-/-}$ mice before PTX ad-

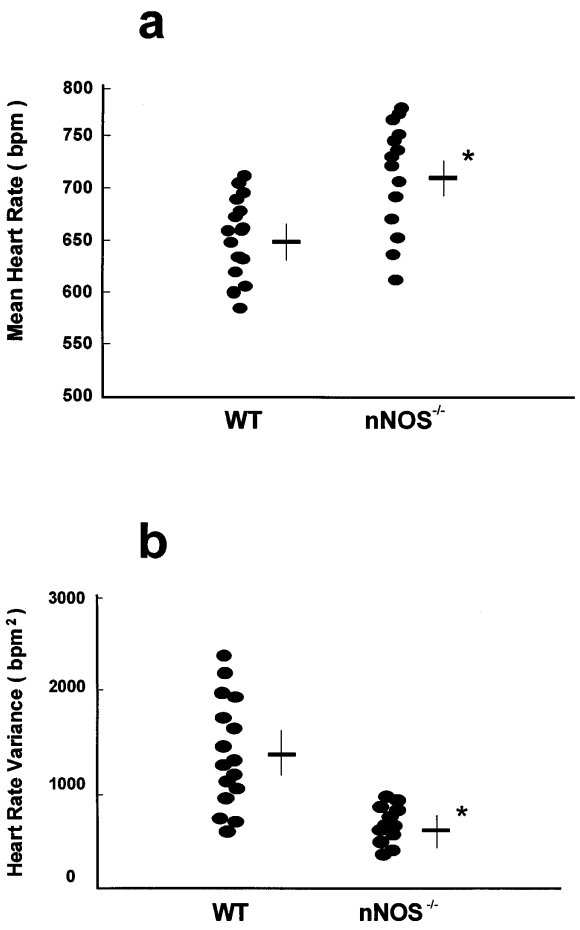

Figure 1. Mean HR (*a*) and HR variance (*b*) in WT and nNOS$^{-/-}$ mice. *$P < 0.05$ compared with WT.

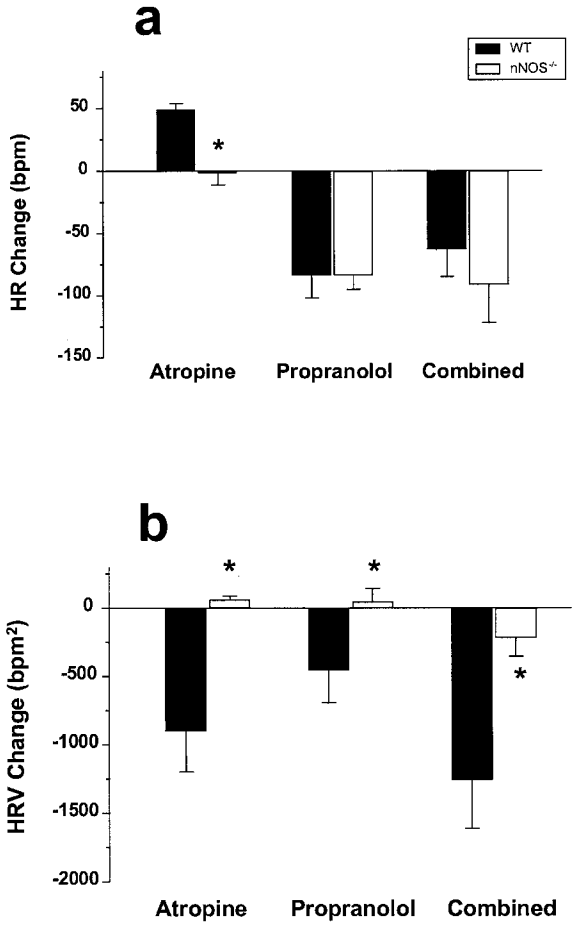

Figure 2. Change in mean HR (*a*) and HR variance (*b*) in WT and nNOS$^{-/-}$ mice after atropine, propranolol, and combined autonomic blockade. *$P < 0.05$ compared with WT.

ministration similar to that in WT mice (mean HR change −284±11 bpm, HR variance change 1,481±386 bpm^2, P = NS in both parameters compared with WT mice). However in PTX-treated nNOS$^{-/-}$ mice, phenylephrine elicited a much smaller change in mean HR (−106±26 bpm) and HR variance (107±73 bpm^2) than in either PTX-treated WT mice ($P = 0.04$ for mean HR, $P = 0.001$ for HR variance) or nNOS$^{-/-}$ mice with intact G protein activity ($P = 0.002$ for mean HR, $P = 0.001$ for HR variance).

Acute blood pressure measurements. In the acutely instrumented anesthetized state, baseline systolic blood pressure was similar in the two groups (77±6 mmHg in nNOS$^{-/-}$ mice vs. 87±6 mmHg in WT mice, P = NS). With phenylephrine administration, systolic blood pressure rose identically (17±7 mmHg in nNOS$^{-/-}$ mice vs. 17±4 mmHg in WT mice, P = NS), and the HR fell by similar amounts (82±18 bpm in nNOS$^{-/-}$ mice vs. 67±10 bpm in WT mice, P = NS). The calculated baroreceptor sensitivity was also not significantly different (7.1±3.3 bpm/mmHg in nNOS$^{-/-}$ mice vs. 5.0±1.6 bpm/mmHg in WT mice, P = NS).

Discussion

To our knowledge, this is the first study to evaluate the role of neuronally derived NO in autonomic HR regulation. Our use of mice with a targeted disruption of the nNOS gene allowed us to distinguish the effects of NO synthesized in neurons from those due to NO produced in the sinus node, myocardium, and vasculature. This model also enabled investigation of the role of NO in autonomic activity without the need for administration of nonspecific NOS inhibitors, whose effects are protean. Importantly, mice were studied in the intact conscious state so that autonomic function could be observed free of the confounding effects of anesthetic agents as well. A similar approach was used by Mansier et al., who studied HR variability in freely moving transgenic mice overexpressing atrial β_1-adrenoceptors (19), and by Uechi et al., who studied transgenic mice overexpressing cardiac $G_{s\alpha}$ (20).

The main findings of this study are that (*a*) nNOS activity mediates an inhibitory effect on sinus rate in the mouse at baseline, (*b*) an inhibitory effect on sinus rate can be elicited by the baroreflex even in mice with a targeted disruption for the nNOS gene, and (*c*) inactivation of inhibitory G proteins results in partial blunting of the HR baroreflex, while (*d*) absence of both nNOS activity and inhibitory G protein activity leads to near abolition of baroreflex HR inhibition. Collectively, these findings suggest nNOS activity and cardiac inhibitory G protein activity work in parallel to reduce sinus nodal rate and mediate HR variability. A role for NO in the mediation of cardioinhibitory mechanisms has been suggested by

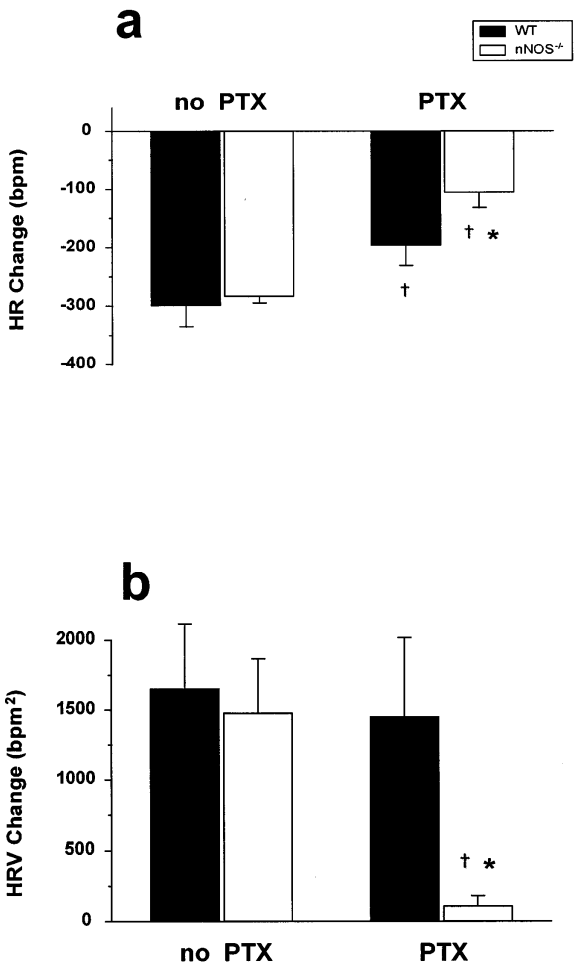

Figure 3. Change in mean HR (*a*) and HR variance (*b*) in WT and nNOS$^{-/-}$ mice in response to phenylephrine challenge in the setting of prior β-adrenergic blockade. Data are shown both before treatment with PTX (*no PTX*) and after PTX administration (*PTX*). Inhibitory G protein inactivation with PTX led to attenuation of the response to pressor challenge in WT mice, but almost completely abolished the response in nNOS$^{-/-}$ mice. *$P < 0.05$ compared with WT. †$P < 0.05$ compared with the no-PTX state.

others (11, 12), but the relationship between nNOS activity and cholinergic regulation of HR has not been explored previously.

Baseline effects of neuronal NO on HR. In the baseline state, nNOS$^{-/-}$ mice had higher mean HR and lower HR variance than WT mice had. While the elevated mean HR could reflect either increased sympathetic or reduced parasympathetic tone, the diminished HR variance strongly suggests the latter. HR variability has been shown to depend on fluctuations in both limbs of the autonomic nervous system (25, 26). The abnormalities in baseline HR dynamics observed in the nNOS$^{-/-}$ mice are similar to those seen in human heart failure (27) that have been interpreted to represent diminished vagal tone (24). In concert with this interpretation of the present data, atropine increased mean HR and greatly reduced HR variability in WT mice, while it had little effect on HR mean or variance in the nNOS$^{-/-}$ mice. This suggests that muscarinic-cholinergic tone was already minimal in the nNOS$^{-/-}$ mice before the atropine was given. On the other hand, propranolol produced an identical fall in mean HR in the two groups of mice, suggesting baseline sympathetic tone was intact in the nNOS$^{-/-}$ mice. Interestingly, β-adrenergic blockade caused a significant reduction in HR variability in WT mice, while it led to little change in this parameter in nNOS$^{-/-}$ mice. This is probably explained by the fact that nNOS$^{-/-}$ mice had such low baseline HR variability, it was difficult to detect a consistent reduction in this parameter resulting from any intervention. Since vagal activity is a modulator of sympathetic tone (28), HR variability may be dramatically reduced with parasympathetic withdrawal or blockade even in the setting of intact sympathetic tone.

Combined sympathetic and parasympathetic autonomic blockade abolished virtually all HR variability in both WT and nNOS$^{-/-}$ mice, demonstrating the lack of other mechanisms in the mediation of short-term HR fluctuations. The resulting mean HR was similar in the two groups, suggesting nNOS activity has little or no effect on intrinsic sinus nodal rate. It is unclear whether the intrinsic rate depends on NO produced within cardiac cells. Han et al. described an obligatory role for cardiac-derived NO in cholinergic control of mammalian HR (10, 29), while Kennedy et al. found that inhibitors of arginine-derived NO synthesis had no chronotropic effect in isolated rat atria (30).

We considered the possibility that reduced parasympathetic tone in the nNOS$^{-/-}$ mice might be a reflexogenic response to a lower baseline blood pressure or altered baroreceptor sensitivity. While our chronic mouse preparation precluded making invasive blood pressure measurements in the conscious state, our findings in acutely instrumented anesthetized mice suggest baseline blood pressure and baroreceptor sensitivity are not significantly different between WT and nNOS$^{-/-}$ mice. These findings are consistent with those of Huang et al. (31), who also found no difference in blood pressure between WT and nNOS$^{-/-}$ mice. Thus, the reduction in baseline cardiac parasympathetic activity in the nNOS$^{-/-}$ mice appears to be the result of an alteration in the efferent limb of the baroreflex, either centrally or at the end organ (i.e., sinus node).

Interaction between inhibitory G proteins and neuronal NO. After finding that mice lacking nNOS activity had reduced baseline parasympathetic tone, we tested whether an increase in parasympathetic tone could be elicited in these mice by activation of the baroreflex. To exclude the effects of baroreceptor-mediated sympathetic withdrawal, baroreflex activation in the chronically instrumented animals was performed after β-adrenergic blockade. We found that with administration of the pressor agent phenylephrine, nNOS$^{-/-}$ mice exhibited a fall in mean HR and an increase in HR variance equal to those in WT mice. These findings are in concert with those obtained in the acutely instrumented anesthetized mice and suggest that the absence of nNOS activity does not prevent a typical baroreflex-mediated rise in parasympathetic tone.

We then tested whether the pressor response depends on intact G protein activity, and found a striking difference between conscious nNOS$^{-/-}$ and WT mice after G_i and G_o inactivation with PTX. At baseline, inhibitory G protein inactivation led to a slight increase in mean HR and decrease in HR variance in both WT and nNOS$^{-/-}$ mice. With phenylephrine administration, the PTX-treated WT mice exhibited a blunted decrease in mean HR and a near normal rise in HR variance, while the PTX-treated nNOS$^{-/-}$ mice had a dramatically at-

tenuated response. In fact, the PTX-treated nNOS$^{-/-}$ mice had nearly complete abolition of HR variability after β-adrenergic blockade, and they exhibited almost no subsequent increase in HR variability after phenylephrine injection. These findings suggest that enhancement in parasympathetic tone elicited by the baroreflex requires either intact cardiac G protein activity or the presence of neuronally derived NO, but does not require the presence of both.

The known pathway for cholinergic modulation of HR begins with release of acetylcholine from vagal nerve terminals that impinge on sinus nodal cells. Acetylcholine binds to the muscarinic receptor, causing activation of G proteins G_i and G_o, leading to both direct effects on membrane-bound ion channel proteins and indirect effects coupled to adenylyl cyclase inhibition and other cytosolic pathways (32). G_i and G_o inhibit the hyperpolarization-activated pacemaker current I_f through both direct and indirect effects (33), and G_i inhibits L-type calcium current $I_{Ca[L]}$ through its effect on adenylyl cyclase (34), both resulting in decreased sinus nodal firing rate. Muscarinic activation also inhibits sinoatrial automaticity through modulation of the polarizing acetylcholine-activated potassium channel $I_{K(ACh)}$ (35), although it is not known whether G_i is the G protein that couples this effect to the muscarinic receptor.

PTX has been shown to inactivate G_i and G_o by catalyzing ADP ribosylation of these proteins (22), resulting in a potent anticholinergic effect on HR regulation (36). We found a rise in mean HR and a fall in HR variability at baseline in both WT and nNOS$^{-/-}$ mice with PTX administration, consistent with an anticholinergic effect. Surprisingly, however, the PTX-treated WT mice exhibited a significant (although partially attenuated) fall in mean HR and a normal rise in HR variance with pressor challenge. Thus, in the mouse, baroreflex activation alters HR dynamics in a manner consistent with enhanced vagal tone, even in the absence of cardiac inhibitory G protein activity.

Based on our findings in the WT mice alone, several mechanisms may be postulated to explain the partially preserved baroreflex after PTX administration. G_i and G_o inactivation might have been incomplete, muscarinic activation of other G proteins might have mediated the observed cardioinhibition, or some alternative pathway may participate in the efferent limb of the baroreflex. Data from the nNOS$^{-/-}$ mice provide insight into the likely mechanism. Since pressor-induced cardioinhibition was nearly absent in the PTX-treated nNOS$^{-/-}$ mice, nNOS activity must be responsible for the pressor-induced change in HR dynamics observed in the PTX-treated WT mice. If inhibitory G protein inactivation were merely incomplete or another non–nNOS-dependent mechanism mediated the preserved baroreflex in the PTX-treated WT mice, then the same effects should have been present in the nNOS$^{-/-}$ mice.

NO within the central nervous system has been shown to modulate baroreflex-mediated sympathetic nerve activity (4, 21, 37), so abnormal baroreflex function in the nNOS$^{-/-}$ mice might have been expected. Despite this, we found no significant differences between WT and nNOS$^{-/-}$ mice in baseline blood pressure or baroreflex sensitivity in the anesthetized acutely instrumented state. In addition, we found that in the awake β-adrenergic blocked state, nNOS$^{-/-}$ mice exhibited nearly normal pressor-induced HR changes until inhibitory G proteins were inactivated. This suggests that in the mouse neuronally derived NO participates in the efferent limb of autonomic cardioinhibition in a fashion that is independent of β-adrenergic activity and that bypasses the inhibitory G protein pathway.

Recently, Han et al. proposed a model for cholinergic HR modulation in which inhibitory G protein activity and NO provide parallel cytosolic pathways within sinus nodal cells to couple the muscarinic receptor to ion channel function (29). In their model, G_i exerts its effects through inhibition of adenylyl cyclase, while NO, synthesized within sinus nodal cells presumably by endothelial NOS, stimulates soluble guanylyl cyclase. Both of these effects lead to reduced L-type calcium current, resulting in diminished cellular automaticity. We hypothesize that NO, synthesized by nNOS in vagal nerve fibers, diffuses from vagal cells into sinus nodal cells to mediate the NO effects on HR, as schematized in Fig. 4. NO may also interact with cholinergic signaling by potentiating the release of acetylcholine from vagal nerve terminals. Lack of this potentiation in nNOS$^{-/-}$ mice would explain their apparent loss of resting parasympathetic tone and minimal response to atropine in the baseline state.

The concept of NO acting as a neurotransmitter is not new, as there is evidence that NO produced in myenteric neurons diffuses into smooth muscle cells of the intestines and modulates intestinal relaxation (17). A similar mechanism may be present in cardiac ganglia. Sosunov et al. have shown by immunocytochemical analysis that neurons within cardiac ganglia of the rat and guinea pig contain NOS (38). They further argue,

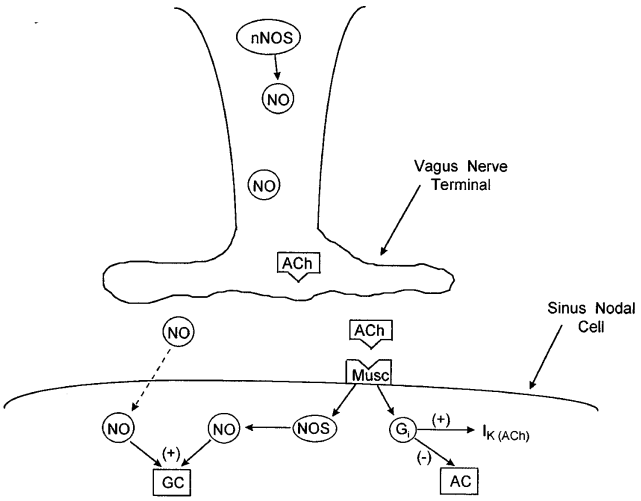

Figure 4. Proposed model demonstrating parallel signaling pathways for parasympathetic control of HR. Acetylcholine (*ACh*) released by vagus nerve fibers mediates parasympathetic signaling by binding with the muscarinic receptor (*Musc*) on the sinus nodal cell surface. Muscarinic activation leads to increased G_i activity, and may enhance NO synthesis within the sinus nodal cell, presumably by endothelial NO synthase. G_i exerts its inhibitory effects on HR predominantly by inhibition of adenylyl cyclase (*AC*) and augmentation of $I_{K(ACh)}$. In this model, NO synthesized by nNOS within the vagus nerve diffuses across the synapse and into the sinus nodal cell. NO may also potentiate the release of acetylcholine from the vagus nerve terminal. NO within the sinus nodal cell, whether synthesized there or having diffused into the cell from the vagus nerve, enhances soluble guanylyl cyclase (*GC*) activity to exert an inhibitory effect on HR through modulation of L-type calcium current.

on the basis that NOS and vasoactive intestinal peptide are colocalized in some cardiac nerve fibers (39, 40), that NOS-immunoreactive nerves in the heart are parasympathetic in origin. If parasympathetic nerve cells impinging on or within the heart represent a significant source of NO in the mouse, then the parallel actions of NO and inhibitory G proteins proposed in Fig. 4 could explain the observed difference in baroreflex-mediated HR changes between WT and nNOS$^{-/-}$ mice after inhibitory G protein inactivation.

As an alternative explanation for our findings, NO may act centrally within the cardioinhibitory center of the brainstem, although this mechanism would require participation by a second messenger other than G_i in order to explain the presence of a pressor response, even if blunted, in the PTX-treated WT mice. Of note, we did not test whether pressor-induced cardioinhibition in WT mice after PTX administration would be abolished by atropine. Thus, it remains unclear whether the cardioinhibitory effect of neuronally derived NO requires intact muscarinic receptor function. This unresolved issue awaits clarification with further study.

Limitations. In our interpretation of the results of pharmacologic blockade, we assumed complete effect of the atropine, propranolol, and PTX dosages used, although we did not specifically test for the completeness of blockade. While the dosages we used were similar to those used by others to achieve complete autonomic or G protein blockade (19, 20, 22), it is possible that the WT and nNOS$^{-/-}$ mice had differential sensitivities to these agents, which could have confounded our findings. In addition, in this study we did not consider interactions between cholinergic signaling and the α-adrenergic system, the latter of which may also be influenced by the presence or absence of nNOS activity.

This study is also limited by the imperfections in HR variability analysis. The work assumes that changes in autonomic tone are accurately reflected in the sinus nodal rate. Conceivably, nNOS$^{-/-}$ mice may have altered sinus nodal function independent of changes in autonomic activity. However, this appears unlikely since the intrinsic rate of the sinus node did not differ significantly between WT and nNOS$^{-/-}$ mice. In addition, in the setting of intact inhibitory G protein activity, pressor challenge elicited the same change in HR mean and variance in the nNOS$^{-/-}$ mice as in WT mice, further supporting intact sinus nodal function in the nNOS$^{-/-}$ mice.

Conclusions. By studying HR dynamics in this conscious transgenic mouse model, we have found a role for neuronally derived NO in parasympathetic HR regulation. Absence of nNOS activity leads to reduced baseline parasympathetic tone, but does not prevent baroreflex-mediated cardioinhibition unless cardiac inhibitory G proteins are concomitantly inactivated. These findings provide evidence for parallel pathways of cardioinhibition using nNOS activity on the one hand, and inhibitory G protein activity on the other.

Acknowledgments

The nNOS$^{-/-}$ breeding colony used in this study was obtained through a collaborative agreement between Johns Hopkins University and Massachusetts General Hospital. The mice were originally made by homologous recombination in the laboratories of Drs. Paul Huang and Marc Fishman.

R. Berger is the recipient of a FIRST Award from the National Institutes of Health (HL-54584) and is a Solo Cup Clinician Scientist. T. Dawson is an Established Investigator of the American Heart Association and is supported by US Public Health Service (USPHS) grant NS33277 and the Paul Beeson Physician's Faculty Scholar Award. V. Dawson is supported by the American Heart Association and USPHS grant NS33142. S. Snyder is supported by USPHS grant MH18501 and Research Scientist Award DA00074. J. Hare is supported by USPHS grant K08 HL03228 and a grant-in-aid from the American Heart Association.

References

1. Moncada, S., R.M.J. Palmer, and E.A. Higgs. 1991. Nitric oxide: physiology, pathophysiology and pharmacology. *Pharmacol. Rev.* 43:109–142.

2. Shen, W., M. Ochoa, X. Xu, J. Wang, and T.H. Hintze. 1994. Role of EDRF/NO in parasympathetic coronary vasodilation following carotid chemoreflex activation in conscious dogs. *Am. J. Physiol.* 267:H605–H613.

3. Broten, T.P., J.K. Miyashiro, S. Moncada, and E.O. Feigl. 1992. Role of endothelium-derived relaxing factor in parasympathetic coronary vasodilation. *Am. J. Physiol.* 262:H1579–H1584.

4. Zanzinger, J., J. Czachurski, and H. Seller. 1994. Inhibition of sympathetic vasoconstriction is a major principle of vasodilation by nitric oxide in vivo. *Circ. Res.* 75:1073–1077.

5. Goodson, A.R., J.M. Leibold, and D.D. Gutterman. 1994. Inhibition of nitric oxide synthesis augments centrally induced sympathetic coronary vasoconstriction in cats. *Am. J. Physiol.* 267:H1272–H1278.

6. Hare, J.M., J.F. Keaney, J.L. Balligand, J. Loscalzo, T.W. Smith, and W.S. Colucci. 1995. Role of nitric oxide in parasympathetic modulation of β-adrenergic myocardial contractility in normal dogs. *J. Clin. Invest.* 95:360–366.

7. Hare, J.M., B. Kim, N.A. Flavahan, K.M. Ricker, X. Peng, L. Colman, R.G. Weiss, and D.A. Kass. 1998. Pertussis toxin-sensitive G proteins influence nitric oxide synthase III activity and protein levels in rat heart. *J. Clin. Invest.* 101:1424–1431.

8. Keaney, J.F., J.M. Hare, J.L. Balligand, J. Loscalzo, T.W. Smith, and W.S. Colucci. 1996. Inhibition of nitric oxide synthase augments myocardial contractile responses to β-adrenergic stimulation. *Am. J. Physiol.* 271:H2646–H2652.

9. Balligand, J.L., R.A. Kelly, P.A. Marsden, T.W. Smith, and T. Michel. 1993. Control of cardiac muscle cell function by an endogenous nitric oxide signaling system. *Proc. Natl. Acad. Sci.* 90:347–351.

10. Han, X., Y. Shimoni, and W.R. Giles. 1994. An obligatory role for nitric oxide in autonomic control of mammalian heart rate. *J. Physiol.* 476:309–314.

11. Elvan, A., M. Rubart, and D.P. Zipes. 1997. NO modulates autonomic effects on sinus discharge rate and AV nodal conduction in open-chest dogs. *Am. J. Physiol.* 272:H263–H271.

12. Conlon, K., T. Collins, and C. Kidd. 1996. Modulation of vagal actions on heart rate produced by inhibition of nitric oxide synthase in the anaesthetized ferret. *Exp. Physiol.* 81:547–550.

13. Ignarro, L.J., T.M. Burke, K.S. Wood, M.S. Wolin, and P.J. Kadowitz. 1984. Association between cyclic GMP accumulation and acetylcholine-elicited relaxation of bovine intrapulmonary artery. *J. Pharmacol. Exp. Ther.* 228:682–690.

14. Radomski, M.W., R.M.J. Palmer, and S. Moncada. 1987. The role of nitric oxide and cGMP in platelet adhesion to the vascular endothelium. *Biochem. Biophys. Res. Commun.* 148:1482–1489.

15. George, W.J., R.D. Wilkerson, and P.J. Kadowitz. 1973. Influence of acetylcholine on contractile force and cyclic nucleotide levels in the isolated perfused rat heart. *J. Pharmacol. Exp. Ther.* 184:228–235.

16. Watanabe, A.M., and J.R. Besch. 1975. Interaction between cyclic adenosine monophosphate and cyclic guanosine monophosphate in guinea pig ventricular myocardium. *Circ. Res.* 37:309–317.

17. Christopherson, K.S., and D.S. Bredt. 1997. Nitric oxide in excitable tissues: physiological roles and disease. *J. Clin. Invest.* 100:2424–2429.

18. Huang, P.L., T.M. Dawson, D.S. Bredt, S.H. Snyder, and M.C. Fishman. 1993. Targeted disruption of the neuronal nitric oxide synthase gene. *Cell.* 75:1273–1286.

19. Mansier, P., C. Medigue, N. Charlotte, C. Vermeiren, E. Coraboeuf, E. Deroubai, E. Ratner, B. Chevalier, J. Clairambault, F. Carre, et al. 1996. Decreased heart rate variability in transgenic mice overexpressing atrial β_1-adrenoceptors. *Am. J. Physiol.* 271:H1465–H1472.

20. Uechi, M., K. Asai, M. Osaka, A. Smith, N. Sato, T.E. Wagner, Y. Ishikawa, H. Hayakawa, D.E. Vatner, R.P. Shannon, et al. 1998. Depressed heart rate variability and arterial baroreflex in conscious transgenic mice with overexpression of cardiac $G_{s\alpha}$. *Circ. Res.* 82:416–423.

21. Liu, J.L., H. Murakami, and I.H. Zucker. 1996. Effects of NO on baroreflex control of heart rate and renal nerve activity in conscious rabbits. *Am. J. Physiol.* 270:R1361–R1370.

22. Fleming, J.W., T.D. Hodges, and A.M. Watanabe. 1988. Pertussis toxin-treated dog: a whole animal model of impaired inhibitory regulation of adenylate cyclase. *Circ. Res.* 62:992–1000.

23. Berger, R.D., S. Akselrod, D. Gordon, and R.J. Cohen. 1986. An effi-

cient algorithm for spectral analysis of heart rate variability. *IEEE Trans. Biomed. Eng.* BME-33:900–904.

24. Saul, J.P., Y. Arai, R.D. Berger, L.S. Lilly, W.S. Colucci, and R.J. Cohen. 1988. Assessment of autonomic regulation in chronic congestive heart failure by heart rate spectral analysis. *Am. J. Cardiol.* 61:1292–1299.

25. Akselrod, S., D. Gordon, F.A. Ubel, D.C. Shannon, A.C. Barger, and R.J. Cohen. 1981. Power spectrum analysis of heart rate fluctuations: a quantitative probe of beat-to-beat cardiovascular control. *Science.* 213:220–222.

26. Akselrod, S., D. Gordon, J.B. Madwed, N.C. Snidman, D.C. Shannon, and R.J. Cohen. 1985. Hemodynamic regulation: investigation by spectral analysis. *Am. J. Physiol.* 249:H867–H875.

27. Woo, M.A., W.G. Stevenson, D.K. Moser, R.B. Trelease, and R.M. Harper. 1992. Patterns of beat-to-beat heart rate variability in advanced heart failure. *Am. Heart J.* 123:704–710.

28. Levy, M.N. 1971. Sympathetic-parasympathetic interactions in the heart. *Circ. Res.* 29:437–445.

29. Han, X., Y. Shimoni, and W.R. Giles. 1995. A cellular mechanism for nitric oxide-mediated cholinergic control of mammalian heart rate. *J. Gen. Physiol.* 106:45–65.

30. Kennedy, R.H., K.K. Hicks, J.E. Brian, and E. Seifen. 1994. Nitric oxide has no chronotropic effect in right atria isolated from rat heart. *Eur. J. Pharmacol.* 255:149–156.

31. Huang, Z., P.L. Huang, N. Panahian, T. Dalkara, M.C. Fishman, and M.A. Moskowitz. 1994. Effects of cerebral ischemia in mice deficient in neuronal nitric oxide synthase. *Science.* 265:1883–1885.

32. Brown, A.M. 1990. Regulation of heartbeat by G protein-coupled ion channels. *Am. J. Physiol.* 259:H1621–H1628.

33. Yatani, A., K. Okabe, J. Codina, L. Birnbaumer, and A.M. Brown. 1990. Heart rate regulation by G proteins acting on the cardiac pacemaker channel. *Science.* 249:1163–1166.

34. Hartzell, H.C. 1988. Regulation of cardiac ion channels by catecholamines, acetylcholine and second messenger systems. *Prog. Biophys. Mol. Biol.* 52:165–247.

35. Yatani, A., J. Codina, A.M. Brown, and L. Birnbaumer. 1987. Direct activation of mammalian atrial muscarinic potassium channels by GTP regulatory protein G_k. *Science.* 235:207–211.

36. Adamson, P.B., S.S. Hull, E. Vanoli, G.M. de Ferrari, P. Wisler, R.D. Foreman, A.M. Watanabe, and P.J. Schwartz. 1993. Pertussis toxin-induced ADP ribosylation of inhibitor G proteins alters vagal control of heart rate in vivo. *Am. J. Physiol.* 265:H734–H740.

37. Jimbo, M., H. Suzuki, M. Ichikawa, K. Kumagai, M. Nishizawa, and T. Saruta. 1994. Role of nitric oxide in regulation of baroreceptor reflex. *J. Auton. Nerv. Syst.* 50:209–219.

38. Sosunov, A.A., C.J.S. Hassall, A. Loesch, M. Turmaine, and G. Burnstock. 1996. Nitric oxide synthase-containing neurones and nerve fibres within cardiac ganglia of rat and guinea-pig: an electron-microscopic immunocytochemical study. *Cell Tissue Res.* 284:19–28.

39. Klimaschewski, L., W. Kummer, B. Mayer, J.Y. Couraud, U. Preissler, B. Philippin, and C. Heym. 1992. Nitric oxide synthase in cardiac nerve fibers and neurons of rat and guinea pig heart. *Circ. Res.* 71:1533–1537.

40. Klimaschewski, L., W. Kummer, and B. Mayer. 1994. Co-localization of nitric oxide synthase with vasoactive intestinal polypeptide and neuropeptide Y in the guinea pig heart. *Cell. Vision.* 1:131–137.