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Peripheral vagal control of heart rate is impaired in neuronal NOS knockout mice

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Choate, J. K., E. J. F. Danson, J. F. Morris, and D. J. Paterson. Peripheral vagal control of heart rate is impaired in neuronal NOS knockout mice. *Am J Physiol Heart Circ Physiol* 281: H2310–H2317, 2001.—The role of nitric oxide (NO) in the vagal control of heart rate (HR) is controversial. We investigated the cholinergic regulation of HR in isolated atrial preparations with an intact right vagus nerve from wild-type (nNOS+/+, $n = 81$) and neuronal NO synthase (nNOS) knockout (nNOS−/−, $n = 43$) mice. nNOS was immunofluorescently colocalized within choline-acetyltransferase-positive neurons in nNOS+/+ atria. The rate of decline in HR during vagal nerve stimulation (VNS, 3 and 5 Hz) was slower in nNOS−/− compared with nNOS+/+ atria in vitro ($P < 0.01$). There was no difference between the HR responses to carbamylcholine in nNOS+/+ and nNOS−/− atria. Selective nNOS inhibitors, vinyl-L-niohydrochloride or 1–2-trifluoromethylphenyl imidazole, or the guanylyl cyclase inhibitor, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one significantly ($P < 0.05$) attenuated the decrease in HR with VNS at 3 Hz in nNOS+/+ atria. NOS inhibition had no effect in nNOS−/− atria during VNS. In all atria, the NO donor sodium nitroprusside significantly enhanced the magnitude of the vagal-induced bradycardia, showing the downstream intracellular pathways activated by NO were intact. These results suggest that neuronal NO facilitates vagally induced bradycardia via a presynaptic modulation of neurotransmission.

nitric oxide; parasympathetic; sinoatrial node

NITRIC OXIDE (NO) is an important signaling molecule in the regulation of vascular resistance and myocardial contraction; however, its role in the cholinergic modulation of cardiac excitability is controversial. Balligand et al. (2) first reported that NO synthase (NOS) inhibition blocked the negative chronotropic effects of cholinergic agonists in spontaneously beating rat neonatal myocytes. It was subsequently found that inhibition of NOS also prevented the cholinergic inhibition of the L-type Ca^{2+} current ($I_{Ca,L}$) in adrenergically prestimulated sinoatrial node cells (13), an effect absent in endothelial NOS knockout mice (12). This has led to speculation that NO plays an obligatory role in the autonomic control of heart rate (13), although others fail to confirm this idea (38) and have shown that endothelial NOS (eNOS) knockout mice exhibit normal autonomic activation of $I_{Ca,L}$ (3, 39). Interpretation of these data are further complicated by the observation that activation of the NO-cGMP pathway can also stimulate the hyperpolarization-activated pacemaking current (I_f) in sinoatrial node myocytes (24).

Data reporting the heart rate response to cholinergic activation using putatively selective neuronal NOS (nNOS) inhibitors and nonisoform-specific NOS inhibitors are as controversial as that for the cellular studies. Inhibition of nNOS causes a dramatic reduction in the vagally mediated bradycardia in the ferret and guinea pig in vivo (6) and a modest effect in the dog (10), whereas others report no significant effect in the guinea pig in vitro (32) or rabbit in vivo (21, 32). However, NOS inhibition slows the rate of decay in heart rate with vagal stimulation in the guinea pig following adrenergic prestimulation (33), suggesting a small modulatory role for NO in the indirect control of vagally mediated bradycardia (i.e., accentuated antagonism). Increasing the bioavailability of NO with NO donors or cGMP analogs enhances the drop in heart rate caused by vagal nerve stimulation (VNS) in vitro and in vivo (34). This effect is not mimicked by bath-applied acetylcholine (ACh), suggesting that NO may act presynaptically (16, 34).

The degree of variability between many studies may be related to the lack of specificity of some NOS inhibitors for the nNOS isoform (29), significant differences in NOS protein expression due to the developmental state of the animal (16), and additional actions of NOS inhibitors in vivo on vascular resistance and baroreflex activation. Therefore, the aims of this study were twofold. First, to localize nNOS in the vagal innervation of the sinoatrial node region of the adult mouse heart. Second, to determine whether the heart rate response to VNS in the isolated atria is impaired in the nNOS knockout mouse compared with its wild-type control.

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Some of these results have been communicated in abstract form (5).

MATERIALS AND METHODS

Animals

All experiments were performed in accordance with Home Office license requirements (PPL 30/16060, Queen Anne's Gate, London, UK) and the United Kingdom's Animals (Scientific Procedures) Act 1986. Mice homozygous for targeted disruption of the nNOS gene (B6,129-NOS1tm1plh, nNOS−/−) (15) were purchased from Jackson Laboratories (Bar Harbor, Maine). Because the nNOS−/− came from a mixed background, we used the C57BL/6J as our homozygous wild-type control (nNOS+/+) (e.g., Refs. 18 and 41). Animals were genotyped postmortem from tail clippings. DNA extracted from the tail clippings was amplified in a PCR reaction, with the use of one pair of oligonucleotide primers complimentary to part of the neomycin resistance sequence (present in nNOS−/− and nNOS+/−) and a second pair complimentary to part of the nNOS gene sequence (present in nNOS+/+ and nNOS+/− mice). Amplified DNA products were then identified by agarose gel electrophoresis and used to determine the mouse genotype.

Adult (3–4 mo old) male mice were used in all experiments.

Anatomy

Tissue processing. Animals (10 nNOS+/+, 4 nNOS−/−) were terminally anesthetized (pentobarbitone ip) and then fixed by perfusion of the left ventricle with 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 mol/l phosphate buffer solution (PBS, pH 7.1, 20 min). The mouse sinoatrial node has been anatomically located near the crista terminalis at the junction between the superior vena cava and the intercaval region of the right atrium (26). To isolate the sinoatrial node region, the posterior aspect of the right atrium was dissected free. To provide a positive control for the nNOS antibody, slices of the brain (40 μm thick) were prepared by vibratome. The tissues were treated for immunohistochemistry as has been previously described (40).

Immunohistochemistry. After processing was completed, tissue (nNOS+/+, $n = 5$, nNOS−/−, $n = 2$) was incubated in primary antiserum against nNOS (raised in sheep against whole recombinant rat nNOS, 1:400 dilution, a gift from Dr. P. C. Emson and Dr. I. Charles) for 12 h. The tissue was then washed in 1% chicken egg albumin-PBS solution (30 min), incubated in a biotinylated secondary antiserum (2 h, 1:200 dilution), and then immersed in an avidin-biotin peroxidase complex (1 h). Immunoreactivity was revealed by the chromogenic substrate diaminobenzidene with hydrogen peroxide. As a negative control, tissues (nNOS+/+, $n = 2$) were treated without primary antiserum.

To colocalize nNOS and choline acetyltransferase (ChAT), a marker for parasympathetic neurons, atrial tissue ($n = 3$, nNOS+/+ and $n = 2$, nNOS−/−) was incubated with primary antisera against nNOS (1:400) and ChAT (dilution 1:250, raised in goats, obtained from Vector Laboratories). Rhodamine-conjugated anti-goat (1:200) and fluorescein-conjugated anti-sheep (1:200) were used as secondary antisera. Immunoreactivity for ChAT was viewed using 565 nm/590 nm filters and for nNOS using 485 nm/520 nm filters and a laser-scanning confocal microscope (Leica).

Physiology

Mouse atrial preparation. Mice ($n = 73$ nNOS+/+, $n = 39$ nNOS−/−) were killed by cervical dislocation. The thorax and mediastinum were removed, placed in mouse physiological saline (mmol/l: 118 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 0.5 Na_2EDTA, 1.2 KH_2PO_4, 25 $NaHCO_3$, 11 glucose, and 1.75 $CaCl_2$, pH 7.4), and aerated with carbogen (95% O_2-5% CO_2) at room temperature (~22°C). A double atrial-right vagal preparation was dissected free and placed into an organ bath (5 ml vol) maintained at 37 ± 0.5°C. The preparation was attached to a silicone resin base in the bath via fine pins inserted into the inferior vena cava and the pericardium below the right atrium. A silk suture (0.6 μm diameter, Mersilk) was placed into the left auricle, and this was attached to an isometric force transducer (F30, Hugo Sachs Electronik). The force response (mN) was amplifed, and data were acquired (150 Hz sampling rate) with a Power Macintosh 7500 computer using a Biopac MP100 data acquisition system and Acknowledge 3.5 software. Heart rate was triggered from the upstroke of each contraction. The right vagus was tied onto a pair of fine silver electrodes connected to a stimulator, and the preparation was left to equilibrate (45 min) until the heart rate did not alter by >5 beats/min for 20 min. Postequilibration, the vagus was stimulated at 10 V, 1 ms pulse width, 3 Hz for 30 s at 2- to 3-min intervals, and experimental protocols were commenced when three consistent consecutive vagal heart rate responses were produced.

In Vitro Protocols

Vagal nerve stimulation. The changes in heart rate with right vagal stimulation at 3 and 5 Hz (10 V, 1 ms duration, 30 s, in random order) were determined. These were measured as the difference in average baseline heart rate between a 5-s period before the onset and offset of nerve stimulation. The average of three heart rate responses was determined for each stimulation frequency. To calculate any differences in vagal bradycardia over the time course of nerve stimulation, the time taken to reach 50% of the maximum heart rate response to vagal stimulation ($TT_{50\%}$) was also calculated.

Bath-applied carbamylcholine. To assess changes in the postsynaptic regulation of heart rate, the chronotropic responses to the cumulative bath application of the ACh analog, carbamylcholine (CCh) (0.01–100 μmol/l) were investigated. The responses to the doses were measured in the same way as with VNS and used to calculate the the concentration that produced a half-maximal response (IC_{50}) for heart rate responses to CCh.

EFFECTS OF NNOS GENE KNOCKOUT ON HEART RATE RESPONSES. Heart rate responses to VNS at 3 and 5 Hz were compared in nNOS+/+ ($n = 56$) and nNOS−/− ($n = 39$) atria, and IC_{50} values were calculated for the response to bath-applied CCh.

EFFECTS OF NOS AND GUANYLYL CYCLASE INHIBITION ON HEART RATE RESPONSES. 1-(2-Trifluoromethylphenyl)imidazole (TRIM, 100 μmol/l; nNOS+/+, $n = 8$; nNOS−/−, $n = 8$) or vinyl-L-niohydrochloride (L-VNIO, 100 μmol/l; nNOS+/+, $n = 8$; nNOS−/−, $n = 8$) were used as selective inhibitors of nNOS. TRIM and L-VNIO were chosen because they have different specificities for nNOS over eNOS and have a different mechanism of inhibition {TRIM: $IC_{50[nNOS]} = 27$ μmol/l; $IC_{50[eNOS]} = 1.06$ mmol/l (14). L-VNIO: inhibitory constant for nNOS ($K_{i,[nNOS]}$) = 90 nmol/l; $K_{i,[eNOS]} = 12$ μmol/l (1)}. nNOS inhibitors were equilibrated for 20 min and tested on control responses to VNS at 3 and 5 Hz. The substrate for NOS enzymes L-arginine (1 mmol/l, 20 min equilibration, Sigma) was used to reverse the effect of nNOS inhibition.

Higher concentrations of L-arginine were avoided because they can change baseline heart rate by affecting the pH of the Tyrode's solution (25). To investigate the proposed presynaptic action (16) of the nNOS inhibitors, the effects of TRIM on the chronotropic responses to different doses of carbamylcholine (0.01–100 μmol/l) were also investigated.

Also, to establish whether the effects of NOS inhibition were likely to be via the cGMP pathway, the effect of the soluble guanylyl cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a] quinoxalin-1-one (ODQ, 10 μmol/l, 40 min equilibration, Calbiochem Novabiochem) on the vagal bradycardia (3 and 5 Hz stimulation) were determined (nNOS+/+, $n = 6$).

Time control experiments (nNOS+/+, $n = 4$) were performed to ensure that changes in the responses during equilibration for the drugs were not due to time-dependent rundown.

EFFECTS OF AN NO DONOR. An NO donor was used to bypass the endogenous production of NO to determine whether the downstream signaling pathway was intact in nNOS−/− atria. Low doses of sodium nitroprusside (SNP, 10 μmol/l) release NO and stimulate guanylyl cyclase, rather than causing nitrosylation or the generation of superoxide radicals, which is a property of many other donors (30). The effects of SNP on the vagal bradycardia (1, 3 and 5 Hz stimulation) were therefore investigated in nNOS+/+ ($n = 10$) and nNOS−/− ($n = 7$) atria.

EFFECTS OF ATROPINE AND PROPRANOLOL ON BASELINE HEART RATE. To assess whether background release of ACh or norepinephrine made significant contributions to the heart rates recorded in atria, doses of 0.1 μmol/l atropine and 0.1 μmol/l propranolol were added at the end of each experiment to block cardiac muscarinic and β-adrenergic receptors, respectively (nNOS+/+, $n = 9$; nNOS−/−, $n = 4$).

EFFECTS OF BLOCKADE OF I_F ON BASELINE HEART RATE. From the evidence that NO can activate I_f via production of cGMP (24), we assessed whether blockade of I_f in the nNOS−/− atria had a smaller effect on baseline heart rate. Cs^+ (2 mmol/l) can produce complete pharmacological blockade of I_f in isolated sinoatrial node cells (8) and was therefore administered at this dose in nNOS+/+ ($n = 8$) and nNOS−/− ($n = 8$) atria.

Statistics

Data are presented as means ± SE. All data were normally distributed. Between-group comparisons were performed by one-way ANOVA. Comparisons among multiple groups were performed by repeated-measures ANOVA (one or two factor) where appropriate, followed by Dunn's multiple comparison post hoc tests. Statistical significance was accepted at $P < 0.05$.

Drugs

The NOS inhibitors and ODQ were obtained from Calbiochem Novabiochem (Nottingham, UK). Carbamylcholine, SNP, and atropine were obtained from Sigma. All solutions were made up immediately before use.

RESULTS

There were no differences in the body weights of nNOS+/+ (27 ± 1 g; $n = 56$) and nNOS−/− (26 ± 1 g; $n = 39$) mice. Similarly, the ventricular weight-to-body weight ratios were not different for nNOS+/+ (5.56 ± 0.33 mg/g) and nNOS−/− (5.41 ± 0.39 mg/g) mice.

Localization of nNOS in Mouse Right Atrium and Hypothalamus

nNOS-positive neurons were found in the sinoatrial node region of the right atrium ($n = 8$, nNOS+/+). These neurons were all unipolar, with an unstained nucleus and a thin axon (see Fig. 1*A*). At the light and electron microscope level there was no evidence of nNOS staining within the atrial myocytes. Fluorescent

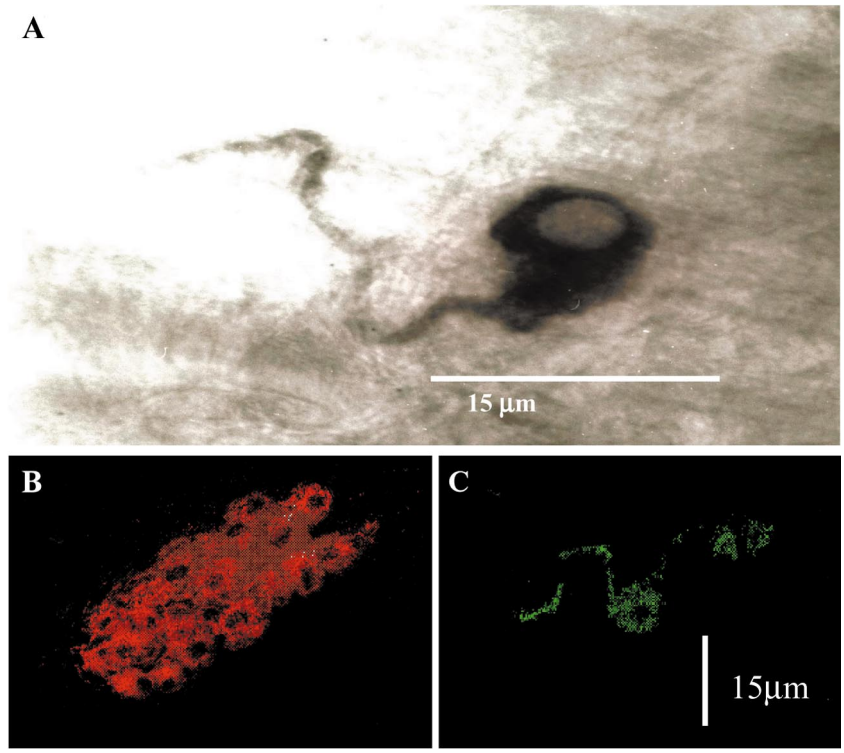

Fig. 1. *A*: diaminobenzidine-stained neuronal nitric oxide synthase (nNOS)-immunopositive neuron found in intercaval region of right atrium ($n = 6$). *B*: double-labeled tissue from intercaval region showing choline acetyltransferase (ChAT)-positive intrinsic ganglion (red/rhodamine labeled). *C*: nNOS-positive neurons (green/fluorescein labeled) colocalize within the ganglion.

staining for ChAT was revealed in neuronal cell bodies of cardiac ganglia (see Fig. 1*B*) as well as within nerve fibers coursing throughout the atrium. In 16% of the ChAT-positive neuronal cell bodies in 20 different ganglia, nNOS was colocalized (see Fig. 1*C*).

In nNOS+/+ mice ($n = 3$), nNOS stained positive in the neurons of the supraoptic nucleus, but as expected (40), not in neurons of the adjacent preoptic area of the hypothalamus. This observation was used as a positive control for nNOS staining because it is consistent with the results obtained by other investigators (28, 40). No positive staining for nNOS was found in atrial or hypothalamic tissue from nNOS−/− mice ($n = 4$) or in nNOS+/+ tissue ($n = 2$) in which primary antiserum was omitted from the immunostaining protocol.

Effects of nNOS Gene Knockout on Heart Rate Responses

nNOS−/− atria had significantly higher baseline heart rates (360 ± 7 beats/min, $n = 39$) than the nNOS+/+ atria (322 ± 6 beats/min, $n = 56$) (one-way ANOVA, $P < 0.01$).

At both 3 and 5 Hz stimulation frequency, the average rates of decline in the heart rate response to VNS (measured as the $TT_{50\%}$) were significantly slower in the nNOS−/− ($n = 39$) compared with the nNOS+/+ ($n = 56$) atria (one-way ANOVA, $P < 0.01$) (see Fig. 2).

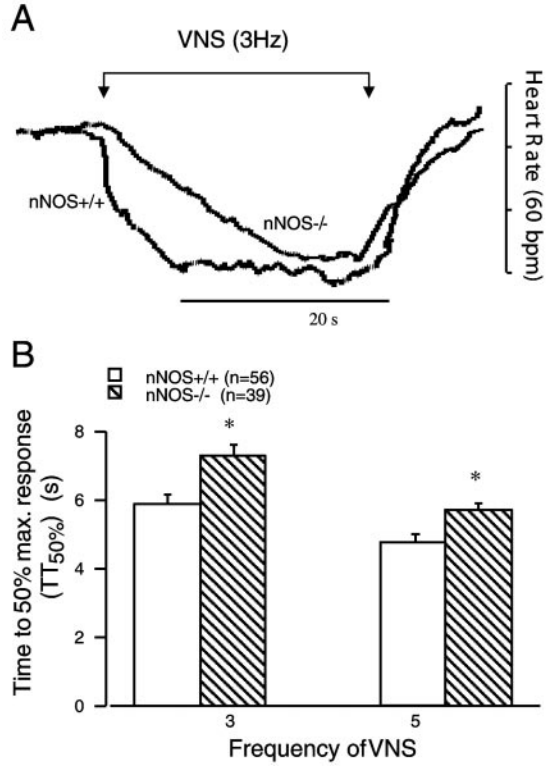

Fig. 2. *A*: raw data traces showing different transient rates of decrease in heart rate with in nNOS wild-type (+/+, $n = 56$) and nNOS knockout (−/−, $n = 39$) atria with vagal nerve stimulation (VNS) at 3 Hz. *B*: histogram showing nNOS−/− atria have a significantly delayed decrease in heart rate with VNS at 3 and 5 Hz compared with nNOS+/+ atria (means ± SE, *$P < 0.05$, nNOS−/− vs. nNOS+/+).

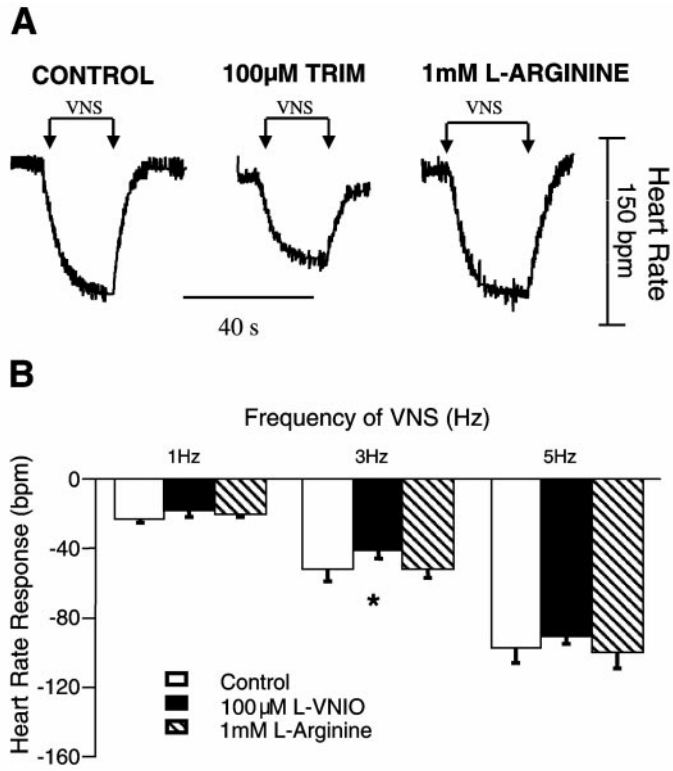

Fig. 3. *A*: raw data trace from nNOS+/+ atria showing the decrease in heart rate with VNS at 3 Hz before and after inhibition of nNOS with 100 μM 1-(2-trifluoromethylphenyl)imidazole (TRIM) and reversal with 1 mM L-arginine ($P < 0.05$, $n = 9$). *B*: histogram showing a significant decrease of vagal-induced bradycardia at 3 Hz VNS in nNOS+/+ atria after inhibition of nNOS with vinyl-1-niohydrochloride (L-VNIO) and reversal with L-arginine (means ± SE, *$P < 0.05$, $n = 8$).

In contrast, the heart rate responses to carbamylcholine (0.01–100 μmol/l) were not different for the nNOS+/+ ($n = 16$; $IC_{50} = 6.00 \pm 0.37$ μmol/l) and nNOS−/− ($n = 12$; $IC_{50} = 6.37 \pm 0.39$ μmol/l) atria.

Effects of NOS and Guanylyl Cyclase Inhibition on Heart Rate Responses

Inhibition of nNOS with TRIM (100 μM) or L-VNIO (100 μM) significantly attenuated the magnitude of the decrease in heart rate with vagal stimulation at 3 Hz in nNOS+/+; an effect reversed by excess L-arginine (see Fig. 3, Table 1). All NOS inhibitors used had no significant effect on the response to VNS in nNOS−/− atria (Table 1).

Inhibition of soluble guanylyl cyclase with ODQ also significantly attenuated the vagal heart rate response (3 and 5 Hz) in nNOS+/+ atria ($n = 6$) (see Fig. 4).

TRIM had no significant effect on the chronotropic responses to bath-applied carbamylcholine (0.01–100 μmol/l), suggesting that NO was working on a presynaptic pathway (see Fig. 5).

Time-control experiments ($n = 4$) on nNOS+/+ atria confirmed that the baseline heart rate, vagal bradycardia, and $TT_{50\%}$ values (3 Hz, 30 s) were not altered over a 40-min experimental period (baseline heart rate: 0 min = 339 ± 22 beats/min, 20 min = 326 ± 19 beats/

Table 1. *nNOS inhibition attenuates HR response to VNS in nNOS+/+ but not nNOS−/− atria*

	L-VNIO				Trim			
	nNOS+/+		nNOS−/−		nNOS+/+		nNOS−/−	
	3 Hz	5 Hz	3 Hz	5 Hz	3 Hz	5 Hz	3 Hz	5 Hz
Control	58.4 ± 10.3	99.6 ± 11.7	64.5 ± 13.4	111.3 ± 16.7	60.5 ± 18.2	89.8 ± 12.2	67.1 ± 10.0	110.3 ± 14.5
Inhibitor	41.6 ± 9.5*	88.0 ± 9.1	59.0 ± 11.8	97.7 ± 12.5	42.4 ± 10.1*	84.3 ± 11.1	58.5 ± 8.7	96.9 ± 11.3
L-Arginine	53.2 ± 8.8	100.3 ± 14.7	59.3 ± 10.8	100.4 ± 12.4	55.9 ± 14.4	97.3 ± 12.1	54.5 ± 8.7	97.5 ± 11.6

Values are means ± SE (in beats/min); $n = 8$. L-VNIO, vinyl-L-niohydrochloride; TRIM, HR, heart rate; nNOS, neuronal nitric oxide synthase; +/+, wild type; −/−, knockout. *$P < 0.05$, one-way repeated-measures ANOVA.

min, 40 min = 323 ± 20 beats/min. Decrease in heart rate with VNS at 3 Hz: 0 min = −64 ± 5 beats/min, 20 min = −67 ± 7 beats/min, 40 min = −61 ± 2 beats/min. $TT_{50\%}$: 0 min = 5.47 ± 1.51 s, 20 min = 6.44 ± 0.88 s, 40 min = 5.93 ± 0.80 s).

Effect of an NO Donor

The NO donor SNP (10 μmol/l) significantly enhanced the magnitude of the heart rate responses to vagal stimulation (see Fig. 6). This effect was similar in magnitude in nNOS+/+ ($n = 10$) and nNOS−/− atria ($n = 7$).

Effects of Atropine and Propranolol on Baseline Heart Rate and Responses

All heart rate responses to VNS and bath-applied CCh were abolished by 0.1 μmol/l atropine, confirming their action through cardiac muscarinic receptors. In addition, atropine significantly increased baseline heart rates, suggesting that there was a small amount of background release of ACh from terminals of the sectioned nerve. Furthermore, the tachycardia induced by atropine was less in nNOS−/− (increase in heart rate of 20 ± 1 beats/min or 5.4 ± 0.1%) compared with nNOS+/+ (26 ± 2 beats/min or 8.0 ± 0.1%) atria ($P <$ 0.05, one-way ANOVA), indicating that background release of ACh from vagal terminals may be impaired in nNOS−/− atria. However, heart rates of nNOS−/− atria remained significantly elevated compared with the nNOS+/+ atria following muscarinic receptor inhibition. Propranolol (0.1 μmol/l) had no effect on heart rates.

Effects of Blockade of I_f on Baseline Heart Rate

As expected (8), heart rate was significantly reduced after blockade of I_f with 2 mmol/l Cs^+ in both nNOS+/+ (from 322 ± 6 beats/min to 249 ± 9 beats/min, $n = 8$) and nNOS−/− (from 361 ± 7 to 286 ± 14, $n = 8$) atria. There was no statistical difference between the responses to Cs^+ in nNOS+/+ and nNOS−/− atria (one-way ANOVA), indicating that the contribution of I_f to the spontaneous beating rate was unchanged in nNOS−/− compared with nNOS+/+ atria.

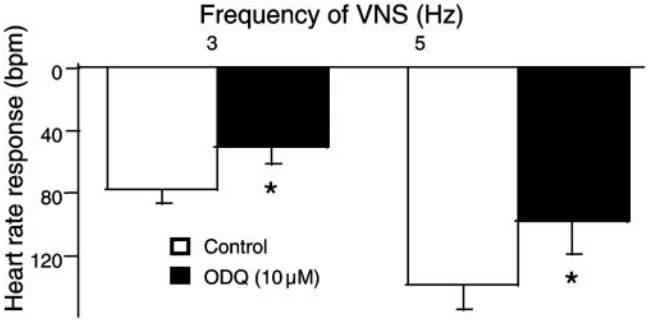

Fig. 4. Graph of the decrease in heart rate with VNS at 3 and 5 Hz before and after inhibition of soluble guanylyl cyclase with 10 μM 1H(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one (ODQ) showing a significant attenuation of vagal-induced bradycardia in nNOS+/+ (means ± SE, *$P < 0.05$, $n = 6$).

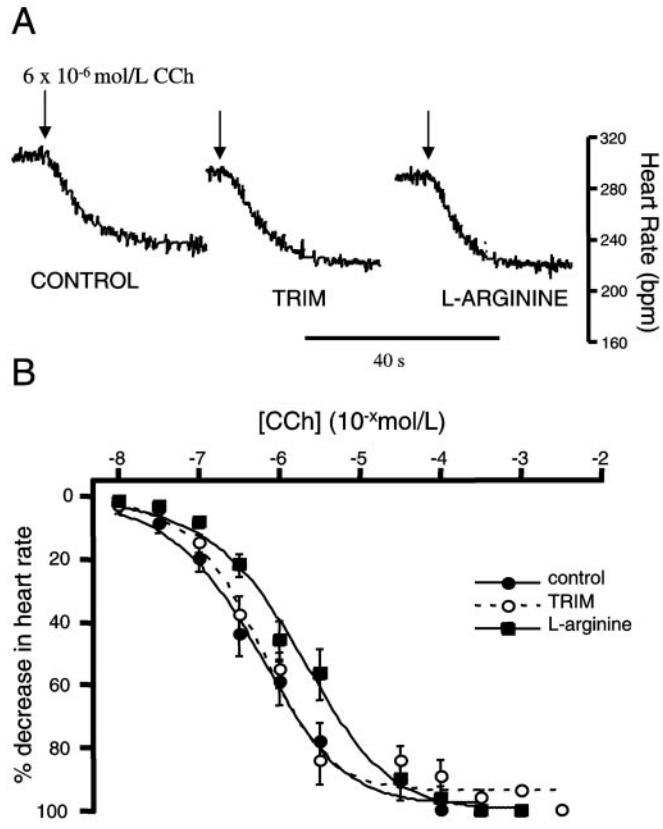

Fig. 5. *A*: raw data trace from nNOS+/+ atria showing the decrease in heart rate with the IC_{50} dose of carbamylcholine (CCh). There is no change in this response with 100 μM TRIM or 1 mM L-arginine. *B*: log dose-response curve for CCh before and after addition of TRIM or L-arginine ($n = 8$).

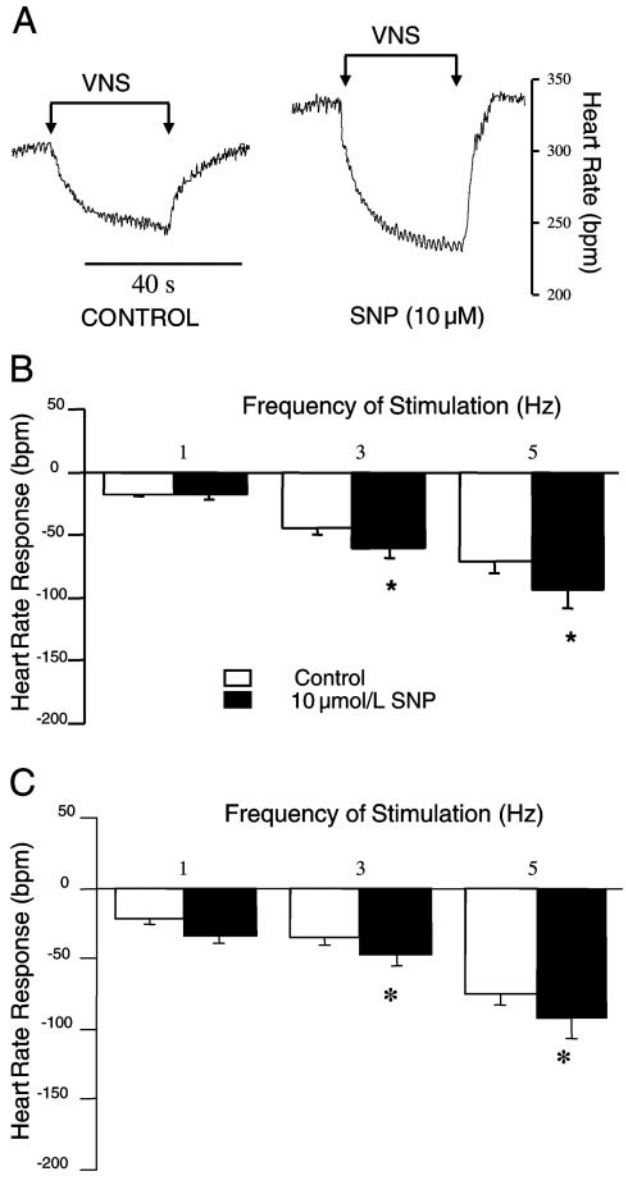

Fig. 6. *A*: raw data trace from nNOS+/+ atria showing the effects of 10 μM sodium nitroprusside (SNP) on heart rate and response to VNS at 3 Hz. *B*: graph showing significant increase in vagal-induced bradycardia with VNS at 3 and 5 Hz after addition of SNP in nNOS+/+ atria (means ± SE,*$P < 0.05$, $n = 10$). *C*: graph showing that the response to SNP in nNOS−/− atria (means ± SE,*$P < 0.05$, $n = 7$) is the same as that in nNOS+/+ atria.

DISCUSSION

The novel findings of this study are the following: *1*) in mouse atria, nNOS is coexpressed only in ChAT-positive neurons innervating the sinoatrial node region of the right atrium; *2*) nNOS−/− atria have significantly slower HR responses to VNS compared with nNOS+/+ atria; 3) HR responses to VNS are attenuated by nNOS inhibition in nNOS+/+, but not in nNOS−/− atria; *4*) responses to CCh are unaffected by nNOS inhibition in nNOS+/+ atria, suggesting the vagal bradycardia is modulated by NO via a presynaptic mechanism; and *5*) increasing NO production with a NO donor enhances vagal-induced bradycardia in both nNOS+/+ and nNOS−/−, showing that the downstream NO-cGMP pathway is intact in nNOS−/− atria.

Location of nNOS in Mouse Atria

We have identified for the first time positive staining for nNOS within intrinsic neurons innervating the sinoatrial node region of the mouse heart. These were the only cells that stained immunopositive for nNOS in the whole mount preparation of the right atrium. No cross-reactivity of the nNOS antibody for other NOS isoforms has been observed using this antibody (28). Immunoelectron microscopy has been able to identify nNOS in the cardiac sarcoplasmic reticulum of ventricular myocytes (42); however, sarcoplasmic reticulum staining was not evident in this study at the electron microscopic level, though size and prevalence of the sarcoplasmic reticulum may be less in atrial tissue.

Other immunohistochemical studies have reported nNOS staining in intrinsic neurons of the atria in the rat (31, 35) and guinea pig (35, 36). Vagotomy studies in the guinea pig suggest preganglionic vagal fibers terminate on these neurons (37). Furthermore, Mawe et al. (22) found that in guinea pig atria, all of the cardiac neurons containing nNOS also stained for ChAT. However, these data suggested that only 5% of the population of ChAT cell bodies were nitrergic. From the reports that 37% of right atrial ganglia label positive for NADPH diaphorase, a marker for nNOS (19), 5% may be an underestimate. Also, recent evidence suggests that many of the commercially available nNOS antibodies have a limited specificity (20), and poor penetration of antisera into intact atrial tissue may impair the visualization of nNOS-positive staining. Using laser-scanning confocal microscopy, our estimation that 16% of cholinergic neurons are nitrergic is based on counts of double-labeled neurons at different focal points within the tissue in 20 different ganglia.

Effects of nNOS Gene Knockout

We have shown that isolated nNOS−/− atria, devoid of circulating factors and reflexes, have an elevated heart rate compared with nNOS+/+ atria. This finding is consistent with data from the conscious and anesthetized nNOS−/− mice (18). This previous study also demonstrated that the nNOS−/− mouse has a blunted response to atropine, suggesting an impaired vagal tone. The small positive chronotropic response to atropine in the nNOS−/− compared with nNOS+/+ atria in vitro is also consistent with the idea that background release of ACh from nerve terminals may be impaired in nNOS−/− atria. However, a reduction in peripheral parasympathetic nerve activity alone was unable to account for the elevated heart rates in nNOS−/− compared with nNOS+/+. Further experiments with propranolol and the sinoatrial node I_f inhibitor, Cs^+ indicated that the activation of β-adrenoceptors or I_f was unlikely to contribute to the elevated

heart rate in the nNOS−/− atria. This suggests that other ionic pacemaking currents may be upregulated in nNOS−/− atria.

Upon vagal nerve stimulation at 3 and 5 Hz, the rate of decrease in heart rate was significantly slowed in nNOS−/− compared with nNOS+/+ atria. The effect of nNOS gene knockout is therefore similar to that of pharmacological NOS inhibition in the isolated guinea pig atria [see Sears et al. (33)]. Sears et al. also demonstrated that transient decreases in HR can be artificially accelerated by inhibition of I_f or slowed by inhibition of $I_{Ca,L}$. Direct evidence for these possibilities comes from experiments on isolated sinoatrial node cells showing that postsynaptically, NO-cGMP can activate both I_f (24) and inhibit $I_{Ca,L}$ (13).

However, postsynaptic muscarinic (M_2)-receptor-coupled NO-cGMP-dependent modulation of HR may not be as physiologically important in the vagal control of heart rate as was first thought, because we saw no effect of NOS inhibitors on the bradycardia induced by bath-applied CCh in nNOS+/+ atria. Similar results have been reported from experiments on guinea pig atria (16, 34).

Bypassing endogenous NOS and amplifying NO production enhanced the HR response to VNS, showing that the downstream NO signaling mechanism was intact in nNOS−/− and nNOS+/+ atria. In isolated guinea pig atria, NO donors were without effect on the HR responses to CCh (16, 34). Inhibition of protein kinase A, a mediator through which NO-cGMP may act (23), diminishes the effect of SNP on vagally induced bradycardia (16). Conversely, inhibition of phosphodiesterase III, which prevents the breakdown of cAMP, mimics the effect of SNP (17). This suggests that SNP may work by activating cAMP-dependent protein kinase A and analogs of cAMP as well as activators of adenylate cyclase have been shown to increase exocytotic release of ACh in mouse atria (7). Furthermore, NO donors have recently been reported to increase the release of radiolabeled ACh from nerve terminals during field stimulation in isolated guinea pig atria; an effect abolished by inhibition of guanylyl cyclase (17).

Effects of NOS Inhibition on Vagal Modulation of Heart Rate

Previous studies investigating the effects of NOS inhibition on the vagal control over cardiac function have produced inconsistent results (6, 10, 16, 21, 32, 33). Here we present evidence showing consistent attenuation of vagal bradycardia using two different selective nNOS inhibitors. For both compounds, this was only seen when the nerve was stimulated at 3 Hz, although a nonsignificant trend was observed at 5 Hz. Significant attenuation of responses by NOS inhibitors have been revealed at these stimulation frequencies in the majority of other studies of this kind (16). Our finding is therefore consistent with the idea that pathways involving NOS have a more visible role in pacemaking when cholinergic activation is low (18) and when activation of the acetylcholine-activated potassium current (I_{KACh}), on which NOS inhibitors have no effect (13), is minimized.

Nonspecific constitutive NOS inhibition has only a small modulatory effect on the transient bradycardia in guinea pig atria in vitro at 3 Hz vagal stimulation (33). Though it is known that the mechanism of inhibition of the constitutive NOS isoforms by N^G-nitro-L-arginine (L-NMMA) is different, it is not known whether this drug is more isoform specific for eNOS than was previously thought (29). Selective nNOS inhibitors L-VNIO or TRIM caused a more pronounced inhibition of the vagal bradycardia than L-NMMA did in guinea pig atria (33). TRIM binds to L-arginine and tetrahydrobiopterin sites (14), whereas L-VNIO binds to L-arginine and hemecofactor sites (1). L-VNIO may be a more potent inhibitor of nNOS than TRIM, but we found no differences between the decrease in response to nerve stimulation when using either drug, suggesting that we achieved complete inhibition in both cases. More importantly, however, all effects were reversed with excess L-arginine. In addition, NOS inhibitors caused no change in the HR response to bath-applied CCh and had no effect on the response to VNS in nNOS−/− atria. Our results therefore suggest that these drugs work presynaptically and that they are specific for nNOS.

The vagal HR responses after NOS inhibition were quantitatively smaller compared with the responses in the nNOS knockout atria. This could either reflect the nonspecificity of the NOS inhibitors, although all responses were reversed by L-arginine, or that acute administration of pharmacological inhibitors may demonstrate more pronounced effects than gene knockout models, where compensatory changes might be brought about by gene deletion. Evidence suggests that signaling cascades involving atrial natriuretic peptide, which is also coupled to cGMP production (11), and inhibitory G proteins (9) may compensate for redundant NO signaling in transgenic mouse hearts.

In conclusion, we present anatomic and pharmacological evidence that implicates a modulatory role for neuronally derived NO in the peripheral presynaptic facilitation of vagal bradycardia.

The functional differences resulting from the genetic knockout of neuronal NOS support this hypothesis.

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