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Pre-synaptic NO-cGMP Pathway Modulates Vagal Control of Heart Rate in Isolated Adult Guinea Pig Atria

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N. Herring, S. Golding and D. J. Paterson. Pre-synaptic NO-cGMP Pathway Modulates Vagal Control of Heart Rate in Isolated Adult Guinea Pig Atria. *Journal of Molecular and Cellular Cardiology* (2000) **32**, 1795–1804. The role of nitric oxide (NO) in the vagal modulation of heart rate (HR) is controversial. We tested the hypothesis that NO acts via a pre-synaptic, guanylyl cyclase (GC) dependent pathway. The effects of inhibiting NO synthase (NOS) and GC were evaluated in isolated atrial/right vagal nerve preparations from adult (550–750 g) and young (150–250 g) female guinea pigs. Levels of NOS protein were quantified in right atria using Western blotting and densitometry. The non-specific NOS inhibitor N-ω-nitro-L-arginine (L-NA, 100 μM, $n=5$) significantly reduced the negative chronotropic response to vagal nerve stimulation (VNS) at 3 and 5 Hz in the adult guinea pig. This effect was reversed with 1 mM L-arginine. Similar results were observed with the specific neuronal NOS inhibitor vinyl-N5-(1-imino-3-butenyl)-L-ornithine (L-VNIO, 100 μM, $n=7$). Inhibition of GC with 1H-(1,2,4)-oxadiazolo-(4,3-a)-quinoxalin-1-one (ODQ, 10 μM, $n=7$) also significantly reduced the negative chronotropic response to VNS at 3 and 5 Hz in adult guinea pigs. Neither L-NA ($n=6$), L-VNIO ($n=5$) nor ODQ ($n=6$) changed the HR response to cumulative doses of carbamylcholine in adult guinea pig atria suggesting that the action of NO is pre-synaptic. The HR response to VNS was unaffected by L-NA ($n=7$) or ODQ ($n=7$) in young guinea pigs and Western blot analysis showed significantly lower levels of nNOS protein in right atria from young animals. These results suggest a pre-synaptic NO-cGMP pathway modulates cardiac cholinergic transmission, although this may depend on the developmental stage of the guinea pig.

Key Words: Nitric oxide; Autonomic nervous system; Acetylcholine; Heart rate; Guinea pig.

Introduction

A role for nitric oxide (NO) in the vagal control of heart rate (HR) is controversial. Endothelial nitric oxide synthase (eNOS) has been identified in sino-atrial node cells[1] and neuronal NOS (nNOS) has been immunohistochemically located in para-sympathetic ganglion around the pacemaker of the heart.[2] Han *et al.*[3] proposed a post-synaptic role for NO whereby muscarinic receptor stimulation of eNOS activity is essential for cholinergic inhibition of adrenergically stimulated L-type calcium current (I_{CaL}). However, whilst some groups show that NOS inhibition[4] or eNOS gene knockout[5] abolishes the reduction in I_{CaL} to acetylcholine after prior adrenergic stimulation, others do not observe this.[6,7]

Functionally, inhibition of nNOS has no effect on the HR response to vagal nerve stimulation in the rabbit *in vivo*[8,9] or young guinea pig *in vitro*.[9] However, high concentrations of NO or cGMP are still able to increase the HR response to vagal nerve stimulation in these preparations.[10] This suggests that the pathway by which NO acts is intact despite there being no significant endogenous effect of NO. In contrast, inhibition of nNOS in the adult guinea pig[8] and ferret[11] *in vivo* causes a marked reduction in the vagally mediated bradycardia, although whether neuronal NO acts post-synaptically as a

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co-transmitter or pre-synaptically to control the release of acetylcholine had not been established. One possible explanation for these differences is that the expression of nNOS in the cardiac autonomic nervous system may be related to the developmental stage of the animal as more intra-cardiac ganglia positively stain for NOS in older animals.[2] *v* [12]

Therefore two hypotheses were tested. First, does the functional role of NO in cholinergic modulation of HR depend on the developmental stage of the guinea pig and the expression of the nNOS protein? Secondly, does NO act pre or post-synaptic via a guanylyl cyclase dependent pathway to modulate the vagal control of heart rate?

Methods

Experiments conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), and the Animals (Scientific Procedures) Act 1986 (UK) and were performed under British Home Office Project License PPL 30/1133.

Isolated guinea pig sino-atrial node/right vagus nerve preparation

Young (150–250 g) and adult (550–750 g) female guinea pigs were killed by cervical dislocation and exsanguinated. The thorax was opened and ventricles removed so that heparinized Tyrode's solution (1000 U/ml) could be rapidly perfused into both atria. The heart was removed with the rib cage and mediasternum and placed in a dissecting dish with Tyrode's solution aerated with 95% O_2/5% CO_2 at room temperature. Both lungs and any remaining ventricle were carefully removed and the atria and mediasternum were dissected free from the thorax. The right vagus was carefully separated from the carotid artery and tied. Sutures (Ethicon 5/0 silk) were placed at the lateral edges of the two atria. The preparation was then transferred to a preheated (37 ± 0.2°C), continuously oxygenated, water-jacketed organ bath containing 60 ml of Tyrode's solution. The atria were mounted vertically with the suture in the left atrium attached to a stainless steel hook and the right atrium attached to an isometric force transducer (HDE F30) connected to an amplifier. Data were acquired on a Power Macintosh 8500 computer using a Biopac Systems MP100 data acquisition system and Acqknowledge 3.5 software. Beating rate was triggered from contraction, and the signals displayed in real time. Data were stored on optical disk for off-line analysis.

Solutions and drugs

The Tyrode solution contained (mmol/l) NaCl: 120; KCl: 4; $MgCl_2$: 2; $NaHCO_3$: 25; $CaCl_2$: 2; Na_2HPO_4: 0.1; glucose: 11. The solution was aerated with 95% O_2/5% CO_2 (pH 7.4) and its temperature was continuously monitored (Digitron 1408-K gauge) and kept at 37 ± 0.2°C.

N-ω-nitro-L-arginine (L-NA, 100 μM, Sigma) was used as a commercially available potent inhibitor of nNOS ($IC_{50} = 0.025$ μM for the isolated enzyme[13]) although it can also inhibit eNOS ($IC_{50} = 0.09$ μM). The dose used has previously been shown to significantly reduce the release of norepinephrine[14] and the HR response to sympathetic nerve stimulation[15] in a similar preparation. To investigate the role of nNOS in vagal control of HR, vinyl-N5-(1-imino-3-butenyl)-L-ornithine (L-VNIO, 100 μM, Sigma) which has a similar potency for nNOS as L-NA but is twenty times less potent for eNOS[16] was used. The substrate for the NOS enzyme L-arginine (1 mM, Sigma) was used to reverse the effects of L-NA and L-VNIO. Higher concentrations of L-arginine were avoided as they can change baseline HR by affecting the pH of the Tyrode's solution.[17] In addition, 1H-(1,2,4)-oxadiazolo-(4,3-a)-quinoxalin-1-one (ODQ, 10 μM, Tocris Cookson, UK) was used to inhibit GC. This dose has also been shown to produce full block of the isolated enzyme[18] and reduce the HR response to sympathetic nerve stimulation *in vitro*.[15] The effects of these inhibitors on the HR response to vagal nerve stimulation were also compared to those of the stable analogue of acetylcholine, carbamylcholine chloride (Sigma), to determine if their likely effects on the vagal modulation of HR are pre or post-synaptic.

Drugs were dissolved in reagent grade water from an Elga purification system except ODQ that was dissolved in Dimethylsulphoxide (DMSO). A control experiment showed that DMSO at the concentrations used (<0.02%) did not effect the HR response to vagal nerve stimulation at 1, 3 or 5 Hz.

Protocols

Before starting each protocol, the mounted atria were kept in Tyrode's solution for at least 60 min until their beating rate stabilized (± 5 beats/min over 20 min). The Tyrode's solution in the organ

bath was replaced approximately every 30 min throughout each protocol. The vagus nerve was stimulated at 1, 3 or 5 Hz, 10–15 V, 1 ms pulse duration for 30 s every minute until three consistent responses were obtained. We have previously shown that all heart rate changes from vagal nerve stimulation are completely abolished by hyoscine in this preparation and are therefore due to release of acetylcholine.[19] A control experiment showed that the HR response to vagal nerve stimulation remained constant (± 1 beats/min at 5 Hz) over a 2-h period. Since ODQ is light sensitive, these experiments were carried out in a darkened room.

The effect of the non-specific NOS inhibitor L-NA on the HR response to vagal nerve stimulation or carbamylcholine in adult guinea pigs

To determine if any effect of NOS inhibition in adult guinea pigs was pre or post-synaptic, the HR responses to vagal stimulation and carbamylcholine (100 nM) were evaluated in the same preparation. Carbamylcholine produces a similar degree of bradycardia to 5 Hz vagal nerve stimulation. Immediately after the equilibration period, the HR response to carbamylcholine was measured. When HR had recovered, the vagus nerve was stimulated at 1, 3 and 5 Hz and the preparation was incubated for 20 min with L-NA (100 μM, $n=5$). The vagus nerve was again stimulated at 1, 3, and 5 Hz and the HR response to carbamylcholine evaluated. The preparation was incubated for a further 20 min with L-arginine (1 mM) in the presence of L-NA and the vagus nerve stimulated as before. To investigate more thoroughly whether L-NA could be having a post-synaptic effect, in a separate set of experiments, increasing concentrations of carbamylcholine (50, 100, 150 and 200 nM) were cumulatively added to the organ bath as soon as the HR response to one concentration had stabilized. When HR had recovered the preparation was incubated for 20 min with L-NA (100 μM, $n=6$) and then carbamylcholine was added again in a cumulative manner as before.

The effect of the specific nNOS inhibitor L-VNIO on the HR response to vagal nerve stimulation in adult guinea pigs

L-VNIO was used to investigate the role of nNOS in the vagal control of HR. The vagus nerve was stimulated at 1, 3 and 5 Hz and the preparation was incubated for 20 min with L-VNIO (100 μM, $n=7$). The vagus nerve was again stimulated at 1, 3, and 5 Hz and then the preparation was incubated for a further 20 min with L-arginine (1 mM) in the presence of L-VNIO before stimulating the vagus nerve as before. The HR responses to increasing concentrations of carbamylcholine (50, 100, 150 and 200 nM) were also evaluated before and after nNOS inhibition by a 20 min incubation with L-VNIO (100 μM, $n=5$) in a separate set of experiments.

The effect of the GC inhibitor ODQ on the HR response to vagal nerve stimulation and cumulative doses of carbamylcholine in adult guinea pigs

NO has been shown to produce its effects by stimulating GC to raise levels of cGMP (see Kelly *et al.*[20] for a review). To see if engodenous NO acts via this pathway, the HR responses to vagal nerve stimulation at 1, 3 and 5 Hz were evaluated before and after a 40 min incubation with ODQ (10 μM, $n=7$). Pre-synaptically generated NO may modulate the HR response to vagal nerve stimulation by acting on the acetylcholine release mechanism or by diffusing across the synaptic cleft as a co-transmitter to mediate its GC dependent actions. To investigate this, the HR responses to increasing concentrations of carbamylcholine (50, 100, 150 and 200 nM) were evaluated before and after GC inhibition by a 40 min incubation with ODQ (10 μM, $n=6$).

The effects of L-NA and ODQ on the HR response to vagal nerve stimulation in young guinea pigs

To determine if L-NA or ODQ had any effect on vagal control of HR in young guinea pigs, the vagus nerve was stimulated at 1, 3, and 5 Hz before and after incubating the preparation for 20 min with L-NA (100 μM, $n=7$) or 40 min with ODQ (10 μM, $n=7$).

Protein extraction and Western blotting

Freshly dissected right atria, forebrain and gut were lysed in buffer containing 50 mM HEPES pH 7.4, 1 mM EDTA, 0.5% Triton X-100, and a cocktail of protease inhibitors ("Complete", Roche Diagnostics, UK). Protein concentrations were measured using the Bio-Rad DC protein assay kit. Then 200 μg of total protein was separated on 7.5% SDS-polyacrylamide (SDS-PAGE) gels at a constant 35 mA and resolved proteins transferred to a MSI PVDF membrane (GRI, UK) using a semi-dry transfer cell following manufacturers protocols (Bio-Rad, UK). Membranes were blocked for 12 h in 3% dried milk

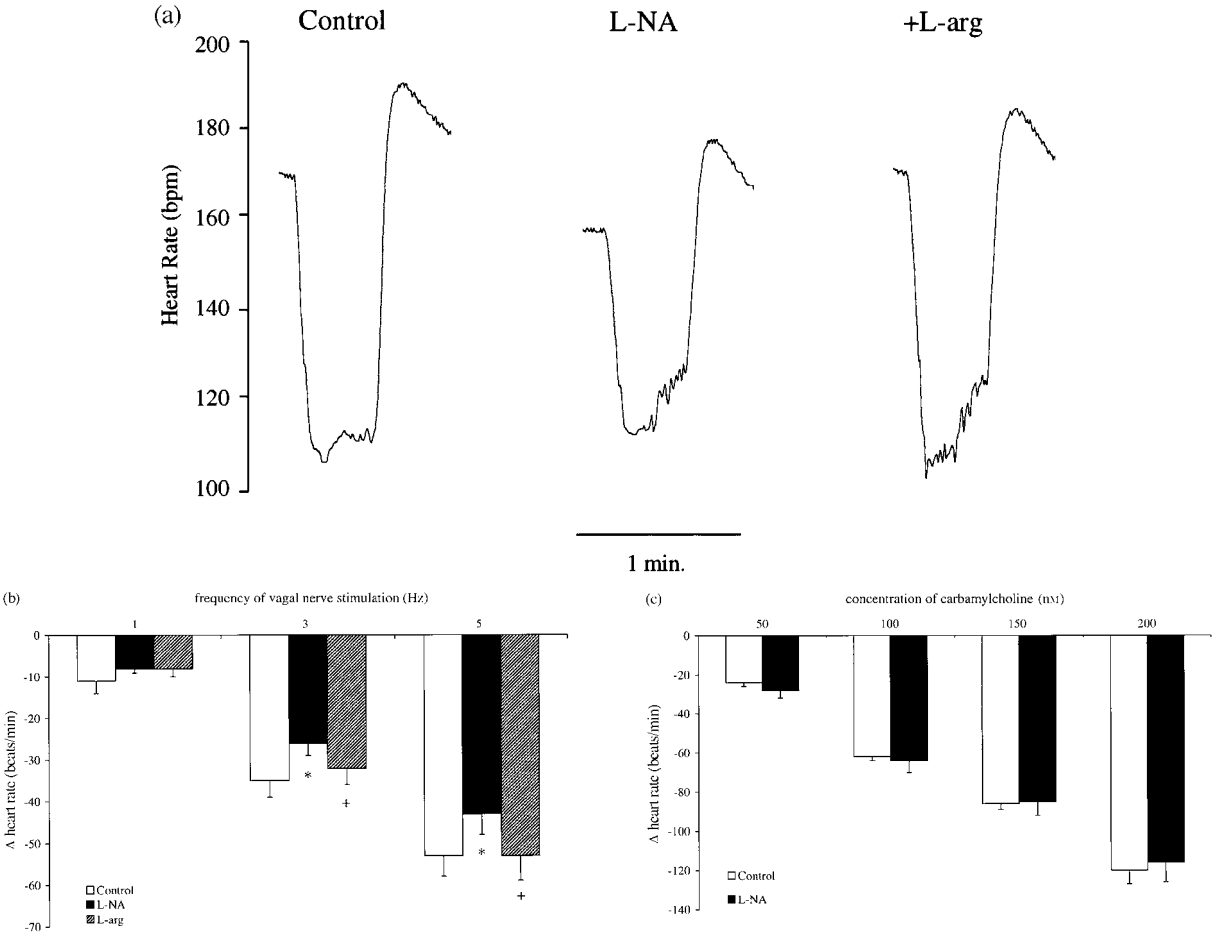

Figure 1 (a) Representative raw data trace showing the effect of NOS inhibition (100 μM L-NA) and its reversal with 1 mM L-arginine on the heart rate response (beats/min) to vagal nerve stimulation (5 Hz, 10 V, 1 ms pulse width, 30 s duration) in a double atrial/right vagal nerve preparation from an adult guinea pig. (b) Frequency response graph for the decrease in heart rate (beats/min) with right vagal nerve stimulation (1, 3 and 5 Hz) in atria from adult guinea pigs ($n=5$). NOS inhibition (100 μM L-NA) significantly attenuated (*$P<0.05$) the negative chronotropic responses at 3 and 5 Hz and this effect was significantly reversed by 1 mM L-arginine (+$P<0.05$). (c) NOS inhibition (100 μM L-NA) had no effect on the heart rate response (beats/min) to cumulative doses of carbamylcholine in adult guinea pig atria ($n=6$).

in PBS/0.1% Tween-20 (PBST) at 4°C, and then incubated for 2 h in PBST containing 1% dried milk powder and primary antibody. Blots were washed three times for 10 min in PBST and incubated with the appropriate secondary HRP-conjugated antibody for 30 min and then washed as before. All incubations were done at room temperature. Antibody-bound proteins were detected using luminol-based chemiluminescent detection reagents ("Renaissance", NEN Life Science, UK) and autoradiography. Images were then digitized for analysis.

Antibodies

Purified polyclonal anti-eNOS (N30030) and monoclonal anti-nNOS (N31020) both obtained from Transduction Labs (via Affiniti UK) were used as the primary antibodies. Secondary horseradish peroxidase (HRP) conjugated anti-mouse IgG was used for Western blot analysis (Amersham plc, UK).

Statistical analysis

Data are presented as mean ± S.E.M. One-way repeated measures ANOVA followed by Tukey's *post hoc* analysis was used to evaluate the effect of an intervention. An unpaired Student's *t*-test was used to evaluate differences in equilibration HR. The HR responses to vagal nerve stimulation and the optical densities of Western blot protein bands between young and adult guinea pigs were also evaluated by an unpaired Student's *t*-test. Statistical significance was accepted at $P<0.05$.

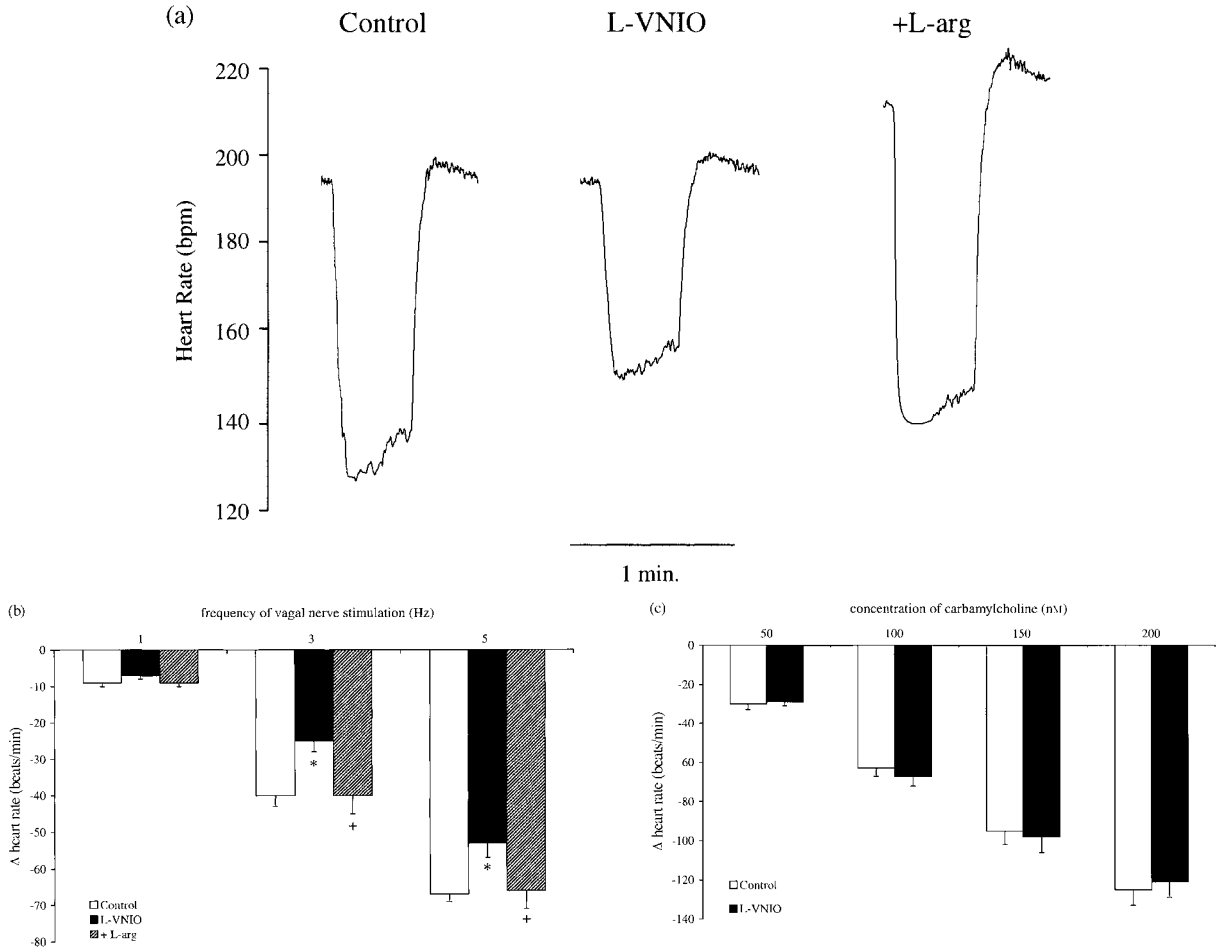

Figure 2 (a) Representative raw data trace showing the effect of neuronal NOS inhibition (100 μM L-VNIO) and its reversal with 1 mM L-arginine on the heart rate response (beats/min) to vagal nerve stimulation (5 Hz, 10 V, 1 ms pulse width, 30 s duration) in a double atrial/right vagal nerve preparation from an adult guinea pig. (b) Frequency response graph for the decrease in heart rate (beats/min) with right vagal nerve stimulation (1, 3 and 5 Hz) in atria from adult guinea pigs ($n=7$). Neuronal NOS inhibition (100 μM L-VNIO) significantly attenuated (*$P<0.05$) the negative chronotropic responses at 3 and 5 Hz and this effect was significantly reversed by 1 mM L-arginine (+$P<0.05$). (c) nNOS inhibition (100 μM L-VNIO) had no effect on the heart rate response (beats/min) to cumulative doses of carbamylcholine in adult guinea pig atria ($n=5$).

Results

After the equilibration period, mean baseline HR was significantly higher in atria from small ($n=14$) compared to large ($n=36$) guinea pigs (211 ± 8 compared to 168 ± 3 beats/min). The decreases in HR (beats/min) to vagal nerve stimulation at all frequencies were similar between young and adult guinea pigs. There was a trend for inhibitors of NOS and GC inhibitors to slightly decrease baseline HR (significant with ODQ in atria from young guinea pigs, 214 ± 11 to 207 ± 10 beats/min, $n=7$) and this was seen equally in atria from small and large guinea pigs. There was also a small, significant increase in baseline HR to L-arginine from 172 ± 8 to 184 ± 7 beats/min ($n=12$). This has previously been shown to be due to L-arginine alkalinizing the pH of the Tyrode's solution.[17]

The effect of NOS and guanylyl cyclase inhibition on the HR response to vagal nerve stimulation or carbamylcholine in adult guinea pigs

L-NA significantly reduced the HR response to vagal nerve stimulation at both 3 and 5 Hz and this effect was reversed by L-arginine [Fig. 1(a and b)]. In the same preparations, L-NA did not effect the response to carbamylcholine. In a further set of experiments,

L-NA had no effect on the HR response to cumulative doses of carbamylcholine [Fig. 1(c)].

The specific nNOS inhibitor L-VNIO also significantly reduced the size of the HR response to vagal nerve stimulation at 3 and 5 Hz and this effect was reversed by L-arginine [Fig. 2(a and b)]. L-VNIO did not change the HR response to cumulative doses of carbamylcholine [Fig. 2(c)]. This suggests that pre-synaptic nNOS modulates the vagal control of HR.

NO generated pre-synaptically may modulate the HR response to vagal nerve stimulation via post-synaptic GC, diffusing across the synaptic cleft and acting as a co-transmitter, or by acting on pre-synaptic GC to modulate acetylcholine release. Inhibiting GC with ODQ significantly reduced the HR response to vagal nerve stimulation at 3 and 5 Hz respectively [Fig. 3(a and b)] but did not change the HR response to cumulative doses of carbamylcholine [Fig. 3(c)], suggesting that NO acts via a pre-synaptic guanylyl cyclase dependent pathway.

The effect of NOS or GC inhibition on the HR response to vagal nerve stimulation in young guinea pigs

Inhibition of NOS with L-NA, or GC with ODQ failed to produce any effect on the magnitude of the HR response to vagal nerve stimulation in young guinea pigs (Table 1).

Western blot analysis of neuronal and endothelial NOS protein levels in right atria from young and adult guinea pigs

Figure 4 shows the results of Western blot analysis of nNOS protein expression, using a specific monoclonal antibody, in right atria from young and adult guinea pigs. The nNOS antibody recognized protein bands at approximately 120 kDa in the total protein sample from both adult and young atria that were not recognized by the eNOS antibody. nNOS of this molecular weight has previously been detected (e.g. in rat ileum[21]). The forebrain sample showed nNOS bands at both 120 and 160 kDa. These blots were exposed to autoradiography film featuring a high resolution and linearity and the blots were analyzed by densitometry using NIH-Image software and an optical density (O.D.) step tablet. The levels of 120 kDa nNOS protein were significantly lower in the young compared to adult atria (young atria, 146 ± 32 O.D., $n = 3$ adult atria, 620 ± 111 O.D., $n = 3$; and brain 120 kDa 162 O.D.). A similar pattern was found in a second blot where adult atria showed distinct 120 kDa bands ($n = 5$), but no detectable bands were seen in young right atria ($n = 4$). We detected very low levels of eNOS protein (140 kDa) in similar quantities in right atria from young ($n = 4$) and adult ($n = 5$) guinea pigs but optical densities were too low to quantify accurately.

Discussion

The main new findings of this study are that: (1) inhibition of nNOS reduced the HR response to vagal nerve stimulation in adult guinea pigs via a pre-synaptic GC dependent pathway; and (2) this effect was not seen in young guinea pigs where right atrial nNOS levels were significantly lower than in adult guinea pigs.

A developmental role for NO in vagal control of heart rate

It has been shown here that whilst potent inhibitors of nNOS and GC reduce the HR response to vagal nerve stimulation at physiological stimulation frequencies (3–5 Hz) in adult guinea pigs, they have no effect in young guinea pigs. A previous report from this laboratory[9] found no effect of nNOS inhibiton on the HR response to vagal nerve stimulation in the young guinea pig *in vitro* whilst others have reported a dramatic reduction in adult guinea pigs *in vivo*.[8] Bypassing endogenous NO and amplifying its production with the NO donor sodium nitroprusside, or the cGMP analogue 8Br-cGMP, increases the HR response to vagal nerve stimulation in young guinea pig atria.[10] Therefore, the intracellular pathway for NO is still intact in young animals despite there being no significant basal NO production.

Our results also show that whilst there were no differences in the levels of eNOS in right atria from young and adult guinea pigs, there were lower levels of nNOS in young guinea pigs. This suggests a possible molecular mechanism for the different effects of NOS and GC inhibitors on the HR response to vagal nerve stimulation in young and adult animals. The monoclonal nNOS antibody identified a 120 kDa band in atria that was not recognized by the eNOS antibody. A 120 kDa band was also detected in guinea pig forebrain as well as a 160 kDa band. Brain nNOS was originally identified as a 160 kDa protein,[22] but several splice variants of nNOS have since been identified (some of 120 kDa molecular weight) and shown to be differentially expressed in a tissue specific and developmental

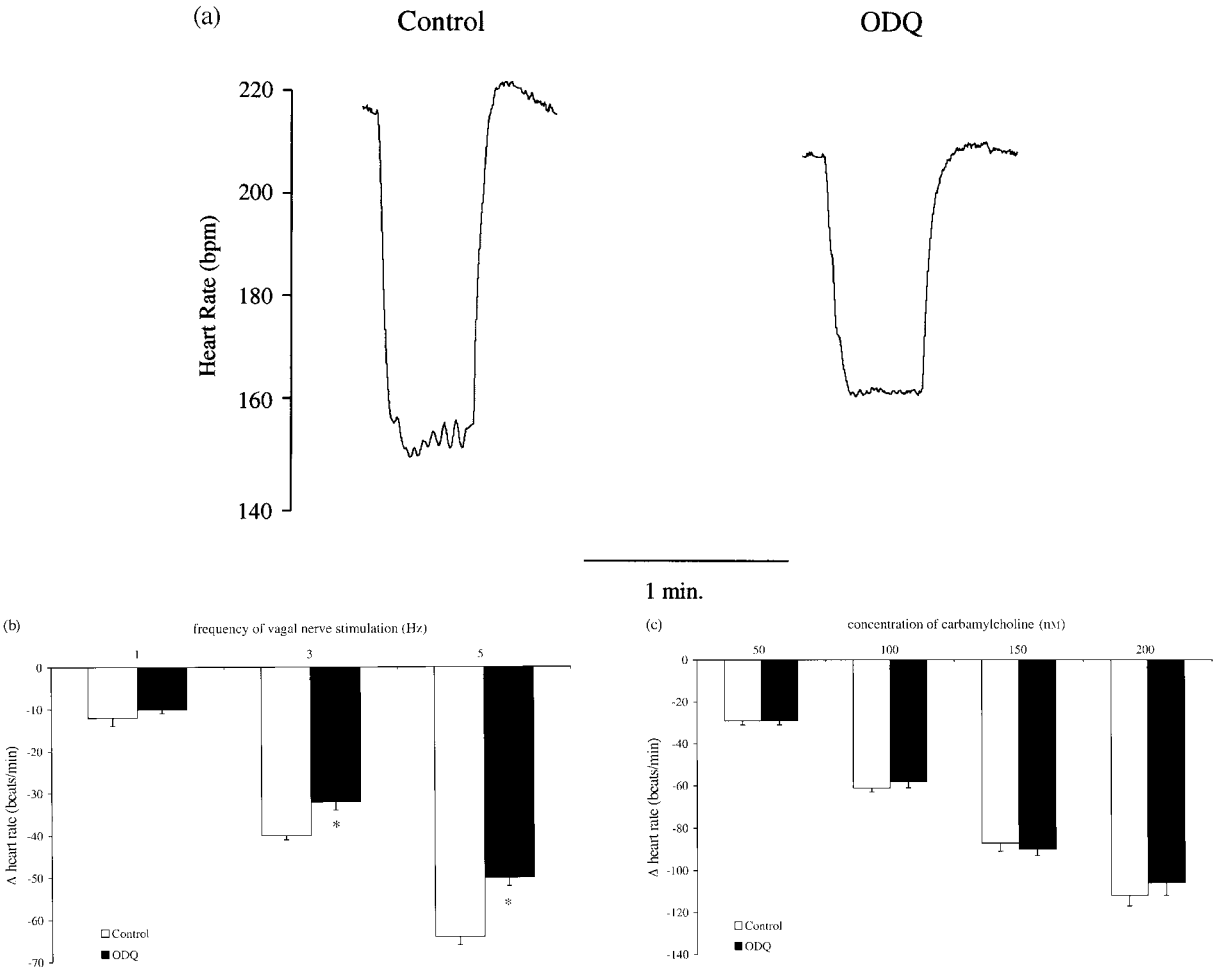

Figure 3 (a) Representative raw data trace showing the effect of guanylyl cyclase inhibition (10 μM ODQ) on the heart rate response (beats/min) to vagal nerve stimulation (5 Hz, 10 V, 1 ms pulse width, 30 s duration) in a double atrial/right vagal nerve preparation from an adult guinea pig. (b) Frequency response graph for the decrease in heart rate (beats/min) with right vagal nerve stimulation (1, 3 and 5 Hz) in atria from adult guinea pigs ($n=7$). Guanylyl cyclase inhibition (with 10 μM ODQ) significantly attenuated the negative chronotropic responses at 3 and 5 Hz (*$P<0.05$). (c) Guanylyl cyclase inhibition (10 μM ODQ) had no effect on the heart rate response (beats/min) to cumulative doses of carbamylcholine in adult guinea pig atria ($n=6$).

Table 1 Both L-NA (100 μM, $n=7$) and ODQ (10 μM, $n=7$) have no effect on the heart rate response (beats/min) to vagal nerve stimulation (1, 3 and 5 Hz) in young guinea pigs

Stimulation frequency (Hz)	Control (beats/min)	L-NA (beats/min)	Control (beats/min)	ODQ (beats/min)
1	-10 ± 1	-11 ± 1	-12 ± 2	-11 ± 2
3	-34 ± 3	-35 ± 3	-39 ± 5	-41 ± 3
5	-68 ± 7	-67 ± 6	-71 ± 6	-73 ± 6

manner in the rat.[23] A 120 kDa nNOS isoform has been identified in the rat ileum[21] and nNOS-γ, a 120 kDa variant, has been identified in the skeletal muscle of mutant mice.[24] Whether the 120 kDa nNOS protein we describe here is related to the rat splice variants or is encoded by a separate gene awaits confirmation with its cloning. The source of the right atrial nNOS is likely to be intrinsic cholinergic cardiac ganglia. Postsynaptic nNOS has been identified at the sarcoplasmic reticulum[25] but this has been shown to be of 160 kDa molecular weight, similar to the brain isoform. The source of eNOS protein is likely to be mainly from the microvasculature as well as from cardiac myocytes.

Reports on the degree of nNOS staining in neurons of intrinsic cardiac ganglia also vary. Mawe *et*

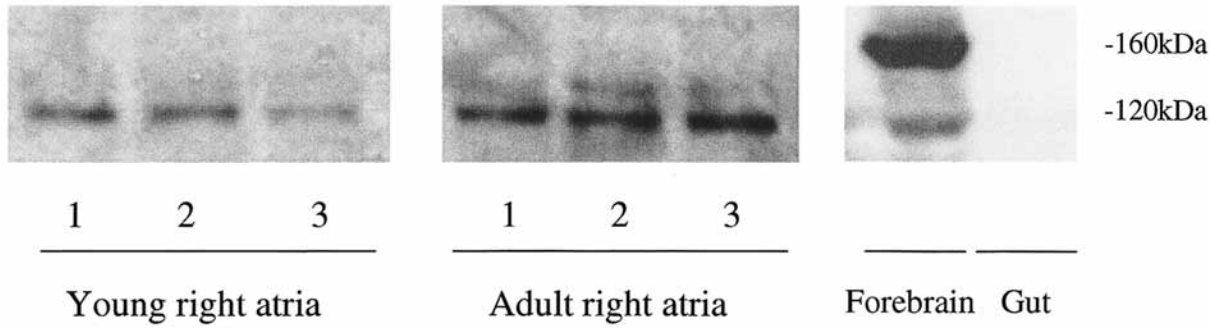

Figure 4 Western blot showing significantly lower levels of nNOS (120 kDa) protein in right atria isolated from three young compared to three adult guinea pigs. nNOS was found at both 120 kDa and 160 kDa in guinea pig fore-brain. Equal amounts of protein (200 μg) were loaded into each lane.

al.[12] show that in 250–500 g guinea pigs, all post-ganglionic neurons in atrial cardiac ganglia are cholinergic and contain choline acetyl-transferase, but only 5% of these co-stain for nNOS. In adult guinea pig atria however, Klimaschewski *et al.*[2] report that 37% of cardiac ganglia are positively labelled by NADPH diaphorase (a marker for NOS). Yoshida and Toda[26] also show that in elderly human, and adult dog and monkey atria, nearly all neurons in intracardiac plexi co-stain for both acetylcholine-esterase and NADPH diaphorase. Cardiac nNOS activity is also 47% higher in 6-month-(adult) compared to 2-month-old mice.[27] Little is known about the transcriptional regulation of the nNOS gene and how the expression of the NOS enzyme is controlled, especially during development.

A pre-synaptic role for NO in the vagal control of HR

Our results suggest that in adult guinea pigs, NO from nNOS acts pre-synaptically on GC to increase vagal neurotransmission since inhibition of NOS or GC had no effect on the HR response to a range of concentrations of carbamylcholine. Indirect evidence from the nNOS knockout mouse suggests that NO regulates the pre-synaptic control of cholinergic neurotransmission.[28] The membrane permeable cGMP analogue, 8Br-cGMP has also been shown to increase the magnitude of the vagal bradycardia in atria from young guinea pigs, although it does not effect the HR response to 100 nM carbamylcholine which produces a similar degree of bradycardia to 5 Hz vagal nerve stimulation.[10]

The NO-cGMP pathway may increase the release, or synthesis and vesicular storage of acetylcholine to enhance vagal neurotransmission. The rapid increase in the HR response to vagal nerve stimulation caused by NO donors[10] suggests that an effect on transmitter release is more likely. NO has been implicated in increasing the release of acetylcholine in the rat forebrain.[29] L-NA also decreases the release of radioactively labelled acetylcholine in guinea pig gastric fundus.[30] NO can stimulate calcium currents via inhibition of phosphodiesterase3 (PDE3) to raise cAMP and increase the activity of protein kinase A in cardiac myocytes.[31] Raising pre-synaptic cAMP levels with pituitary adenylate cyclase activating protein (PACAP) increases the release of radioactively labelled acetylcholine in guinea pig atria.[32] As PKA phosphorylates pre-synaptic N and P-type calcium channels in hippocampal neurons,[33] it is therefore conceivable that pre-synaptic NO facilitates vagal neurotransmission via a PDE3 pathway increasing cAMP-PKA-dependent phosphorylation of pre-synaptic calcium channels to increase calcium influx and vesicular release of acetylcholine.

Overall role of NO in the vagal modulation of HR in the peripheral nervous system

Post-synaptically, muscarinic receptor stimulation of eNOS activity may have a role in the cholinergic inhibition of adrenergically stimulated I_{CaL},[3] although this is controversial.[6,7] NO also stimulates the hyperpolarization activated current, I_f, and increases HR via a GC dependent pathway.[34] Functionally, post-synaptic NOS inhibition appears only to slow the HR response to low doses of acetylcholine following adrenergic stimulation as NO generated by muscarinic receptor stimulation of eNOS reduces I_{CaL} but also stimulates I_f.[19] Therefore, the main role of NO in the vagal modulation of HR (at physiological stimulation frequencies in the guinea pig) appears to be pre-synaptic with a NO-cGMP dependent pathway facilitating vagal neurotransmission.

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