Vagus nerve stimulation protects against ventricular fibrillation independent of muscarinic receptor activation

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Received 8 December 2010; revised 23 March 2011; accepted 6 April 2011; online publish-ahead-of-print 16 May 2011

Time for primary review: 25 days

Aims
The role of the vagus in the ventricle is controversial, although the vagus can protect against ventricular fibrillation (VF) via nitric oxide (NO). This study aims to determine whether the mechanisms involved are dependent on post-ganglionic release and muscarinic receptor activation. For this purpose, NO release and electrophysiological effects of vagus nerve stimulation (VNS) were evaluated in relation to acetylcholine and vasoactive intestinal peptide (VIP). In addition, the role of the coronary endothelium and afferent nerves was tested.

Methods and results
Using the isolated innervated rabbit heart, we measured ventricular NO release using 4,5-diaminofluorescein (DAF-2) fluorescence and ventricular fibrillation threshold (VFT) during VNS after muscarinic, ganglionic, and VIP inhibition [atropine, hexamethonium, and VIP (6–28), respectively] and after Triton-X endothelial functional dysfunction. The vagal-mediated increases in NO and VFT were not significantly affected (P > 0.05) during (i) atropine perfusion [increase in NO: 196.8 ± 35.2 mV (control) vs. 156.1 ± 20.3 mV (atropine) and VFT 3.1 ± 0.5 mA (control) vs. 2.7 ± 0.4 mA (atropine)], (ii) VIP inhibition—increase in NO: 243.0 ± 42.4 mV (control) vs. 203.9 ± 28.5 mV [VIP(6–28)] and VFT 3.3 ± 0.3 mA (control) vs. 3.9 ± 0.6 mA [VIP(6–28)], or (iii) after endothelial functional dysfunction [increase in NO: 127.7 ± 31.7 mV (control) vs. 172.1 ± 31.5 mV (Triton-X) and VFT 2.6 ± 0.4 mA (control) vs. 2.5 ± 0.5 mA (Triton-X)]. However, the vagal effects were inhibited during ganglionic blockade [increase in NO: 175.1 ± 38.1 mV (control) vs. 0.6 ± 25.3 mV (hexamethonium) and VFT 3.3 ± 0.5 mA (control) vs. −0.3 ± 0.3 mA (hexamethonium)].

Conclusions
We show that the vagal anti-fibrillatory action in the rabbit ventricle occurs via post-ganglionic efferent nerve fibres, independent of muscarinic receptor activation, VIP, and the endothelium. Together with our previous publications, our data support the possibility of a novel ventricular nitrergic parasympathetic innervation and highlight potential for new therapeutic targets to treat ventricular dysrhythmias.

Keywords
Vagal stimulation • Ventricular Fibrillation • Acetylcholine • Nitric oxide

1. Introduction
Clinical studies have shown that abnormal autonomic states with impaired heart rate (HR) variability and baroreflex sensitivity—both measures of vagal activity—are strong prognostic markers in patients with heart failure with or without β-blockers,⁵,⁶ or in patients with a previous myocardial infarction.³ There is strong evidence that the relationship between impaired cardiac autonomic control and mortality is a result of an increased susceptibility to lethal ventricular arrhythmias,⁴ particularly ventricular fibrillation (VF) that leads to sudden cardiac death (SCD). The underlying mechanisms are poorly understood and SCD remains a major clinical problem.

The dogma of physiology teaching is that vagal post-ganglionic nerve terminals modulate heart function via acetylcholine (ACh) acting on muscarinic receptors. It is accepted that the vagus slows HR, atrioventricular conduction, and decreases atrial contraction; however, there is ongoing controversy as to whether the vagus has any significant direct effect on ventricular inotropy.⁵–⁷ However,
Despite this controversy, there is increasing evidence to support that stimulation of the vagus nerve is protective against induced and spontaneous VF occurrence. The first study over 100 years ago, by Eidenbrodt, using an inductorium demonstrated that direct cervical vagus nerve stimulation (VNS) increased the threshold for VF induction. An elegant study in conscious dogs with a healed myocardial infarct, demonstrated that VNS suppressed spontaneous VF development during simultaneous exercise testing and a 2-s circumflex artery occlusion. However, the mechanisms underlying these effects in vivo are complex and not well understood. Understanding these effects are limited due to confounding factors such as circulatory neurohumoral factors and autonomic reflexes that are present in vivo.

To circumvent these factors, using a unique isolated in vitro innervated heart preparation without underlying sympathetic tone, we have previously shown that stimulation of the cervical vagus nerve makes induction of VF more difficult, underscoring that VNS has a direct and prominent electrophysiological effect on the ventricular myocardium. We have also shown that this anti-fibrillatory effect is associated with a change in the electrical restitution property of the heart, which is considered a key mechanistic factor in the initiation of fibrillation. These effects are blocked during nitric oxide synthase (NOS) inhibition, providing indirect evidence that nitric oxide (NO) is involved. We have since provided direct evidence that VNS leads to the release of NO in the ventricle via neuronal nitric oxide synthase (nNOS/NOS 1) activation.

However, the question remains as to whether the anti-arrhythmic effect of VNS and effect on electrical restitution are mediated in the classical sense by the release of ACh and muscarinic receptor activation, with a synergistic involvement of NO—which is evident at the atrial level. This study aims to address this by investigating the effects of VNS during muscarinic receptor blockade, which if present may suggest a parallel nitrergic protective pathway in the ventricle. In addition, we aim to investigate (i) if the anti-fibrillatory effects and release of NO involve the cardioactive co-transmitter, vasoactive intestinal peptide (VIP) which is known to act in part via NO and is released along with Ach, (ii) whether the changes are due to the cervical vagus nerve electrodes antidromically activating afferent nerves, a large number of which have ventricular origin, and (iii) if the VNS effects are present after functional disruption of the coronary endothelium and thereby determining if the endothelium is a source of vagally released NO in the ventricle.

2. Methods

Methods are described in full in the Supplementary material online.

2.1 Experimental techniques

All procedures were undertaken after local Ethics approval at the University of Leicester and were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and the Guide for the Care and Use of Laboratory Animals Published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985).

2.1.1 Isolation of heart with intact autonomic nerves

This preparation has been described previously. In brief, adult male New Zealand White rabbits (2.8 ± 0.1 kg, n = 21) were premedicated with an i.m. Medetomidine Hydrochloride (0.2 mg/kg), Ketamine (10 mg/kg), and Butorphanol (0.05 mg/kg) mixture, and anaesthetized with i.v. Propofol (1 mg/kg). Surgical anaesthetic depth was confirmed with the absence of pedal/corneal reflexes. The rabbit was ventilated, after tracheotomy (60 breaths/min) with air. The cervical vagi were isolated and cut, while the blood vessels leading to and from the rib cage were ligated and dissected. The rabbit was killed with 800 mg Sodium Pentobarbitone with 1000 IU heparin i.v.

2.1.2 Langendorff perfusion

The preparation extending from the neck to thorax was dissected and attached for Langendorff perfusion via the descending aorta using Tyrode solution at a rate of 100 mL/min. Intraventricular and perfusion pressure was monitored throughout.

2.1.3 Vagus nerve stimulation

The left cervical vagus nerve stimulated using a custom-made bipolar silver electrode at 10.8 ± 2.4 Hz, 10.0 ± 2.2 V, 2 ms pulse width.

2.1.4 Cardiac electrical recording and pacing

Monophasic action potentials (MAPs) were recorded from left ventricular epicardial-free wall, at sites matched to that during NO fluorescence testing. A bipolar catheter at the right ventricular apex was used for pacing at ×2 threshold, using a 2 ms pulse width.

2.1.5 Electrical restitution

Standard restitution of MAP duration was obtained with right ventricular pacing using the S1–S2 extrastimulus method. MAP duration was measured at 90% repolarization monophasic action potential duration (MAPD90) and restitution was examined by the analysis of the relationship between S2–MAPD90 and preceding diastolic intervals. An exponential curve was fitted to this data and the maximum restitution slope measured.

2.1.6 Ventricular fibrillation threshold

Ventricular fibrillation threshold (VFT) was obtained with right ventricular pacing using a train of 30 stimuli (30 ms interval) spanning the refractory period at the end of a 20-beat drive train and was defined as the minimum current required producing sustained VF. Hearts were cardioverted with a bolus injection of KCl.

2.1.7 Fluorescence system and loading for nitric oxide measurements

As described previously, NO fluorescence was measured at 490 ± 10 nm (F490) using a bifurcated light guide positioned on the left ventricular-free wall after hearts were loaded with 4,5-diaminofluorescein diacetate (DAF-2 DA).

2.2 Experimental protocols

Two separate experimental protocols were used.

2.2.1 Vagus nerve stimulation and ventricular electrophysiology

Effective refractory period (ERP), VFT, and electrical restitution were determined at baseline and during VNS under control conditions, during perfusion with drug and following a 10-min period of washout in sinus rhythm.

2.2.2 Vagus nerve stimulation and ventricular DAF-2 NO-dependent fluorescence (F490)

The effect of VNS on F490 was studied during 30 s of nerve stimulation with a 60 s rest period between stimulation protocols. After control changes were obtained, protocols were repeated with drug, followed by washout and hearts were paced at 200 b.p.m.
2.3 Drugs

2.3.1 Muscarinic receptor blockade
The first part of the study was designed to determine whether the effects from VNS were dependent on ACh. All measurements were obtained in the absence and presence of atropine (0.1 μM).  

2.3.2 Alternative neurotransmitter, neural pathways, and NO source
The second part of the study investigated if: (i) the effect of VNS involved the VIP signalling pathway using the VIP antagonist VIP(6–28) (20 nM), (ii) the effects are mediated via afferent nerve fibres by using 0.5 mM hexamethonium bromide for ganglionic blockade, or (iii) if the coronary endothelium is a source of vagally released NO by chemically disrupting the vascular and microvascular endothelium using Triton-X (0.1%). Functional endothelial disruption was confirmed using Bradykinin (100 μM).

2.4 Statistical analysis
Data are mean ± SEM and analysed using single or two-factor repeated measures ANOVA with Bonferroni post hoc tests for comparisons where appropriate. P < 0.05 was considered significant.

3. Results

3.1 The effect of VNS during muscarinic receptor blockade

3.1.1 VNS effects on ERP, VFT, and electrical restitution
The electrophysiological effects of VNS were examined in nine hearts where HR, ventricular ERP, VFT, and electrical restitution were measured.

During control, VNS decreased HR, which was abolished with atropine (Figure 1A). As seen previously, ERP (Figure 1B) and VFT (Figure 1C) were significantly increased with vagal stimulation. However, during atropine perfusion, the prolongation of ERP was abolished while the protection afforded against VF was notably preserved, suggesting a novel divergence in the parasympathetic control of HR and ventricular refractoriness when compared with effects on VF initiation. Of note, there was no effect of atropine infusion alone on these parameters.

The effects of vagal stimulation in the absence and presence of atropine on MAPD restitution in a typical experiment are illustrated in Figure 2A. During control, the restitution curve was shifted upwards during VNS with increased MAPD₉₀ at corresponding diastolic intervals compared with baseline. Maximum MAPD₉₀ duration (MAPD₉₀max) was increased from a baseline of 153.2 ± 4.3 to 166.7 ± 5.2 ms (P < 0.05) with VNS. The maximum slope of the restitution curve (dotted lines in Figure 2A) was significantly reduced with VNS (Figure 2B). In the presence of atropine, there was no vertical shift of the restitution curve with no change in MAPD₉₀max [154.2 ± 4.6 ms (baseline) to 154.9 ± 9.0 ms (VNS), P > 0.05]. However, the decrease in the maximum slope of the restitution curve with VNS was preserved in the presence of atropine. After washout of atropine, the increase in MAPD₉₀max with VNS returned [150.2 ± 2.6 ms (baseline), 164.1 ± 4.2 ms (VNS), P < 0.05] with the persistent increase in maximum slope of the restitution curve.

3.1.2 VNS effects on ventricular NO release
As shown in Figure 3A (left panel), during control VNS increased DAF-2 fluorescence indicative of NO release. This increase in

![Figure 1](image_url) Effect of atropine on the VNS-dependent changes in HR, ERP, and VFT. Mean data representing heart rate (HR, A) effective refractory period (ERP, B) and ventricular fibrillation threshold (VFT, C) at baseline and during vagus nerve stimulation (VNS), during control, perfusion of 0.1 μM atropine and washout. Data mean ± SEM. Two factor ANOVA with Bonferroni post hoc test. ***P < 0.001, NSP > 0.05, n = 9.
NO-dependent fluorescence was not attenuated during muscarinic receptor blockade (right panel and mean data in Figure 3B, n = 8). Of note, this occurred at a dose where the chronotropic effect with VNS was abolished confirming muscarinic receptor blockade although we have tested a higher concentration of atropine (1.5 mM) with similar results (data not shown). There was no effect of VNS on either left ventricular pressure or perfusion pressure during any treatment group throughout the experiments (data not shown).

3.2 Exploring alternative neurotransmitter, neural pathways, and NO source

3.2.1 VIP inhibition

The effects of inhibiting the VIP signalling pathway on electrophysiological changes and NO release from VNS are summarized in Figure 4. While the peak HR achieved during VNS in the presence of VIP(6–28) was not significantly different (Figure 4A, P = 0.08) from control, the change in HR during VNS was significantly larger during VIP inhibition—decrease in HR of 71.8 ± 8.9 (control) vs. 83.7 ± 8.1 b.p.m. [VIP(6–28)], P < 0.05. Figure 4B–C summarizes the effects of VNS on ERP and VFT, respectively and illustrates that the effect of VNS was unchanged during VIP inhibition. As before and in Figure 4Di (left panel), VNS increased DAF-2 fluorescence during control, indicative of NO release.14 This increase in NO-dependent fluorescence was not attenuated in the presence of VIP(6–28) (Figure 4Di, right panel and Figure 4Dii, n = 5).

3.2.2 Ganglionic blockade

Figure 5 summarizes the experimental data obtained during ganglionic inhibition. Hexamethonium abolished the vagally mediated effects on HR (Figure 5A), ERP (Figure 5B), and VFT (Figure 5C), without affecting HR, ERP, or VF induction in unstimulated conditions. As before, VNS in increased DAF-2 fluorescence during control indicative of NO release (Figure 5D).14 This increase in NO-dependent fluorescence was abolished during ganglionic blockade (Figure 5Di, right panel and Figure 5Dii, n = 6).

3.2.3 Endothelial functional disruption

Endothelial functional disruption was achieved using a bolus injection of 0.1% Triton-X (0.1–0.3 mL).22,23 Functional disruption was confirmed with an increase in perfusion pressure and the abolition of
endothelium-dependent vasodilatation using bradykinin. During control, bradykinin decreased perfusion pressure from 39.0 ± 1.9 mmHg to 37.0 ± 1.8 mmHg, a decrease in 5.0 ± 0.5% (n = 10, P < 0.001). After the bolus injection of Triton X, perfusion pressure was significantly increased (P < 0.05) while left ventricular pressure was significantly decreased (42.9 ± 3.2 to 32.7 ± 1.7 mmHg, P < 0.001), and bradykinin failed to elicit a significant vasodilatory response (43.8 ± 1.8 to 43.9 ± 1.9 mmHg, change of 0.2 ± 0.4%, P > 0.05, n = 10). In five of these hearts, NO was measured with DAF-2 fluorescence and bradykinin significantly increased NO-dependent fluorescence before Triton-X from 9.424 ± 0.033 to 9.551 ± 0.026 V, an increase in 127.2 ± 31.5 mV (1.35 ± 0.34%, P < 0.001). Corresponding with the increase in perfusion pressure, basal NO fluorescence after Triton-X was significantly (P < 0.01) decreased and the bradykinin-dependent increase in NO fluorescence was abolished (9.148 ± 0.072 to 9.133 ± 0.076 V, change of 14.9 ± 33.1 mV, P > 0.05, n = 5) thus confirming endothelial functional disruption.

Figure 6A–C summarizes the effects of endothelium functional disruption on the effect of VNS on HR, ERP, VFT, and NO fluorescence (n = 7). The VNS-mediated changes in HR, ERP, and VFT were preserved after treatment with Triton-X. Of note, baseline ERP and VFT were significantly decreased (P < 0.05) after endothelial functional disruption with Triton-X. The increase in NO fluorescence during VNS during control (Figure 6Di, left panel) was still present after Triton-X treatment (Figure 6Dii; right panel and Figure 6Diii, n = 8).

4. Discussion

To the best of our knowledge, this is the first demonstration that the increase in VFT during direct stimulation of the cervical vagus nerve, which is entirely parasympathetic in origin in the rabbit, and the effect on electrical restitution are preserved during muscarinic receptor blockade and are thus independent of activation of muscarinic receptors. In addition, we have provided evidence to support the
conclusion that these effects of VNS do not involve VIP or the endothelium and not due to the cervical vagus stimulation antidromically activating afferent fibres to cause release of endogenous chemicals from their terminals in the heart. Therefore, we conclude that the anti-fibrillatory effects are mediated via activation of efferent nerve fibres.

The results support the hypothesis that non-muscarinic vagal protection against VF is mediated by neuronal release of NO independently of ACh as summarized here.

- The action of the vagus on VFT and RT is prevented by reducing NO production using NOS inhibitors.
- The action of the vagus is not prevented by atropine given in sufficient doses to block the bradycardic and atrioventricular nodal conduction effect of VNS.
- Furthermore, the increase of NO by applied ACh was not blocked by an nNOS-specific inhibitor where as this blocker abolished the action of the vagus indicating the vagal effect was independent of ACh.
- We have previously shown that nNOS inhibitors block the increase in NO fluorescence while NOS inhibitors block the electrophysiological effects of VNS demonstrating an association between NO release and the anti-fibrillatory action.
- The increase in NO and electrophysiological effects during cervical VNS is blocked by hexamethonium and is therefore not due to antidromic afferent nerve activation. This also indicates that nACH receptors are involved in the efferent pathway that lead to the release of NO.
- The VNS effects are not due to endothelial NO release as they are nNOS dependent, and not eNOS dependent and they are unaffected by rendering the cardiac endothelium functionally dysfunctional.

The implication that NO is involved in mediating vagal activity in the ventricle is a recent finding although there is established data that this occurs at the atrial level where NO is primarily involved in modulating pre-synaptic ACh release at the sinoatrial node. However, other evidence suggests that vagally released NO and ACh may act...
through parallel independent signalling pathways.\textsuperscript{25} We have also obtained preliminary evidence using the NO donor sodium nitroprusside\textsuperscript{26} that SNP perfusion increases ERP, VFT and reduces the maximal slope of the restitution curve. These effects mimic the response to direct VNS, supporting the notion that NO underlies the effect from direct VNS. Our data from the current study raise a novel possibility that there may be independent mechanisms for ACh and NO release via different populations of efferent parasympathetic fibres. However, to robustly substantiate this novel finding, more direct evidence to demonstrate a lack of nAChR involvement may be needed.

The importance of the vagus nerve in the ventricle of most mammalian species has previously been underappreciated although the therapeutic use of VNS is becoming better known and the beneficial effects recognized by a wider audience. Despite the wealth of early evidence there has been the view that the vagus nerve does not significantly innervate the ventricle. However, recent histochemical evidence in the pig ventricle\textsuperscript{27} demonstrated that dense parasympathetic innervation which clearly refutes this viewpoint. In view of the results in the present study and our previous published work,\textsuperscript{13} some of this parasympathetic innervation may be non-cholinergic. Interestingly, electrical stimulation of the vagus nerve, which is currently an approved therapy for certain neurological disorders,\textsuperscript{28} has more recently been used to improve heart failure outcomes and arrhythmic status in both animal\textsuperscript{29,30} and human studies\textsuperscript{31,32} of cardiac disease. This approach is supported by the results of the present study which together with recent evidence from our group show that an anti-fibrillatory effect is abolished during NO synthase inhibition\textsuperscript{13} and that VNS releases NO in the ventricle,\textsuperscript{14} indicating that the vagus nerve has a direct NO-dependent effect on important electrophysiological effects in the cardiac ventricle.

4.1 Mechanism of NO release

4.1.1 VIP signalling pathways

One particular mechanism that may be involved in the increase in ventricular NO during VNS concerns VIP. VIP is known to be co-released from the vagus nerve together with ACh.\textsuperscript{16,17} Data support that VIP is co-localized with nNOS in epicardially located intracardiac ganglia of parasympathetic neurones in the atria and ventricles of a number of species.\textsuperscript{18} Functionally, vagally released VIP promotes tachycardia\textsuperscript{19} and...
coronary vasodilatation while simultaneously increasing right ventricular contractility without affecting left ventricular performance.\textsuperscript{34} The vasodilatation caused may be mediated via a NO-dependent pathway\textsuperscript{16} since VIP receptors are known to couple to membrane bound NO synthase.\textsuperscript{35} However in our study, VIP inhibition using VIP(6-28) at a concentration sufficient to modulate vagal-mediated bradycardia did not block the release of NO suggesting that this pathway is not involved in the effect of the vagus in the ventricle.

4.1.2 Efferent nerve or afferent nerve dependent release of NO

Some 80% of the axons in the cervical vagus nerve are afferent fibres, many of which have a cardiac origin.\textsuperscript{18} More importantly, ventricular afferent sensory fibres are known to contain NOS\textsuperscript{19} and this raises the possibility that the parameters used to stimulate the cervical vagus nerve present in our study may antidromically promote NO release in the ventricle from cardiac vagal afferent nerve terminals, rather than resulting from orthodromic stimulation of pre-ganglionic efferent nerve fibres. We investigated this possibility directly by blocking efferent ganglionic synaptic neurotransmission using the nicotinic ACh-specific receptor blocker hexamethonium. Parasympathetic ganglionic blockade was confirmed using a concentration that abolished the vagally induced bradycardia. In these experiments, the release of NO and electrophysiological effects of VNS in the rabbit ventricle was similarly abolished suggesting that the effects observed were not mediated via afferent nerve activation, which would not be expected to be blocked in the presence of hexamethonium. This suggests that the effects observed occurred via cardiac nerves after activation of efferent cervical vagal pre-ganglionic nerve fibres. Modulation of and the role of the efferent nicotinic-ganglionic-dependent mechanisms, which may underpin the vagal-mediated effects on arrhythmogenesis, requires further investigation and the possibility of afferent nerve processing within intracardiac ganglia remains to be elucidated. Furthermore, nACh receptors are present on cardiomyocytes, particularly nACh\textsuperscript{7,36} which are known to be linked to NO production in sensory dorsal root neurons.\textsuperscript{37} This raises an additional possibility that ACh may be involved in release NO and reduce the vulnerability towards VF via a post-junctional nACh pathway present on cardiomyocytes. While this possibility seems unlikely, since the action of applied ACh on NO release was not blocked by an nNOS inhibitor that prevented the effects of VNS it warrants further investigation.

4.1.3 Origin of NO release

Despite these findings, questions remain as to the origin of this nerve-induced release of NO in the ventricle. NO is synthesised by three isoforms of NOS: endothelial (eNOS/NOS III), neuronal (nNOS/NOS I), and inducible NOS (iNOS/NOS II). All three isoforms are present in the heart; however, receptor-mediated NOS-dependent mechanisms only appear to involve nNOS or eNOS since iNOS is stress activated.\textsuperscript{38}

Our previous work\textsuperscript{14} demonstrated that the vagally induced release of NO in the ventricle was prevented by the selective nNOS inhibitor, TRIM. In contrast, TRIM did not block an increase in NO fluorescence induced by ACh although the latter effect was blocked by a non-selective NOS inhibitor, indicating a clear difference in the action of VNS and infused ACh. It was concluded that the VNS effect was independent of eNOS. In the current study, we confirmed this finding by showing that the release of NO and associated electrophysiological effects was preserved after impairing the function of the endothelium of the cardiac vasculature using a bolus injection of Triton-X that completely abolished the vasodilatory effects of the endothelium-contingent drug, bradykinin. Triton-X also caused a significant reduction in ERP and VFT suggesting that cardiac cells may have also been affected by Triton-X and therefore that microvascular endothelium would have been similarly impaired. This supports the conclusion of Hassanabad\textsuperscript{22} and Li et al.\textsuperscript{23} based on morphological studies at the electron microscopy level that showed in rat heart a bolus injection of Triton-X caused extensive disruption of the endothelium in both large and small cardiac vessels. These data would of course not rule out a contribution of eNOS from a different source, such as plasmasema or T-Tubule caveola cell membranes,\textsuperscript{39,40} but this seems unlikely in view of the action of TRIM in the previous experiments. Therefore, the data strongly suggest that the release of NO associated with protection of the myocardium from VF is neural in origin. This accords with the presence of nNOS in ventricular intracardiac nerve fibres,\textsuperscript{41,42} but it also indicates that these may be a unique group of neurones in which nNOS is not co-located with ACh. This arrangement would be unlike that described by Richardson et al.\textsuperscript{13} for neurones in the atrial ganglia surrounding the pulmonary vessels of the rat but more in accord with the demonstration in human-derived cardiac ganglia of non-cholinergic neurones that are immunoreactive for nNOS and neuropeptides some of which seemed to project towards the ventricles.\textsuperscript{19} Thus the overall evidence indicates that in addition to the nAChR vagal cholinergic supply to the cardiac ventricle, there is an independent vagal innervation that acts via NO to significantly influence physiological mechanisms related to ventricular dysrhythmias. The precise ionic mechanisms by which the vagal-NO pathway might alter ventricular electrophysiology are unknown and is the subject of ongoing investigations. However, there is emerging evidence that there is a direct relationship between the handling of the chemical molecule NO and ventricular myocardial repolarization. The QT interval on surface ECG which signifies ventricular repolarization is prolonged by a genetic variant of the NOS1 regulatory protein, NOS1AP.\textsuperscript{44-46} Additionally, a recent study by Crottie et al.\textsuperscript{47} demonstrates that a variation in NOS1AP is associated with an increased risk of SCD in patients with Long-QT syndrome representing direct evidence that these abnormalities in NO signalling are involved in arrhythmogenesis. Further work on the details of the subcellular compartments where this occurs and how this pathway may be exploited to develop effective anti-fibrillatory therapy in the ventricle are urgently warranted.

4.2 Study limitations

In this study, we applied an electrical current to induce fibrillation as a surrogate marker of the susceptibility of hearts to VF. Although this method may not be considered to be representative of what occurs spontaneously in the intact human, and that the link between VFT and electrical restitution may not be a causal one, VFT is considered a useful marker of electrical stability. In addition to our previous work,\textsuperscript{1,17} other groups have demonstrated predictable, consistent and reliable changes in VFT in conditions of autonomic nerve stimulation,\textsuperscript{48} ventricular dilation,\textsuperscript{49} and myocardial ischaemia.\textsuperscript{50} We intentionally did not use any predisposing factors to promote spontaneous VF development, such as hypoxia or adrenergic activation as this would make it more difficult to delineate the effects from direct VNS. In addition, the exogenous current that spans the refractory
period promotes electrical alternans development\textsuperscript{51} that degenerates into wavebreaks and ultimately VF. This type of wavebreak leading to fibrillation may exist naturally and is considered to be dependent on electrical restitution.\textsuperscript{12}

MAPs were measured from only one site of the left ventricular epicardial surface. The contribution of dispersion of repolarization—either transmural or spatial over the epicardial surface—was not assessed. Regional differences in electrical restitution may have an additional impact on arrhythmogenesis. The current study was aimed at obtaining data on an effect of direct nerve stimulation on ventricular electrophysiology at one epicardial site, to form a base from which studies on potential mechanisms and other electrophysiological effects may be developed. One such avenue of investigation is underway applying optical mapping techniques to the innervated heart preparation to study the spatial heterogeneity of electrophysiological parameters over a wide area of the heart.\textsuperscript{51}

4.3 Conclusion

In summary, the protective effect of VNS against VF and the concomitant reduction in the slope of electrical restitution is associated with NO release in the ventricle which is preserved during muscarinic receptor blockade. This effect occurs via post-ganglionic efferent fibres and does not involve the VIP pathway or the endothelium. This reinforces the notion that there is a direct nitrergic action in cardiac surface. The contribution of dispersion of repolarization—

Supplementary material

Supplementary material is available at Cardiovascular Research online.

Acknowledgements

The study is part of the research portfolio supported by the Leicester NIHR Biomedical Research Unit in Cardiovascular Disease.

Funding

This work was supported by Project Grants from the British Heart Foundation (PG/02/088) and Garfield Weston Trust (PMS/MM8-08/09-3023).

Conflict of interest

None to declare.

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