Neuronal Nitric Oxide Synthase Gene Transfer Promotes Cardiac Vagal Gain of Function

R.M. Mohan, D.A. Heaton, E.J.F. Danson, S.P.R. Krishnan, S. Cai, K.M. Channon, D.J. Paterson

Nitric oxide (NO) generated from neuronal nitric oxide synthase (NOS-1) in intrinsic cardiac ganglia has been implicated in parasympathetic-induced bradycardia. We provide direct evidence that NOS-1 acts in a site-specific manner to promote cardiac vagal neurotransmission and bradycardia. NOS-1 gene transfer to the guinea pig right atrium increased protein expression and NOS-1 immunolocalization in cholinergic ganglia. It also increased the release of acetylcholine and enhanced the heart rate (HR) response to vagal nerve stimulation (VNS) in vitro and in vivo. NOS inhibition normalized the HR response to VNS in the NOS-1–treated group compared with the control groups (enhanced green fluorescent protein and sham) in vitro. In contrast, an acetylcholine analogue reduced HR to the same extent in all groups before and during NOS inhibition. These results demonstrate that NOS-1–derived NO acts presynaptically to facilitate vagally induced bradycardia and that upregulation of NOS-1 via gene transfer may provide a novel method for increasing cardiac vagal function.

Moreover, the vagal heart rate (HR) response to modulators of the NO-cGMP pathway is not mimicked by the stable analogue of ACh, carbachol, suggesting that the dominant functional role of this pathway is presynaptic to the neuroeffector junction. We tested the hypothesis that NOS-1 gene transfer into the right atrium would enhance vagal-induced neurotransmission and bradycardia but would be ineffective when HR was decreased by carbachol.

Materials and Methods

Detailed methods for gene transfer, immunohistochemistry, fluorescence microscopy, confocal imaging, immunoblotting, measurements of ACh release, and in vivo or in vitro autonomic phenotyping can be found in the online data supplement, available at http://www.circresaha.org.

Results and Discussion

Qualitative examination using NADPH-diaphorase staining of tissue cryosections showed greater expression in atrial tissue after replication-deficient adenoviral vector transfection with neuronal NOS (Ad.NOS-1) (Figure 1A). This was confirmed by Western blotting in which Ad.NOS-1–treated atria (n=8) showed significantly greater expression of NO protein compared with atria infected with an adenoviral vector encoding recombinant enhanced green fluorescent protein (Ad.eGFP) (n=8, P=0.01; Figure 1B). eGFP was present only in atria from Ad.eGFP-treated animals, using both Western blotting and fluorescence microscopy (Figures 1A and 1B). Moreover, NOS-1 transfer was markedly localized to NOS-1–positive stained neurons that colocalized with choline acetyltransferase compared with the eGFP control indicating site specific pickup of the transgene into neurons (Figure 1C).

Basal HR was not affected by gene transfer (see online Table 1, available in the data supplement at http://www.circresaha.org). Ad.NOS-1 treatment increased (P≤0.001) the HR response to vagal nerve stimulation (VNS) in vivo (n=6 Ad.NOS-1; n=7 Ad.eGFP) and in vitro (n=15 Ad.NOS-1; n=17 Ad.eGFP; n=5 sham injection) when compared with Ad.eGFP atria and sham (Figures 2A, 2B, and 3A; raw data, online Figure 1a), indicating physiological significance. Responses between Ad.eGFP and sham preparations were not different. NOS-1–treated atria (n=6) had enhanced (P<0.01) release of ACh during field stimulation compared with eGFP-treated atria (n=8). This difference was abolished by inhibition of guanylyl cyclase with 1H-(1,2,4)oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (Figure 2C).

Pretreatment of isolated atria with the NOS inhibitor N-nitro-L-arginine (L-NA) significantly attenuated the enhanced vagal bradycardia in Ad.NOS-1–treated atria (*P<0.001; Figure 3B) and the HR responses of Ad.eGFP-treated (*P<0.01, n=13) and sham preparations (*P<0.01, n=5). After NOS inhibition, responses of Ad.NOS-1, Ad.eGFP, and sham-preparations to 3-Hz VNS (data not shown) and 5-Hz VNS were not significantly different (Figure 3B; raw data, online Figure 1b). In contrast, there were no significant differences among responses of the three treated groups to bath-applied carbachol (see online Figure...
Furthermore, we observed no significant difference among responses of the three groups to carbachol in atria pretreated with L-NA (see online Figure 2b). This suggests that NO generated from NOS-1 acts predominantly within intracardiac ganglia to enhance cholinergic regulation of cardiac function without affecting postsynaptic cholinergic signaling. These data are consistent with recent findings that report pharmacological enhancement of the

Figure 1. A, Representative cryosections from right atrium harvested 5 days after gene transfer or sham procedure. A and D are sections from atria treated with medium alone; B and E, atria treated with Ad.eGFP; and C and F, treated with Ad. NOS-1. Note that histochemical localization of eGFP was detected in Ad.eGFP-treated atria only (panel B, white arrow) and that intense NOS diaphorase staining was present in Ad.NOS-1 atria (panel F, black arrows). White bar in top panels = 50 μm. Black bar in bottom panels = 25 μm. Red color represents tissue autofluorescence. B, Western blot analysis (17.5 μg protein loaded) shows atrial NOS-1 gene transfer (Ad.NOS-1, n=8) resulted in a 110% increase (OD indicates optical density) in expression of NOS-1 in atria compared with Ad.eGFP (n=8, P<0.01; unpaired t test). eGFP (27 kDa) expression was found only in Ad.eGFP-treated animals. Levels of NOS-1 protein expression in the sham group were similar to that observed in the Ad.eGFP-treated group (data not shown). Expression of β-actin was equal between all groups. C, Confocal imaging of atria from Ad.eGFP group (top row) and Ad.NOS-1 group (bottom row) stained for NOS-1 (left column, green) and choline acetyltransferase (Chat, middle column, red). NOS-1 staining in cholinergic ganglia was greatly increased in Ad.NOS-1 compared with Ad.eGFP groups. Shown in the right column in yellow is the colocalization of NOS-1 and Chat. Note that we cannot rule out that Ad.NOS-1 did not transfect other cell types.
NO-cGMP pathway during right atrial field stimulation facilitates the release of ACh by activating the guanylate cyclase/cGMP pathway. Moreover, the vagal HR response to pharmacological modulators of the NO-cGMP pathway is not mimicked by bath-applied cholinergic analogues, confirming that the main functional response of the NO-cGMP pathway resides presynaptically.

The implication of our findings extends our present understanding of cholinergic modulation of HR by NO in intracardiac ganglia and underscores the importance of spatial localization of NOS activity in the cholinergic modulation of cardiac function. Functionally, high vagal tone is a positive prognostic indicator against sudden cardiac death, whereas impaired activity is a strong independent predictor of mortality. Emerging evidence suggests that the NOS-1-regulated vagal pathway is involved in the enhanced cardiac parasympathetic response after exercise training, because NOS-1 inhibition abolishes the gain of function in trained mice that have increased atrial NOS-1 expression. It remains to be established, however, whether impaired cardiac vagal signaling seen in hypertension, heart failure, and after myocardial infarction is a consequence of a defective NOS-1--NO-cGMP pathway and decreased NO bioavailability.

Understanding how NO regulates cardiac vagal activity is a fundamental step toward the development of new targets that may enhance the therapeutic action of the vagus in pathophysiological states where its action is impaired. In principle, NOS-1 gene transfer could be used to increase cardiac vagal gain of function in these patient groups where exercise training is often poorly tolerated.

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**References**


**Key Words**: vagus, nitric oxide, gene transfer, heart rate, autonomic control.
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Methods

Replication deficient adenoviral vectors encoding recombinant enhanced green fluorescent protein (Ad.eGFP) or neuronal NOS (Ad.NOS-1) were generated and purified as previously described\(^1\). Percutaneous gene transfer to the atrial wall of \(5 \times 10^9 - 5 \times 10^{10}\) virus particles of Ad.eGFP (n=34) or Ad.NOS-1 (n=33) in 300\(\mu\)l phosphate-buffered saline (PBS) or sham injections (n=9, PBS only) was performed in halothane-anaesthetised male guinea pigs (mean wt, 374±5g).

Following circa 5 days incubation, in-vitro\(^2\) and in-vivo\(^3\) heart rate (HR) responses to vagal nerve stimulation (VNS) were measured (3 & 5Hz; 10V, 1ms pulse interval) Heart rate was also decreased by a similar amount with bath-applied carbachol (CCh, Sigma UK) in-vitro \((10^{-7} & 3 \times 10^{-7}\) mol/L). Isolated atria from all groups were treated in-vitro with the NOS inhibitor, Nω-nitro-L-arginine (L-NA, Sigma UK). Acetylcholine release was measured in vitro before and after inhibition of guananyl cyclase as previously described\(^2\). Samples of atrial tissue were snap frozen in either OCT medium after equilibration in 4% paraformaldehyde/ 2% sucrose (for NADPH-diaphorase and fluorescence microscopy) or liquid nitrogen (for Western immunoblotting). Fresh atrial tissue was used for confocal microscopy to determine cellular localisation of NOS-1.

Atrial cryosections were fixed and evaluated for NOS activity and the presence of eGFP by NADPH-diaphorase or fluorescence microscopy\(^4\). To assess NOS-1 and eGFP protein expression (Western Blot), right atria were homogenised, equalised for protein content and separated by SDS-PAGE. Following transfer to polyvinyl membranes, NOS-1 and eGFP protein were detected using rabbit α-eGFP and mouse α-NOS-1 primary
antisera (Transduction Labs., UK). Atria from Ad.NOS-1 and Ad.eGFP groups were processed for immunohistochemistry. Colocalisation of NOS-1 and cholinergic ganglia was revealed by confocal microscopy using immunofluorescence in whole atria against sheep α-NOS-1 and goat α-ChAT (Chemicon, UK); and fluorescein isothiocyanate (FITC) or texas red (ITC) conjugated secondary antisera.

References


Supplementary Figure: 1a) Raw data traces showing Ad.NOS-1 enhances the heart rate response to 3&5 Hz vagal nerve stimulation compared to sham and Ad.eGFP treated atria.

b) Raw data trace showing NOS inhibition with L-NA attenuates the heart rate response to vagal activation in all groups.
Supplementary Figure: 2  a) In contrast to vagal nerve stimulation, there were no significant differences among responses of the three groups to 0.1-0.2 μM bath-applied carbachol (CCh; 2 mins incubation).

b) Similarly, in atria pre-treated with L-NA (100μM, 20 min incubation), there were also no significant differences between groups to bath-applied CCh (0.2 μM, 2 mins incubation). The similarity of heart rate responses between groups to CCh before and during NOS inhibition highlights that the main functional action of the NO-cGMP pathway resides pre-synaptically.
Table 1

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<th>Sham</th>
<th>Ad.eGFP</th>
<th>Ad.NOS-1</th>
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<tr>
<td><strong>In vivo</strong></td>
<td>N/A</td>
<td>205±9 (n=8)</td>
<td>208±10 (n=8)</td>
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<tr>
<td><strong>In vitro</strong></td>
<td>186±11 (n=9)</td>
<td>186±6 (n=22)</td>
<td>179±5 (n=23)</td>
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Basal heart rate (bpm; mean ± S.E.) following treatment – no significant effect seen *in vivo* or *in vitro* (un-paired t-tests).