Report

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

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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.

The biological messenger nitric oxide (NO) is thought to be a fundamental signaling molecule in the regulation of cardiac cholinergic function.[1–4] Neuronal nitric oxide synthase (NOS-1), the enzyme responsible for NO synthesis, colocalizes with choline acetyltransferase in the intracardiac ganglia.[5] Functionally, pharmacological evidence suggests that NO generated from NOS-1 directly enhances the negative chronotropic effect of cholinergic stimulation[6,7] by activating the guanylate cyclase/cGMP pathway[7,8] to facilitate the release of acetylcholine (ACh),[9] and indirectly via endothelial NOS-3 M_2 receptor coupled inhibition of $I_{Ca\text{-}L}$ in pacemaking cells,[10] although this latter point is disputed.[11] 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.[7,8] 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.

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Materials and Methods

Detailed methods for gene transfer,[12] immunohistochemistry,[13] fluorescence microscopy,[14] confocal imaging,[5] immunoblotting,[7] measurements of ACh release,[9] and in vivo or in vitro autonomic phenotyping[7,8] 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 NOS-1 protein compared with atria infected with an adenoviral vector encoding recombinant enhanced green fluorescent protein (Ad.eGFP) (n=8, $P \leq 0.01$; 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 ($P \leq 0.001$) 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 1*H*-(1,2,4)oxadiazolo(4,3-a)quinoxaline-1-one (ODQ) (Figure 2C).

Pretreatment of isolated atria with the NOS inhibitor N^{ω}-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-treated 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 2a). Furthermore, we observed no significant difference among responses of the three groups to bath-applied 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

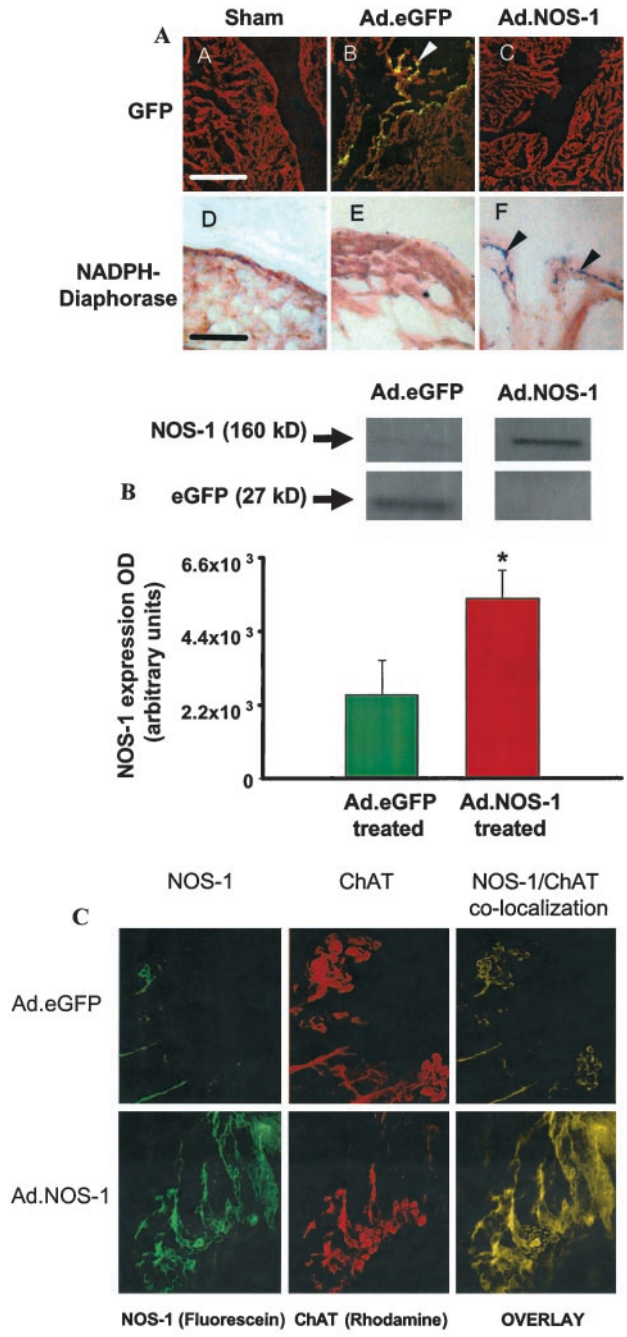

Figure 1. A, Representative cryosections from right atrium harvested 5 days after gene transfer or sham procedure. A and D are sections from atria treated with medium alone; B and E, atria treated with Ad.eGFP; and C and F, treated with Ad. NOS-1. Note that histochemical localization of eGFP was detected in Ad.eGFP-treated atria only (panel B, white arrow) and that intense NADPH 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.

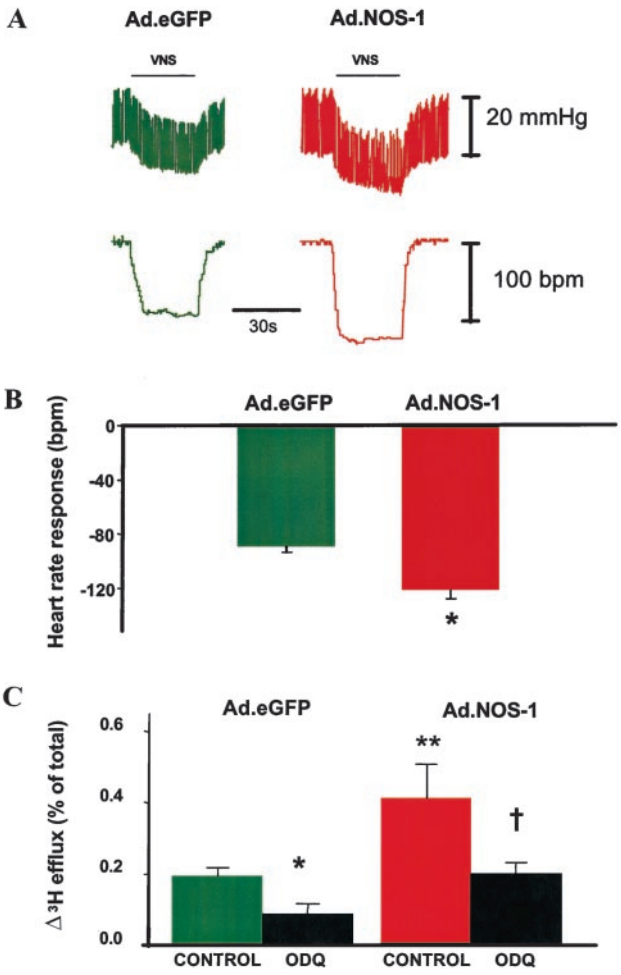

Figure 2. A and B, Ad.NOS-1 significantly increased (*$P<0.05$, unpaired t test; n=6) the HR response to vagal nerve stimulation (5 Hz) in vivo compared with Ad.eGFP-treated animals. C, Ad.NOS-1 also significantly increased (**$P<0.05$, unpaired t test; n=6) the release of ACh compared with Ad.eGFP control (n=8). The guanylyl cyclase inhibitor ODQ (10 μmol/L) significantly attenuated the release of ACh in both Ad.eGFR (*$P<0.01$, paired t test) and Ad.NOS-1 atria (†$P<0.05$, χ^2).

function without affecting postsynaptic cholinergic signaling. These data are consistent with recent findings that report pharmacological enhancement of the NO-cGMP pathway during right atrial field stimulation facilitates the release of ACh[9] by activating the guanylate cyclase/cGMP pathway.[7,8] 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.[7,8]

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.[15]

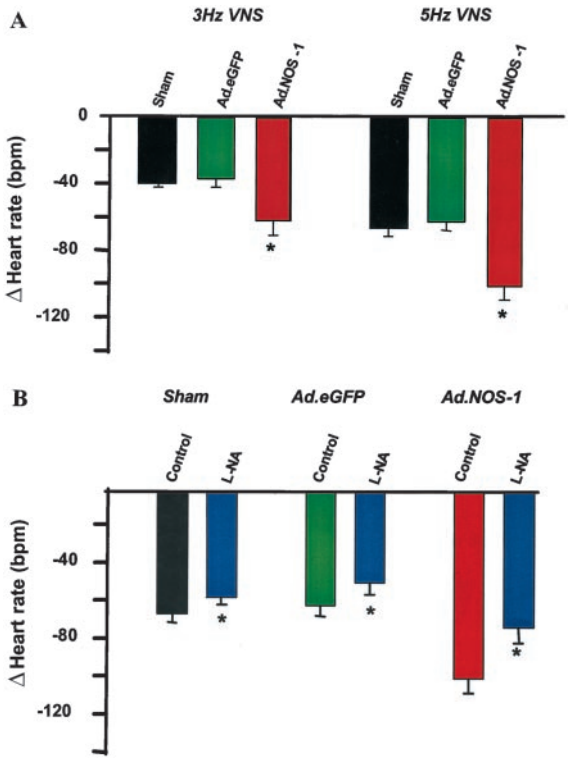

Figure 3. A, Ad.NOS-1 treatment significantly increased (*$P<0.001$, unpaired t test) the HR response to 3- and 5-Hz VNS in vitro. Responses of Ad.eGFP-treated and sham preparations were not significantly different. B, Pretreatment of isolated atria with the NOS inhibitor L-NA (100 μmol/L, 20-minute incubation) significantly attenuated the enhanced vagal bradycardia in Ad.NOS-1–treated atria at 3 Hz (data not shown) and 5 Hz (*$P<0.001$; n=15). NOS inhibition also significantly attenuated 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-treated preparations to 5-Hz VNS were not significantly different. L-NA reduced the HR response by the same relative amount (Ad.eGFP: $-27.3\pm6.5\%$ vs Ad.NOS-1: $-28.4\pm6.1\%$). Taken together, these results show that the increase in vagal function after NOS-1 gene transfer was due to enhanced NO bioavailability.

Functionally, high vagal tone is a positive prognostic indicator against sudden cardiac death, whereas impaired activity is a strong independent predictor of mortality.[16] 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.[17] It remains to be established, however, whether impaired cardiac vagal signaling seen in hypertension,[18] heart failure,[19] and after myocardial infarction[20] 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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