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Disruption of inhibitory G-proteins mediates a reduction in atrial β-adrenergic signaling by enhancing eNOS expression☆ DANSON: Augmented eNOS signaling in atrial myocytes

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Abstract

Objective: Cardiac parasympathetic nerve activity is reduced in most cardiovascular disease states, and this may contribute to enhanced cardiac sympathetic responsiveness. Disruption of inhibitory G-proteins (Gi) ablates the cholinergic pathway and increases cardiac endothelial nitric oxide (NO) synthase (eNOS) expression, suggesting that NO may offset the impaired attenuation of β-adrenergic regulation of supraventricular excitability. To test this, we investigated the role of endogenous NO production on β-adrenergic regulation of rate (HR), contraction (CR) and calcium (Ca^{2+}) handling in atria following blockade of Gi-coupled muscarinic receptors.
Methods: Mice were administered pertussis toxin (PTx, $n=105$) or saline (C, $n=100$) intraperitoneally. After 3 days, we measured CR, HR, and NOS protein levels in isolated atria. Intracellular calcium (Ca^{2+}) transients and Ca^{2+} current density (I_{Ca}) were also measured in atrial myocytes.
Results: PTx treatment increased atrial myocyte eNOS protein levels compared to C ($P<0.05$). This did not affect basal atrial function but was associated with a significant reduction in the CR and HR response to isoprenaline (ISO) compared with C. NOS inhibition normalized responses in PTx atria with respect to responses in C atria ($P<0.05$), which were unaffected. Furthermore, PTx did not affect ISO-stimulated HR and CR in eNOS gene knockout mice ($n=40$). In agreement with these findings, the ISO-mediated increase in Ca^{2+} transient was suppressed in PTx-treated myocytes ($P<0.05$), whereas I_{Ca} did not differ between groups.
Conclusion: eNOS-derived NO inhibits β-adrenergic responses following disruption of Gi signaling. This suggests that increased eNOS expression may be a compensatory mechanism which reduces β-adrenergic regulation of heart rate when cardiac parasympathetic control is impaired.

Keywords: Nitric oxide synthase; Contractility; Calcium; Catecholamines; Acetylcholine

1. Introduction

Impaired cardiac parasympathetic regulation and enhanced sympathetic activity characterize cardiac disease states. Although this neurohumoral response may initially act to compensate for impaired left ventricular (LV) function, ultimately the effects are detrimental and impaired vagal and augmented sympathetic regulation of heart rate are well-established independent predictors of mortality in patients with myocardial infarction or heart failure [1–4].

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Furthermore, vagal stimulation in rats with chronic heart failure has dramatic effects in terms of reducing ventricular remodeling and mortality [5]. Anti-arrhythmic effects are also observed acutely following vagal nerve stimulation in animals [6] in which vagal stimulation normally has no independent effects on ventricular function if heart rate is kept constant [7], suggesting the beneficial effects of vagal activation are mediated through the control of heart rate, supraventricular cardiac excitability and the resulting impact on myocardial oxygen demand. However, little is known about whether any endogenous signaling pathways are capable of compensating for cholinergic control of heart rate and atrial excitability when vagal neural activity is impaired, as seen in heart failure [8].

Emerging evidence suggests that myocardial nitric oxide (NO) may facilitate parasympathetic signaling and attenuate β-adrenergic responses in ventricular myocytes, atrial myocytes and at nerve endings (reviewed in Massion et al., 2003 [9]), and as such, it may help to maintain a favorable autonomic balance in all excitable tissue during cardiovascular disease. Although some consensus exists that NO synthesized by endothelial NO-synthase (eNOS) attenuates cardiac β-adrenergic ventricular developed pressure in isolated hearts and in vivo [10–12], it is not clear whether this is brought about by inhibition of calcium (Ca^{2+}) entry into ventricular myocytes or by decreased myofilament sensitivity to Ca^{2+} [13]. The extent to which eNOS derived NO is involved in cholinergic signaling in atrial and ventricular myocytes is also a matter of debate [11,14–16].

Recently, it has been shown that augmented eNOS signaling, induced by α-myosin heavy chain (α-MHC) promoter-driven eNOS protein overexpression attenuates β-adrenergically-stimulated inotropy and augments muscarinic reduction of chronotropy in mice in vivo and in vitro [16]. Similarly, adenoviral transfection of eNOS into the left ventricle (LV) of eNOS gene knockout ($eNOS^{-/-}$) mice restores the autocrine effects of eNOS observed in isolated ventricular myocytes [17]. However, both studies leave open the question of the pathophysiological significance of eNOS gene upregulation with respect to the autocrine control of autonomic signaling and Ca^{2+} cycling in cardiomyocytes. Importantly, it remains to be established whether increased endogenous NO production may replace cholinergic inhibition of β-adrenergic heart rate responses when cardiac parasympathetic responsiveness is diminished.

In support of this hypothesis, myocardial eNOS expression has been found to be increased in human heart failure [18,19] where myocardial cholinergic signaling is severely impaired [20]. Similarly, Hare and others (1998) [21] have shown that increased expression of myocardial eNOS can be induced experimentally by blocking the muscarinic receptor coupled G-protein (Gi) with pertussis toxin (PTx), suggesting the presence of an important regulatory link between Gi and eNOS expression. However, unlike transgenic myocardial overexpression of eNOS [16,22] the PTx-mediated increase in cardiac eNOS protein was associated with an augmented response to β-adrenergic stimulation on ventricular developed pressure in vivo [21].

Paradoxically, blunted parasympathetic activity in cardiac disease usually leads to an increase in expression of both Gi and the muscarinic receptor[23], presumably because vagal impairment occurs via a neural mechanism rather than at cardiomyocytes [8]. However, here we show that abolition of cholinergic signaling by blockade of Gi with PTx leads to attenuation of β-adrenergic inotropy via a reduction in intracellular Ca^{2+} transients in murine atrial myocytes. This effect is attributed to an increase in eNOS-signaling in myocytes. These results highlight a potentially beneficial role of eNOS gene induction in the control of β-adrenergic signaling and intracellular Ca^{2+} cycling following derangement of sympathovagal balance similar to that seen in cardiovascular disease states.

2. Methods

2.1. Animals

The treatment of all animals (male; C57BL/6 mice, 4–6 months old, $n=205$; $eNOS^{-/-}$ mice, 3–6 months old, $n=40$) was in accordance with the Home Office *Guidance on the Operation of Animals (Scientific Procedures) Act, 1986* (H.M.S.O.) and National Institute of Health (*Guide for the Care and Use of Laboratory Animals*). Mice were administered either pertussis toxin (30 μg/kg, PTx i.p.) or saline alone (C; 100 μl, i.p.), 3 days before experiments were commenced.

2.2. Atrial myocyte isolation

To isolate atrial myocytes for experiments concerning Western blot analysis and immunolocalisation of eNOS protein expression; and measurement of intracellular Ca^{2+} transient and Ca^{2+} current (I_{Ca}), mice were killed by cervical dislocation and the heart was initially perfused for 3 min with a nominally Ca^{2+}-free solution, followed by a further 9 min with an enzyme-containing solution (collagenase type II, 1 mg/ml, Worthington Biochemical Co., protease, 0.1 mg ml^{-1}, Sigma; Ca^{2+} 0.05 mmol/L). The atria were dissected free and placed in a separate flask containing fresh enzyme solution for 4 min. Atria were then diced and shaken in solution at room temperature for 10 min. In order to purify atrial myocytes, the digested atrial myocardium underwent several processes of filtering and spinning. This method yielded a high percentage of myocytes. For experiments measuring I_{Ca}, cells were kept in Kraft–Brühe medium containing (in mmol/L): L-glutamate 100, KCl 30, Na-pyruvate 5, taurine 20, creatine 5, succinic acid 5, Na_2ATP 2, β-OH butyrate 5, glucose 20, $MgCl_2$5, EGTA 1, HEPES 10 (pH 7.2, KOH), 2–3 h at 4 °C. For experiments measuring intracellular Ca^{2+} transients, cells were stored in

a modified Tyrode (in mmol/L; NaCl 120, KCl 5.4, $MgSO_4$ 5, $CaCl_2$ 0.2, Na-pyruvate 5, glucose 20, taurine 20, Hepes 10, pH 7.4, NaOH) at room temperature.

2.3. Western Blot analysis of NOS protein

In order to determine the effect of Gi-blockade on NOS protein levels, atrial myocytes from C and PTx mice were isolated 3 days following i.p. injections and were immersed in iced CellLytic™ MT lysis buffer (Sigma) containing a 1/40 v/v dilution of a proprietary mixture of mammalian protease inhibitors: 4-(2-aminoethyl)benzenesulfonyl fluoride, bestatin, pepstatin A, E-64, leupeptin and aprotinin (P8340, Sigma), then homogenized for 30 s in a Polytron homogenizer and homogenates centrifuged for 10 min at 10,000 $\times g$. Total protein levels were determined by the Bradford method and 200 μg protein loaded per well. Western blot analysis was performed as described previously [24] using commercially available polyclonal antibodies to eNOS, neuronal NO-synthase (nNOS) and β-actin (BD Biosciences), and the Western Lightening detection system (Perkin Elmer Life Sciences). Protein levels were expressed as a ratio of the optical densities of the eNOS and nNOS bands and the β-actin band to control for any inaccuracies in the protein loading.

2.4. Immunocytochemistry and confocal imaging of eNOS and caveolin-3

Laminin (Sigma) was diluted in PBS to a concentration of 25 μg/ml and used to coat microscope-slide cover slips. These were then incubated at 37 °C in 95% O_2/5 CO_2 for 2 h. Atrial myocytes were then isolated as described, and suspended in storage solution. Of the cell suspension, 100 μl, was spread on to the laminin-coated cover slips and incubated as before for 2 h to allow cell adhesion. These were then fixed and permeabilised with a 3% paraformaldehyde/0.1% Triton-X100 solution in the incubation chamber for 1 h, followed by a 1% BSA solution for 1 h. Cells were then incubated with (or without for negative control) primary antibodies (α-caveolin-3 mAb, Transduction Labs.) and α-eNOS (rabbit pAb, Transduction Labs) for 12 h and FITC or TRITC-conjugated secondary antisera. Immunoreactivity was viewed using a laser-scanning confocal microscope (Leica).

Confocal image (106.5 × 106.5 μm) and pinhole dimensions (2.23 μm) were set by viewing atrial myocytes processed with omission of the primary antibodies and photomultiplication of fluorescence was set at intensities (PMT FITC 647; PMT TRITC 591) at which these cells did not fluoresce. Images of cells were then acquired.

2.5. Measurement of atrial contractility and rate in-vitro

Mice were killed by cervical dislocation, the thorax was opened, and the ventricles perfused with heparinised physiological saline. The thorax and mediastinum were then removed and placed in physiological saline (mmol/L: NaCl 118, KCl 4.7, $MgSO_4$ 1.2, Na_2EDTA 0.5, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11, $CaCl_2$ 1.75, pH 7.4) and aerated with carbogen (95% O_2, 5% CO_2) at room temperature (~22 °C). A double atrial preparation was dissected free and placed into an organ bath (3 ml volume) maintained at 37±0.5 °C. Atria were used either for the measurement of spontaneous beating rate or contractile force. Heart rate (HR) was measured as previously described [25]. For the measurement of contractile force (CR), atria were paced at 540 beats per minute (bpm), (10 V, 1 ms pulse duration).

Cumulative doses of isoprenaline were applied to the organ bath to measure the increase in HR and CR (ISO, 0.01–10 μmol/L). This was repeated a total of three times. In separate experiments, singles doses of ISO (0.3 μmol/L) were applied followed by singles doses of carbamylcholine (CCh, 0.3 μmol/L). After a steady-state response was achieved to CCh, the bath was washed twice with fresh saline and this protocol was repeated a total of three times. The NOS inhibitor L-nitroarginine (L-NA, 0.1 mmol/L) was then added to the organ bath and allowed to equilibrate for 20 min before doses of ISO and CCh were applied as previously. L-arginine (1 mmol/L) was added to the organ bath in order to reverse the inhibition of eNOS by L-NA and allowed to equilibrate for 20 min. After equilibration the protocol was repeated.

2.6. Measurement of intracellular Ca^{2+} transient

Atrial myocytes were preincubated with the acetoxymethyl ester of fura-2 (5 μmol/L, Molecular Probes) for 15 min in perfusion solution (250 μmol/L Ca^{2+}) at room temperature after which they were washed and resuspended in perfusion solution containing 500 μmol/L Ca^{2+}. Myocytes were then transferred to the experimental chamber and field-stimulated to contract at 1 Hz. Cells were excited alternately at 365 and 380 nm by light from a xenon arc lamp (75 W). A barrier filter (510±15 nm) reflected the emission fluorescence to a photomultiplier tube. Background fluorescence was subtracted from the 365 and 380 signals, and the Ca^{2+} transient displayed as the 365/380 ratio. Measurements were taken from the average of 5 steady-state transients obtained both in control conditions and after application of ISO (0.3 μmol/L). All experiments were carried out at 35±1 °C.

2.7. Measurement of I_{Ca}

Membrane current was recorded from atrial myocytes using the whole-cell configuration of the patch-clamp technique (Axopatch 200A, Axon Instruments). Peak inward calcium current (I_{Ca}) was measured with respect to the current at the end of the step depolarization both in control condition and in the presence of ISO (0.3 μM). The

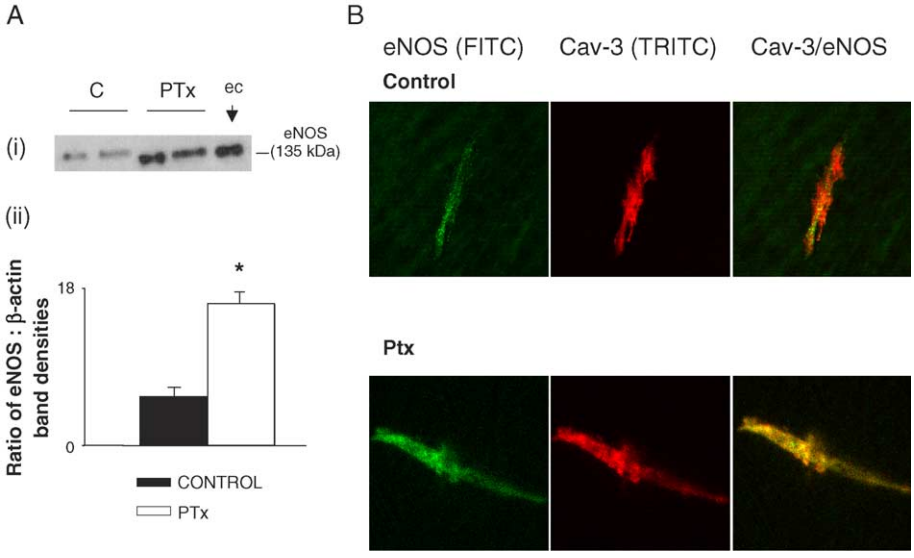
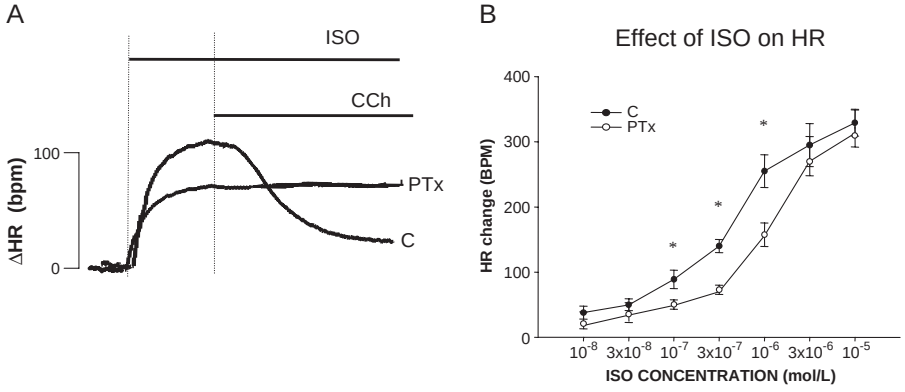


Fig. 1. Western blot analysis revealed augmented expression of endothelial NO-synthase (eNOS) protein in homogenized atrial myocytes from pertussis toxin-treated mice (PTx, $n=8$) compared to saline-treated (C, $n=8$). Photographic film of protein bands (A) (i) shows enhanced eNOS band densities in PTx-treated atrial myocytes compared to C; (ii) Mean band optical densities (eNOS:β-actin) demonstrates that eNOS protein is significantly increased in PTx compared to C atrial myocytes (*$P<0.05$, unpaired t-test; ec: endothelial cell homogenate used as positive control for eNOS protein). (B) Representative confocal micrographs from PTx and C atrial myocytes illustrate distribution and overlap of eNOS and caveolin-3 protein.

patch electrode solution to measure I_{Ca} contained (mmol/L): CsCl 110, TEA-Cl 20, HEPES 10, NaCl 10, $MgCl_2$ 1, MgATP 5, pH 7.2, CsOH. 1 mmol/L 4-aminopyridine (4-AP) was added to the external solution to block I_{to}.

I_{Ca} was elicited by 200 ms step depolarizations from a holding potential of −40 mV to test potential 0 mV. Cell membrane capacitance (C_m, which gives a measure of cell size) was measured in all cells by applying a −5 mV pulse



C

ISO (mol/L)	10^{-8}	$3x10^{-8}$	10^{-7}
C (ΔmN)	0.3 ± 0.1	0.6 ± 0.1	1.0 ± 0.2
PTx	0.3 ± 0.1	0.3 ± 0.1*	0.7 ± 0.1
ISO (mol/L)	**$3x10^{-7}$**	**10^{-6}**	**$3x10^{-6}$**
C (ΔmN)	1.5 ± 0.1	2.3 ± 0.1	2.9 ± 0.2
PTx	1.1 ± 0.1*	1.6 ± 0.1*	2.6 ± 0.2

Fig. 2. A: Raw heart rate (HR) trace obtained from spontaneously beating isolated atria from pertussis-toxin (PTx, $n=12$) and saline (C, $n=12$) treated mouse atria in vitro. (B): Dose–response curve to isoprenaline (ISO, 0.01–10 μmol/L) showing responses were significantly attenuated by PTx-treatment (*$P<0.05$) at several concentrations of ISO. (C): Table with values for changes in contraction amplitude (in mN) in response to doses of ISO showing significantly attenuated responses in PTx atria ($n=12$) compared to C atria ($n=12$, *$P<0.05$).

of 50 ms duration, from a holding potential of −40 mV. For each cell I_{Ca} was normalized to capacitance and expressed as current density in pA/pF. Analog signals were digitized (Digidata 1320A, Axon Instruments) and stored on-line to computer for subsequent off-line analysis.

The composition of the perfusion solution used during the measurements of I_{Ca} and Ca^{2+} transients contained in mmol/L: NaCl 134, KCl 5.4, $MgCl_2$ 1.2, $CaCl_2$ 1.75, glucose 11.1, HEPES 5, 4-AP 1, pH 7.4, NaOH. All experiments were carried out at 35±1 °C.

2.8. Statistics

Data are presented as mean+standard error of the mean (SEM). Comparisons between groups of data were performed using Student's unpaired *t*-test, and comparisons among multiple groups were performed by ANOVA (one factor). Statistical significance was accepted for values of $P<0.05$.

3. Results

3.1. Effect of PTx on constitutive NOS expression

Gi blockade with PTx resulted in an increase in total eNOS protein in homogenized whole atria compared to C ($P<0.05$). In addition, PTx-treatment resulted in a 2.9 fold increase in the eNOS protein level in murine atrial myocytes compared with C (eNOS:β-actin band density ratios: C ($n=8$): 5.5±1.8 vs. PTx ($n=8$): 16.0±2.2; Fig. 1A; $P<0.05$). Conversely, PTx treatment did not elicit any change in nNOS protein expression (nNOS:β-actin band density ratios: C: 0.24±0.01 vs. PTx: 0.28±0.01; $P=0.34$ unpaired *t*-test).

3.2. Effect of PTx treatment on eNOS distribution in atrial myocytes

Confocal images of C ($n=4$) and PTx ($n=4$) atrial myocytes stained for eNOS and caveolin-3 are shown in Fig. 1B. Co-localization of eNOS with caveolin-3 was only partial in C cells, since caveolin-3 was abundant throughout the cell whereas eNOS was focally distributed. The distribution of eNOS throughout the cell was more diffuse in PTx cells and co-localization of eNOS and caveolin-3 appeared greater.

3.3. Effect of PTx on atrial rate and contractility

The spontaneous beating rate of isolated mice atria (HR) was not affected by PTx (C ($n=12$): 378+5 bpm vs. PTx ($n=12$): 367±7 bpm). Similarly, basal CR did not differ between PTx-treated and C atria paced at 540 bpm (C ($n=12$): 1.22±0.07 mN vs. PTx ($n=12$) 1.10±0.08 mN). In contrast both HR (Fig. 2) and CR responses (Fig. 2C) to ISO were attenuated at a range of concentrations in PTx atria

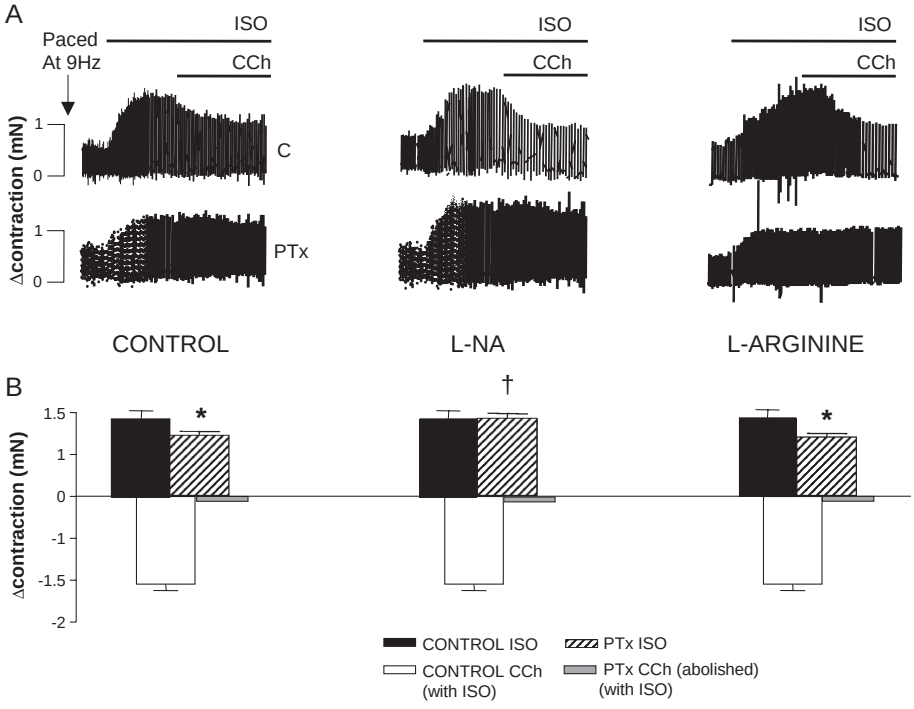


Fig. 3. Contractile-force (CR) data trace (A) from isolated saline (C, $n=20$, upper panel) and pertussis toxin-treated (PTx, $n=20$) atria (lower panel) showing responses to isoprenaline (ISO, 0.3 μmol/L) and carbachol (CCh, 0.3 μmol/L) before and after NOS inhibition with L-nitroarginine (L-NA, 100 μmol/L); and competitive reversal of inhibition with excess L-arginine (1 mmol/L). CR responses to CCh were abolished whereas ISO responses were attenuated in PTx compared to C (B: *$P<0.05$, unpaired *t*-test). NOS inhibition reversed the attenuated CR responses to ISO, and this was restored by L-arginine (†$P<0.05$, repeated-measures ANOVA).

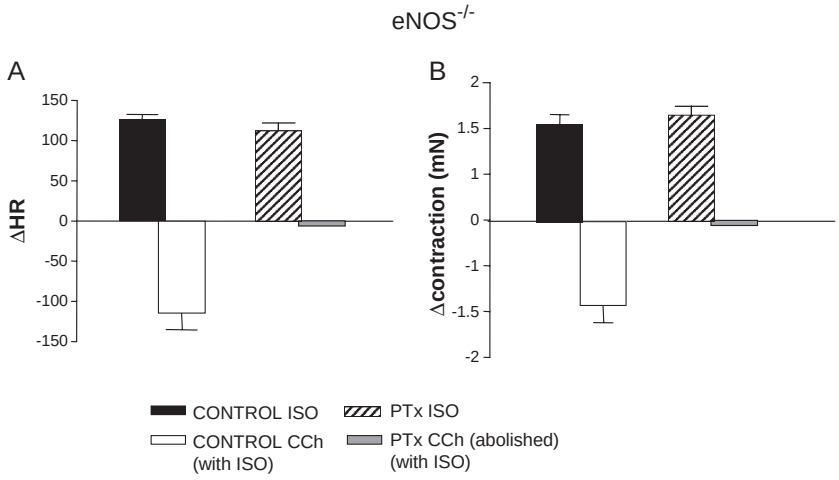


Fig. 4. In contrast to what was seen in normal mouse atria, heart rate (HR, A) and contractile force (CR, B) responses to isoprenaline (ISO, 0.3 μmol/L) were not affected by pertussis-toxin (PTx, $n=22$) treatment compared to C ($n=18$) in endothelial NO-synthase (eNOS) knockout atria in vitro, although responses to carbachol (CCh, 0.3 μmol/L) were still abolished by PTx.

compared with C, although not at the maximal dose (10^{-5} mol/L; EC_{50}C: 3.7×10^{-7} vs. EC_{50}PTx: 1.3×10^{-6} mol/L, $P<0.05$). CCh (0.3 μmol/L) mediated suppression of ISO responses was present in C but was abolished in all PTx atria (see raw traces in Figs. 2 and 3), demonstrating that Gi is obligatory for cholinergic inhibition of β-adrenergic signaling.

3.4. Effect of NOS inhibition or eNOS gene deletion on the β-adrenergic responses

Non-isoform specific NOS inhibition with L-NA had no effect on basal HR or CR in either C ($n=40$; 20 for HR and 20 for CR experiments) or PTx ($n=40$; 20 for HR and 20 for CR experiments) atria. In C atria, L-NA also had no

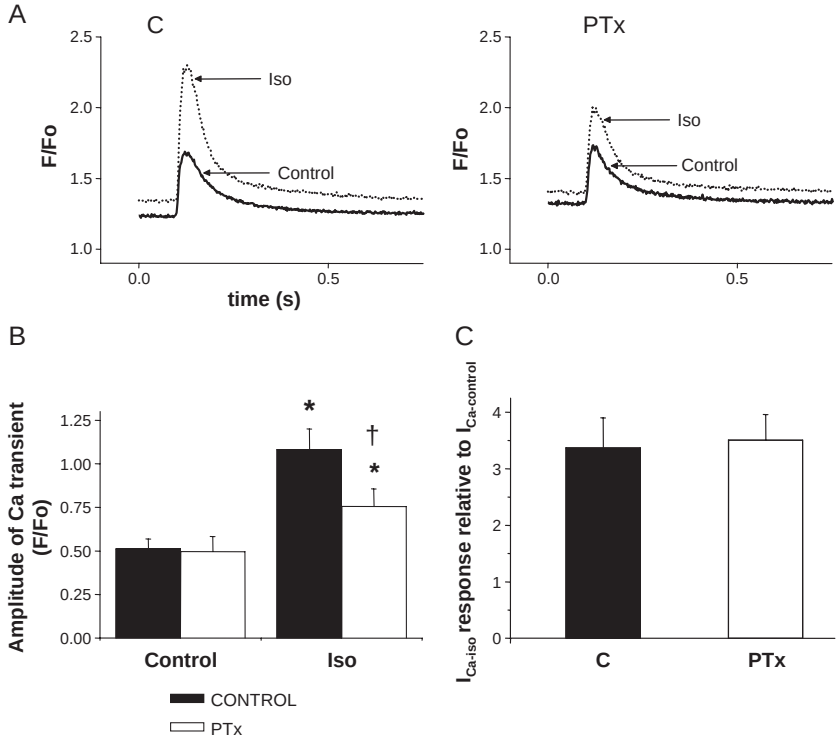


Fig. 5. A: Intracellular calcium transients in saline (C, $n=16$) and pertussis toxin (PTx, $n=18$) atrial myocytes before and after stimulation with isoprenaline (ISO, 0.3 μmol/L). 5B: Grouped data illustrating that baseline calcium transients were not affected by PTx treatment, but the amplitude of transients were significantly less in PTx atrial myocytes compared to C after they had been significant augmented by ISO (*$P<0.05$, unpaired t-test). 5C: Calcium current density in atrial myocytes measured before and after augmentation by isoprenaline (ISO, 0.3 μmol/L). The augmentation caused by ISO was not different in myocytes from pertussis toxin (PTx, $n=11$) and saline-treated (C, $n=8$) mice.

effect on the HR (ISO: 121±11 bpm; CCh: 116±9 bpm; control HR responses see Fig. 2) or CR response (Fig. 3) to ISO or CCh. However, in PTx atria the HR and CR responses to ISO were significantly enhanced by L-NA (HR response: ISO: 106±3; control HR response see Fig. 2; CR responses see Fig. 3) such that they no longer differed from C. This effect was reversed by L-arginine (HR response: ISO: 71±6 bpm; for CR response see Fig. 3; repeated measures ANOVA, $P<0.05$). Similar responses were seen between the effects of noradrenaline on HR and CR in PTx and C atria. These effects were also reversible by NOS inhibition ($P<0.05$, data not shown).

eNOS gene deletion did not affect the HR or CR response to ISO or CCh in C atria ($n=18$; 8 for HR and 10 for CR measurements) (Fig. 4). Moreover, PTx treatment did not suppress the HR ($n=10$) and CR ($n=12$) response to ISO in eNOS$^{-/-}$ mice, indicating that eNOS is instrumental in mediating the reduction in β-adrenergic responses in PTx treated mice.

3.5. Effect of PTx treatment on intracellular Ca^{2+} transient

In basal conditions, resting and peak intracellular Ca^{2+} did not differ between atrial myocytes from PTx ($n=18$) treated mice compared to C ($n=16$). However, the increase in amplitude of the Ca^{2+} transient in response to ISO was significantly smaller in atrial myocytes from PTx-treated mice compared to C (Fig. 5A and B; $P<0.05$, unpaired t-test). The rate of decline of the Ca^{2+} transient was also not different between C and PTx myocytes (Tau C: control 72.7±6 ms; ISO 60.7±7 ms; PTx: control 67.6±10; ISO 54.9±7).

3.6. Effect of PTx treatment on I_{Ca}

To establish whether a reduction in Ca^{2+} entry via the L-type Ca^{2+} channels contributed to the suppressed β-adrenergic Ca^{2+} transient in PTx atrial myocytes, we compared basal and ISO-stimulated I_{Ca} density in C ($n=8$) and PTx ($n=11$) treated myocytes. Our findings show that neither basal (C: −2.81+0.46 pA/pF; PTx: −3.36+0.40 pA/pF) nor β-adrenergically stimulated I_{Ca} (C: −7.77±0.19 pA/pF; PTx: −10.51=1.63 pA/pF) differs between PTx and C myocyte (Fig. 5C).

4. Discussion

In this study, we have attempted to elucidate the responsive role of eNOS in the maintenance of cardiac autonomic regulation (primarily in the control of beating rate and atrial contractility by autonomic transmitters), after the cholinergic signaling pathway has been ablated. Emerging evidence suggests that myocardial NO can augment parasympathetic and diminish sympathetic responses in most excitable cardiac tissue. This work provides novel evidence that atrial eNOS gene expression augments in response to Gi-blockade, and that this acts to attenuate β-adrenergic responses in atria, and thus regulation of heart rate. Whether eNOS upregulation may have a protective role in clinical scenarios where cardiac vagal tone is depressed remains purely speculative. However, this work places eNOS in the context of potential mediators capable of dampening cardiac sympathetic regulation of heart rate and signaling in atrial myocytes in disease states characterized by vagal-impairment.

The idea that eNOS-derived NO plays an important part in the regulation of cardiac autonomic responsiveness has recently been a matter of debate. Whilst several reports have documented modest effects of eNOS-derived NO on basal, cholinergic or adrenergically-stimulated cardiac responses [10–12,17,22], other investigators have been unable to demonstrate an eNOS-dependent regulation of cardiac function [15,26]. Differences in experimental conditions and models, which in turn may affect NO bioavailability within cardiac myocytes, may contribute to these discrepancies [9,27].

Our findings indicate that eNOS has a negligible role in the regulation of basal and β-adrenergic atrial contractility and heart rate in isolated atria from saline-treated mice. This conclusion is based on the evidence that both acute NOS inhibition with L-NA and selective gene disruption of eNOS did not alter basal or ISO-stimulated contraction or rate in isolated murine atria. However, the increase in atrial eNOS expression that followed disruption of cholinergic signaling by PTx treatment was accompanied by a significant attenuation of β-adrenergic responsiveness, which was reversed by pharmacological NOS inhibition and was absent in eNOS knockout mice. In addition, PTx attenuated the increase in peak intracellular calcium in response to ISO without affecting the basal or ISO-stimulated calcium current in murine atrial myocytes. Taken together these findings indicate that eNOS-derived NO can inhibit β-adrenergic contractility and heart rate responses in an autocrine fashion when the expression of eNOS is increased following Gi inhibition. This mechanism might protect the myocardium from catecholamine toxicity in pathophysiological states where parasympathetic responsiveness is suppressed.

4.1. Regulation of eNOS expression in cardiac myocytes

Increased myocardial eNOS expression has been observed in human failing hearts [19,28] and in an animal model of dilated cardiomyopathy [18], however the mechanism underlying these findings has not been established. Although there is substantial in vitro evidence indicating that eNOS promoter activity can be modulated by a host of different factors (reviewed in Fleming and Busse, 2003 [29]), the stimuli that have the largest effects on myocardial eNOS protein level are those that affect eNOS mRNA stability [30]. In particular, β-adrenergic/adenylate

cyclase stimulation and a sustained increase in intracellular cAMP have been shown to decrease eNOS mRNA levels and protein abundance in ventricular myocytes resulting in an increased contractile responsiveness to β-adrenergic agonists [31]. Similarly, myocardial eNOS expression in explanted failing human hearts has been shown to be greater in patients treated with β-blockers [19].

The molecular mechanism by which PTx treatment stimulates eNOS expression remains unclear, although this may be related to direct interactions between the cardiac muscarinic receptor (M_2) and eNOS protein within caveolae [32]. In particular, evidence produced by others has implicated a role for acetylcholine-M_2 interactions as a trigger for eNOS synthesis of NO [33]. Here we show that association between caveolin-3 and eNOS is greater in the PTx-treated animal, suggesting that trafficking of eNOS protein to the caveolar complex may be regulated by activity of the downstream cholinergic pathway. Although caveolin-3 is a known inhibitor of NOS activity, augmented NO-synthesis has now repeatedly been observed where association of caveolin-3 and NOS is increased [34,35]. The reason for this is unknown although it may be a result of an increased availability of NOS substrates and co-factors within the caveolar microdomain.

The functional significance of physiological stimuli in eliciting changes in eNOS protein expression and trafficking in atria is not understood; however it has become clear recently that genetic polymorphisms of the eNOS gene are associated with impaired regulation of eNOS in coronary vasculature during stress [36,37] and poorer prognosis in patients with coronary artery disease [38]. Whether these gene polymorphisms are also associated with impaired myocardial eNOS protein expression and β-adrenergic signaling may be of significant future interest.

4.2. eNOS-dependent regulation of cardiac β-adrenergic responsiveness

Evidence supporting a role for eNOS in the regulation of adrenergic responsiveness in cardiomyocytes in unstressed animals is highly contradictory, although it establishes a pattern that potentially indicates that the level of eNOS expression may be crucial in defining its functional role. In particular, the most dramatic effects of eNOS blockade are often seen when eNOS activity or expression are augmented compared to when basal eNOS activity is inhibited.

LV contractile responses to ISO in $eNOS^{-/-}$ are modestly enhanced both in isolated hearts [10,11] and in vivo [10,12]. This effect, however, is not seen in our atrial preparations and in most [11,14,15,26] but not all [12] studies in isolated LV myocytes, suggesting that the contribution of endothelial-derived NO may dominate eNOS-mediated inhibition of β-adrenergic responses in normal hearts [39]. Conversely, transgenic myocyte-specific overexpression of eNOS results in the attenuation of both basal and β-stimulated LV inotropy [16,22]. These findings are in partial agreement with our data, as we saw attenuation of β-adrenergic but not of basal atrial inotropy with PTx-induced eNOS overexpression. Furthermore, whilst the chronotropic response to β-adrenergic stimulation was not affected in hearts with transgenic overexpression of eNOS [22], we observed a significant reduction in the HR response to ISO in PTx treated atria, which was abolished by NOS inhibition. These differences are likely to be related to the method used to increase eNOS expression. In particular, in Brunner et al. [22] eNOS expression was driven by α-MHC promoter, which resulted in a 40–90 fold increase in eNOS activity and expression compared with a 2–3 fold increase in eNOS protein in our PTx-treated atria. This quantitative difference in eNOS expression may explain the lack of effect on basal HR or contractility in our study. Similarly, there may be regional differences in eNOS expression between models. In particular, as the sinoatrial node is known to contain a significant proportion of β-MHC and of a fetal-like myosin isoform [40], it is conceivable that α-MHC-driven eNOS overexpression may be less efficient in this region. Conversely, the preferential location of Go/Gi in the central sinoatrial node region [41], may make PTx-driven upregulation of eNOS more effective at augmenting NO-mediated effects on pacemaking.

The functional consequence of PTx-driven eNOS upregulation however has not been fully described. In contrast with our findings, Hare et al. (1998) [21] reported an increase in basal and β-adrenergic peak LV dP/dt max in isolated rat hearts pre-treated with PTx, despite seeing a similar increase in eNOS expression as we do. Although it is conceivable that eNOS activity was significantly limited by the availability of substrate in their hearts, since significant negative inotropic effects were brought about by L-arginine, additional evidence exists that PTx can increase the ability of β_2 receptors to stimulate adenylate cyclase in atrial membranes [42]. However, it has also recently been suggested that the contribution of β_2 or β_3 adrenoreceptor signaling to the inotropic and HR response to physiological β-agonists in murine atria may be negligible under these conditions [41]. Furthermore, the β_1 signaling pathway, which may play a dominant role in physiological β-adrenergic responsiveness, is not affected by acute Gi-blockade (for <24 h) with PTx [43], suggesting the cardioinhibitory effects we see develop over the course of the 3 day incubation period, during which time eNOS gene upregulation occurs.

4.3. Intracellular targets for eNOS in cardiomyocytes

The mechanisms through which NO inhibits β-adrenergic inotropy are complex and may vary depending on the experimental conditions. A cGMP-mediated inhibition of the L-type calcium current via PKG or through enhanced breakdown of cAMP via cGMP-sensitive cAMP phosphodiesterase (PDEII) has been described by Campbell et al. (1996) [44] by using high concentrations of NO donors.

This mechanism, however, is an unlikely candidate in our experiments given that basal and β-adrenergic calcium current density were unchanged in PTx atrial myocytes compared to control.

PKG-mediated phosphorylation of troponin I [45] resulting in inhibition of myofilament calcium sensitivity has also been advocated as the mechanism through which NO may reduce myocardial inotropy and facilitate relaxation. We cannot exclude an eNOS-mediated inhibition of myofilament calcium sensitivity in our preparation, however, the smaller increase in peak intracellular calcium observed in PTx-treated atrial myocytes in response to ISO, suggest that other mechanisms may contribute to the NO-mediated inhibition of β-adrenergic inotropy in PTx treated atrial myocytes.

Zahradnikova et al. (1997) [46] have shown that endogenous NO production may decrease the open probability of the ryanodine receptor (RyR) and thus calcium release from the sarcoplasmic reticulum (SR) Others, however, have demonstrated that eNOS-derived NO increases RyR open-probability and SR calcium release in response to stretch in isolated rat ventricular myocytes [47]. The interaction between NO and β-adrenergic stimulation on RyR function in the heart is similarly complex and dependent on the degree of adrenergic stimulation [48]. Specifically, application of NO donors decreases RyR activity and contraction in myocytes pre-stimulated with high concentrations of ISO. However, in the presence of mild β-adrenergic stimulation, addition of an NO donor increases both cell shortening and RyR open probability in rat ventricular myocytes [49]. Given that the time constant of the decay (tau) of the transient is similar in both groups, it is unlikely that there is any effect of eNOS on calcium removal from the cytosol, favoring inhibition of SR calcium release from RyR as a more likely target. However, as experiments to measure calcium spark frequency or myofilament-sensitivity to calcium were not undertaken in this study, it remains to be established the precise mode of action of NO in this context.

5. Limitations of this study

Although data collected regarding the autonomic regulation of heart rate and atrial function is important (and as all indices of autonomic function are important) as a correlate of mortality and cardiac arrhythmia, it is not necessarily applicable to ventricular function or the autonomic regulation of other cardiac tissues perturbed by cardiac diseases. In particular, the vagal innervation of the ventricles is significantly sparser than that of the atria. Although decreased LV contractility in response to vagal nerve stimulation has been shown in humans and pigs [50], effects of vagal stimulation on ventricular performance at constant heart rates are absent in rats [7]. In addition, whether similar effects of PTx-treatment to those observed in atrial myocytes on eNOS expression and autonomic function would be observed in LV myocytes is not known, although in vivo recordings of LV developed pressure in the rat after PTx has previously shown enhanced contractility to β-agonists [21]. Furthermore, whether the functional effect of eNOS is the same in LV and atrial myocytes or on SA node function is not unequivocally known, although the majority of previous reports have documented similar directional effects of NO on autonomic signaling in most excitable cardiac tissues [9]. Ventricular myocytes were not used in this study because autonomic control of rate may be more important in the development of arrhythmias throughout the heart than control of ventricular excitability per se [6], although this may vary between species.

In conclusion, although excluding a role for basally expressed eNOS in the modulation of β-adrenergic responsiveness, our data implicate a significant role for enhanced eNOS in the suppression of β-adrenergic signaling via the modulation of calcium handling in atria and in attenuating β-adrenergic regulation of heart rate. This may have some homeostatic importance in compensating for vagal nerve dysfunction. Whether the absence of this mechanism affects the progression of cardiac disease states in individuals with dysfunctional eNOS gene regulation isn't clear and warrants further investigation.

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