Nitric Oxide and Cardiac Muscarinic Control in Humans

Saqib Chowdhary, Anna M. Marsh, John H. Coote, Jonathan N. Townend

Abstract—Cardiac parasympathetic activity reduces susceptibility to potentially lethal ventricular arrhythmias in heart failure and ischemic heart disease. This influence is mediated in large part by antagonism of the adverse cardiac effects of sympathetic overactivity (“indirect” parasympathetic activity) in addition to the “direct” effects of muscarinic stimulation. Nitric oxide modulates parasympathetic cardiac signaling in some animal models, but human data are lacking. We have investigated the influence of endogenous nitric oxide on cardiac responses to parasympathetic stimulation in healthy humans. In 18 volunteers, we studied chronotropic and inotropic responses to muscarinic stimulation, both before and after prestimulation with isoproterenol. Cardiac muscarinic stimulation was achieved using an intravenous bolus of the short-acting cholinesterase inhibitor, edrophonium. Responses were assessed during a background infusion of a nitric oxide synthase inhibitor (N\textsuperscript{G}-monomethyl-L-arginine [L-NMMA]), placebo (saline), or phenylephrine (vasoconstrictor control) in a single-blind, random order, crossover protocol. L-NMMA did not affect chronotropic responses to edrophonium alone (direct parasympathetic activity). The decrease in heart rate attributable to “indirect” parasympathetic activity (derived by comparison with the effect of edrophonium during concurrent adrenergic stimulation) was substantially attenuated by L-NMMA in comparison to both control infusions. No modification of muscarinic inotropic responses by L-NMMA was apparent in comparison to the vasoconstrictor control. Nitric oxide exerts a powerful facilitating influence on indirect (antiadrenergic) but not direct human cardiac parasympathetic control. Stimulation of the endogenous nitric oxide pathway might enhance parasympathetic protection against the adverse influences of cardiac sympathetic overactivity. (Hypertension. 2004;43:1023-1028.)

Key Words: nitric oxide • autonomic nervous system • receptors • heart rate • myocardial contraction • acetylcholine • catecholamines

Autonomic regulation of the heart has an important influence on prognosis in cardiac disease. Although elevated sympathetic activity is associated with an adverse prognosis, high levels of parasympathetic tone appear to confer cardioprotection, in part by protection against potentially fatal ventricular arrhythmia. Such parasympathetic actions on the heart are mediated not only by the direct consequences of cardiac muscarinic receptor stimulation ("direct" parasympathetic activity) but also by an indirect mechanism, whereby muscarinic stimulation inhibits cardiac adrenergic signaling. This latter influence, termed “indirect” parasympathetic activity, or “accentuated antagonism,” may be of clinical importance by reducing the adverse cardiac consequences of sympathetic overactivity in disease states such as heart failure and myocardial infarction. The factors that modulate this peripheral interaction of cardiac adrenergic and muscarinic signaling have, however, received little attention.

Animal data suggest that nitric oxide (NO) acts as a neuromodulator not only within the central nervous system but also within peripheral autonomic pathways controlling cardiac function to provide a net enhancement of parasympathetic and inhibition of sympathetic control. Up to 40% of intrinsic cardiac neurons contain neuronal NO synthase (nNOS), including those innervating the nodal regions. Endothelial NO synthase (eNOS) has been identified within sinoatrial node (SAN) myocytes and their adjacent capillaries. The NO generated at these peripheral cardiac autonomic sites appears to enhance not only the heart rate response to vagus (parasympathetic) nerve stimulation (although not in all species) but also parasympathetic antagonism of cardiac sympathetic responses (indirect activity). Furthermore, NO has also been shown to directly inhibit cardiac responses to sympathetic stimulation in animals.

In conscious humans, we have previously demonstrated that NO appears to play a tonic facilitatory role in the baroreflex control of cardiac parasympathetic activity (both in health and heart failure); however, these experiments were not able to distinguish between central and peripheral actions. This study aims to define the physiological role of endogenous NO in modulating peripheral parasympathetic control of the heart and also its influence on adrenergic–muscarinic interaction (indirect parasympathetic activity) in healthy, conscious human subjects.

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Methods

Eighteen healthy male nonsmokers aged 18 to 32 (mean = 21) years were studied. All subjects were normotensive (blood pressure <140/90 mm Hg) with no symptoms or signs of disease and were not medicated. Subjects abstained from food or drink for 2 hours before study and from caffeine and alcohol for 24 hours. Experimental protocols were approved by our local research ethics committee and individual written consent was obtained.

Experimental Protocol

The primary aim of this study was to examine the effects of systemic NO inhibition (N\textsuperscript{\textdegree}-monomethyl-L-arginine [L-NMMA] infusion) on direct and indirect muscarinic control of heart rate. Inotropic responses were examined as a secondary endpoint. Because L-NMMA is a vasoconstrictor and causes a reflex bradycardia, its modulating effect on muscarinic responses was compared not only with an inactive placebo (saline infusion) but also with a control vasoconstrictor (the \textalpha\textsubscript{2}-agonist phenylephrine) titrated to produce an equivalent baseline bradycardia to that seen with L-NMMA.

Protocols were of a single-blind, random order, crossover design, with each subject randomly assigned to receive a continuous infusion of either intravenous L-NMMA (3 mg/kg per hour, maximum 250 mg), phenylephrine (0.1 to 0.4 \mu g/kg per minute), or 0.9% saline during the first of 3 study visits. Randomization was repeated for the remaining agents at each subsequent study visit (separated by 7 to 14 days) until each subject had been studied during all 3 agents.

Subject preparation and experimental conditions were as described previously.

Analysis

Data were processed and analyzed off-line, as previously described, by an observer blinded to the agent and the volunteer undergoing study. An instantaneous heart rate tachogram was plotted, and the maximal deviation from baseline (an average of the 30-second pre-injection interval) for a running 4-sample mean was calculated by an automated procedure for each recording. The mean arterial pressure response to each bolus injection was similarly derived.

Because the protocol allows potentially different loading conditions (with matching of baseline heart rates rather than baseline blood pressures), the following "pre-ejection phase" indices of left ventricular contractility were used, reducing the influence of changes in afterload: isovolumetric contraction time (IVCT) and the mean rate of pressure increase (LV dp/dt) calculated as (diastolic blood pressure – LV end-diastolic pressure)/IVCT. LV end-diastolic pressure was indirectly assessed using the Palomo formula.

As depicted in Figure 1, the maximal decrease in heart rate (or contractility) from baseline values with edrophonium alone was taken as a measure of direct muscarinic activity (\Delta M_{\text{d}}). The maximum negative chronotropic (inotropic) response to edrophonium given during background infusion of isoproterenol represents the summation of both direct and indirect (anti-adrenergic) muscarinic activity (\Delta M_{\text{d}+\text{i}}). Indirect muscarinic activity (\Delta M_{\text{i}}) was therefore determined as the difference between responses to the 2 boluses of edrophonium (ie, \Delta M_{\text{d}+\text{i}} – \Delta M_{\text{d}}). As a secondary analysis, adrenergic response (\Delta S) was taken as the change from baseline values with isoproterenol infusion.

After tests for normality, autonomic heart rate, blood pressure, and echocardiographic responses were compared between each of the 3 infusions by repeated measures 1-way ANOVA with the Bonferroni posttest correction. The individual influence of each infusion was tested by paired Student \textit{t} test. Values are mean ± SE.

Results

Infusion of L-NMMA caused a significantpressor response and a reflex bradycardia (Table). This bradycardia was matched to within 1 beat per minute (bpm) by dose titration of the phenylephrine infusion (average dose = 7.8 \mu g/kg per

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**Hemodynamic Variables at Steady-State for Each Drug**

<table>
<thead>
<tr>
<th>Index</th>
<th>Saline</th>
<th>L-NMMA</th>
<th>Phe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before isoproterenol</td>
<td>HR (bpm)</td>
<td>64±3</td>
<td>58±2†</td>
</tr>
<tr>
<td></td>
<td>(Predrug mean HR)</td>
<td>(64±3)</td>
<td>(65±3)</td>
</tr>
<tr>
<td></td>
<td>Mean BP (mm Hg)</td>
<td>80±2</td>
<td>84±1‡</td>
</tr>
<tr>
<td></td>
<td>(Predrug mean BP)</td>
<td>(80±2)</td>
<td>(80±1)</td>
</tr>
<tr>
<td></td>
<td>LVEDP (mm Hg)</td>
<td>9.8±0.4</td>
<td>9.6±0.5</td>
</tr>
<tr>
<td></td>
<td>IVCT (ms)</td>
<td>58±2</td>
<td>62±3</td>
</tr>
<tr>
<td></td>
<td>LV dP/dt (mm Hg/s)</td>
<td>988±49</td>
<td>946±37</td>
</tr>
<tr>
<td>During isoproterenol</td>
<td>HR (bpm)</td>
<td>91±4</td>
<td>79±3‡</td>
</tr>
<tr>
<td></td>
<td>Mean BP (mm Hg)</td>
<td>86±2</td>
<td>87±1</td>
</tr>
<tr>
<td></td>
<td>LVEDP (mm Hg)</td>
<td>10.2±0.4</td>
<td>9.8±0.5</td>
</tr>
<tr>
<td></td>
<td>IVCT (ms)</td>
<td>14±1</td>
<td>17±1</td>
</tr>
<tr>
<td></td>
<td>LV dP/dt (mm Hg/s)</td>
<td>4262±424</td>
<td>3367±269</td>
</tr>
</tbody>
</table>

Data represent baseline conditions before the first (before isoproterenol) and until the second (during isoproterenol) edrophonium boluses. The influence of the background saline, L-NMMA, and phenylephrine infusion on heart rate and mean blood pressure is given as bracketed values before the drug compared to the steady-state values during the drug (and before isoproterenol).

Phe indicates phenylephrine; HR, heart rate; BP, blood pressure; LVEDP, derived left ventricular end-diastolic pressure; IVCT, isovolumetric contraction time; LV dP/dt, mean rate of pressure rise.

\*P<0.05 vs pre-drug level; †P<0.01 vs pre-drug level; ‡P<0.001 vs pre-drug level.

Between drug comparisons are \|P<0.05, ||P<0.01 vs saline.
hour). A significant bradycardia compared with saline was maintained for both vasoconstrictors during isoproterenol infusion.

**Direct Muscarinic Response**
Injection of edrophonium alone resulted in a bradycardia, the direct muscarinic response ($\Delta M_d$), that was not significantly altered by L-NMMA in comparison to either saline or phenylephrine controls (Figure 2A). Responses to edrophonium were also similar between the 2 control infusions (phenylephrine and saline; Figure 2A), despite the significantly lower baseline heart rate with phenylephrine (Table). Edrophonium did not produce any significant change in mean arterial pressure (Figure 2). No bradycardic response was observed with placebo (saline) bolus injection (data not shown).

**Indirect Muscarinic Response**
The negative chronotropic response to edrophonium was significantly increased during concurrent isoproterenol infusion (Figure 2B). This accentuated response, the sum of both direct and indirect muscarinic action ($\Delta M_{d+i}$), was significantly reduced by L-NMMA in comparison to either saline or phenylephrine (Figure 2B). Subtracting the direct response ($\Delta M_d$) from this combined measure ($\Delta M_{d+i}$) reveals modulation of indirect muscarinic action alone ($\Delta M_i$). A >50% reduction in this component is seen during L-NMMA ($\Delta M_i = -5 \pm 2$ bpm) in comparison to either control agent ($P<0.05$ versus both saline, $\Delta M_i = -12 \pm 2$ bpm and phenylephrine, $\Delta M_i = -11 \pm 2$ bpm). In contrast, responses during phenylephrine closely matched those during saline infusion (Figure 2B) despite the lower baseline heart rate with phenylephrine (Table).

**Adrenergic Response**
The positive chronotropic effect of isoproterenol infusion was reduced by L-NMMA in comparison to saline but was not significantly different from effects during phenylephrine, the control vasoconstrictor (Figure 3).

**Inotropic Responses**
Edrophonium given alone failed to produce any measurable change in echocardiographic measures of left ventricular contractility (Figure 4). When administered during concurrent adrenergic stimulation, edrophonium did produce a small but statistically significant lengthening of IVCT (negative inotropic response), which was not modified by L-NMMA (Figure 4). Correcting for changes in diastolic blood pressure by calculation of LV dP/dt revealed that the decrease in this index of contractility with edrophonium was reduced during L-NMMA in comparison to saline but not in comparison to phenylephrine (Figure 4).

Isoproterenol infusion significantly shortened IVCT and increased calculated LV dP/dt (positive inotropic responses), although this response was unaffected by L-NMMA in comparison to either control agent (Figure 4).

**Discussion**
These experiments demonstrate for the first time in humans that endogenous NO acts at a postsynaptic level to facilitate cardiac responses to muscarinic stimulation when background levels of adrenergic activity are high. In healthy subjects, direct muscarinic control of heart rate was not influenced by systemic inhibition of NO synthesis. In contrast, indirect muscarinic chronotropic responses were reduced by >50%, suggesting an important role for endogenous NO in this antiadrenergic component of parasympathetic activity.
Effects on Muscarinic Response

The study of human muscarinic chronotropic control is complicated by the fact that systemic administration of muscarinic agonists results not in a bradycardia, but in a baroreflex-mediated tachycardia caused by profound (NO-mediated) vasodilatation. In these studies, a muscarinically induced bradycardia was successfully achieved with cholinesterase inhibition through the accumulation of acetylcholine (ACh) tonically released at the SAN (a mechanism not seen in resistance vessels). Effects were compared before and during concurrent adrenergic stimulation to demonstrate both direct and indirect components in a novel model of muscarinic chronotropic control.

Direct muscarinic responses were closely matched during L-NMMA and the non-NO–dependent control vasoconstrictor (phenylephrine). However, the indirect muscarinic chronotropic response was attenuated by L-NMMA, and this remained significant when compared with phenylephrine, suggesting that this was not merely a consequence of vasoconstriction.

In contrast, muscarinic control of left ventricular contractility was demonstrable only during concurrent adrenergic stimulation, suggesting a predominantly indirect mechanism of inotropic control in these healthy subjects that is in agreement with previous animal and human data. However, although statistically significant, the magnitude of this negative inotropic response was close to the limits of echocardiographic assessment with respect to the measured variable (IVCT). It is therefore difficult to draw any firm conclusions regarding the existence of nitrergic modulation of this inotropic response, and this question requires further study.

Our primary findings of nitrergic facilitation of indirect but not direct muscarinic control of heart rate are in agreement with the majority of the animal data. Nitric oxide was first shown to modulate muscarinic signal transduction in isolated, spontaneously beating rat myocytes. In contrast to our results, both the direct and the indirect chronotropic responses to muscarinic receptor stimulation were facilitated by endogenous NO. These observations were, however, in neonatal myocytes not under SAN control. Subsequent animal experiments have suggested that within the cardiac myocyte (ie, postsynaptically), endogenous NO specifically augments indirect, but not direct, muscarinic signal transduction in agreement with our findings in the human SAN. In an in vitro guinea pig atrial preparation, the indirect effect of muscarinic agonists was enhanced by endogenous NO. In anesthetized dogs prestimulated with isoproterenol or sympathetic nerve stimulation, NOS inhibition increased the minimum concentration of ACh required to induce a negative chronotropic effect. However, these effects were not seen in the human SAN.

### Figure 4

Changes in IVCT and in derived dP/dt caused by autonomic receptor stimulation during background infusion of each study agent. 

- **ΔMₐ** = change with edrophonium alone,
- **ΔMₐ+i** = change with edrophonium given during isoproterenol,
- **ΔS** = change with isoproterenol.

<table>
<thead>
<tr>
<th>Agent</th>
<th>ΔMₐ</th>
<th>ΔMₐ+i</th>
<th>ΔS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-NMMA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phe</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

- Change with edrophonium alone: 
  - ΔMₐ
- Change with edrophonium given during isoproterenol: 
  - ΔMₐ+i
- Change with isoproterenol: 
  - ΔS

*p<0.05* p=0.6
response,\textsuperscript{12} again implying a tonic facilitatory role for NO. By contrast, no modulation of the direct bradycardic response to muscarinic agonists has been demonstrated in adult guinea pig atria or anesthetized dogs.\textsuperscript{12,13,24} Similarly, nNOS knock-out mice revealed no alteration of bradycardic responses to carbamylcholine in comparison to their wild-type litter mates.\textsuperscript{14} Augmentation of the bradycardic response to vagus nerve stimulation was, however, shown in all of these experiments,\textsuperscript{12–14,24} suggesting that NO may indeed control direct parasympathetic activity but by presynaptic rather than postsynaptic mechanisms. This presynaptic action of NO has recently been confirmed by evidence that both NO donors and more specifically adenoviral nNOS gene transfer to the guinea pig right atrium increase acetylcholine release from vagal nerve terminals in response to electrical stimulation.\textsuperscript{12,24} Our data do not exclude a similar influence of NO in the human SAN, because only postsynaptic effects (ie, those distal to the muscarinic receptor) were studied. Our previous studies would suggest that the effect of NO within the entirety of the cardiac baroreflex is to produce a net enhancement of direct parasympathetic activity in humans, because vagotonic effects were shown under conditions of low to absent tonic sympathetic drive.\textsuperscript{17} Given the results of this current study, this modulation would have to occur before the cardiac muscarinic receptor, although whether it is central or an influence on acetylcholine release at vagal nerve endings remains unknown.

Our findings are also consistent with mechanistic data at the cellular level. Muscarinic receptor stimulation is known to activate caveolin-bound eNOS in the cardiac myocyte.\textsuperscript{25} The resultant generation of NO has been shown to cause a cGMP-dependent inhibition of the adrenergically (isoproterenol) stimulated inward calcium current (\(I_{Ca-L}\)), a mechanism believed to mediate, at least in part, the indirect muscarinic response. No effect of NO on the stimulation of the Ach-gated outward potassium current (direct muscarinic pathway) was evident.\textsuperscript{10}

**Effects on Adrenergic Response**

The majority of animal data suggest that NOS inhibitors augment (and thus endogenous NO antagonizes) both the chronotropic and the inotropic responses to \(\beta\)-adrenergic stimulation.\textsuperscript{16} We found no evidence of a similar response in humans, although it should be noted that our protocol was not primarily designed to examine these influences. The attenuated positive chronotropic response to isoproterenol induced by \(L\)-NMMA in comparison to saline may have been a nonspecific influence related to vasoconstriction, because this effect was not significantly different from that seen with phenylephrine.

In contrast to findings with muscarinic stimulation, \(\beta\)-adrenergic inotropic responses were clearly documented by the echocardiographic methodology used in our studies. The lack of any modulation by NOS inhibition here is in agreement with a previous human study by Hare et al\textsuperscript{26} in a group of subjects with normal cardiac function. This group did, however, obtain results consistent with NO attenuating \(\alpha\)-adrenergic inotropy in chronic heart failure,\textsuperscript{28} a disease state accompanied by increased myocardial NO production.\textsuperscript{27}

**Limitations**

The avoidance of NO-mediated vasodilation, common to all cholinergic analogues, necessitated an indirect approach to cardiac muscarinic stimulation. Rather than using a fixed amount of muscarinic agonist, the protocol had to rely on the accumulation of endogenously released ACh in response to a fixed dose of cholinesterase inhibitor. It is therefore possible that the response to a given dose of edrophonium could be influenced by the underlying rate of ACh release, ie, parasympathetic tone. However, a similar negative chronotropic response to edrophonium during saline infusion as during baroreflex mediated parasympathetic activation with phenylephrine argues against this as a significant factor in these experiments.

We used the effect of edrophonium given alone as a measure of the direct muscarinic response. Our subjects were by necessity not under \(\beta\)-adrenergic blockade, allowing the possibility of a component of indirect activity as a result of interaction with basal sympathetic tone. As an approximation of direct muscarinic response, our method is, however, reasonable, because levels of baseline sympathetic tone can be assumed to be minimal in these healthy subjects, rested supine.\textsuperscript{28}

\(L\)-NMMA is a competitive inhibitor and as such it is unlikely that we achieved total inhibition of NOS in these studies. It may be argued that a higher degree of NOS inhibition might have produced a modulation of direct muscarinic heart rate control (or muscarinic inotropic responses). We believe that this is unlikely, because the clearly apparent modulation of indirect muscarinic activity suggests that NOS within the SAN was inhibited to a physiologically significant degree. However, the possibility that the modulation of direct and indirect actions differ greatly in their sensitivity to NO cannot be excluded.

Finally, our ability to detect modulation of muscarinic inotropic responses was limited by the low sensitivity of noninvasive echo Doppler measurement of left ventricular contractility and also by a protocol designed to study chronotropic modulation. To fully address the question of inotropic control would require the use of sensitive invasive methodology (eg, intracardiac conductance catheters) to measure load-independent contractility. The invasive nature of such a protocol would, however, have disrupted the autonomic stability of our subjects.

**Perspectives**

The data here provide the first demonstration that endogenous NO tonically and specifically facilitates the indirect, \(\alpha\)-adrenergic component of muscarinic signal transduction in the healthy human heart. Sympathetic activation and high levels of cardiac norepinephrine frequently accompany cardiac pathophysiology, and demonstrated associations include arrhythmic death and death from progressive heart failure.\textsuperscript{6} Even though frequently attenuated in cardiac disease states, cardiac parasympathetic activity still retains a significant clinical impact with the degree of preservation of parasympathetic control related to protection against both life-threatening arrhythmia and death from progressive heart failure.\textsuperscript{1,2} These mechanisms of cardiac protection point to the
importance of indirect parasympathetic activity (blocking the adverse effects of adrenergic stimulation) with strong supportive evidence provided by animal studies.\textsuperscript{4} It is notable that even modest amounts of cardiac parasympathetic activity (as seen in cardiac disease states) are effective in inhibiting sympathetic cardiac signaling.\textsuperscript{5} Augmenting endogenous NO pathway activity may be of therapeutic benefit as a means of favorably modifying the pattern of reduced parasympathetic and elevated sympathetic cardiac tone that contributes to mortality in cardiac disease. Further exploration of this mechanism in disease states accompanied by this pattern of autonomic dysfunction is required.

**Acknowledgment**

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**References**


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