

## Interaction between cholinergic and nitrenergic vasodilation: a novel mechanism of blood pressure control<sup>☆</sup>

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### Abstract

**Objective:** Cholinergic vasodilation has been thought to play little if any role in the regulation of blood pressure in humans. Autonomic denervation potentiates the vasoconstriction evoked by nitric oxide synthase inhibition in humans, but the mechanism is unclear. We hypothesized that this may be related to loss of neuronal, non-nitric-oxide-dependent vasodilation. **Methods:** To test this hypothesis, we examined effects of cholinergic blockade on blood pressure, heart rate and peripheral vascular responses to systemic infusion of the nitric-oxide-dependent vasoconstrictor L-NMMA (0.5 mg/kg/min over 15 min) in eight normal subjects. **Results:** The L-NMMA-induced increase in mean ( $\pm$ S.E.) arterial pressure was roughly three times larger ( $P=0.002$ ) in the presence than in the absence of cholinergic blockade ( $38\pm 6$  vs.  $13\pm 2$  mmHg). Similarly, the increase in systemic and calf vascular resistance was more than twofold larger during L-NMMA–atropine. This potentiation was specific for nitric-oxide-dependent vasoconstriction, because atropine did not alter the responses to phenylephrine infusion. Cholinergic blockade also altered ( $P=0.004$ ) the heart rate response to nitric oxide synthase inhibition; during L-NMMA alone heart rate decreased by  $10\pm 2$  beats/min, whereas during L-NMMA–atropine infusion it increased by  $14\pm 4$  beats/min. **Conclusion:** Cholinergic mechanisms play an important hitherto unrecognized role in offsetting the hypertension and cardiac sympathetic activation caused by nitric oxide synthase inhibition in humans. Decreased parasympathetic activity and impaired nitric oxide synthesis characterize several cardiovascular disease states, as well as normal aging. The conjunction of these two defects could trigger sudden death and contribute to the hypertension of the elderly. © 2001 Elsevier Science B.V. All rights reserved.

**Keywords:** Blood pressure; Muscarinic (ant)agonists; Nitric oxide; Regional blood flow; Vasoconstriction/dilation

### 1. Introduction

The vascular endothelium and the autonomic nervous system interact between each other and play a key role in the regulation of the cardiovascular system [1,2]. Major cardiovascular disease states such as hypertension, heart failure and myocardial infarction are associated with endothelial [3,4] and autonomic dysfunction [5], as is aging, another risk factor for cardiovascular disease. Nitric oxide (NO) which is synthesized from the amino acid

L-arginine by the ubiquitous enzyme nitric oxide synthase (NOS) [6], regulates vascular tone by a direct action at the vasculature [7], by modulating central neural vasoconstrictor outflow [7–13], and via its release from sympathetic nitrenergic vasodilator nerves [14]. While the importance of parasympathetic control of heart rate (HR) is well established [15], little is known regarding the role of cholinergic vasodilator mechanisms in the regulation of blood pressure in humans [16], and the possibility of an interaction between the cholinergic and the NO–L-arginine system. Autonomic denervation potentiates the vasoconstrictor effect of NOS inhibition by  $N^G$ -monomethyl-L-arginine (L-NMMA) infusion in humans, but the underlying mechanism is not known [17]. We hypothesized that cholinergic

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mechanisms attenuate the vasoconstrictor effects of NOS inhibition in humans. To test this hypothesis, we examined the effects of cholinergic blockade on the pressor response to systemic infusion of the NO-dependent vasoconstrictor L-NMMA, and compared this response with the one evoked by the non-NO-dependent vasoconstrictor phenylephrine.

## 2. Methods

### 2.1. Subjects

Eight healthy subjects [three women and five men, mean ( $\pm$ S.D.) weight  $67\pm 3$  kg, height  $175\pm 3$  cm, body mass index  $21.9\pm 0.5$  kg/m<sup>2</sup>, age  $25\pm 2$  years] participated in this study after providing informed written consent. All subjects were normotensive, were taking no medications, and had no evidence of metabolic or cardiovascular disease. The studies were performed in the morning after an overnight fast. The subjects were asked to abstain from alcohol, caffeine and tobacco for at least 24 h before each study. The experimental protocol was approved by the Institutional Review Board on Human Investigation and the investigation conforms with the principles outlined in the Declaration of Helsinki.

### 2.2. General procedures

The subjects were studied in the supine position. Heart rate (electrocardiogram) and blood pressure (Finapres) were measured continuously. For drug infusion, an intravenous catheter was inserted in an antecubital vein.

### 2.3. Measurement of calf blood flow

Calf blood flow was measured by venous occlusion plethysmography, using mercury-in-Silastic strain gauges. The calf was elevated 10–15 cm above the level of the right atrium to collapse the veins. The circulation to the foot was arrested by inflating a cuff around the ankle during blood flow determinations, which were performed at 15-s intervals over a 5-min period [18].

### 2.4. Measurement of cardiac output

Cardiac output was measured using echocardiography [19]. The cross sectional area of the left ventricular outflow tract was multiplied by the velocity time integral of the systolic flow to obtain the stroke volume. The cardiac output was then calculated by multiplying the stroke volume by the HR. The diameter of the left ventricular outflow tract was determined in a parasternal long axis view and the systolic flow by a pulse Doppler sample from an apical 5 chamber view.

### 2.5. Drugs

Drugs were dissolved in physiological saline immediately before use. L-NMMA, L- and D-Arginine were obtained from Clinalfa (Läufelfingen, Switzerland), atropine from Syntetica (Mendrisio, Switzerland), phenylephrine from Winthrop (Zurich, Switzerland) and propranolol from Zeneca (Luzern, Switzerland).

### 2.6. Experimental protocols

#### 2.6.1. Protocol 1: cardiovascular effects of L-NMMA infusion alone

After instrumentation the subject rested quietly for 30 min. The subject then received sequential infusions of normal saline (1 ml/min for 20 min), L-NMMA (0.5 mg/kg/min for 15 min), D-arginine (100 mg/kg over 10 min,  $n=5$ ) and L-arginine (100 mg/kg over 10 min). Hemodynamic measurements were recorded during two 5-min periods of the saline infusion and during the last 5 min of each drug infusion. In four of the subjects we also measured cardiac output (echocardiography). The present dose of L-NMMA was chosen, because it was found to consistently increase calf vascular resistance [9]. L-Arginine at the dose used in these studies was found to consistently reverse the hemodynamic effect of L-NMMA [9].

#### 2.6.2. Protocol 2: cardiovascular effects of L-NMMA infusion during cholinergic blockade

After instrumentation the subject rested quietly for 30 min. After resting control values had been measured, a prime (0.4 mg/m<sup>2</sup> over 10 min) continuous (0.3 mg/m<sup>2</sup>/h) atropine infusion was started. 30 min after its start, sequential infusions of L-NMMA (0.5 mg/kg/min for 15 min), D-arginine (100 mg/kg over 10 min,  $n=5$ ) and L-arginine (100 mg/kg over 10 min) were superimposed on the atropine infusion. Hemodynamic measurements were recorded during two 5-min periods of baseline and atropine infusion, and during the last 5 min of the L-NMMA infusion. The present dose of atropine was chosen, because we had previously found that it abolished the vasodilator response to intra-arterial acetylcholine infusion [20].

#### 2.6.3. Protocol 3: cardiovascular effects of an equipressive dose of phenylephrine infusion performed alone and during cholinergic blockade

The aim of this protocol was to test whether the potentiation of the vasoconstriction by cholinergic blockade was specific for the NO-dependent vasoconstrictor L-NMMA or not. To this end we examined the effects of cholinergic blockade on the vasoconstrictor response evoked by the non-NO-dependent vasoconstrictor phenylephrine. Five of the subjects returned for this protocol. The timing of the phenylephrine infusions was

identical to that of the L-NMMA infusions in protocols 1 and 2. In a first session, after 30 min of normal saline infusion (1 ml/min), they received a 15-min phenylephrine infusion titrated at a dose to match the increase in arterial pressure observed during the L-NMMA infusion. Hemodynamic measurements were recorded during two 5-min periods of the saline infusion and during the last 5 min of the phenylephrine infusion.

In a second session, on a separate day, the same dose of phenylephrine was superimposed on an atropine infusion at the same dose as that used in protocol 2. Hemodynamic measurements were recorded during two 5-min periods of baseline and atropine infusion, and during the last 5 min of the phenylephrine–atropine infusion.

#### 2.6.4. Protocol 4: hemodynamic effects of L-NMMA and atropine during concomitant propranolol infusion

Since we found that in protocol 2 HR increased during the L-NMMA infusion, the aim of this protocol was to test whether this effect was caused by  $\beta$ -adrenergic stimulation. Four of the subjects returned for this study. The protocol was identical to protocol 2, except that together with atropine, propranolol was co-infused as a prime (0.1 mg/kg over 10 min) continuous (0.01 mg/kg/min) infusion. This dose of propranolol abolishes isoproterenol-induced hemodynamic and chronotropic responses [20].

#### 2.7. Data analysis

Mean arterial pressure was calculated as diastolic pressure plus one third pulse pressure. Vascular resistance in the calf was calculated as mean arterial pressure in millimeters of mercury divided by blood flow in milliliters per min per 100 milliliters of tissue and expressed in units. Systemic vascular resistance was calculated as mean arterial pressure divided by the cardiac output and expressed in dynes s per  $\text{cm}^5$ . The measurements of calf blood flow, arterial pressure and HR that were collected over 5-min periods were averaged to a single value.

Statistical analysis was performed with paired two-tailed *t*-tests and the Wilcoxon signed-rank test, as appropriate. A *P* value of  $<0.05$  was considered to indicate statistical significance. Unless stated otherwise data are expressed as mean  $\pm$  S.D.

### 3. Results

#### 3.1. Effects of cholinergic blockade on the cardiovascular responses to L-NMMA infusion

Cholinergic blockade markedly potentiated the L-NMMA induced pressor and vasoconstrictor effects (Fig. 1, Table 1). The L-NMMA-induced increase in mean

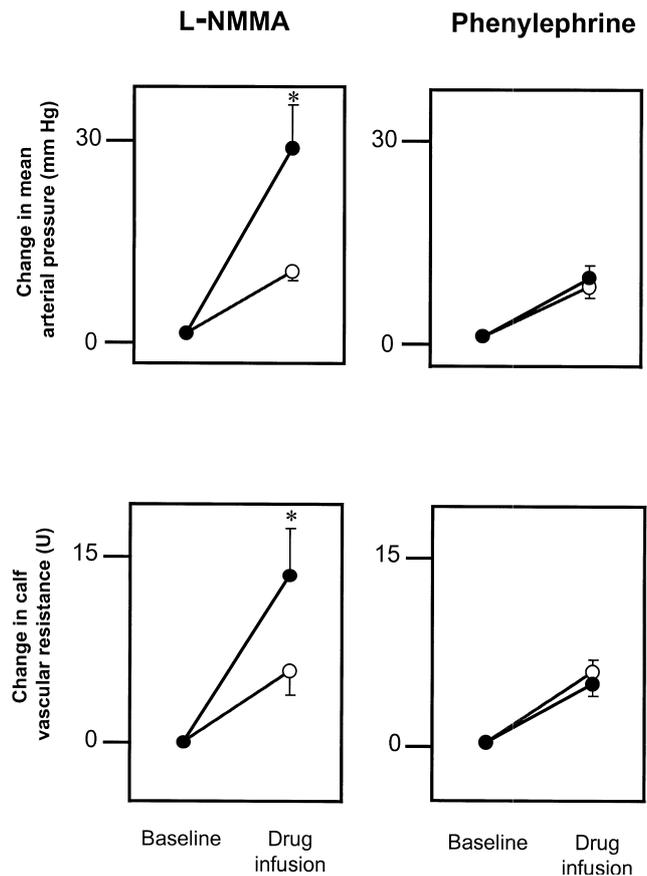


Fig. 1. Mean ( $\pm$ S.E.) effects in the same five normal subjects, of L-NMMA or phenylephrine infusion performed alone (open circles), and performed during concomitant atropine infusion (closed circles) on mean arterial pressure and calf vascular resistance. Cholinergic blockade potentiated the pressor and vasoconstrictor responses to infusion of the NO-dependent vasoconstrictor L-NMMA (\*,  $P<0.05$  vs. L-NMMA alone), but did not alter the responses to the non-NO-dependent vasoconstrictor phenylephrine ( $P<0.05$ , for the comparisons between L-NMMA–atropine and both phenylephrine–atropine and phenylephrine alone).

arterial pressure was roughly three times larger in the presence than in the absence of cholinergic blockade ( $38\pm 17$  vs.  $13\pm 6$  mmHg,  $P=0.002$ ). Similarly, the increase in systemic ( $437\pm 44$  vs.  $303\pm 23$  dynes s/ $\text{cm}^5$ ,  $P=0.039$ ) and calf vascular resistance ( $18\pm 11$  vs.  $8\pm 4$  units,  $P=0.02$ ) was significantly larger during L-NMMA–atropine infusion than during L-NMMA infusion alone. Cholinergic blockade also altered the HR response to NOS inhibition. During L-NMMA alone, the HR decreased by  $10\pm 5$  beats/min, whereas when L-NMMA was administered during atropine infusion, the HR increased by  $14\pm 12$  beats/min ( $P=0.004$  for the comparison between L-NMMA alone and L-NMMA–atropine). The L-NMMA-induced hemodynamic effects plateaued after the 10th minute of infusion (data not shown) and were related to NO inhibition, because they were reversed by L-arginine (but not D-arginine, data not shown) infusion (Table 1).

Table 1  
Responses to L-NMMA infusions given alone or during concomitant atropine infusion<sup>a</sup>

Variable	Baseline	L-NMMA infusion	L-Arginine infusion	Baseline	Atropine infusion	L-NMMA + atropine infusion	L-Arginine + atropine infusion	P value <sup>b</sup>
Heart rate (beats/min)	62±6	52±4	61±7	67±8	92±10	106±18	94±11	0.004
Mean arterial pressure (mmHg)	80±11	93±9	81±9	81±11	91±13	129±26	95±10	0.002
Calf blood flow (ml/min/100 ml)	1.6±0.6	1.6±0.7	1.7±0.8	1.8±0.6	2.0±0.9	2.0±0.8	1.9±0.8	NS
Calf vascular resistance (units)	51±14	60±17	48±11	46±6	46±9	64±18	50±12	0.02

<sup>a</sup> Values are mean±S.D. for eight subjects.

<sup>b</sup> P values are for the comparisons of the L-NMMA-induced changes from baseline, respectively from atropine infusion alone.

### 3.2. Effects of cholinergic blockade on the cardiovascular responses to an equipressive phenylephrine infusion

Fig. 1 shows that atropine infusion did not alter the pressor and calf vasoconstrictor responses to phenylephrine infusion. Mean arterial pressure increased by 9±2 mmHg ( $P=0.001$ ) during phenylephrine alone and by 9±2 mmHg ( $P=0.002$ ) during phenylephrine–atropine infusion. Atropine prevented the phenylephrine-induced decrease in HR; HR decreased during phenylephrine alone, whereas it remained unchanged during phenylephrine–atropine infusion (Table 2).

### 3.3. Effects of propranolol on HR responses to concomitant L-NMMA–atropine infusion

Propranolol did not alter the pressor response to L-NMMA–atropine infusion (mean arterial pressure increased by 29±9 and 32±14 mmHg in the presence and absence of propranolol, respectively), but abolished the L-NMMA-induced increase HR; when L-NMMA was infused during atropine alone, it increased the HR from 93±5 to 103±9 beats/min ( $P<0.05$ ), whereas when it was infused during atropine–propranolol, HR remained unchanged at 83±5 and 82±6 beats/min, respectively ( $P=$

0.03, for the comparison between L-NMMA–atropine and L-NMMA–atropine–propranolol).

## 4. Discussion

Cholinergic vasodilation has been thought to play little, if any, role in the regulation of blood pressure in humans [16,21]. We found that cholinergic blockade had a dramatic effect on the pressor response to NOS inhibition in these healthy subjects, since the L-NMMA-induced increase in mean arterial pressure was roughly three times larger in the presence than in the absence of atropine infusion. Our data indicate that cholinergic vasodilation is much more important than previously thought, and offsets roughly two thirds of the pressor effect caused by NOS inhibition. The potentiation of the pressor effect by cholinergic blockade was specific for NO-dependent vasoconstriction (as evidenced by the phenylephrine studies). Moreover, the L-NMMA-induced hemodynamic effects were related specifically to inhibition of NO synthesis, since they were reversed by L-arginine (but not D-arginine) infusion. Autonomic denervation potentiates the vasoconstrictor effect of NOS inhibition in humans, but it is not clear whether this observation is related to augmented contribution of NO to local vascular resistance regulation after denervation or to

Table 2  
Responses to phenylephrine infusions given alone or during concomitant atropine infusion<sup>a</sup>

Variable	Baseline	Phenylephrine infusion	Baseline	Atropine infusion	Phenylephrine+ atropine infusion	P value <sup>b</sup>
Heart rate (beats/min)	66±9	56±4	69±8	93±14	94±16	0.03
Mean arterial pressure (mmHg)	83±5	92±5	81±11	89±8	98±10	NS
Calf blood flow (ml/min/100 ml)	1.5±0.9	1.4±0.8	1.6±0.8	1.8±0.8	1.8±0.8	NS
Calf vascular resistance (units)	58±15	64±18	52±12	50±12	55±13	NS

<sup>a</sup> Values are mean±S.D. for five subjects.

<sup>b</sup> P values are for the comparisons of the phenylephrine-induced changes from baseline, respectively from atropine infusion alone.

loss of neuronal non-nitric-oxide-dependent vasodilator mechanisms [17]. The present findings could be consistent with the hypothesis that this augmented vasoconstriction was related, at least in part, to the latter mechanism. However, since cholinergic vasodilator nerves in skeletal muscle have not been demonstrated by histological techniques in humans, alternative explanations need to be considered. Cultured human endothelial cells can release acetylcholine in response to increased flow [22], and local cholinergic mechanisms mediate NO dependent vasorelaxation in canine coronary artery ring preparations in vitro [23]. Thus, local cholinergic mechanisms, which may be upregulated in denervated limbs, could attenuate the L-NMMA induced vasoconstriction.

Cholinergic blockade not only had dramatic effects on the vascular responses to NOS inhibition, but also altered the heart response to L-NMMA infusion. When infused alone, the L-NMMA induced increase in arterial pressure, as expected [8,9], was accompanied by a reflex decrease in HR. In contrast, when L-NMMA was infused during atropine, even though the increase in arterial pressure was much larger, it was now accompanied by an increase in HR. In humans, NOS inhibition stimulates sympathetic outflow to the peripheral vasculature, an effect which is masked by inhibitory baroreflexes [8]. Here we show that L-NMMA infusion also stimulates sympathetic outflow to the heart, as evidenced by the propranolol studies. Consistent with this hypothesis, NOS inhibition enhances the norepinephrine release from rat heart sympathetic nerves evoked by electrical stimulation [24].

We do not know yet the exact underlying mechanism(s) by which cholinergic and nitrenergic pathways interact. What this study shows, however, is that cholinergic mechanisms play an essential, hitherto unrecognized role in attenuating the increase in blood pressure and the cardiac sympathetic activation induced by NOS inhibition in normal subjects. Decreased cholinergic control of the heart (which is mediated by the parasympathetic nervous system [4,25]) and impaired NO synthesis [3,26,27] characterize cardiovascular disease states such as hypertension, myocardial infarction and heart failure, as well as aging, another cardiovascular risk factor. We speculate that the conjunction of these two defects may trigger cardiac sympathetic overactivity and sudden death and (if the defect holds true for this novel cholinergic vasodilator pathway) may represent a candidate mechanism for the hypertension of the elderly.

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