Endothelium, Nitric Oxide, Oxidative Stress

Contribution of Endothelial Nitric Oxide to Blood Pressure in Humans

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Abstract—Impaired endothelial-derived NO (eNO) is invoked in the development of many pathological conditions. Systemic inhibition of NO synthesis, used to assess the importance of NO to blood pressure (BP) regulation, increases BP by \( \approx 15 \) mm Hg. This approach underestimates the importance of eNO, because BP is restrained by baroreflex mechanisms and does not account for a role of neurally derived NO. To overcome these limitations, we induced complete autonomic blockade with trimethaphan in 17 normotensive healthy control subjects to eliminate baroreflex mechanisms and contribution of neurally derived NO. Under these conditions, the increase in BP reflects mostly blockade of tonic eNO. \( \text{N}^6\)-Monomethyl-L-arginine (250 \( \mu \)g/kg per minute IV) increased mean BP by 6.3.7 mm Hg (from 77 to 82 mm Hg) in intact subjects and by 21.8.4 mm Hg (from 75 to 96 mm Hg) during autonomic blockade. We did not find a significant contribution of neurally derived NO to BP regulation after accounting for baroreflex buffering. To further validate this approach, we compared the effect of NOS inhibition during autonomic blockade in 10 normotensive individuals with that of 6 normotensive smokers known to have endothelial dysfunction but who were otherwise normal. As expected, normotensive smokers showed a significantly lower increase in systolic BP during selective eNO block (11.4.5 versus 30.2.3 mm Hg in normotensive individuals; \( P<0.005 \)). Thus, we report a novel approach to preferentially evaluate the role of eNO on BP control in normal and disease states. Our results suggest that eNO is one of the most potent metabolic determinants of BP in humans, tonically restraining it by \( \approx 30 \) mm Hg.

(Hypertension. 2007;49:170-177.)

Key Words: baroreflex ■ trimethaphan ■ phenylephrine ■ nitric oxide synthase ■ L-NMMA

Nitric oxide is one of the most widely studied substances in biology. It is produced from L-arginine by NO synthase (NOS) and modulates vascular tone, platelet activation, and neural function, among other actions. NO is produced by 3 isoforms of NOS. An inducible form is found mostly in macrophages (NOS2). Two constitutively expressed isoforms are expressed, one in epithelial and neural cells (NOS1), and another in endothelial cells, platelets, and myocardial cells (NOS3). There is great interest in defining the contribution of endogenous NO to blood pressure modulation. Endothelial cells tonically produce NO (eNO), which lowers basal blood pressure by producing vasodilation. Neurally derived NO (nNO) modulates blood pressure primarily through its interaction with the autonomic nervous system, mainly through inhibition of central sympathetic outflow.1,2 There is controversy about the relative contribution of eNO and nNO in the regulation of blood pressure. Mice made deficient of NOS3 have a significant increase in baseline blood pressure,3 whereas mice lacking NOS1 do not.4 In humans, the contribution of the sympathetic nervous system to the increase in blood pressure produced by systemic administration of NOS inhibitors has been difficult to assess. In some studies, the increase in blood pressure produced by systemic administration of the NOS inhibitor \( \text{N}^6\)-monomethyl-L-arginine (l-NMMA) results in a reflex decrease in muscle sympathetic nerve activity5 that is similar to that produced by an equipressor dose of phenylephrine, suggesting that NO does not tonically restrain central sympathetic outflow in humans.6 On the other hand, in other studies, NOS inhibition with \( \text{N}^6\)-nitro-L-arginine methyl-ester resulted in a larger increase in blood pressure that was partially reversed with the \( \alpha \)-adrenergic antagonist phentolamine, most likely reflecting a tonic inhibition of sympathetic tone by nNOS.7

The relative contribution of endothelial-derived NO to blood pressure, therefore, remains difficult to assess. In humans, this could be achieved by specific inhibition of...
eNOS. Unfortunately, selective eNOS inhibitors are not available for use in humans; currently available inhibitors act on both eNOS and nNOS. An alternative approach has been to study an isolated vascular bed, assuming that the vascular effects of NO-dependent vasodilators reflect eNOS function. The forearm is commonly used, and forearm blood flow is monitored in response to intrabrachial infusions of endothelium-dependent and -independent vasodilators and to nonselective NOS inhibitors. There are, however, limitations to this approach. Commonly used “endothelium-dependent vasodilators” can also induce vasodilation through mechanisms independent of NO, including endothelium-derived hyperpolarizing factor.8,9 Also, this approach studies only one vascular bed with limited influence to overall blood pressure levels, and it is not certain that this approach selectively examines eNOS function. nNOS is also expressed in skeletal muscle cells and has been suggested to be the source of the NO-mediated inhibition of sympathetic vasoconstriction in contracting muscle.10 nNOS is also expressed in presynaptic noradrenergic nerve terminals, and its inhibition could theoretically increase NE release and contribute to forearm vasoconstriction.

An alternative approach has been to measure flow-mediated dilation, usually by monitoring the brachial artery diameter in response to reactive hyperemia.11 This response, however, seems to be influenced by sympathetic activity.12,13 Therefore, it is difficult to estimate how important eNOS is to overall blood pressure regulation from the use of these conventional methods.

The goal of this study was to develop an experimental approach that will allow us to preferentially examine the role of endothelial NO in blood pressure regulation. We used blockade of autonomic ganglia with the NN-nicotinic receptor antagonist trimethaphan to eliminate the restraining effect of the baroreflex, thus allowing for the full expression of the effect of NOS blockade on blood pressure. Furthermore, the effects of nNOS become irrelevant, because they depend on their interaction with the autonomic nervous system, which is no longer operative (Figure 1). Under these experimental conditions, the increase in blood pressure induced by systemic NOS inhibition can be assigned to preferential inhibition of tonic eNOS.

Methods

Subjects

We studied a total of 25 subjects. In protocol 1, we studied 17 healthy nonsmoking normotensive subjects (9 women and 8 men; 32±8.8 years; 25±3.9 kg/m² body mass index [BMI]) on 2 separate
days. In protocol 2, we compared 10 healthy nonsmoking normoten-
sive subjects with no family history of hypertension (6 women and
4 men; 30 ± 3.1 years; 25 ± 2.0 kg/m² BMI), with 6 normotensive
heavy smokers (≥2 packs a day) but otherwise healthy subjects (2
women and 4 men; 31 ± 2.5 years; 25 ± 1.1 kg/m² BMI). Some
healthy controls from the first protocol also participated in the
second protocol. Participants were recruited from the Vanderbilt
University General Clinical Research Center volunteer database.
Subjects abstained from all drugs, including caffeine and nicotine,
for ≥72 hours before testing. All of the subjects underwent a
thorough clinical examination, ECG, and admission urinalysis and
blood work. Written informed consent was obtained before study
entry. All of the studies were approved by the Vanderbilt University
Institutional Review Board.

Study Design

Protocol 1: To Determine the Contribution of eNO to
Blood Pressure Regulation

Seventeen healthy volunteers were studied on 2 separate study days
randomly assigned ≥1 week apart using a crossover design. One day
was designed to assess the effect of NOS blockade with the
autonomic nervous system intact (intact study day) and the other one
with the autonomic nervous system temporarily blocked with tri-
methaphan (blocked study day).

Four days before any study, volunteers were on a diet free of food
containing methyloxanthines. The volunteers were admitted to the
Elliot V. Newman Clinical Research Center at Vanderbilt University
Medical Center the day that testing was performed.

The studies were conducted in the morning with the subject in the
recumbent position ≥8 hours after their last meal. Heart rate was
determined with continuous ECG monitoring, blood pressure
through the volume clamp method (Finapres 2300; Ohmeda), and
also automated brachial cuff pressure with standard sphygmo-
nometry (Dinamap). Two intravenous lines were placed in different
arms. Three infusion ports were connected to the cather placed in a
large antecubital vein in the left arm, one for trimethaphan infusion, the
second for infusion of phenylephrine, and the third for L-NMMA.
In the other arm, 1 heparin lock was placed to access cardiovascular
responses to phenylephrine before and during tri-
methaphan to ensure complete autonomic blockade.

After a stable baseline was reached, phenylephrine boluses
were started with 25 µg. The dose of phenylephrine was increased
every 3 minutes until an increase in systolic blood pressure (SBP)
≥20 mm Hg was achieved. The changes in SBP were used to
calculate baseline dose–response curves to phenylephrine. After
this, Nα-cholinergic receptors were blocked by continuous infu-
sion of trimethaphan (Cambridge Pharmaceuticals) at 4 µg/min.
We have shown previously that this dose induces complete
autonomic blockade.14 During ganglionic blockade, phenyl-
ephrine boluses were repeated with adjustment of the dose to account
for the loss of baroreflex buffering, starting at a dose of 2.5 µg.
The dose of phenylephrine was increased every 3 minutes until an
increase in SBP of ≥20 mm Hg was achieved to calculate the
dose–response curve to phenylephrine during autonomic block-
ade. Blood pressure was then restored by infusing phenylephrine
at individually titrated doses, starting with 0.05 µg/kg per minute.
L-NMMA was then infused at 2 different doses for 15 minutes
each (250 and 500 µg/kg per minute) or until SBP reached
150 mm Hg. On the “intact” day, saline was infused instead of
trimethaphan and phenylephrine.

Estimation of the Relative Contribution of eNO and nNO
to Blood Pressure Regulation

The increase in blood pressure induced by systemic NO inhibition
in the presence of intact sympathetic tone is the resultant of 3
components: (1) blockade of eNO synthesis resulting in vasocon-
striction; (2) blockade of nNO synthesis resulting in an increase
in sympathetic tone; and (3) the presence of baroreflexes that restrain
the increase in blood pressure that would otherwise result from the
first 2 components. These interactions are shown in the following
equations for illustration purposes only:

\[ \uparrow BP = \left( eNO \text{ inhibition} + nNO \text{ inhibition} \right) - \text{baroreflex restrain} \]

From our experiment we can determine the value of the left side of
the equation (“\( \uparrow BP \)”) by measuring the increase in blood pressure
produced by 250 µg/kg per minute IV of L-NMMA in autonomically
intact subjects (+eNO + nNO− baroreflex restraint). We can also
measure the increase in blood pressure produced by the same dose in
autonomically blocked subjects (“eNO inhibition” in the equation).
Baroreflex restraint can be individually estimated in each subject by
taking into account the potentiation of the pressor response to
phenylephrine during autonomic blockade compared with baseline15
(baroreflex restraint). We can, therefore, resolve the unknown
component of the equation (“nNO inhibition”) to estimate the
contribution of nNO to blood pressure regulation (see Results for
further explanation).

Protocol 2: To Compare the Importance of eNO on Blood
Pressure Regulation in Normal Individuals and Smokers

A parallel group design was used to compare the effects of selective
eNOS inhibition between control subjects and smokers. The inclu-
sion criteria for controls were refined to include only normotensive
subjects with no hypertensive genetic background (normotensive
with no hypertensive parents), or heavy smokers (≥2 packs per day),
a group known to have endothelial dysfunction. Subjects were
excluded if they met criteria for stage 1 hypertension (SBP >140 or
DBP ≥90 mm Hg) or were on any medication. Some volunteers
from protocol 1 were also included in this study in the normotensive
subjects with no hypertensive genetic background group. Instrumenta-
tion and pharmacological testing were identical to the “blocked”
day of protocol 1.

Data Acquisition

The surface ECG was amplified, and no additional filters were
applied. ECG, blood pressure, and impedance recordings were
digitized with 14-bit resolution and 500 Hz sample frequency and
recorded using the WINDAQ data acquisition system (DATAQ).
Data were analyzed offline using a customized program for data
analysis (DIANA, Dr Andrée Diedrich, Vanderbilt University, Nash-
ville, TN) written in PV-Wave (VNI).

Heart Rate and Blood Pressure Variability

A robust QRS detection algorithm, modified from Pan and Tomp-
kins,16 was used to generate beat-to-beat values. The nonquasidistant
event time series of RR intervals and blood pressure values were
interpolated, low-pass filtered (cutoff: 2 Hz), and resampled at 4 Hz.
The estimation of the power spectral density was done by the Welch
method, which is a fast Fourier transform–based algorithm. Data
segments of 128 s recorded at the end of baseline and were used for
spectral analysis. Linear trends were removed, and power spectral
density was estimated with the fast Fourier transform–based Welch
algorithm using 3 segments of 256 data points with 50% overlapping
and Hanning window.17 The Hanning window was applied to
previous estimation of the power spectral density. The power in the
frequency ranges for very low frequencies (0.003 to <0.04 Hz), low
frequencies (0.04 to <0.15 Hz), and high frequencies (0.15 to <0.40
Hz) were calculated for each interval in according to task force
recommendations.18

Statistical Analysis

Unless otherwise noted, data are presented as mean ± SEM. A
preliminary comparison of outcomes by study days was performed
by Wilcoxon signed-rank test, and comparisons of demographics and
outcomes by smoking status were performed using Mann–Whitney
U test.

Random-effects models were used to take into account a correla-
tion between measurements taken over time within a subject. The

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preliminary comparison of outcomes by study days was performed
by Wilcoxon signed-rank test, and comparisons of demographics and
outcomes by smoking status were performed using Mann–Whitney
U test.

Random-effects models were used to take into account a correla-
tion between measurements taken over time within a subject. The
null hypotheses were that there was no difference in outcomes in the response to systemic infusion of L-NMMA between the intact and blocked study days for protocol 1 or between nonsmokers and smokers for protocol 2. The primary end point for both protocols was SBP achieved during interventions. More specific hypothesis tests were performed to answer to the following questions: (1) how the overall dose effects of L-NMMA would differ between the intact and blocked study days for protocol 1 and between nonsmokers and smokers for protocol 2; and (2) if any difference was found between 2 intervention days or smoking status as a function of dose, at what doses the difference has arisen. The major outcomes of interests were systolic blood pressure (SBP) and diastolic blood pressure (DBP). Two random-effects models were built to answer to the above 2 questions for each protocol, model 1 for question 1 and model 2 for question 2. The blocked day indicator (smoking status for protocol 2) as covariates, considering the limited sample size and that no evidence of association between the variables in model 2) were the main effects, and the interaction between them was also analyzed. To adjust for the potential confounding factors and carryover effects for protocol 1, the blocked study days for protocol 1 and between nonsmokers and smokers for protocol 2. The primary end point for both protocols was SBP but higher DBP while sitting; hence, pulse pressure was found to be significantly lower in the smokers group (P=0.01 using Mann–Whitney U test). Smokers also had reduced baroreflex sensitivity at baseline, in accordance to previous studies.19

Effect of Autonomic Blockade on Resting Cardiovascular Parameters and Pressor Response to Phenylephrine

As expected, trimethaphan eliminated heart rate and blood pressure variability, as evidence by a significant decrease in the power spectra parameters (Table 2). There was only a modest decrease in mean arterial blood pressure, from 78±2.1 to 74±2.0 mm Hg (P=0.01), consistent with the low contribution of the sympathetic nervous system to blood pressure in the supine position. In contrast, heart rate increased from 58±2.0 to 84±2.0 bpm (P<0.001) consistent with parasympathetic withdrawal. Baroreflex sensitivity also was abolished, with a reduction in the baroreflex gain from 12.1±1.7 to 1.8±0.2 ms/mm Hg (P<0.001). In the absence of baroreflex buffering, there was a significant increase in the pressor response to phenylephrine, as evidenced by a decrease in the dose required to increase blood pressure by 20 mm Hg (205±19.6 µg during saline versus 22±1.8 µg during trimethaphan; P<0.005).

Contribution of eNO to Blood Pressure Regulation

For protocol 1, during the blocked day, the increases in both SBP and DBP induced by L-NMMA were higher compared with the intact day (Figure 2 and Table 3). There were also statistically significant linear positive dose effects on both SBP and DBP for the intact day (SBP: P<0.001; DBP: P<0.001) and for the blocked day (SBP: P<0.001; DBP: P<0.001), and the linear dose–response rate in the blocked day was increased 3-fold compared with that of the intact day (SBP, P<0.001; DBP, P<0.001). The mean responses during the blocked day at dose 250 were significantly greater (SBP:

### Table 1. Demographics and Baseline Characteristics of Subjects Studied

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Protocol 1 (n=17)</th>
<th>NTN (n=10)</th>
<th>SMK (n=6)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>32±2.1</td>
<td>30±3.1</td>
<td>31±2.5</td>
<td>0.562</td>
</tr>
<tr>
<td>Height, cm</td>
<td>171±2.5</td>
<td>170±3.9</td>
<td>172±4.1</td>
<td>0.958</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>75±4.0</td>
<td>73±4.7</td>
<td>73±3.1</td>
<td>0.958</td>
</tr>
<tr>
<td>BMI</td>
<td>25±1.0</td>
<td>25±0.7</td>
<td>25±1.1</td>
<td>0.875</td>
</tr>
<tr>
<td>Seated SBP, mm Hg</td>
<td>120±3.1</td>
<td>116±4.2</td>
<td>108±3.3</td>
<td>0.181</td>
</tr>
<tr>
<td>Seated DBP, mm Hg</td>
<td>70±2.2</td>
<td>68±2.4</td>
<td>76±2.7</td>
<td>0.050</td>
</tr>
<tr>
<td>Seated HR, bpm</td>
<td>73±2.4</td>
<td>77±3.8</td>
<td>78±5.0</td>
<td>0.529</td>
</tr>
<tr>
<td>Pulse pressure</td>
<td>50±3.2</td>
<td>48±4.6</td>
<td>32±3.4</td>
<td>0.008</td>
</tr>
<tr>
<td>LF RRI, ms²</td>
<td>1768±428.1</td>
<td>2267±689.7</td>
<td>704±61.5</td>
<td>0.066</td>
</tr>
<tr>
<td>HF RRI, ms²</td>
<td>1633±487.7</td>
<td>1375±732.0</td>
<td>736±289.1</td>
<td>0.328</td>
</tr>
<tr>
<td>LF/HF RRI</td>
<td>1.7±0.4</td>
<td>2.5±0.8</td>
<td>1.9±0.7</td>
<td>0.529</td>
</tr>
<tr>
<td>LF Sys, mm Hg²</td>
<td>9.4±2.7</td>
<td>12.1±5.7</td>
<td>7.6±1.8</td>
<td>0.955</td>
</tr>
<tr>
<td>BRS, ms/mm Hg</td>
<td>14.4±2.0</td>
<td>15.5±2.4</td>
<td>10.0±1.2</td>
<td>0.050</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>172±6.8</td>
<td>166±8.3</td>
<td>168±17.4</td>
<td>0.950</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>92±7.9</td>
<td>102±8.3</td>
<td>100±22.7</td>
<td>0.662</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dL</td>
<td>53±4.2</td>
<td>52±7.3</td>
<td>60±3.5</td>
<td>0.108</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dL</td>
<td>100±7.5</td>
<td>94±13.3</td>
<td>88±15.6</td>
<td>0.950</td>
</tr>
</tbody>
</table>

NTN indicates normotensive subjects; SMK, smokers; HR, heart rate; LF RRI, low frequency variability of RR interval; HF RRI, high frequency variability of RR interval; LF sys, low frequency variability of systolic blood pressure; BRS, baroreflex sensitivity; HDL, high-density lipoprotein; LDL, low-density lipoprotein. Values are expressed as mean±SD. P values are for the differences between NTN and SMK, by Mann–Whitney U test.

### Table 2. Changes in Spectra Analysis Parameters Before and After Ganglionic Blockade

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Intact</th>
<th>Blocked</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>LF RRI, ms²</td>
<td>1506±392.7</td>
<td>4.8±1.4</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>HF RRI, ms²</td>
<td>1439±490.8</td>
<td>6.7±2.6</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>LF Sys, mm Hg²</td>
<td>12±2.5</td>
<td>2.2±0.6</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>BRS, ms/mm Hg</td>
<td>12±1.7</td>
<td>1.8±0.2</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

LF RRI indicates low-frequency variability of RR interval; HF RRI, high-frequency variability of RR interval; LF sys, low-frequency variability of SBP; BRS, baroreflex sensitivity. Values are expressed as mean±SEM. P values are from Wilcoxon signed-rank test.
Estimation of the Contribution of nNO to Blood Pressure Regulation

To estimate the relative contribution of eNO and nNO to blood pressure, we constructed log dose SBP responses to phenylephrine before and after autonomic blockade in each subject. The potentiation of the pressor effects of phenylephrine observed after autonomic blockade estimates the magnitude of baroreflex buffering\(^1\) and can be used to predict how much blood pressure should have increased if only the autonomic nervous system (eNO\(^{-}\)) baroreflex restraint, and an infusion of trimethaphan followed by phenylephrine to restore blood pressure to baseline values. This could be because of missing measurements at this dose for many subjects in the blocked day because of safety concerns. The missing measurements were informative in the sense that they would have been much larger values than the nonmissing measurements at dose 500, because they were not able to be taken from subjects who already showed high blood pressures at dose 250, and, hence, the next dose could not be given to them to maintain the blood pressures within the safe limit of 150 mm Hg of SBP.

mean: 20.4 mm Hg; 95% CI: 15.8 to 25.0; \(P<0.001\); DBP mean: 11.2 mm Hg; 95% CI 7.8 to 14.7; \(P<0.001\)) compared with those of the intact day after controlling for sequence order, period, BMI, sex, and baseline blood pressure. Although the mean responses on the blocked day at dose 500 were also greater than those of the intact day, they were not statistically significant (SBP: \(P=0.376\); DBP: \(P=0.389\)). This could be because of missing measurements at this dose for many subjects in the blocked day because of safety concerns. The missing measurements were informative in the sense that they would have been much larger values than the nonmissing measurements at dose 500, because they were not able to be taken from subjects who already showed high blood pressures at dose 250, and, hence, the next dose could not be given to them to maintain the blood pressures within the safe limit of 150 mm Hg of SBP.

rined observed after autonomic blockade estimates the magnitude of baroreflex buffering\(^1\) and can be used to predict how much blood pressure should have increased if only the baroreflex was removed. An example is given in Figure 3. The measured increase in blood pressure induced by 250 \(\mu\)g/kg per minute of \(\text{L-NMMA}\) in the presence of an intact autonomic nervous system (eNO\(^{+}\)nNO\(^{-}\)baroreflex restraint, from the equation shown in the Methods) was 5 mm Hg in this subject (Figure 3, top). This increase is equivalent to that produced by 1.67 log dose phenylephrine. By extrapolation to the phenylephrine dose response curve during autonomic blockade, we can predict that \(\text{L-NMMA}\) should have produced an increase in blood pressure of 18.2 mm Hg if only the baroreflex restraint was removed (eNO\(^{+}\)nNO\(^{-}\)). A predicted increase in blood pressure during autonomic blockade

\[\text{SBP, mm Hg } 108 \pm 2.5 \quad 108 \pm 2.6 \quad 0.619\]
\[\text{DBP, mm Hg } 62 \pm 2.2 \quad 62 \pm 2.2 \quad 0.717\]
\[\text{HR, bpm } 61 \pm 2.2 \quad 59 \pm 2.0 \quad 0.102\]
\[\text{MAP, mm Hg } 77 \pm 2.2 \quad 78 \pm 2.1 \quad 0.507\]

\[\text{Pre-L-NMMA* Postsaline Posttrimethaphan}\]

\[\begin{array}{l|c|c|c}
\text{SBP, mm Hg} & 107 \pm 2.5 & 107 \pm 2.3 & 0.795 \\
\text{DBP, mm Hg} & 62 \pm 2.3 & 59 \pm 1.5 & 0.227 \\
\text{HR, bpm} & 61 \pm 2.3 & 81 \pm 2.1 & <0.001 \\
\text{MAP, mm Hg} & 77 \pm 2.2 & 75 \pm 1.6 & 0.435 \\
\end{array}\]

\[\text{L-NMMA dose 250}\]

\[\begin{array}{l|c|c|c}
\text{SBP, mm Hg} & 114 \pm 2.7 & 134 \pm 3.1 & 0.001 \\
\text{DBP, mm Hg} & 67 \pm 2.2 & 76 \pm 2.3 & 0.002 \\
\text{HR, bpm} & 52 \pm 2.4 & 77 \pm 1.7 & <0.001 \\
\text{MAP, mm Hg} & 82 \pm 2.3 & 96 \pm 2.4 & 0.001 \\
\end{array}\]

\[\text{L-NMMA dose 500, all subjects (n=16)}\]

\[\begin{array}{l|c|c|c}
\text{SBP, mm Hg} & 121 \pm 3.4 & \quad \quad & \\
\text{DBP, mm Hg} & 71 \pm 2.7 & \quad \quad & \\
\text{HR, bpm} & 49 \pm 2.3 & \quad \quad & \\
\text{MAP, mm Hg} & 87 \pm 2.9 & \quad \quad & \\
\end{array}\]

\[\text{L-NMMA dose 500, subjects able to receive this dose after trimethaphan (n=5)}\]

\[\begin{array}{l|c|c|c}
\text{SBP, mm Hg} & 123 \pm 4.0 & 144 \pm 2.6 & \\
\text{DBP, mm Hg} & 73 \pm 3.5 & 84 \pm 3.8 & \\
\text{HR, bpm} & 52 \pm 2.4 & 74 \pm 3.2 & \\
\text{MAP, mm Hg} & 90 \pm 3.6 & 104 \pm 3.2 & \\
\end{array}\]

\(HR\) indicates heart rate; MAP, mean arterial pressure; \(\text{L-NMMA dose 250: IV L-NMMA infusion of 250 } \mu\text{g/kg per minute; L-NMMA dose 500: IV L-NMMA infusion of 500 } \mu\text{g/kg per minute; } P\text{ values are from Wilcoxon signed-rank test.}\)

\(\text{\(P\) values from paired } t\text{ test.}\)

\(\text{Pre-L-NMMA values are those after an infusion of saline on the “intact day” and an infusion of trimethaphan followed by phenylephrine to restore blood pressure to baseline values.}\)

\(\text{\(P\) values from Wilcoxon signed-rank test.}\)

\(\text{Pre-L-NMMA values are those after an infusion of saline on the “intact day” and an infusion of trimethaphan followed by phenylephrine to restore blood pressure to baseline values.}\)

\(\text{\(P\) values from Wilcoxon signed-rank test.}\)
greater than the measured increase in blood pressure produced by L-NMMA during autonomic blockade (selective eNO) would imply that nNO tonically contributes to blood pressure. However, when individual responses were averaged, we saw no differences between the measured and the predicted responses to L-NMMA during autonomic blockade (Figure 3, bottom), implying that nNO did not significantly contributed to the increase in blood pressure induced by L-NMMA in normal subjects.

(eNO+nNO) greater than the measured increase in blood pressure produced by L-NMMA during autonomic blockade (selective eNO) would imply that nNO tonically contributes to blood pressure. However, when individual responses were averaged, we saw no differences between the measured and the predicted responses to L-NMMA during autonomic blockade (Figure 3, bottom), implying that nNO did not significantly contributed to the increase in blood pressure induced by L-NMMA in normal subjects.

**Figure 3.** Estimation of the contribution of endothelial-derived and nNO on SBP. Blood pressure responses to phenylephrine before and after autonomic blockade were constructed in each subject. In the example shown at the top, L-NMMA increased blood pressure by 5 mm Hg when the autonomic nervous system was intact (average value of all subjects is shown at the bottom as "INTACT"; eNO+nNO—baroreflex restraint). By extrapolation from the phenylephrine curves, we can predict how much blood pressure should have increased in the absence of baroreflex restraint ("DEBUFFERED PREDICTED"; eNO+nNO). Average responses are depicted at the bottom and show no differences between the measured and the predicted responses to L-NMMA during autonomic blockade, implying little contribution of nNO to blood pressure regulation in these normal subjects.

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<th>Parameters</th>
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Posttrimethaphan/phenylephrine

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**Effect of eNO Inhibition in Smokers**

For protocol 2, no differences were found in the decrease in blood pressure induced by trimethaphan in normal controls compared with smokers (Figure 4, left). Baseline blood pressures were not significantly different between nonsmokers and smokers, but SBP was significantly greater in nonsmokers after 250 µg/kg per minute of L-NMMA (P=0.02 by Mann–Whitney U test; Table 4).

Analysis using the random-effects model showed a statistically significant linear positive dose effects of L-NMMA on both SBP and DBP for both nonsmokers (SBP: P<0.001; DBP: P<0.001) and smokers (SBP: P<0.001; DBP: P=0.001), but the linear dose–response rate for nonsmokers was increased ~2-fold compared with that of smokers in SBP (P=0.007) but not in DBP (P=0.493) indicating a greater effect of L-NMMA on nonsmokers. The mean SBP of nonsmokers at dose 250 was increased

![Figure 3](https://hyper.ahajournals.org/)

**Figure 3.** Estimation of the contribution of endothelial-derived and nNO on SBP. Blood pressure responses to phenylephrine before and after autonomic blockade were constructed in each subject. In the example shown at the top, L-NMMA increased blood pressure by 5 mm Hg when the autonomic nervous system was intact (average value of all subjects is shown at the bottom as "INTACT"; eNO+nNO—baroreflex restraint). By extrapolation from the phenylephrine curves, we can predict how much blood pressure should have increased in the absence of baroreflex restraint ("DEBUFFERED PREDICTED"; eNO+nNO). Average responses are depicted at the bottom and show no differences between the measured and the predicted responses to L-NMMA during autonomic blockade, implying little contribution of nNO to blood pressure regulation in these normal subjects.

![Figure 4](https://hyper.ahajournals.org/)

**Figure 4.** Effect of autonomic blockade and preferential eNO inhibition in normal controls and smokers. Changes in SBP induced by autonomic blockade (left) and L-NMMA during autonomic blockade (right) in normal controls (NTN) and smokers (SMK). Values are shown as mean±SEM. Comparisons were made using the Mann–Whitney U test.
compared with smokers (mean: 11.6 mm Hg; 95% CI: 0.3 to −23.4; P<0.056), but it was marginally nonsignificant. No difference in the means for DBP between nonsmokers and smokers was found. Statistical analysis was not performed with the higher dose of L-NMMA (500 μg/kg per minute), because it could be given only to 4 subjects in the nonsmoker groups. Blood pressure exceeded our safety limit in the remainder.

Discussion
We report that endogenous endothelial-derived NO tonically restrains blood pressure in normal subjects by ≥30 mm Hg (Figure 2). We based this conclusion on the increase in blood pressure achieved by systemic blockade of NOS with L-NMMA in the presence of the ganglion blocker trimethaphan. This approach allowed us to observe the full expression of the increase in blood pressure in the absence of the restraining effect of baroreflex mechanisms. Furthermore, the contribution of neurally derived NO to blood pressure regulation is greatly diminished in the presence of autonomic blockade, because it is mediated mostly by its interaction with the autonomic nervous system, which is no longer operative. Therefore, the increase in blood pressure with L-NMMA can be ascribed to preferential inhibition of eNO production. It should be noted that our findings reflect only partial inhibition of eNO, because during ganglionic blockade, we clearly did not reach a maximal dose effect of NOS inhibition. We deemed it unsafe to increase blood pressure >150 mm Hg in our subjects, and this limited the dose of L-NMMA during autonomic blockade. It is likely, therefore, that our results underestimate the real importance of eNO in restraining blood pressure in normal subjects.

There is little doubt that NO tonically restrains blood pressure in normal subjects, but the magnitude of this effect has been difficult to estimate. Previous studies using systemic inhibition of NO production have consistently found an increase in mean arterial pressure of ∼10%, even when L-NMMA was given at higher doses than the one used in the present study. Our results indicate that the actual importance of eNO in restraining blood pressure can be gauged if the autonomic nervous system is blocked. Under these conditions, eNO inhibition produced at least a 25% increase in mean arterial blood pressure, for an increase in SBP of ≥27 mm Hg and mean arterial blood pressure of ≥21 mm Hg. An increase in mean arterial blood pressure of similar magnitude was reported by Halliwell et al in normal subjects infused with L-NMMA after α-adrenergic blockade with phentolamine. Therefore, eNO is arguably the most important metabolic regulator of blood pressure in normal subjects.

We have validated this approach by studying heavy smokers, which are widely accepted to have an impaired production of NO, based on impaired forearm vasodilatory response to acetylcholine, impaired flow-mediated dilation, and impaired activity of vascular NOS. As expected, we found that the increase in blood pressure produced by L-NMMA in the presence of trimethaphan was significantly lower in smokers than in normal control subjects, reflecting an impaired contribution of eNO to their blood pressure. Despite the documented eNO deficiency in smokers, it is important to note that they had a normal blood pressure. This has been observed in other studies and implies that other mechanisms compensate for impaired eNO function to maintain blood pressure within a reference range.

There is controversy about the contribution of nNO to blood pressure regulation. We cannot directly measure the importance of nNO on blood pressure, but we can at least provide an estimate after individually taking into account the buffering capacity provided by the baroreflex (see Results for details). Using this approach we did not find a significant contribution of nNO to the increase in blood pressure induced by L-NMMA in normal subjects. This is in agreement with the finding that blood pressure is not increased in mice lacking nNOS.

We do not claim that we have achieved selective inhibition of the NOS3 enzyme over NOS1, merely that the contribution of nNO to blood pressure is minimized under the condition of our experiments, because it is primarily mediated by inhibition of the sympathetic nervous system. We cannot rule out the possibility that NO, synthesized by nNOS, can have a direct effect on vascular tone unrelated to autonomic interactions. Similarly, we cannot exclude a potential role of NO derived from inducible NOS (NOS2). The lack of corroboration of endothelial dysfunction in the smokers group is a limitation of the study that needs to be considered.

The paradigm developed in this study, the use of systemic inhibition of NOS in the presence of autonomic blockade, complements other techniques currently used to evaluate eNO dysfunction in disease states. Flow-mediated dilation is commonly used for this purpose, but measures mostly the role of eNO in conduit arteries and may be influenced by sympathetic tone. Forearm blood flow responses to intra-brachial infusion of endothelial-dependent vasodilators are also used for this purpose. This approach provides important information about endothelial function, and forearm responses correlate with those of other vascular beds, most notably the coronary circulation. However, none of the vasodilators available act exclusively via NO mechanisms, and it is difficult to quantify from these results the effect that eNO may have to overall blood pressure levels.

Perspectives
We describe a novel approach to preferentially gauge the importance of eNO on blood pressure by measuring the effect of systemic inhibition of NOS during autonomic blockade, thus eliminating the confounding effect of baroreflex mechanisms, and diminishing the role of nNO on blood pressure, which depends on its interaction with the autonomic nervous system. Using this approach, we found that eNO is likely the most important metabolic regulator of blood pressure, tonically restraining it by ∼30 mm Hg or more. On the other hand, we did not find nNO to be as important in the regulation of blood pressure in normal subjects, with the caveat that this conclusion is based on an indirect assessment. Our approach was validated by the observation that the increase in blood pressure during selective eNO inhibition was impaired in smokers, a group known to have eNO deficiency. The
paradigm presented here can be used to gauge the importance of eNO in other pathological conditions.

**Acknowledgment**

We dedicate this work in memory of Dr Bojan Pohar.

**Sources of Funding**

This work was supported in part by National Institutes of Health grants 1RO1 HL67232, 1PO1 HL56693, and RR00095, and by Deutsche Forschungsgemeinschaft grants Jo 284/1-1, and Jo 284/3-1.

**Disclosures**

None.

**References**


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**Endothelial Nitric Oxide and Blood Pressure**

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Hypertension. 2007;49:170-177; originally published online November 27, 2006; doi: 10.1161/01.HYP.0000252425.06216.26
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2006 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

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