Enhanced Blood Pressure Variability in eNOS Knockout Mice

Harald M. Stauss, Axel Gödecke, Ralf Mrowka, Jürgen Schrader, Pontus B. Persson

Abstract—It has been shown previously that endogenous nitric oxide can buffer arterial blood pressure variability in dogs and rats. In these former studies, all isoforms of the nitric oxide synthase were blocked pharmacologically and an increased blood pressure variability was observed. Thus the question as to which isoform of the nitric oxide synthase is responsible for the blood pressure buffering effect of endogenous nitric oxide remains unraveled. In the present study, we therefore compared blood pressure variability in knockout mice that lack specifically the gene for endothelial nitric oxide synthase with their respective wild-type controls. One day after carotid artery cannulation, blood pressure was recorded in these conscious mice. During resting conditions, blood pressure variability was markedly enhanced in knockout mice compared with wild-type mice (10.5±1.5 mm Hg^2 vs 6.0±0.8 mm Hg^2, P<0.05). Power spectral analysis revealed that this increase in blood pressure variability is manifested at low frequencies that range from 0.05 to 0.40 s^-1 (Hz) (5.1±1.0 mm Hg^2 vs 2.5±0.5 mm Hg^2, P<0.05). On the basis of these results, we conclude that the blood pressure buffering effect of endogenous nitric oxide is mediated by the endothelial isoform of the nitric oxide synthase. In addition, endothelial nitric oxide is most effective in buffering blood pressure oscillations at frequencies that range from 0.05 to 0.40 s^-1 (Hz) in conscious mice. (Hypertension. 1999;33:1359-1363.)

Key Words: hypertension, arterial blood pressure spectrum analysis endothelium nitric oxide

Chronically elevated blood pressure has been identified as an important risk factor for cardiovascular death in several clinical trials. Furthermore, it has been demonstrated recently that enhanced blood pressure variability represents an additional risk factor for end-organ damage that is independent of the average blood pressure level. It is therefore not surprising that the cardiovascular system is equipped with a powerful mechanism to buffer short-term blood pressure fluctuations: the arterial baroreceptor reflex. In addition, recent studies have identified a second short-term blood pressure buffer that operates by the release of nitric oxide (NO) in response to an increase in arterial blood pressure. In these former studies, endogenous NO production was blocked pharmacologically by the false substrate NG-nitro-L-arginine in conscious dogs and rats, and an increased blood pressure variability was found. Thus endogenously produced NO constitutes an important blood pressure buffer. This blood pressure buffer was found to be most effective in buffering blood pressure fluctuations in a frequency range of 0.1 to 0.5 s^-1 (Hz) in dogs and 0.2 to 0.6 s^-1 (Hz) in rats.

The efferent arch of this blood pressure buffer depends on NO, which causes vasodilation in response to a blood pressure increase. However, it remains unknown by which pathway the initial blood pressure increase is detected. Theoretically, at least 2 mechanisms can be postulated. First, the same mechanoreceptors that are involved in the classic baroreceptor reflex may detect pressure changes and signal this information to the central nervous system. There, a neuronal pathway may be initiated that activates the neuronal isoform of NO synthase (nNOS) that subsequently causes NO production. Second, a local vascular pathway may be possible. In this local vascular pathway, changes in arterial blood pressure lead to changes in vascular shear stress. This mechanical stimulus would increase the cytosolic Ca^2+ content in the endothelial cells, which in turn would activate the endothelial isoform of NO synthase (eNOS). The subsequently formed NO reaches the adjacent vascular smooth muscle cells, where it modulates vascular resistance to maintain blood pressure at its initial level. Because all isoforms of NO synthase have been blocked by NG-nitro-L-arginine in the former studies, it remained unclear whether the blood pressure–buffering effect of NO is mediated by NO that is formed by the eNOS or nNOS isoforms.

The purpose of the present study, therefore, was 2-fold. First, with the use of knockout mice in which the eNOS isoform was specifically mutated, we investigated whether NO that is generated by the endothelial isoform of NO synthase is involved in the blood pressure–buffering effect of NO. Second, with the use of power spectral analysis,
we determined the frequency range in which endothelium-derived NO can buffer blood pressure variability in mice. As an experimental approach, spontaneous blood pressure fluctuations were compared in 13 wild-type and 10 eNOS mutant mice. To eliminate artifacts of anesthesia, experiments were performed in conscious animals, at least 24 hours after implantation of a carotid artery catheter. The animals were not restrained to avoid psychological stress, which has a large impact on blood pressure variations.\(^\text{16}\)

**Methods**

**Animals**

The experiments were conducted in 13 wild-type C57 Bl6 mice and 10 eNOS mutant animals derived from the same strain, that weighed 26.5\(\pm\)1.3 g and 28.4\(\pm\)1.6 g, respectively. The eNOS gene was inactivated by replacing exons 24 and 25 with the neomycin-resistance gene in the embryonic stem cell line E14 to 1.\(^\text{14,15}\) Aortic endothelial cells obtained from these eNOS mutant mice have been shown to produce only background levels of NO.\(^\text{15}\) After surgery, mice were housed individually in clear plastic cages. Temperature (24\(\pm\)2 °C), humidity (60\(\pm\)10%), and light periods (12:12 hours light-dark cycle; light 6:00 AM to 6:00 PM) were controlled. Mice had free access to a standard mouse chow diet and were provided with tap water ad libitum. All experiments were approved by the federal animal rights committee and were performed in accordance with institutional guidelines for health and care of experimental animals.

**Catheter Implantation and Blood Pressure Recording**

For catheter implantation, the mice were anesthetized by a single intraperitoneal dose of 4 mg/10 g body wt chloral hydrate. The common carotid artery was exposed by a midline incision in the vicinity and the tip was tapered on a length of 5 mm to ease cannulation of the arterial blood pressure variations,\(^\text{16}\) was minimized. The external line was exteriorized at the dorsum of the neck through subcutaneous tunneling.

All experiments were performed in conscious mice at least 24 hours after implantation of a carotid artery catheter. During the experimental protocol, the animals were not restrained and could move freely in the same cages where they were housed after surgery. For blood pressure recordings, the carotid artery catheter was attached to an external line. This external line was brought straight out of the cage and guided around a metal bar that was placed 300 mm horizontally above the cage. Thus tethering of the mice was not necessary and psychological stress, which has a large impact on blood pressure variations,\(^\text{16}\) was minimized. The external line was attached to a pressure transducer (DTX Plus, Ohmeda Inc) that was located, at the same level as the mouse. Finally, the pressure transducer was connected to a pressure processor amplifier (Gould 4600 Series, Gould Instrument Systems Inc) that provided the analog blood pressure signal that was digitized with a computer based monitoring system (XmAD, ftp://sunsite.unc.edu/pub/Linux/science/lab) with a sampling rate of 1000 s\(^{-1}\) (Hz). During the recordings, mice were continuously observed and only episodes of physical rest were saved for further analyzes. Thus >60 minutes and occasionally >120 minutes had to be allowed before a stationary blood pressure signal was monitored. During these resting conditions heart rate was consistently <600 bpm (Table).

**Data Analysis and Statistics**

All blood pressure time series were visually inspected on the computer screen, and 66-second-long stationary segments were selected (Figure 1a) with the use of a freely available analyzing software (XmANA, ftp://sunsite.unc.edu/pub/Linux/science/lab). Power spectra of these segments were calculated by the fast Fourier transform, based on 65 536 values (Figure 1c). Mean blood pressure variances (Table) were determined as the total power of the arterial blood pressure power spectra up to a frequency of 5.0 s\(^{-1}\) (Hz) that was far below the heart rate peak in all cases. Probability distributions of the mean blood pressure amplitude, mm Hg 3 0.8 10.5

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Wild-Type Mice (n=13)</th>
<th>eNOS Mutant Mice (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>123(\pm)4</td>
<td>173(\pm)10*</td>
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<tr>
<td>Mean blood pressure, mm Hg</td>
<td>106(\pm)3</td>
<td>153(\pm)9*</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm Hg</td>
<td>90(\pm)3</td>
<td>136(\pm)10*</td>
</tr>
<tr>
<td>Blood pressure amplitude, mm Hg</td>
<td>33(\pm)3</td>
<td>37(\pm)5</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>545(\pm)23</td>
<td>468(\pm)15*</td>
</tr>
<tr>
<td>Respiration rate, s(^{-1}) (Hz)</td>
<td>3.4(\pm)0.1</td>
<td>3.1(\pm)0.2</td>
</tr>
<tr>
<td>Low frequency power, mm Hg(^2)</td>
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<td>10.5(\pm)1.5*</td>
</tr>
<tr>
<td>High frequency power, mm Hg(^2)</td>
<td>2.5(\pm)0.5</td>
<td>5.1(\pm)1.0*</td>
</tr>
<tr>
<td>Mid frequency power, mm Hg(^2)</td>
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<td>0.5(\pm)0.1</td>
</tr>
<tr>
<td>High frequency power, mm Hg(^2)</td>
<td>1.5(\pm)0.4</td>
<td>2.3(\pm)0.9</td>
</tr>
</tbody>
</table>

Respiration rates were determined as the center frequency of the high frequency power in the blood pressure power spectra. Frequency boundaries were 0.05 to 0.4 s\(^{-1}\) (Hz) for low frequency power, 0.4 to 0.8 s\(^{-1}\) (Hz) for mid frequency power, and 0.5 s\(^{-1}\) (Hz) around the respiration peak for high frequency power.

Data are expressed as mean\(\pm\)SEM. Statistical evaluations were performed by unpaired t tests.

\(*P<0.05\) for eNOS mutant animals vs wild-type mice.

**Figure 1.** Blood pressure variability in wild-type mice and eNOS mutant animals. a, Original blood pressure tracings in representative animals. White lines indicate mean blood pressure. b, Probability distributions of mean blood pressure values. After shifting to the mean blood pressure of each strain, probability distributions of all animals were averaged. c, Arterial blood pressure power spectra. Spectra of all animals were averaged. LF indicates low frequency range [0.05 to 0.4 s\(^{-1}\) (Hz)]; MF, mid frequency range [0.4 to 0.8 s\(^{-1}\) (Hz)].
pressure values were calculated and are presented in Figure 1b. First, mean blood pressure time series were generated by low-pass filtering the pulsatile blood pressure signals [corner frequency 5.0 s⁻¹ (Hz)]. Then, frequency distributions were calculated for all 23 mean blood pressure time series by use of a bin-width of 1.0 mm Hg. Next, the frequency distributions were shifted on the blood pressure axis (x-axis) to the mean blood pressure value of the respective strain and the frequency distributions of all animals from each group were averaged. Finally, the probability distributions were obtained by dividing the frequency distributions by the number of data points in the mean blood pressure time series (n=65 536). Because blood pressure variability may be affected by the chronic increase in arterial blood pressure in eNOS knockout mice, the correlation between mean arterial blood pressure and mean blood pressure variance was calculated for both strains of mice.

Because an enhanced blood pressure variability in eNOS knockout mice may be partly compensated for by the baroreceptor reflex, we calculated baroreceptor reflex sensitivity in both strains of mice by use of the sequence method that was first introduced by Bertinieri et al. This method is based on detecting spontaneously occurring sequences of 3 or more consecutive heartbeats during which both arterial blood pressure and interbeat interval simultaneously increased or decreased. These sequences were detected by use of the same algorithm as described previously. From each of these sequences, linear regressions were calculated for the blood pressure versus interbeat interval relation. Only sequences with a correlation coefficient (r) of >0.85 were included in the analysis. The regression coefficient or slope of the linear relation between blood pressure and interbeat interval was used as a measure for baroreceptor–heart rate reflex sensitivity. If the baroreceptor reflex partly compensates an enhanced blood pressure variability in eNOS knockout mice, one would expect to find more spontaneously occurring sequences in eNOS mutant animals than in wild-type mice. Therefore the number of sequences per 1000 heartbeats was calculated and used as an index for the activity of the baroreceptor reflex.

All data are expressed as mean ± SEM. Statistical comparisons between eNOS knockout mice and wild-type mice were performed by unpaired t tests.

Results
Original recordings of arterial blood pressure obtained from representative wild-type control and eNOS knockout mice are presented in Figure 1a. A higher blood pressure level and a larger spontaneous blood pressure variability can be detected in the blood pressure tracing from the eNOS knockout mouse compared with the recording from the control mouse. In contrast to the first reports regarding mean arterial blood pressure in this mouse model, a more severe hypertension was found in this study (Table). This greater difference between the knockout strain versus the wild-type mice may rely on the experimental protocol, since previous studies either measured blood pressure 1 hour after catheter implantation or the tail-cuff method was used. Heart rate was significantly lower in eNOS mutant animals than in control mice (Table). However, in both groups, heart rate was <600 bpm, which indicates that the recordings have been obtained during resting conditions.

Effects of eNOS Knockout on Blood Pressure Variability
Blood pressure variability is graphically illustrated by the probability distribution of the mean blood pressure time series in Figure 1b. The probability distribution in the eNOS knockout mice is characterized by a broader distribution of the blood pressure values (width=15 mm Hg) than the probability distribution in the control mice (width=10 mm Hg). In addition, blood pressure variability was expressed as the total power in the blood pressure power spectra at frequencies below the heart rate [<5.0 s⁻¹ (Hz)], for example, the variance of the mean blood pressure time series. Mean blood pressure variance was significantly larger in eNOS mutant animals than in wild-type controls (Table), which indicates the lack of a physiologically important blood pressure buffer. To investigate a possible role of the chronically increased blood pressure level in eNOS mutant animals on blood pressure variability, the correlation between mean blood pressure and blood pressure variance was calculated. Figure 2 demonstrates that a negative correlation was found between these 2 parameters in eNOS knockout mice (r=−0.80, P<0.01) but not in the wild-type strain (r=+0.28, not significant). Thus the higher the blood pressure level, the smaller the blood pressure variability was. However, despite the higher blood pressure level, blood pressure variance was significantly larger in eNOS knockout mice than in controls, and only 2 out of 10 knockout mice had smaller blood pressure variances (4.6 and 5.2 mm Hg²) than the average blood pressure variance in the wild-type group (6.0 mm Hg²). This finding suggests that the chronically elevated blood pressure level was not the cause of the increased blood pressure variability in eNOS mutant animals.

Effects of eNOS Knockout on Blood Pressure Power Spectra
NO has been demonstrated to buffer blood pressure fluctuations at frequencies between 0.1 and 0.5 s⁻¹ (Hz) in dogs8 and between 0.2 and 0.6 s⁻¹ (Hz) in rats. In eNOS mutant mice, a marked increase in spectral power of arterial blood pressure was observed below 0.4 s⁻¹ (Hz) compared with control mice (Figure 1c and Table). Spectral power at frequencies >0.4 s⁻¹ (Hz) was comparable in both strains. Therefore endothelium-derived NO can buffer blood pressure fluctuations at frequencies <0.4 s⁻¹ (Hz) in mice.

Effects of eNOS Knockout on Baroreceptor-Heart Rate Reflex Sensitivity
Baroreceptor–heart rate reflex sensitivity was calculated with the sequence method. No significant difference was found between both strains of mice (2.44±0.84 versus 2.69±0.81 ms/mm Hg: eNOS knockouts versus wild-type controls; not significant). The number of sequences per 1000 heartbeats was used as an index for baroreceptor reflex activity. In
eNOS knockout mice there were 3.1±0.9 sequences per 1000 heartbeats, whereas only 1.5±0.5 sequences were found in 1000 heartbeats in wild-type controls. This difference in the number of sequences per 1000 heartbeats did not reach statistical significance (\(P=0.15\)). A greater amount of sequences in the eNOS knockout mice would suggest that the larger blood pressure variability in eNOS knockout mice is partly compensated for by the baroreceptor reflex.

**Discussion**

In resting knock-out mice that lack a functional eNOS gene,14,15 blood pressure fluctuations were more pronounced than in wild-type controls. This larger blood pressure variability was confined to a frequency range of 0.05 to 0.4 s\(^{-1}\) (Hz). Thus it is reasonable to ascribe the blood pressure buffering effect of NO that was reported in former studies7,8 to that generated by the endothelial isoform of the NO synthase. A local endothelial mechanism, therefore, can be postulated for the blood pressure–buffering effect of NO. This local endothelial pathway is initiated by fluctuations in arterial blood pressure that lead to changes in vascular shear stress. This mechanical stimulus activates the endothelial isoform of the NO synthase. Subsequently, NO reaches the adjacent vascular smooth muscle cells, where it modulates vascular resistance to maintain blood pressure at its initial level.

Although we could demonstrate that NO generated by the endothelial isoform of the NO synthase buffers short-term blood pressure fluctuations, we were not able to rule out that NO produced by other isoforms of the NO synthase also contribute to this effect. For example, it has been demonstrated that long-term treatment for 4 weeks with 7-nitroindazole, a specific nNOS antagonist, causes sustained hypertension in rats.21 In this study, it was suggested that the hypertensive effect of nNOS blockade is mediated by an increased tubuloglomerular feedback sensitivity, which leads to decreased glomerular filtration rate and an increased body fluid volume. Similarly, selective neuronal NO synthase inhibition has been shown to block furosemide-stimulated renin secretion in vivo.22 Such renal mechanisms are unlikely to respond to blood pressure fluctuations in the time range of 2 to 20 seconds, since it has been shown that renal autoregulation reaches a maximum at frequencies below 0.01 s\(^{-1}\) (Hz) (\(\tau >100\) seconds) in conscious dogs.23 On the basis of these findings, one would speculate that nNOS-generated NO does not participate in the short-term blood pressure–buffering effects of NO. On the other hand, it has been demonstrated in rabbits that nNOS blockade can modulate baroreflex control of heart rate24 and that neuronal NO reduces sympathetic excitability in pigs.25 These mechanisms, however, may be rapid enough to respond to blood pressure fluctuations within seconds. Therefore an additional blood pressure–buffering effect of NO derived from nNOS cannot be completely ruled out.

Chronically increased blood pressure levels can cause increased blood pressure variability. Therefore the question arises whether the increased blood pressure variability in eNOS knockout mice is secondary to the hypertension observed in these mice. To address this question, we calculated the correlation between mean blood pressure and blood pressure variance and found a negative correlation between these 2 parameters in the eNOS knockout group (Figure 2). In addition, the increased blood pressure variability was not uniformly distributed over the entire frequency domain, as would have been expected if the enhanced blood pressure variability was secondary to hypertension. Instead, the increase in blood pressure variability was confined to a low frequency band between 0.05 and 0.4 s\(^{-1}\) (Hz). Thus the negative correlation between mean blood pressure and blood pressure variance together with the selective increase in low frequency spectral power suggests that in this particular model of hypertension, increased blood pressure variability does not rely on the chronically increased blood pressure level.

Marked fluctuations in blood pressure are hardly compatible with everyday life and can lead to end-organ damage.3,4 The best known and probably most effective mechanism to maintain arterial blood pressure within tight boundaries is the arterial baroreceptor reflex.5,6 The afferent component of this reflex consists of stretch receptors located in the carotid sinus region and the aortic arch, whereas the effector side of the reflex is mediated by the sympathetic and parasympathetic nervous system.26 However, the efferent portion of the reflex that depends on the integrity of the autonomic nervous system may not function properly in various pathophysiological conditions such as diabetic polyneuropathy,27 multiple sclerosis,28 or pure autonomic failure.29 Because nature in general provides organisms with compensatory systems that can step into the breach if critical organs are malfunctioning, it is not surprising that a second short-term blood pressure–buffering mechanism exists that is independent from the autonomic nervous system. Because blood pressure variability is larger in eNOS knockout mice than in wild-type controls and because it has been shown that an increase in vascular shear stress enhances aortic endothelial NO synthase expression in vivo,10,11 it is reasonable to assume that this second blood pressure–buffering mechanism is the local vascular NO system. It most likely operates by a shear stress–induced elevation in cytosolic Ca\(^{2+}\) content, which activates the endothelial isoform of NO synthase.9,12,13 The subsequently formed NO counterbalances the initial increase in arterial blood pressure and thereby maintains blood pressure at its initial level. It is remarkable that it has been demonstrated in conscious rats that the autonomic nervous system does not contribute to tonic NO-mediated vasodilatation.30 Thus this local vascular blood pressure buffer is largely independent from the autonomic nervous system, which may have particular importance in patients with autonomic dysfunctions.

Baroreceptor reflex sensitivity was found to be similar in eNOS mutant and wild-type control mice. Therefore the question arises as to why the baroreceptor reflex does not eliminate the enhanced blood pressure variability in eNOS knockout mice. Twice as many sequences in which blood pressure and interbeat interval simultaneously increased or decreased were found in the blood pressure time series obtained from eNOS knockout mice than in wild-type controls. This finding suggests that the higher blood pressure variability in eNOS knockout mice was partly compensated
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