Sympathetic Modulation of Endothelium-Derived Relaxing Factor

Alfredo A. Pegoraro, Oscar A. Carretero, David H. Sigmon, and William H. Beierwaltes

To determine whether the release of endothelium-derived relaxing factor (EDRF) is sympathetically mediated, we studied the effects of β-blockade by propranolol, ganglionic blockade with hexamethonium, or mechanical pithing on the blood pressure response to EDRF inhibition in anesthetized rats. We inhibited EDRF with 10 mg/kg of either Nω-monomethyl-L-arginine (L-NMMA) or Nω-nitro-L-arginine-methyl ester (L-NAME). In controls, L-NMMA and L-NAME increased blood pressure by 14±1 (p<0.01) and 22±2 mm Hg (p<0.01), respectively. Propranolol lowered blood pressure from 98±3 to 72±4 mm Hg without altering the response to L-NAME (Δ26±3). This response correlated with the resting blood pressure (r=0.87; p<0.001). Hexamethonium (25 mg/kg) lowered blood pressure from 118±6 to 85±4 mm Hg but did not change the response to L-NAME (Δ15±1). In pithed rats, blood pressure was lowered, but the pressor response to L-NAME was unchanged. When blood pressure was returned to normotensive levels by angiotensin II, norepinephrine, or phenylephrine, L-NAME increased blood pressure by 50±2, 68±8, and 109±7 mm Hg, respectively (p<0.001). We conclude that an intact autonomic nervous system is not needed for the pressor response to EDRF inhibition. The enhanced response in pithed rats treated with vasoconstrictors may be due to removal of the buffering effect of the baroreceptors and the absence of EDRF, which would oppose vasoconstriction. The correlation between blood pressure and the L-NAME response in the propranolol group may be due to the degree of preconstriction of the peripheral arterioles or to a direct relation between blood pressure and EDRF. The greater response to EDRF synthesis inhibition observed in the rats pretreated with phenylephrine suggests that EDRF synthesis may be stimulated by α1-receptors.

Key Words • blood pressure • endothelium • nitric oxide • endothelium-derived relaxing factor • arginine • autonomic nervous system • adrenergic receptors

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ince the discovery of endothelium-derived relaxing factor (EDRF) by Furchgott and Zawadzki,1 numerous studies have examined the factors that regulate its synthesis and release. EDRF is thought to be nitric oxide,2 derived from L-arginine,3 or a nitrosothiol compound,4 which is released primarily by endothelial cells. EDRF acts through the second messenger cyclic GMP,5 causing relaxation of vascular smooth muscle. Tonic EDRF vasodilation can be presumed to be nitric oxide and organ perfusion. The release of EDRF has been reported to be regulated by mechanical forces such as shear stress6 and by certain vasoactive factors, including the vasodilators acetylcholine and bradykinin,7 and vasoconstrictors such as catecholamines and serotonin.8 It has been reported that an intact sympathetic nervous system may be required for tonic release of EDRF, because ganglionic blockade completely abolished the pressor response to EDRF synthesis inhibition using Nω-nitro-L-arginine-methyl ester (L-NAME).9 Additionally, infusion of phenylephrine has been shown to increase nitrite production in the isolated mesenteric vascular bed and potentiate the vasoconstriction caused by EDRF inhibitors.10 Although these observations are provocative, they do not explain whether EDRF can be produced in the absence of neural influences. We designed our study to assess the pressor response to inhibition of EDRF synthesis in rats after β-blockade, ganglionic blockade, and pithing.

Methods

Male Sprague-Dawley rats (250–350 g; Charles River Laboratories, Wilmington, Mass.) were placed on normal rat chow (Purina Mills) and given water ad libitum in a 12-hour light/dark cycle. On the day of the study, the rats were anesthetized with an intraperitoneal injection of sodium thiopental (50 mg/kg) and placed on a heating pad to maintain body temperature at 37°C. A PE-240 tube (Clay Adams, Parsippany, N.J.) was inserted into the trachea, the femoral artery was cannulated for intravenous infusions. A solution of isosotic saline was infused through the femoral vein throughout the experiment. For the maintenance of hematocrit and recovery of volume loss, a 2-ml supplement of plasma from a donor rat nephrectomized 12 hours earlier was added during the equilibration period. After the surgical preparation was completed, equilibration was allowed for at least 20 minutes. Blood pressure and heart rate were obtained with a Gould Statham pressure transducer and monitored with a recorder (Gould Inc., Valley View, Ohio) for the duration of the
experiment. This basic preparation was used for three protocols.

Protocol 1: β-Adrenergic Blockade

In the first group of rats, we studied the pressor response to EDRF synthesis inhibition in rats subjected to β-adrenergic blockade by an intravenous bolus of 1 mg/kg propranolol, supplemented by a constant infusion of 0.3 mg/kg/hr. This treatment resulted in a spectrum of basal blood pressures ranging from 40 to 100 mm Hg. Once blood pressure had stabilized, these rats were given an intravenous bolus of 10 mg/kg L-NAME, and the response was monitored for 30 minutes. Control rats treated with vehicle instead of propranolol received the same dose of L-NAME.

Protocol 2: Ganglionic Blockade

In a third group of rats, we tested the effects of an EDRF synthesis inhibitor during ganglionic blockade. Ten minutes after a bolus injection of either the ganglionic blocker hexamethonium chloride (25 mg/kg) or vehicle, each rat received either 10 mg/kg Nω-monomethyl L-arginine (L-NMMA) or vehicle intravenously, and blood pressure and heart rate were monitored for 30 minutes.

Protocol 3: Pithed Rats

In the second group of rats, we tested the effects of an EDRF synthesis inhibitor during ganglionic blockade. Ten minutes after a bolus injection of either the ganglionic blocker hexamethonium chloride (25 mg/kg) or vehicle, each rat received either 10 mg/kg L-NAME, L-NMMA, hexamethonium chloride, norepinephrine (200 ng/50 μl  • min⁻¹), norepinephrine (200 ng/50 μl  • min⁻¹), or phenylephrine (400 ng/5 μl  • min⁻¹) initiated through the femoral vein immediately after pithing and was adjusted to maintain mean arterial pressure at 100±5 mm Hg. The rats treated with phenylephrine received an additional infusion of saline (45 μl/min) for the maintenance of volume. The endotracheal tube was connected to a mechanical rodent respirator (model 680, Harvard Instruments, Harvard, Mass.) with a tidal volume of 2 ml, 60 strokes per minute. Once a stable pressure level of 100 mm Hg was achieved, the infusion of angiotensin II, norepinephrine, or phenylephrine was held constant. EDRF synthesis was blocked with L-NAME. This basic preparation was used for three experiments. This basic preparation was used for three protocols.

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Analysis of Data

Basal pressures and heart rates were obtained within 5 minutes before drug injection. The maximum change in blood pressure was compared with the basal value. Heart rate was monitored concurrently, and the values that corresponded to the maximum change in blood pressures were recorded. Student's paired t test was used to compare changes in blood pressure and heart rate. Pearson's regression analysis was used to correlate changes in blood pressure in the propranolol-treated rats, and analysis of covariance was used to compare the pithed groups. A value of p<0.05 was considered significant.

Results

Protocol 1: β-Adrenergic Blockade

In the first set of experiments, β-adrenergic blockade with propranolol resulted in a spectrum of basal blood pressures ranging between 40 and 100 mm Hg. Figure 1 shows the pressor response to 10 mg/kg L-NAME in propranolol-treated rats (n=19) as a function of basal blood pressure. L-NAME resulted in a mean pressor response of 26±3 mm Hg, but the actual changes correlated highly with basal values (r=0.87; p<0.001): the higher the basal pressure, the greater the pressor response to L-NAME. In contrast, untreated controls (n=20) showed no correlation between the pressor response to L-NAME and basal pressure (Figure 1). Propranolol lowered the heart rate by 12% (from 330±8 to 291±8 beats per minute); however, heart rate was not further decreased by administration of L-NAME.

Protocol 2: Ganglionic Blockade

Table 1 shows blood pressure and heart rate for nontreated control and hexamethonium-treated rats. In the nontreated rats (n=5), a single bolus injection of 10 mg/kg L-NMMA significantly increased blood pressure by 13% and decreased heart rate by 9% (p<0.01). In rats given hexamethonium (n=7), blood pressure fell by 28% (from 118±6 to 85±4 mm Hg) and heart rate by 19% (from 373±12 to 301±12 beats per minute; p<0.01). Despite the changes in blood pressure and heart rate, L-NMMA still raised blood pressure significantly by 18% (p<0.01) without changing heart rate. Instead, the administration of vehicle in hexamethonium-treated rats (n=6) produced no change in either blood pressure or heart rate (80±7 mm Hg and 280±14 beats per minute, respectively). As shown in Table 1, the change in blood pressure produced by L-NMMA in the nontreated controls was not different from the hexamethonium-treated rats. However, we observed a decrease in heart rate in only the nontreated control rats (p<0.01).

Protocol 3: Pithed Rats

Basal blood pressure in nontreated anesthetized control rats (n=8) was 106±5 mm Hg. When 10 mg/kg
L-NAME was administered, blood pressure increased by 22±2 mm Hg, and heart rate decreased by 10±6 beats per minute (from 283±11 to 273±11 beats per minute). After mechanical ablation of the central nervous system by pithing (n=6), blood pressure was 44±2 mm Hg. L-NAME raised blood pressure by 20±2 mm Hg, which was not significantly different from the intact control group. Heart rate was 344±9 beats per minute and did not change significantly after L-NAME (−6±3 beats per minute). In pithed rats treated with angiotensin II (n=7) before administration of L-NAME, blood pressure was maintained at 105±4 mm Hg, with a heart rate of 260±10 beats per minute. However, in pithed rats during constant angiotensin II–maintained blood pressure, the pressor response to L-NAME was significantly potentiated (Figure 2); blood pressure increased by 30±2 mm Hg (p<0.01). Heart rate did not change (264±15 beats per minute). Similarly, in a third set of pithed rats (n=6) in which blood pressure was maintained at 95±5 mm Hg by a constant infusion of norepinephrine, the pressor response to L-NAME was 68±8 mm Hg (Figure 2). Heart rate did not change significantly after administration of L-NAME (from 372±15 to 382±17 beats per minute; p=NS). In the fourth set, during the infusion of phenylephrine (n=7), blood pressure was maintained at 92±3 mm Hg and heart rate was 362±18 beats per minute (n=7). Administration of L-NAME increased blood pressure by 109±7 mm Hg, more than five times the response seen in control and pithed rats and significantly greater than that produced by angiotensin II or norepinephrine (p<0.01). Again, L-NAME did not alter heart rate significantly (365±22 beats per minute).

Discussion

We designed our study to determine whether the nervous system is necessary for the release and subsequent depressor effect of EDRF. We have done this by studying the pressor response to inhibition of EDRF synthesis using either L-NMMA or L-NAME. Previously, it was suggested that an intact nervous system may be required for the tonic release of EDRF13; however, this observation was qualified to suggest that release of EDRF is dependent on blood pressure10 and disappears with hypotension. These authors10 reported that if rats were subjected to ganglionic blockade, blood pressure decreased and the pressor response to L-NAME disappeared; however, if the rats were returned to a normotensive pressure with phenylephrine, the pressor response was restored. This could mean either that release of EDRF is dependent on blood pressure or that phenylephrine could directly stimulate EDRF. However, we found that an intact autonomic nervous system is not necessary for EDRF production, though our results do support the hypothesis that the pressor response to inhibition of EDRF synthesis is related to basal blood pressure.

After β-blockade with propranolol, the pressor response to L-NAME was still present. We also found that the response correlated significantly with the basal blood pressure. However, in pithed rats, whose blood L-NAME was administered, blood pressure increased by 22±2 mm Hg, and heart rate decreased by 10±6 beats per minute (from 283±11 to 273±11 beats per minute). After mechanical ablation of the central nervous system by pithing (n=6), blood pressure was 44±2 mm Hg. L-NAME raised blood pressure by 20±2 mm Hg, which was not significantly different from the intact control group. Heart rate was 344±9 beats per minute and did not change significantly after L-NAME (−6±3 beats per minute). In pithed rats treated with angiotensin II (n=7) before administration of L-NAME, blood pressure was maintained at 105±4 mm Hg, with a heart rate of 260±10 beats per minute. However, in pithed rats during constant angiotensin II–maintained blood pressure, the pressor response to L-NAME was significantly potentiated (Figure 2); blood pressure increased by 30±2 mm Hg (p<0.01). Heart rate did not change (264±15 beats per minute). Similarly, in a third set of pithed rats (n=6) in which blood pressure was maintained at 95±5 mm Hg by a constant infusion of norepinephrine, the pressor response to L-NAME was 68±8 mm Hg (Figure 2). Heart rate did not change significantly after administration of L-NAME (from 372±15 to 382±17 beats per minute; p=NS). In the fourth set, during the infusion of phenylephrine (n=7), blood pressure was maintained at 92±3 mm Hg and heart rate was 362±18 beats per minute (n=7). Administration of L-NAME increased blood pressure by 109±7 mm Hg, more than five times the response seen in control and pithed rats and significantly greater than that produced by angiotensin II or norepinephrine (p<0.01). Again, L-NAME did not alter heart rate significantly (365±22 beats per minute).

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**TABLE 1. Effects of N⁰-Monomethyl L-Arginine in Hexamethonium-Treated Rats**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Before L-NMMA (mm Hg)</th>
<th>Change</th>
<th>Before L-NMMA (bpm)</th>
<th>Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nontreated (n=5)</td>
<td>110±6</td>
<td>124±6</td>
<td>14±1*</td>
<td>363±23</td>
</tr>
<tr>
<td>Hexamethonium-treated (n=7)</td>
<td>85±4</td>
<td>100±4</td>
<td>15±1*</td>
<td>301±12</td>
</tr>
</tbody>
</table>

Blood pressure and heart rate before and after administration of 10 mg/kg N⁰-monomethyl L-arginine (L-NMMA) or vehicle. Both are lower in hexamethonium-treated rats. Despite these differences, L-NMMA increased blood pressure similarly in both groups; however, it did not alter heart rate in the hexamethonium-treated group. bpm, Beats per minute.

*p<0.01 compared with pre-L-NMMA.
pressure was very low, the pressor response remained intact. These differences could be due to some characteristic of \( \beta \)-blockade or due to inherent differences between pharmacological and mechanical models of blocking the nervous system. Regardless, it is clear that the pressor response to EDRF synthesis inhibition is not dependent on an intact nervous system. Because shear stress stimulates EDRF release, the correlation between the pressor response to L-NAME and basal blood pressure could be explained by a decrease in shear stress at lower pressures during \( \beta \)-blockade in our study. Despite the hypotension caused by hexamethonium treatment, the pressor response to EDRF synthesis inhibition remained similar. Thus, ganglionic blockade did not eliminate the apparent release of EDRF, nor did the pressor response appear to diminish as blood pressure dropped. This was probably the result of the lack of baroreceptor reflexes during hexamethonium treatment. Although these studies used L-NMMA rather than L-NAME, we found the pressor response in controls to be similar with either blocker. Because ganglionic blockade may not be fully achieved by hexamethonium, we used pithing to ensure complete elimination of autonomic nervous system activity. As expected, this produced a decrease in both blood pressure and heart rate. In pithed rats, blood pressure dropped to 40–50 mm Hg, and yet the pressor response to L-NAME was similar to that observed in the intact controls, reinforcing our observation that autonomic nervous system activity is not required for production of EDRF. When blood pressure was returned to normal levels with angiotensin II, norepinephrine, or phenylephrine, we found that the pressor response to L-NAME inhibition of EDRF synthesis was twofold, threefold, or fivefold greater, respectively, than in controls with similar blood pressure. This potentiation of the pressor response to L-NAME could be due in part to the fact that their vasoconstrictor influences were not buffered by baroreceptor adaptation in the pithed rats, combined with the increased resistance evoked by the exogenous vasoconstrictors. On the other hand, it is also possible that the potentiation was in part the result of inhibiting greater synthesis of EDRF under these conditions. Increased intracellular calcium, which could result from vasoconstrictors such as angiotensin II, norepinephrine, or phenylephrine, is a stimulus for EDRF. Therefore, vasoconstrictors could stimulate EDRF release either by a direct (calcium-mediated?) action or indirectly by increasing shear stress.

Although potentiation of the pressor response to L-NAME might suggest greater EDRF synthesis under these conditions, our in vivo studies cannot answer this question. However, it has been suggested that angiotensin II or its degradation products can stimulate EDRF release. It has also been shown that clonidine becomes a vasoconstrictor in vitro only after the endothelium has been removed or EDRF has been inhibited with methylene blue or hemoglobin. Additionally, phenylephrine-induced vasoconstriction is potentiated by endothelialization. These authors interpreted these results as showing that there are endothelial \( \alpha \)-adrenergic receptors that can mediate EDRF release, totally counteracting the constrictor effect of clonidine. Our results with phenylephrine suggest that \( \alpha \)-receptors may be particularly important in the stimulation of EDRF release by the endothelium in vivo. Thus, the endothelium may contain both \( \alpha_{1} \) and \( \alpha_{2} \)-receptors, which may be stimulated directly by catecholamines to release EDRF.

One may question why we observed a difference in the pressor response to L-NAME after treatment with the three vasoconstrictors in our study, as blood pressure was returned to the same level by all three. Although the pressor response to L-NAME in the norepinephrine-treated group was more than three times greater than in the controls, this was considerably less than the fivefold potentiation observed after phenylephrine. Phenylephrine only stimulates constrictor \( \alpha \)-adrenergic receptors, whereas norepinephrine stimulates both \( \alpha \) and \( \beta \)-adrenergic receptors. It could be
that α-adrenergic stimulation produces greater EDRF release, whereas its blockade by L-NAME results in a greater pressor effect.

In conclusion, although various components of the autonomic nervous system may modulate EDRF, we have found that it is not necessary to have an intact nervous system for the synthesis and release of EDRF. Additionally, we have found that there is a strong relation between EDRF and basal blood pressure. Although various endogenous pressor or depressor systems or both may modify this relation, basal blood pressure appears to be an important component in the regulation of EDRF synthesis and release.

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