Pergamon

S0306-4522(96)00090-5

Neuroscience Vol. 72, No. 4, pp. 881–887, 1996
Elsevier Science Ltd

0306-4522/96 $15.00 + 0.00

Letter to Neuroscience

STIMULATION OF LUMBAR SYMPATHETIC NERVES MAY PRODUCE HINDLIMB VASODILATION VIA THE RELEASE OF PRE-FORMED STORES OF NITROSYL FACTORS

R. L. DAVISSON,* R. A. SHAFFER,* A. K. JOHNSON† and S. J. LEWIS*‡

The Cardiovascular Center and the Department of Pharmacology* and the Department of Psychology,† The University of Iowa, Iowa City, IA 52242, U.S.A.

Key words: neurogenic vasodilation, nitric oxide, S-nitrosothiols, hindlimb blood flow, rat.

The physiological activation of lumbar sympathetic nerves by air-jet stress produces a hindlimb vasodilation in conscious rats. Although the nitric oxide synthase inhibitor N^G-nitro-L-arginine methyl ester markedly reduces the duration of this air-jet stress-induced vasodilation, it does not prevent the initial fall in resistance. These data suggest that the vasodilation is initiated by the release of an as yet unidentified factor, whereas the vasodilation is sustained by the release of nitric oxide or newly synthesized nitrosyl factors such as S-nitrosothiols. At present, the possibility that neurogenic vasodilation may be initiated by the release of pre-formed pools of nitrosyl factors from storage sites within the hindlimb vasculature has not been addressed. We reasoned that if nitrosyl factors do exist in storage pools, then we should be able to demonstrate a "use-dependent" loss of vasodilation after nitric oxide synthesis inhibition which would be the result of a gradual depletion of the releasable pools of these nitrosyl factors. In the present study, we examined the effects of repeated episodes of direct electrical stimulation of the lumbar sympathetic chain on ipsilateral hindlimb vascular resistance in pentobarbital-anesthetized rats prior to and following administration of the nitric oxide synthesis inhibitors N^G-nitro-L-arginine methyl ester (10, 25 or 100 μmol/kg i.v.) or N^G-nitro-L-arginine (50 μmol/kg i.v.). Three episodes of electrical stimulation at 3.2 $\pm$ 0.4 V (20 Hz, 5 ms duration, 5 ms delay for 10 s given 5 min apart) produced pronounced and reproducible reductions in hindlimb vascular resistance in the ipsilateral hindlimb ($-56 \pm 5\%$, $-55 \pm 5\%$ and $-53 \pm 6\%$, respectively), but no changes in mean arterial pressure. Three episodes of electrical stimulation at 4.8 $\pm$ 0.4 V also caused reproducible decreases in hindlimb resistance ($-59 \pm 7\%$, $-61 \pm 9\%$ and $-64 \pm 12\%$) and minor but reproducible decreases in blood pressure. The vasodilation produced by the first electrical stimulation at 3.2 $\pm$ 0.4 V was completely abolished by a 25 μmol/kg dose of N^G-nitro-L-arginine methyl ester ($-11 \pm 9\%$). The initial episode of electrical stimulation at 4.8 $\pm$ 0.4 V produced a pronounced fall in ipsilateral hindlimb resistance in the N^G-nitro-L-arginine methyl ester-treated animals whereas the second and third stimulations produced progressively smaller vasodilations ($-55 \pm 4\%$, $-34 \pm 3\%$ and $-19 \pm 2\%$, respectively). The 10 μmol/kg dose of N^G-nitro-L-arginine methyl ester was not effective whereas the 100 μmol/kg dose produced similar effects as the 25 μmol/kg dose. The 50 μmol/kg dose of N^G-nitro-L-arginine produced similar effects as the higher doses of N^G-nitro-L-arginine methyl ester.

These results suggest that lower intensity electrical stimulation of the lumbar sympathetic nerves produces vasodilation via the release of nitric oxide or newly synthesized nitrosyl factors such as S-nitrosothiols. In contrast, the vasodilation produced by higher intensity electrical stimulation may involve the mobilization and release of pre-formed pools of nitrosyl factors which undergo a "use-dependent" depletion in the absence of nitric oxide synthesis. These pre-formed pools of nitrosyl factors may exist within the sympathetic nerves themselves. In addition, they may be stored within the vascular endothelium and released by neurogenically-derived neurotransmitters/neuromodulators.

We have reported that air-jet stress produces a pronounced vasodilation within the hindlimb (HL) vasculature of conscious rats, which is markedly reduced by the sympathetic nerve blocker bretylium or a 25 μmol/kg dose of the inhibitor of nitric oxide

‡To whom correspondence should be addressed.

Abbreviations: ATP, adenosine triphosphate; ES, electrical stimulation; HL, hindlimb; HLF, hindlimb blood flow; HLR, hindlimb vascular resistance; HR, heart rate; L-NA, N^G-nitro-L-arginine; L-NAME, N^G-nitro-L-arginine methyl ester; MAP, mean arterial pressure; NE, norepinephrine; NO, nitric oxide; NOFs, nitrosyl factors; SNC, S-nitrosocysteine; SNP, sodium nitroprusside.

(NO) synthesis, N^G-nitro-L-arginine methylester (L-NAME).[6] We also reported that the post-ganglionic lumbar sympathetic nerves innervating the HL vasculature contain NADPH diaphorase,[6] a marker for NO synthase in paraformaldehyde-treated neuronal tissues.[11] These findings suggest that air-jet stress-induced vasodilation within the HL may involve the release of NO or related nitrosyl factors (NOFs) such as S-nitrosothiols[21] or dinitrosyl iron(II) complexes[24] from sympathetic vasodilator nerves. Since it is well-documented that α-adrenoceptor agonists[4,15] and adenosine triphosphate (ATP)[13] also release NO/NOFs from vascular endothelium, it is possible that the air-jet stress-induced HL vasodilation involves a sympathetic nerve-induced norepinephrine- or ATP-mediated release of these factors from endothelial cells.

In our previous study, we found that L-NAME markedly reduced the duration, but not the magnitude of the initial air-jet stress-induced fall in HL vascular resistance.[6] Similar *in vivo* findings have been reported with regard to the effects of NO synthesis inhibitors on the vasodilation produced by the endothelium-dependent agonists acetylcholine[1,20] and bradykinin.[8] Taken together, these results suggest that neurogenic and endothelium-dependent vasodilation may be initiated by the release of an as yet unidentified factor(s), whereas the fall in resistance may be sustained by an increase in NO synthesis and the subsequent release of NO or newly-formed NOFs. Ignarro[12] has postulated that pre-formed pools of S-nitrosothiols may exist within specialized storage vesicles within endothelial cells, and that endothelium-dependent agonists may initiate the Ca^{2+}-dependent exocytotic release of these vesicular stores. At present there is no direct evidence that such NOF-containing organelles exist within vascular endothelial cells or the terminals of sympathetic vasodilator neurons. None the less, Ignarro's postulation prompted us to examine whether neurogenic vasodilation may be initiated by the release of pre-formed stores of NOFs. We reasoned that if such pre-formed pools of NOFs exist within the HL vasculature, then it may be possible to demonstrate a "use-dependent" loss of vasodilation in animals treated with an NO synthesis inhibitor. As such, this loss of vasodilation would be directly related to the progressive depletion of releasable pools of NOFs in the absence of NO synthesis.

We examined the effects produced by successive episodes of electrical stimulation (ES) of the lumbar sympathetic nerves on ipsilateral HL vascular resistance (HLR) prior to and following the administration of the NO synthesis inhibitors L-NAME (10, 25 or 50 μmol/kg, i.v.) or N^G-nitro-L-arginine (L-NA, 50 μmol/kg i.v.), in pentobarbital-anesthetized rats. A typical example of the effects of 3 successive episodes of ES at 2.5 V and 4.5 V (20 Hz, 5 ms duration, 5 ms delay for 10 s given 5 min apart) of the left lumbar sympathetic chain on hemodynamic parameters in a pentobarbital-anesthetized rat prior to and following the administration of L-NAME (25 μmol/kg i.v.) is shown in Fig. 1. Prior to the administration of L-NAME each 2.5 V ES produced pronounced and similar increases in blood flow in the ipsilateral hindlimb (HLF) but no changes in mean arterial pressure (MAP). Each stimulation at 4.5 V produced pronounced and reproducible increases in HLF and small but reproducible decreases in MAP. Following L-NAME, the 2.5 V ES did not alter HLF or MAP. The first ES at 4.5 V produced hemodynamic changes which were similar to those observed prior to administration of the NO synthesis inhibitor. However, each subsequent ES at 4.5 V produced progressively smaller increases in HLF and MAP.

A summary of the effects of successive episodes of ES of the left lumbar sympathetic chain on MAP, HLF and HLR prior to and following the administration of L-NAME (25 μmol/kg i.v.) in pentobarbital-anesthetized rats ($n = 5$) is shown in Fig. 2. Prior to the injection of L-NAME, each of the three episodes of ES at 3.2 ± 0.4 V produced virtually identical increases in HLF and decreases in HLR but no changes in MAP. Each of the three episodes of ES at 4.8 ± 0.4 V also produced virtually identical increases in HLF and decreases in HLR and small falls in MAP of approximately 10%. The administration of L-NAME produced a significant increase in MAP, decrease in HLF and increase in HLR (see Table 1). These values remained constant throughout the experimental period. Following the administration of L-NAME, the first 3.2 ± 0.4 V ES (applied 30 min after the NO synthase inhibitor) did not affect HLF, HLR or MAP. The first ES at 4.8 ± 0.4 V (applied 5 min after the first 3.2 ± 0.4 V ES) produced increases in HLF and decreases in HLR and MAP which were

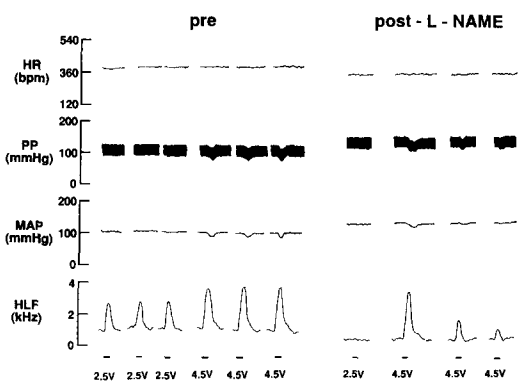

Fig. 1. A typical example of the effects of successive episodes of electrical stimulation (ES) of the left lumbar sympathetic chain given 5 min apart on heart rate (HR), pulsatile (PP) and mean arterial pressure (MAP) and blood flow in the ipsilateral (HLF) hindlimb of a pentobarbital-anesthetized rat prior to and following administration of L-NAME (25 μmol/kg i.v.). The stimulation parameters were 2.5 V and 4.5 V (at 20 Hz, 5 ms duration, 5 ms delay for 10 s). Full details of the surgical and experimental procedures are described in the legend for Fig. 2.

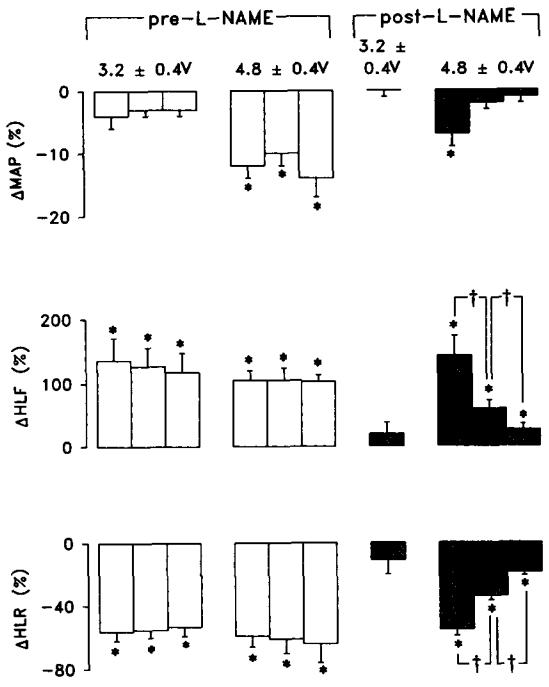

Fig. 2. A summary of the effects of successive episodes of electrical stimulation (ES) of the intact lumbar sympathetic chain given 5 min apart on mean arterial pressure (MAP) and hindlimb blood flow (HLF) and vascular resistance (HLR) in pentobarbital-anesthetized rats prior to and following the administration of L-NAME (25 μmol/kg i.v., $n = 5$). The first ES was applied 30 min after the administration of L-NAME at which time the baseline hemodynamic variables had reached plateau levels for at least 10 min. Data are expressed as percentage ES-induced changes in MAP, HLF and HLR (mean $\pm$ S.E.M.) from baseline and were analyzed by repeated measures analysis of variance (ANOVA) followed by Student's modified t-test with Bonferroni correction for multiple comparisons between means using the modified error mean square term for the ANOVA.[26] $*P < 0.05$, significant ES-induced change from baseline; $\dagger P < 0.05$, significant difference between the first and second or second and third ES-induced responses in L-NAME-treated rats. Male Sprague–Dawley rats (250–300 g) were anesthetized with pentobarbital (50 mg/kg i.p.) and cathethers (PE 50) were placed into the left common carotid artery and jugular vein for the measurement of arterial blood pressure and heart rate, and administration of drugs, respectively. Once the venous catheter was in place, pentobarbital (5 mg/kg i.v.) was given every 30 min. A Doppler flow probe was placed on the left iliac artery for the measurement of blood flow and the determination of hindlimb vascular resistances. Details of the Doppler technique, including construction of the probes, the reliability of the method for the estimation of flow velocity, and quantitative determination of percentage changes in vascular resistance have been described previously.[10] The left lumbar sympathetic chain was identified just dorsal to the abdominal aorta where it passes ventrally over the lumbar veins, and a microbipolar stimulating electrode was placed on the left lumbar sympathetic chain below the level of the last ganglion (L6). The left sympathetic chain was stimulated via square wave pulses from a Grass stimulator at selected voltages at 20 Hz, 5 ms duration, 5 ms delay for 10 s. In each rat, two voltages which produced reliable increases in HLF in the absence of marked changes in MAP were established. In the five rats used in this study, these voltages were 2.0, 2.5, 3.5, 3.5, and 4.5 V, respectively (3.2 ± 0.4 V) and 3.5, 5.5, 4.0, 5.5 and 5.5 V, respectively (4.8 ± 0.4 V, $+1.6 \pm 0.5$ V, $P < 0.05$). After treatment with similar to those produced prior to the administration of L-NAME. However, each successive ES at these higher voltages produced progressively smaller changes in HLF, HLR and MAP. The second ES produced increases in HLF which were $58 \pm 16\%$ less than those produced by the first ES ($P < 0.05$) and the third ES produced increases in HLF which were $56 \pm 7\%$ less than the second ES ($P < 0.05$). As such, the increases in HLF produced by the third ES were $82 \pm 12\%$ less than the first ES ($P < 0.05$). The second ES produced decreases in HLR which were $38 \pm 7\%$ less than the first ES ($P < 0.05$), whereas the third ES produced decreases in HLR which were $44 \pm 9\%$ less than the second ES ($P < 0.05$). As such, the third ES produced decreases in HLR which were $65 \pm 11\%$ less than the first ES ($P < 0.05$).

The administration of 10 μmol/kg L-NAME ($n = 5$) produced minor increases in MAP ($+6 \pm 1\%$, $P < 0.05$) and HLR ($+17 \pm 5\%$, $P < 0.05$). This dose of L-NAME did not inhibit the hindlimb vasodilation produced by lower (3.3 ± 0.2 V) or repetitive ES at higher voltages (4.7 ± 0.1 V) of the lumbar sympathetic nerves (data not shown). The 100 μmol/kg dose of L-NAME ($n = 5$) produced an increase in MAP and HLR of $+16 \pm 4\%$ and $+94 \pm 12\%$, respectively ($P < 0.05$ for both comparisons). These changes were not significantly different from those produced by the 25 μmol/kg dose of L-NAME ($P > 0.05$ for both comparisons). Prior to the administration of the 100 μmol/kg dose of L-NAME, ES at 3.3 ± 0.2 V produced falls in MAP and HLR of $-4 \pm 2\%$ ($P > 0.05$) and $-52 \pm 11\%$ ($P < 0.05$). The three episodes of ES at 4.7 ± 0.1 V produced equivalent falls in MAP of $-11 \pm 3\%$, $-14 \pm 4\%$ and $-12 \pm 2\%$, respectively ($P < 0.05$ for all responses) and equivalent falls in HLR of $-48 \pm 6\%$, $-54 \pm 5\%$ and $-49 \pm 7\%$, respectively ($P < 0.05$ for all responses). Following administration of the 100 μmol/kg dose of L-NAME, ES at 3.3 ± 0.2 V did not effect MAP ($-1 \pm 2\%$, $P > 0.05$) or HLR ($-8 \pm 6\%$, $P > 0.05$). The responses produced by the three episodes of ES at 4.7 ± 0.1 V were, for MAP; $-10 \pm 2\%$, $-1 \pm 1\%$ and $0 \pm 0\%$, respectively ($P < 0.02$, 2nd and 3rd response vs 1st response) and for HLR; $-57 \pm 6\%$, $-27 \pm 4\%$ and $-6 \pm 3\%$, respectively ($P < 0.05$, 2nd vs 1st response and 3rd vs 2nd response). A virtually identical pattern was obtained in rats treated with another NO synthase inhibitor L-NA (50 μmol/kg, i.v., $n = 3$, data not shown).

The hemodynamic effects produced by three episodes of ES at 2.6 ± 0.3 V and 4.1 ± 0.4 V prior to

Fig. 2. (continued).

L-NAME, the effects of ES at these voltages were again tested. In addition, the effects of the NO donor sodium nitroprusside (SNP, 1.25 μg/kg iv) and the S-nitrosothiol S-nitrosocysteine (SNC, 50 nmol/kg, i.v.) on MAP, HLF and HLR were determined after the ES protocols prior to and following the administration of L-NAME.

Table 1. A summary of the resting mean arterial blood pressures, hindlimb blood flows and hindlimb vascular resistances prior to and between 30–60 min following the injection of saline ($n = 6$) or L-NAME (25 μmol/kg i.v., $n = 5$) in pentobarbital-anesthetized rats

Parameter	Treatment	pre	post	Δ(%)
MAP	saline	120 ± 5	119 ± 5	−1 ± 1
(mmHg)	L-NAME	127 ± 7	140 ± 6	+10 ± 3*
HLF	saline	0.88 ± 0.09	0.86 ± 0.10	−4 ± 3
(kHz)	L-NAME	0.84 ± 0.13	0.50 ± 0.05	−39 ± 9*
HLR	saline	142 ± 12	146 ± 14	+3 ± 6
(mmHg/kHz)	L-NAME	169 ± 28	295 ± 37	+83 ± 7*

Each value represents the mean ± S.E.M.
*$P < 0.05$ post- vs pre-treatment.

and following the administration of saline (0.9% NaCl) in pentobarbital-anesthetized rats ($n = 6$) are shown in Fig. 3. Each of the three episodes of ES at 2.6 ± 0.3 V produced virtually identical increases in HLF and decreases in HLR but no changes in MAP. Each of the three episodes of ES at 4.1 ± 0.4 V also produced identical increases in HLF and decreases in HLR and small falls in MAP. These ES-induced responses were not affected by the administration of saline. In addition, the administration of saline did not affect resting hemodynamic parameters (Table 1).

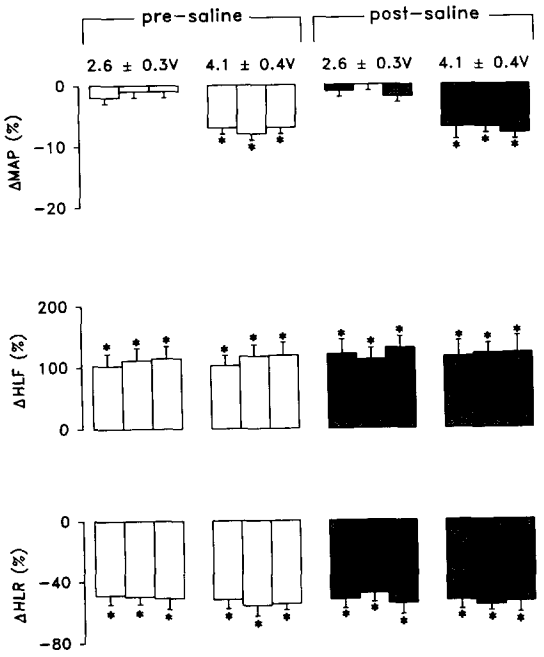

Fig. 3. A summary of the effects of successive episodes of electrical stimulation (ES) of the intact lumbar sympathetic chain given 5 min apart on mean arterial pressure (MAP) and hindlimb blood flow (HLF) and vascular resistance (HLR) in pentobarbital-anesthetized rats prior to and following the administration of saline (0.9% NaCl i.v., $n = 6$). The first ES was applied 30 min after the administration of saline. Data are expressed as percentage ES-induced changes in MAP, HLF and HLR. The experimental procedures were similar to those described in Fig. 2. In each rat, two voltages which produced reliable increases in HLF in the absence of marked changes in MAP were established. In the six rats used in this study, these voltages were 2.0, 2.0, 2.0, 2.5, 3.5 and 3.5 V, respectively (2.6 ± 0.3 V) and 3.0, 3.0, 4.0, 4.5, 4.5 and 5.5 V, respectively (4.1 ± 0.4 V, +1.5 ± 0.2 V, $P < 0.05$). After the injection of saline, the effects of ES at these voltages were again tested.

In the above protocols, the three episodes of 4.8 ± 0.4 V were given 35, 40 and 45 min after the injection of L-NAME, respectively. In order to establish that the progressive loss of the ES-induced vasodilation was not simply due to the time-dependent loss of NO synthase activity between 35–45 min, we again tested the effects of three episodes of ES at 4.5 V after L-NAME (25 μmol/kg, i.v., $n = 4$). In these experiments, the first ES was applied 45 min after L-NAME and the second and third episodes of ES were applied 5 and 10 min later, respectively. The changes in HLR were −64 ± 8%, −36 ± 4% and −12 ± 5%, respectively ($P < 0.05$ for second ES vs first ES and third ES vs second ES). These results suggest that the progressive loss of ES-induced vasodilation is not due to the gradual loss of NO synthase activity over the course of the prior protocol in which the ES was given at 35, 40 and 45 min.

We examined the hypotensive and vasodilator effects of the NO donor sodium nitroprusside (SNP, 1.25 μg/kg, i.v., $n = 5$) and the S-nitrosothiol S-nitrosocysteine (SNC, 50 nmol/kg, i.v., $n = 5$) immediately after the stimulation protocols both prior to and following L-NAME (25 μmol/kg, i.v.). The hypotensive and vasodilator effects of SNP were markedly augmented after the administration of L-NAME. The hemodynamic effects of SNP prior to and following L-NAME were: MAP, −18 ± 2% vs −38 ± 4% (+111 ± 13%, $P < 0.05$); HLF +30 ± 6% vs +61 ± 7%, ([+103 ± 12%, $P < 0.05$); and HLR −37 ± 3% vs −60 ± 5% (+62 ± 7%, $P < 0.05$). The hypotensive and vasodilator effects of SNC were also augmented by L-NAME. The pre- vs post-L-NAME changes produced by SNC were for MAP, −21 ± 2% vs −37 ± 4% (+73 ± 10%, $P < 0.05$), for HLF, +24 ± 4% vs +54 ± 8% (+151 ± 49%, $P < 0.05$) and for HLR, −36 ± 3% vs −58 ± 4% (+63 ± 13%, $P < 0.05$).

There is now considerable evidence that NO-containing factors (NOFs) may be synthesized and released from vascular endothelial cells,[18] bronchial tissue[9] and activated macrophages.[23] Although it is

generally accepted that these factors are released immediately upon their formation, there is evidence that vascular tissue contains pre-formed pools of NOFs. It has been demonstrated that endothelium-denuded rabbit aortic rings relax on exposure to ultra-violet (UV) light.[14,16,25] This relaxation is associated with the release of NO[14] and is inhibited by the NO scavenger hemoglobin and the inhibitor of soluble guanylate cyclase, Methylene Blue.[25] However, this photorelaxation is not affected by inhibitors of NO synthase such as L-NAME.[5,14] The photorelaxation progressively diminishes upon exposure to UV light[14,25] but can be restored by treating the vessels with acidified nitrite or the NO donors S-nitrosopenicillamine and glyceryl trinitrate.[25] These results have prompted the speculation that the photorelaxation is mediated by the release of an exhaustible pool of pre-formed NOFs within vascular smooth muscle.[14,25] In our experiments we have found that the rabbit thoracic aorta is innervated by post-ganglionic sympathetic NO synthase-positive nerve terminals which may contain cytosolic-protected pools of NOFs.[2] As such, it is possible that photorelaxation is mediated by the release of pre-formed pools of NOFs from NO synthase-positive nerve terminals.

Ignarro[12] has postulated that pre-formed pools of NOFs may exist within vascular endothelial cells and that endothelium-dependent agonists may cause the Ca^{2+}-dependent exocytotic release of these stores. At present, there is no direct evidence that NOF-containing vesicles exist within endothelial cells or NO synthase-containing post-ganglionic sympathetic neurons within the HL vasculature. Ignarro's postulation prompted us to examine the possibility that ES of the lumbar sympathetic nerves may produce a HL vasodilation via the release of pre-formed stores of NOFs within resistance vessels in this bed. We reasoned that if pre-formed stores of NOFs exist within the HL vasculature, then we may be able to demonstrate a "use-dependent" loss of neurogenic vasodilation in L-NAME-treated rats since in the absence of NO synthesis there would be a progressive depletion of these stores.

In the present study, we examined the effects of 10, 25 and 100 μmol/kg doses of L-NAME in pentobarbital-anesthetized rats. The lowest dose of L-NAME did not produce marked effects on MAP or HLR and did not alter the hemodynamic responses produced by the ES of the lumbar nerves. The two highest doses of L-NAME produced equivalent effects on baseline parameters and the responses to nerve stimulation. The 25 and 100 μmol/kg doses of L-NAME represent approximately 6.75 and 27 mg/kg, respectively. The 6.75 mg/kg dose is just below the maximally-effective dose reported for thiobutabarbitone-anesthetized rats.[19] The 27 mg/kg dose is in the maximally-effective dose range in the thiobutabarbitone-anesthetized rats.[19]

The principal findings of the present study were that: (i) the successive episodes of ES of the intact lumbar sympathetic chain (e.g 3.2 ± 0.4 V and 4.8 ± 0.4 V) produced highly consistent decreases in vascular resistance in the ipsilateral hindlimb; (ii) the vasodilator responses produced by 3.2 ± 0.4 V ES were completely abolished by the NO synthesis inhibitor L-NAME; (iii) the first 4.8 ± 0.4 ES produced a marked decrease in HLR in the L-NAME-treated rats, whereas each successive ES produced a progressively smaller vasodilation; and (iv) the "use-dependent" loss of ES-induced vasodilation occurred despite the L-NAME-induced exaggeration of the vasodilator effects of the NO-donor SNP and the S-nitrosothiol, SNC. The L-NAME-induced augmentation of the vasodilator effects of SNP and SNC are consistent with the *in vitro* findings that inhibition of NO synthase exaggerates NO-mediated vasorelaxation.[17] As such, our findings suggest that the progressive loss of ES-induced vasodilation in animals treated with NO synthase inhibitors is not due to the diminished biological effectiveness of NO or NOFs.

Taken together, these results suggest that ES at lower voltages may cause the release of newly synthesized NO, newly formed NOFs, or stimulate the Ca^{2+}-dependent exocytotic release of pre-formed NOFs from readily depletable pools. More specifically, these readily depletable pools of NOFs may normally be utilized by sympathetic nerves or vascular endothelial cells to maintain vascular tone. As such, these pools will not be re-generated in the absence of NO synthesis. Therefore, ES at lower voltages will not cause a vasodilation if these pools of NOFs are preferentially mobilized at these voltages. It is well known that there are several types of vesicular pools of NE within sympathetic nerve terminals and that each of these pools responds to different levels of intracellular Ca^{2+}.[22] As such, lower intensity nerve stimulation might mobilize vesicular pools of NOFs which are relatively sensitive to changes in intracellular Ca^{2+}.

The observation that the first ES at higher voltages produced a marked fall in HLR in L-NAME-treated rats suggests that this vasodilation is not due to the release of newly synthesized NO, NOFs or exocytotic release of these readily depletable pools of NOFs. However, the finding that each successive ES produced progressively smaller falls in HLR is consistent with the "use-dependent" depletion of pre-formed stores of a factor(s) whose synthesis requires the incorporation of one or more NO moieties in its structure such as an S-nitrosothiol[21] or a dinitrosyl iron(II) thiol-complex,[24] respectively. As such, ES at higher voltages may under normal circumstances cause the exocytotic release of vesicular stores of NOFs from post-ganglionic sympathetic neurons which require a stronger stimulus, e.g., a greater action potential-induced increase in intracellular Ca^{2+} for them to be mobilized. Alternatively, if these stores originate from within the vascular endothelial cells then these pools may be released exocytotically by a relatively stronger agonist stimulus, i.e., higher concentrations of NE or ATP than is required to

mobilize the readily depletable pools. It is unlikely that the "use-dependent" loss of ES-induced vasodilation is simply due to the progressive time-dependent loss of NO synthase activity over the course of the ES protocols. This is because the loss of ES-induced responses at higher voltages could be elicited between 35–45 min or in separate experiments between 45–55 min following L-NAME. That is, the first ES produced robust falls in HLR either 35 min or, in separate rats, 45 min following L-NAME, whereas each successive episode of ES produced progressively smaller responses in each case.

Another possible explanation for our results is that the entry of L-NAME into the lumbar sympathetic nerves is dependent upon the intensity of ES. That is, the stimulation at higher voltages will increase the rate of entry of L-NAME into the nerves. However, as a methylester, L-NAME would be expected to be highly lipophilic. Indeed, L-NAME is readily soluble in organic solvents such as methanol (30 mg/ml, Sigma Chemical Company, personal communication). Therefore, the entry of L-NAME into the lumbar sympathetic nerve terminals (and vascular endothelium) should not be influenced by the ES of these nerves. Since the lumbar sympathetic nerves were intact in these experiments, it would be expected that L-NAME would readily enter the nerves if there was some dependence upon the integrity of nerve activity. Moreover, it is likely that L-NAME readily enters cells by simple diffusion since this compound does not compete for the L-arginine transporter in endothelial cells.[3]

Although these studies cannot determine whether the pre-formed pools of NOFs exist within the post-ganglionic sympathetic vasodilator nerves and/or the vascular endothelium, we have found that post-ganglionic lumbar sympathetic cell bodies and nerve terminals innervating the HL vasculature contain NADPH diaphorase[6] which is a marker for NO synthase in paraformaldehyde-fixed tissues.[11] Moreover, we have also found that the initial air-jet stress-induced HL vasodilation undergoes a "use-dependent" diminution upon repeated exposure of the rats to this stimulus.[7] The presence of NADPH diaphorase in post-ganglionic lumbar sympathetic terminals[6] raises the possibility that pre-formed vesicular pools of NOFs exist in these nerve terminals and that they are mobilized by action potential-mediated exocytosis. We are currently examining whether the HL vasodilation produced by repeated injections of the endothelium-dependent agonists acetylcholine and bradykinin in pentobarbital-anesthetized rats progressively diminishes in the presence of L-NAME.

In summary, the present study provides evidence that the ES of post-ganglionic lumbar sympathetic neurons produces a vasodilation within the HL vasculature of rats which at lower stimulus intensities may be due to the release of newly synthesized NO or NOFs, whereas at higher stimulus intensities the vasodilation may be mediated by the exocytotic release of vesicular pools of NOFs.

REFERENCES

1. Aisaka K., Gross S. S., Griffith O. W. and Levi R. (1989) N^G-methyl-arginine, an inhibitor of endothelium-derived nitric oxide synthesis, is a pressor agent in the guinea pig: does nitric oxide regulate blood pressure *in vivo? Biochem. Biophys. Res. Commun.* **160,** 881–886.
2. Bates J. N., Davisson R. L., Johnson A. K. and Lewis S. J. (1993) *In vitro* evidence that bretylium (BRE) releases nitric oxide factors (NOFs) from post-ganglionic sympathetic nerves. *Fedn Am. Socs exp. Biol. J.* **7,** A432.
3. Bogle R. G., Moncada S., Pearson J. D. and Mann G. E. (1992) Identification of inhibitors of nitric oxide synthase that do not interact with the endothelial cell L-arginine transporter. *Brit. J. Pharmac.* **105,** 768–770.
4. Cocks T. M. and Angus J. A. (1983) Endothelium-dependent relaxation of coronary arteries by noradrenaline and serotonin. *Nature* **30,** 341–347.
5. Chaudry H., Lynch M., Schomacker K., Birngruber R., Gregory K. and Kochevar I. (1993) Relaxation of vascular smooth muscle induced by low-power laser radiation. *Photochem. Photobiol.* **58,** 661–669.
6. Davisson R. L., Johnson A. K. and Lewis S. J. (1994) Nitrosyl factors mediate active neurogenic hindquarter vasodilation in the conscious rat. *Hypertension* **23,** 962–966.
7. Davisson R. L., Shaffer R. A., Johnson A. K. and Lewis S. J. (1994) *In vitro* evidence that pre-formed stores of nitrosyl factors are released from rat vasculature. *J. Hypertension* **12,** S26.
8. Gardiner S. M., Compton A. M., Kemp P. A. and Bennett T. (1990) Regional and cardiac hemodynamic responses to glyceryl trinitrate, acetylcholine, bradykinin, and endothelin-1 in conscious rats: effects of N^G-nitro-L-arginine methyl ester. *Br. J. Pharmac.* **101,** 632–639.
9. Gaston B., Reilly J., Drazen J. M., Fackler J., Ramdev R., Arnelle D., Mullins M. E., Sugarbaker D. J., Chee C., Singel D. J., Loscalzo J. and Stamler J. S. (1993) Endogenous nitrogen oxides and bronchodilator S-nitrosothiols in human airways. *Proc. natn. Acad. Sci. U.S.A.* **90,** 10957–10961.
10. Haywood J. R., Shaffer R. A., Fastenow C., Fink G. D. and Brody M. J. (1981) Regional blood flow measurement with pulsed Doppler flowmeter in conscious rat. *Am. J. Physiol.* **241,** H273–H278.
11. Hope B. T., Michael G. J., Knigge K. M. and Vincent S. R. (1991) Neuronal NADPH diaphorase is a nitric oxide synthase. *Proc. natn. Acad. Sci. U.S.A.* **88,** 2811–2814.
12. Ignarro L. J. (1990) Nitric oxide: a novel signal transduction mechanism for transcellular communication. *Hypertension* **16,** 477–483.
13. Kennedy C., Delbro K. and Burnstock G. (1985) P_2-purinoceptors mediate both vasodilation (via the endothelium) and vasoconstriction of the isolated rat femoral artery. *Eur. J. Pharmacol.* **107,** 161–168.
14. Kubaszewski E., Peters A., McClain S., Bohr D. and Malinski T. (1994) Light-activated release of nitric oxide from vascular smooth muscle of normotensive and hypertensive rats. *Biochem. biophys. Res. Commun.* **200,** 213–218.

15. Martin W., Furchgott R. F., Villani G. M. and Jothianandan D. (1986) Depression of contractile responses in rat aorta by spontaneously released endothelium-derived relaxing factor. *J. Pharmac. exper. Ther.* **237,** 529–538.
16. Matsunga K. and Furchgott R. F. (1991) Responses of rabbit aorta to nitric oxide and superoxide generated by ultraviolet irradiation of solutions containing inorganic nitrite. *J. Pharmac. exper. Ther.* **259,** 1140–1146.
17. Moncada S., Rees D. D., Schulz R. and Palmer R. M. J. (1991) Development and the mechanism of a specific supersensitivity to nitrovasodilators after inhibition of vascular nitric oxide synthesis *in vivo*. *Proc. natn. Acad. Sci. U.S.A.* **88,** 2166–2170.
18. Myers P. R., Minor R. L., Guerra R., Bates J. N. and Harrison D. G. (1990) Vasorelaxant properties of the endothelium-derived relaxing factor more closely resembles S-nitrosocysteine than nitric oxide. *Nature* **345,** 161–163.
19. Rees D. D., Palmer R. M. J., Schulz R., Hodson H. F. and Moncada S. (1990) Characterization of three inhibitors of endothelial nitric oxide synthase *in vitro* and *in vivo*. *Brit. J. Pharmac.* **101,** 746–752.
20. Ross G., Chaudhuri G., Ignarro L. J. and Chyu K. Y. (1991) Acetylcholine vasodilation of resistance vessels *in vivo* may not entirely depend on newly synthesized nitric oxide. *Eur. J. Pharmac.* **195,** 291–293.
21. Stamler J. S., Simon D. I., Osborne J. A., Mullins M. E., Jaraki O., Michel T., Singel D. J. and Loscalzo J. (1992) S-nitrosylation of proteins with nitric oxide: Synthesis and characterization of biologically active compounds. *Proc. natn. Acad. Sci. U.S.A.* **89,** 444–448.
22. Thureson-Klein A. K. and Klein R. L. (1990) Exocytosis from neuronal large dense-cored vessels. *Int. Rev. Cytol.* **121,** 67–126.
23. Uchizumi H., Hattori R., Sase K., Wei-jing C., Kadota K., Sasayama S., Kawai C. and Yui Y. (1993) A stable L-arginine-dependent relaxing factor released from cytotoxic-activated macrophages. *Am. J. Physiol.* **264,** H1472–H1477.
24. Vedernikov Y. P., Mordvintcev P. I., Malenkova I. V. and Yanin A. F. (1992) Similarity between the vasorelaxing activity of dinitrosyl iron cysteine complexes and endothelium-derived relaxing factor. *Eur. J. Pharmacol.* **211,** 313–317.
25. Venturini C. M., Palmer R. M. J. and Moncada S. (1993) Vascular smooth muscle contains a depletable store of a vasodilator which is light-activated and restored by donors of nitric oxide. *J. Pharmac. exper. Ther.* **266,** 1497–1500.
26. Wallenstein S., Zucker C. L. and Fleiss J. L. (1980) Some statistical methods useful in circulation research. *Circ. Res.* **47,** 1–9.

(*Accepted* 7 *February* 1996)