In vivo mechanisms of acetylcholine-induced vasodilation in rat sciatic nerve

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Thomsen, Kirsten, Inger Rubin, and Martin Lauritzen. In vivo mechanisms of acetylcholine-induced vasodilation in rat sciatic nerve. Am J Physiol Heart Circ Physiol 279: H1044–H1054, 2000.—We examined the importance of nitric oxide (NO), endothelium-derived hyperpolarizing factor (EDHF), and neurogenic activity in agonist-induced vasodilation and baseline blood flow [i.e., nerve microvascular conductance (NMVC)] in rat sciatic nerve using laser Doppler flowmetry. Agonists were acetylcholine (ACh) and 3-morpholinosydnonimine (SIN-1). Vasodilation occurring despite NO synthase (NOS) and cyclooxygenase inhibition and showing dependence on K+ channel activity was taken as being mediated by EDHF. NOS and cyclooxygenase inhibition with Nω-nitro-L-arginine (L-NNA), whereas the specific guanylyl cyclase (sGC) activity was unaffected by L-NNA. TTX affected neither SIN-1 nor ACh-induced vasodilation. In conclusion, ACh-induced vasodilation consisted of two components, the first partially mediated by nitric oxide (NO) and endothelium-derived hyperpolarizing factor (EDHF) (1, 2). EDHF produces hyperpolarization of vascular smooth muscle and accompanying vasodilation via K+ channels, including large conductance K+ (BK) channels (10), whereas NO produces vasodilation via stimulation of soluble guanylyl cyclase (sGC), giving rise to the second messenger cGMP (NO-cGMP pathway) (16). Thus, in an in vitro study, dilatory responses to ACh were abolished by combined inhibition of NO synthase (NOS) and BK channels with Nω-nitro-L-arginine (L-NNA) and iberiotoxin (9). ACh has also been shown to produce vasodilation independently of the endothelium by inhibiting sympathetic vasoconstrictory tone (13, 27). This raises the possibility that blood flow responses to ACh in vivo are dependent on all of these factors, i.e., that ACh-induced vasodilation in vivo is not strictly endothelium mediated.

The circulation of the sciatic nerve is well characterized and, roughly speaking, consists of an endoneurial capillary network supplied by longitudinally oriented arteries in the epi- and perineurium (14). These epineurial blood vessels are richly innervated by sympathetic nerve fibers, although the endoneurial capillary network is not (22). However, endoneurial blood flow is determined by the degree of vasoconstriction and vasodilation of the epi-neurial vessels (11). Thus basal sympathetic vasoconstriction corresponding to ~30% of resting endoneurial blood flow was demonstrated in sciatic nerve after chronic treatment with the ganglion blocker guanethidine (32). Basal NO production is also present in the sciatic nerve, because topical application and endoneurial microinjection of NOS inhibitors resulted in a 30–60% decrease in nerve blood flow (11, 28). Therefore, the sciatic nerve was chosen as a suitable tissue for the study of the factors mediating microcirculatory ACh-induced vasodilation.

The identity of EDHF is not yet established, but in vivo vasodilation induced by agonists such as ACh, which persists in the face of NOS and cyclooxygenase inhibition, is attributed to EDHF (29). Thus we examined the effects of NOS inhibition with and without...
simultaneous cyclooxygenase inhibition as well as the effects of inhibition of voltage-dependent Na\(^+\) channels on vasodilation induced by ACh and the NO donor 3-porphylinosydnonimine (SIN-1) in rat sciatic nerve. We also attempted to elucidate the biochemical basis for the enhanced nitrovasodilator effect seen in the presence of NOS inhibition.

**MATERIALS AND METHODS**

**Animals**

Male Wistar rats (372 ± 34 g) kept in plastic cages with wood shavings, given free access to tap water and Altromin 1314 rat chow, and acclimatized to a 12:12-h light-dark cycle were used in all experiments. Twenty-three animals were used for the in vivo hemodynamic studies and fifteen for the in vitro sGC enzyme assay.

**Hemodynamic Studies**

Anesthesia was induced with halothane (3%) and maintained during surgery (1.8%). The left jugular vein was cannulated for the administration of drugs, and the left carotid artery was cannulated for blood sampling and measurement of arterial blood pressure. The rats were tracheotomized and artery was cannulated for blood sampling and measurement. The left jugular vein was cannulated for the administration of drugs, and the left carotid artery was cannulated for blood sampling and measurement of arterial blood pressure. The rats were tracheotomized and articular ventilation with 30% O\(_2\)–70% N\(_2\)O, ensuring PO\(_2\) of 100 mmHg and PCO\(_2\) of 39 ± 2 mmHg. The lumbar backbone was partially exposed for fixation purposes. The left sciatic nerve was exposed by carefully separating the biceps and quadriceps muscles of the femur, which then formed walls of a well around the sciatic nerve. After surgery, halothane was discontinued and anesthesia was maintained with continuous infusion of intravenous pentobarbital sodium (initial bolus 19.0 mg/kg, infusion 22.9–28.6 mg·kg\(^{-1}\)·h\(^{-1}\)). Suxamethonium (initial bolus 12.9 mg/kg, supplements of 2.6 mg/kg every 30 min) was given through a peritoneal catheter to eliminate the influence of muscle activity. Adequate levels of anesthesia were ensured by regularly verifying the absence of blood pressure responses to tail pinch throughout the experiment.

**Methods**

Variations in nerve blood flow (NBF) were measured with laser Doppler flowmetry (LDF). Four identical needle probes (Probe 411, Perimed) were connected to two PeriFlux 4001 monitors (Perimed). The probes were designed with 150 \(\mu\)m between the transmitting and receiving fibers, and held red laser light with a wavelength of 780 nm allowing the measurement of NBF to a tissue depth of ~0.5 mm. All probes were calibrated using Perimed motility standard (Perimed). The needle probes were held by micromanipulators and placed over the sciatic nerve under microscopic control. All probes were located above the trifurcation within a total distance of 1.5 cm (Fig. 1). Once in place, the probes were kept in the same position for the duration of the experiment.

NBF and blood pressure were measured continuously with sampling rates of 10 and 100 Hz, respectively. Blood flow was measured in arbitrary units of flux with a time constant of 0.2 s. Spike 2 software (Cambridge Electronic Design) was used for data acquisition and later off-line analysis. As NBF measured with the laser Doppler technique can vary greatly from point to point because of local differences in the nerve microcirculation, we averaged the measurements from two stimulations of each vasodilator recorded with the four LDF probes, giving eight sets of data to reduce variability.

**Protocol**

The well was initially filled with Ringer solution (in mM: 145 NaCl, 3.5 KCl, 2 CaCl\(_2\), and 5 HEPES; pH adjusted to 7.4). Baseline NBF was established, and the NBF response to the topical application of two vasodilators, ACh and SIN-1, was recorded (see Fig. 2B). Initially, SIN-1 was applied repeatedly at increasing concentrations until the smallest possible concentration at which NBF increased ~50% was achieved (range: 0.1–0.75 mM). In group 1, the sequence was Ringer solution, L-NNA + indomethacin (Indo) (1 mM + 10\(^{-5}\) M) and L-NNA + Indo + tetraethylammonium (TEA) (1 mM + 10\(^{-4}\) M + 10 mM), and in group 2, the sequence was Ringer solution, TTX (0.02 mM), and TTX + L-NNA (0.02 mM + 1 mM). In group 3 (time controls), the well contained Ringer solution during all 3 steps. At each step, the effects of topically applied ACh (0.1 mM) and 3-porphylinosydnonimine (SIN-1) (0.1–0.75 mM) on nerve blood flow were recorded.
functions as a nonspecific inhibitor of NO donors both in vitro and in vivo (17, 21, 23). Our present study revealed a fourfold increase in the vasodilatory response to NO donors in the presence of Ringer solution. The constancy of the vasodilatory response to ACh in the presence of Ringer solution (step 1) was examined in group 4 (time controls; n = 6 animals). In effect, step 1, in which ACh responses to the vasodilators were measured in the presence of Ringer solution, was repeated three times. Between repetitions, the nerve was allowed to rest for 30 min corresponding to the stabilization periods after application of the inhibitors in groups 1, 2, and 3.

**Homogenate Studies**

NOS inhibition has been shown to accentuate the vasodilatory response to NO donors both in vitro and in vivo (17, 21, 23). Our present study revealed a fourfold increase in the vasodilatory response to SIN-1 during NOS inhibition with L-NNA (see RESULTS). Therefore, we attempted to elucidate the biochemical mechanism responsible for this enhancement using nervous tissue. The NOS inhibitor L-NNA, the specific inhibitor of sGC, ODQ, or both was added to homogenates of sciatic nerve (n = 15 animals for each treatment). Half of each group of homogenates was then stimulated with SIN-1. To confirm our results from the sciatic nerve, we repeated the procedure using homogenates of rat cerebellum (n = 10 animals), a tissue known to contain large amounts of neuronal NOS (4).

Specifically, 15 animals were decapitated under halothane anesthesia (3%). Biopsies were taken from both sciatic nerves and the cerebellum, which were immediately frozen in liquid nitrogen and stored at −80°C until the enzyme assays were performed. The tissue samples were kept in an ice bath during homogenization in 50 mM Tris buffer, 1.15 mM KCl, 1 mM EDTA, 5 mM glucose, 0.1 mM dithiothreitol, 200 units/ml superoxide dismutase, 2 mg/l leupeptin, 2 mg/l pepstatin A, 10 mg/l trypsin inhibitor, and 44 mg/l phenylmethylsulfonyl fluoride (PMSF). The samples were first centrifuged for 10 min at 2,000 g to remove cellular debris. Subsequently, the supernatant was centrifuged for 2 h at 26,700 g to ensure that there was no contamination with membrane-bound guanylyl cyclase. All centrifugation occurred in a refrigerated centrifuge at 2°C. Protein content was estimated according to the method of Groves et al. (7).

Aliquots (40 μl) of supernatant from the tissue homogenate were incubated for 30 min at 37°C with SIN-1 (final concentration 50 μM) in Tris buffer (150 μl) containing the sGC substrate GTP (final concentration 300 μM) in addition to 1.5 mM IBMX and 1 mM theophylline to inhibit degradation of cGMP, 3 mM creatine phosphate and 94 μg/ml creatine kinase to prevent the metabolization of GTP by other enzyme systems, and 5 mM MnCl₂ to maximize sGC activity, as well as 94 μg/ml bovine serum albumin. Before incubation, 10-μl aliquots of L-NNA (final concentration 10 or 100 μM), ODQ (final concentration 10 μM), or solvent without inhibitor (DMSO) were added to the samples. L-NNA was added 30 min before ODQ was added 15 min before incubation; in samples with both L-NNA and ODQ, L-NNA was added 45 min before incubation to allow the NOS inhibitor 30 min of exposure to untreated sGC before the addition of ODQ for the remaining 15 min. The reaction was terminated by adding 10 μl of 1 mM EDTA and boiling for 3 min. After cooling on ice, the samples were centrifuged for 10 min at 10,000 g. The supernatant (25 μl), diluted appropriately, was added to radioimmunoassay buffer (475 μl). The RPA 525 cGMP-(125I) assay system (Amersham International) was used.

**Drugs**

Fresh solutions of all drugs using Ringer solution were made each day; fresh solutions of ACh were made immediately before each application. Substances that were not readily dissolvable in Ringer solution were ultrasonicated for 5 min. ACh chloride, SIN-1, L-NNA, indomethacin, TTX, TEA, and ODQ were all administered topically in the hemodynamic studies.

ACh chloride, SIN-1, L-NNA, GTP, PMSF, IBMX, theophylline, EDTA, and TTX were obtained from Sigma Chemicals. Superoxide dismutase, leupeptin, pepstatin A, trypsin inhibitor, creatine kinase, and creatine phosphate were obtained from Boehringer. ODQ was obtained from Tocris Cookson, and TEA was obtained from ICN Biomedicals. Indomethacin (Confortid) was obtained from Dukex, and suxamethonium (50 mg/l) was obtained from the pharmacy of the National Hospital of Denmark in Copenhagen.

**Calculations and Statistics**

Because NBF varies proportionately to mean arterial blood pressure (15), we converted NBF values into nerve microvascular conductance (NMVC) values to take into account any drift in blood pressure during the course of the procedure. The following formula was used

\[
\text{NMVC} = \frac{\text{NBF}}{\text{BP}}
\]

where NBF and blood pressure (BP) were calculated as the area under the curve using the same time intervals for both; BP was measured as arterial blood pressure. Because LDF measures NBF in arbitrary units of flux, NMVC was calculated as arbitrary units of flux per millimeter of mercury.

Previous studies showed that in vitro vasodilation induced by ACh consists of a transient NO-independent phase that initiates vasodilation followed by a NO-dependent phase that maintains it (1, 3). Therefore, to avoid confounding the data by mixing information from two putatively different vasodilatory mechanisms, the NMVC responses in this study were measured during the last 60 s of each 5-min exposure to ACh in all groups (see Fig. 7) and at the start of vasodilation from 0 to 23 s in group 2 (see RESULTS). To be able to compare responses to both vasodilators with each other, NMVC responses to SIN-1 were also measured during the last 60 s. In this article, vasodilatory NMVC responses to ACh and SIN-1...
RESULTS

Hemodynamic Studies

Between-group comparisons of step 1. In all groups, systemic blood pressure was stable and uninfluenced by applications of topical vasodilators (Fig. 2B). The vasodilatory responses to topical ACh (0.1 mM) in the presence of Ringer solution during step 1 were similar in all four groups of rats, the ACh-induced NMVC increment being 61.1% (CI: 34.4–93.2%) in group 1, 44.7% (CI: 20.7–73.4%) in group 2, 58.5% (CI: 32.2–90.0%) in group 3, and 47.2% (95% CI: 22.8–76.5%) in group 4 (P = 0.7856, ANOVA; see Fig. 5). Likewise, the SIN-1-induced NMVC increment was 35.2% (CI: 16.3–57.3%) in group 1 vs. 40.0% (CI: 20.4–62.9%) in group 2 vs. 34.6% (CI: 15.7–56.6%) in group 3 vs. 29.3% (CI: 11.2–50.4%) in group 4 (P = 0.904, ANOVA; see Fig. 6). Thus the initial NMVC responses in step 1 were similar for all three experimental groups of rats, allowing comparisons between them.

Time controls (group 4). In the time control group (n = 6), systemic blood pressure remained stable throughout the experimental procedure (P = 0.9973, ANOVA; see Fig. 3). The ACh-induced NMVC increment declined gradually to 66.3% of the value in step 1 (P = 0.3077, ANOVA; see Fig. 5); this decrement was statistically insignificant. In comparison, the SIN-1-induced NMVC increment increased significantly to double the value in step 1 (P = 0.0026, ANOVA; see Fig. 6). During the two periods of rest, baseline NMVC values tended to increase slightly by 15.2% (CI: –5.9 to 40.8%) and 17.1% (CI: –11.7 to 55.3%), respectively (see Fig. 4); these increments were not statistically significant (P = 0.1316 for 1st rest period and P = 0.2109 for 2nd rest period). All results presented below were statistically compared with the corresponding time control values to determine the level of significance.

Group 1: Rats receiving L-NNA followed by ODQ + L-NNA. Blood pressure in group 1 (n = 6 animals) was not affected by the topical application of either L-NNA or ODQ (P = 0.9668 vs. time controls, ANOVA; Fig. 3). Thus there was no systemic effect of these substances. In the presence of topical L-NNA (1 mM), baseline NMVC decreased by 54.8% (CI: 41.1–65.3%, P = 1.619 × 10^-4 vs. time controls; Fig. 4), revealing a basal production of NO. There was no further decrease in baseline NMVC after subsequent application of ODQ (0.1 mM; P = 1.000 vs. time controls; data not shown).

The NMVC increment induced by ACh (0.1 mM) decreased by 68.7% during the treatment sequence of Ringer solution, L-NNA (1 mM) alone, and L-NNA + ODQ combined (1 mM and 0.1 mM, respectively), but this decrease did not achieve statistical significance compared with time controls (P = 0.193165, ANOVA; Fig. 5). These treatments, on the other hand, greatly affected the NMVC response to SIN-1 (0.125–0.75 mM; P = 9.035 × 10^-9 vs. time controls, ANOVA). The NMVC increment above baseline in response to SIN-1...
increased from 35.3% (CI: 15.9–57.9%) in the presence of Ringer solution to 160.8% (CI: 123.5–204.4%) in the presence of L-NNA ($P = 0.0096$ vs. time controls), demonstrating an enhanced activity of the NO-cGMP cascade at or distal to sGC. With the addition of ODQ, the NMVC increment caused by SIN-1 fell to 3.2% (CI: 2–11.6 to 20.4%; $P = 1.648 \times 10^{-3}$ compared with response in the presence of L-NNA with respect to time controls; Fig. 6). Thus ODQ completely inhibited SIN-1-induced vasodilation.

Group 2: Rats receiving L-NNA followed by TEA. In group 2 ($n = 5$ animals), L-NNA + indomethacin (1 mM and $10^{-5}$ M, respectively), with or without TEA (10 mM), had no effect on mean arterial blood pressure when applied topically ($P = 0.8855$ vs. time controls, ANOVA). There is a significant difference in the trend in blood pressure levels between time controls and group 3 ($P = 3.935 \times 10^{-7}$, ANOVA), showing a systemic effect of TTX. Comparisons between the individual time points in these 2 groups, however, revealed a significant difference in value only at 1 point ($P = 0.0444$). The data are given as means. See MATERIALS AND METHODS for description of steps 1–3.
l-NNA + indomethacin, ACh-induced vasodilation became transient, peaking at 23 ± 6 s (means ± SD) with a duration of only 145 ± 69 s (means ± SD) despite the continued exposure to ACh throughout a total of 5 min. Similar responses were found in all the animals in this group. Thus, in the presence of l-NNA + indomethacin, the NMVC response to ACh consisted of two phases, an initial phase with transient l-NNA + indomethacin-resistant vasodilation followed by a phase of total inhibition by these two inhibitors of the expected vasodilatory response.

Comparing ACh-induced vasodilation in the presence of l-NNA + indomethacin with that found in their absence, we found that the time-to-peak interval of the transient peak (from 0 to 23 s) seen with l-NNA + indomethacin corresponded to the initial rise in NBF after application of ACh in the presence of Ringer solution. The phase of abolished vasodilation seen with l-NNA + indomethacin corresponded to the sustained vasodilation found in the presence of Ringer solution. This implies that the initial vasodilatory response to ACh in the presence of Ringer solution is l-NNA +

![Image](http://ajpheart.physiology.org/)
indomethacin resistant, whereas sustained vasodilation in the presence of Ringer solution is highly sensitive to l-NNa + indomethacin.

Thus the ACh-induced NMVC increment measured during the time-to-peak interval from 0 to 23 s was 20.3% (CI: 10.7–30.7%) in the presence of Ringer solution and 32.9% (CI: 22.4–44.4%) in the presence of l-NNa + indomethacin, falling significantly to 13.9% after the addition of TEA (CI: 4.8–23.7%; \( P = 0.0330 \) vs. l-NNa + indomethacin with regard to time controls, data not shown). In comparison, the NMVC increment measured during the last 60 s of the 5-min exposure to ACh fell from 44.7% (CI: 29.4–61.8%) in the presence of Ringer solution to −8.9% (CI: −18.6–19%) in the presence of l-NNa + indomethacin and was unchanged, remaining at −10.3% (CI: −19.8–0.3%), in the presence of TEA + l-NNa + indomethacin (\( P = 0.0063 \) vs. time controls, ANOVA; Fig. 5). Thus, although blockade of K+ channels did not affect the second phase of the ACh response, K+ channel activity was responsible for 57.8% of the initial phase of ACh-induced vasodilation.

In contrast to ACh, the NMVC increment caused by SIN-1 was potentiated more than fourfold by treatment with l-NNa + indomethacin, being 40.0% (CI: 20.0–63.5%) in the presence of Ringer solution, rising to 179.7% (CI: 139.7–223.5%) in the presence of l-NNa + indomethacin, and remaining at 160.4% (CI: 123.1–204.0%) in the presence of TEA + l-NNa + indomethacin (\( P = 1.074 \times 10^{-5} \) vs. time controls, ANOVA; Fig. 5). Thus, the potentiation found with l-NNa + indomethacin was not different from that found with l-NNa alone in group 1 (\( P = 1.000 \) vs. time controls). As shown above, subsequent application of TEA had no effect on the SIN-1 response, indicating that vasodilation caused by the NO-cGMP pathway is not dependent on K+ channels.

Group 3: Rats receiving TTX followed by l-NNa. The blood pressure profile throughout the experimental procedure in group 3 (\( n = 6 \) animals) revealed a systemic effect of topical TTX (0.02 mM), decreasing gradually and significantly to 69.6 ± 9.4% (mean ± SE) of the initial blood pressure levels in step 1 (\( P = 3.935 \times 10^{-7} \) vs. time controls, ANOVA; Fig. 3). Application of topical TTX did not alter baseline NMVC significantly compared with time controls (\( P = 1.000 \); Fig. 4). Combined treatment with TTX + l-NNa (0.02 and 1 mM, respectively) resulted in a decrease in baseline NMVC of 51.2% (CI: 13.9–72.4%; \( P = 0.0349 \) vs. time controls), which was of the same magnitude as the decrement in baseline NMVC caused by l-NNa alone in group 1.

In group 3, sequential treatment consisting of Ringer solution, TTX (0.02 mM), and TTX + l-NNa (0.02 and 1 mM, respectively) did not result in statistically significant changes in NMVC responses to ACh (\( P = 0.0908 \) vs. time controls, ANOVA; Fig. 5), nor were significant changes in the NMVC responses to SIN-1 (\( P = 0.1712 \) in comparison with time controls, ANOVA; Fig. 6) seen during the same treatment sequence. Combined treatment with TTX + l-NNa tended to potentiate the SIN-1 response and attenuate the ACh response without achieving statistical significance.

**Homogenate Studies**

In this part of the study, we tested the hypothesis that enhancement of the in vivo NMVC response to SIN-1 during NOS-inhibition was caused by potentiation of sGC activity by l-NNa.

As Fig. 8 shows, SIN-1 (50 \( \mu \text{M} \)) potentiated the activity of sGC in nerve homogenates weakly, by 35.1% compared with controls (\( P = 0.0035 \), \( n = 15 \)). ODQ (10 \( \mu \text{M} \)) inhibited the SIN-1-stimulated activity of sGC by 24.9% (\( P = 0.0007 \), \( n = 15 \)) but did not influence the basal activity of sGC in nerve homogenates (\( P = 1.000 \), \( n = 15 \)). In contrast, l-NNa had no effect on either the basal or SIN-1-stimulated activity of sGC. The activity of basal sGC was 119.6% of control in the presence of 10 \( \mu \text{M} \) l-NNa (\( P = 1.000 \), \( n = 10 \)) and 87.7% of control in the presence of 100 \( \mu \text{M} \) l-NNa (\( P = 0.6489 \), \( n = 5 \); data not shown). Neither 10 \( \mu \text{M} \) or 100 \( \mu \text{M} \) l-NNa enhanced SIN-1-stimulated activity, because the activity of sGC at these concentrations of l-NNa was 98.0% (\( P = 1.000 \), \( n = 10 \)) and 93.2% (\( P = 1.000 \), \( n = 5 \); data not shown), respectively, of the SIN-1-stimulated levels.

To verify our method, we repeated this procedure using homogenates of rat cerebellum (Fig. 9), a tissue known to contain large amounts of NOS (4). In cerebellum, the activity of the enzyme after stimulation with SIN-1 by 24.9% (SIN-1 L-NNA) nor the SIN-1-stimulated activity (SIN-1 L-NNA) of basal sGC (ODQ) but did inhibit the activity of the enzyme after stimulation with SIN-1 by 24.9% (SIN-1 + ODQ). **P < 0.005 vs. control; ***P < 0.001 vs. SIN-1. The data are given as means and 95% confidence intervals.

![Fig. 8. Effects of L-NNA (10 \( \mu \text{M} \)) and ODQ (0.1 \( \mu \text{M} \)) on the activity of sGC in rat sciatic nerve. Two series of assays were performed, the concentration of l-NNa in the first being 10 \( \mu \text{M} \) (\( n = 10 \)) and 100 \( \mu \text{M} \) in the second (\( n = 5 \); data not shown). Values from similarly treated assays in the 2 series did not differ significantly from each other and were therefore pooled (n = 15). sGC responded with a 35.1% increase in activity when stimulated with SIN-1. l-NNa affected neither the basal activity of soluble guanylyl cyclase (sGC) (l-NNa) nor the SIN-1-stimulated activity (SIN-1 + l-NNa). ODQ did not affect the basal activity of sGC (ODQ) but did inhibit the activity of the enzyme after stimulation with SIN-1 by 24.9% (SIN-1 + ODQ). **P < 0.005 vs. control; ***P < 0.001 vs. SIN-1. The data are given as means and 95% confidence intervals.**

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**Fig. 8. Effects of L-NNA (10 \( \mu \text{M} \)) and ODQ (0.1 \( \mu \text{M} \)) on the activity of sGC in rat sciatic nerve. Two series of assays were performed, the concentration of l-NNa in the first being 10 \( \mu \text{M} \) (\( n = 10 \)) and 100 \( \mu \text{M} \) in the second (\( n = 5 \); data not shown). Values from similarly treated assays in the 2 series did not differ significantly from each other and were therefore pooled (n = 15). sGC responded with a 35.1% increase in activity when stimulated with SIN-1. l-NNa affected neither the basal activity of soluble guanylyl cyclase (sGC) (l-NNa) nor the SIN-1-stimulated activity (SIN-1 + l-NNa). ODQ did not affect the basal activity of sGC (ODQ) but did inhibit the activity of the enzyme after stimulation with SIN-1 by 24.9% (SIN-1 + ODQ). **P < 0.005 vs. control; ***P < 0.001 vs. SIN-1. The data are given as means and 95% confidence intervals.**
Fig. 9. Effects of l-NNA (10 μM) and ODQ (0.1 μM) on the activity of sGC in rat cerebellum. Two series of assays were performed, the concentration of l-NNA in the first being 10 μM (n = 5) and 100 μM in the second (n = 10). sGC activity increased 598.7% when stimulated with SIN-1. As in rat sciatic nerve, l-NNA stimulated activity (SIN-1) of the enzyme by 66.0% (SIN-1) but inhibited the SIN-1-stimulated activity of sGC (ODQ). Likewise, ODQ did not affect the basal activity of sGC (ODQ) but inhibited the SIN-1-stimulated activity of the enzyme by 66.0% (SIN-1 + ODQ). *P < 0.05, ****P = 2.022 \times 10^{-8} vs. control; ++++P = 4.653 \times 10^{-8} in comparison to SIN-1. The data are given as means and 95% confidence intervals.

bellar homogenates, SIN-1 (50 μM) potentiated the activity of sGC by 598.7% (P = 0.0000, n = 10). ODQ (10 μM) inhibited the SIN-1-stimulated activity of sGC by 66.0% (P = 0.0000, n = 10) but did not inhibit the activity of basal sGC. In contrast, ODQ significantly potentiated the activity of basal sGC by 15.6% in this assay (P = 0.0434, n = 10). Again, l-NNA had no effect on either the SIN-1-stimulated or basal activity of sGC in cerebellar homogenates. In the presence of 10 μM l-NNA, the activity of sGC was 105.3% of control (P = 1.000, n = 5) and 103.2% of SIN-1-stimulated values (P = 1.000, n = 5). In the presence of 100 μM l-NNA, the corresponding values were found to be 88.8% (P = 1.000, n = 5; data not shown) and 94.3% (P = 1.000, n = 5; data not shown), respectively. Thus ODQ and l-NNA produced similar, but much more extreme, effects in the cerebellar homogenates compared with the sciatic nerve homogenates. In conclusion, the activity of sGC was not enhanced by l-NNA at any concentration in either basal or in SIN-1-stimulated homogenates of sciatic nerve or cerebellum.

**DISCUSSION**

This study has focused on the regulation of the microcirculation in peripheral nervous tissue, both during baseline conditions and in response to the vasodilators ACh and SIN-1. Our findings include demonstration of a basal release of NO, accounting for ~50% of baseline NMVC. Subsequent treatment with TEA reduced the remaining baseline NMVC by ~25%. The NMVC increment caused by the NO donor SIN-1 was enhanced by NOS inhibition with l-NNA and subsequently totally inhibited by the sGC inhibitor ODQ. However, the in vitro enzyme assay did not reveal any potentiation of sGC activity during NOS inhibition to account for this enhanced in vivo SIN-1 response. In vivo vasodilation in response to ACh was found to consist of two components: an initial, transient vasodilation partially mediated by EDHF followed by a phase of sustained vasodilation. ACh-induced sustained vasodilation was not affected by l-NNA alone but was abolished entirely by the combination of l-NNA with the cyclooxygenase inhibitor indomethacin. Finally, neither K⁺ channel blockade with TEA nor inhibition of voltage-dependent Na⁺ channels with TTX was found to have any effect on the sustained vasodilatory responses to ACh and SIN-1.

In the following discussion, the effects of the inhibitors l-NNA, TTX, and TEA on baseline NMVC are considered. The in vivo potentiation of the vasodilatory response to SIN-1 during NOS inhibition is then related to the effects of SIN-1 and l-NNA on the biochemical activity of sGC found in the in vitro assay. Finally, mechanisms responsible for ACh-induced in vivo vasodilation as demonstrated in this study are discussed.

In our study, NOS inhibition by topically applied l-NNA with or without indomethacin resulted in a 55% decrease in baseline NMVC, indicating that NO is continuously released in the sciatic nerve microcirculation. This finding is in good agreement with recent studies in which superfusion of the nerve and enneurial injection with NOS inhibitors caused the baseline NBF to decrease by 35% and 64%, respectively (11, 28). Thus resting blood flow in the rat sciatic nerve is largely dependent on NO production and release. Subsequent addition of TEA resulted in a 28% decrease of the baseline NMVC found in the presence of l-NNA + indomethacin, indicating that EDHF plays a role in maintaining basal nerve blood flow.

We found that inhibition of neurogenic activity with TTX did not alter baseline NMVC. In contrast, ganglion blockade with guanethidine was previously shown to increase NBF by 35% (32). However, in the present study, topically applied TTX had a systemic effect on mean arterial blood pressure, causing a substantial decrease over time. Thus a putative increase in baseline NMVC caused by blockade of neurogenic activity by TTX could conceivably have been counteracted by the falling blood pressure because peripheral nerves have no autoregulation (15, 25).

There is some controversy regarding the interaction between sympathetic tone and NO release. Some in vivo studies have shown that ganglion blockade almost abolished the hypertensive response to systemic NOS inhibition (12), and sectioning the lumbar sympathetic trunk greatly reduced vasoconstriction in skeletal muscle induced by systemic NOS inhibition (8). The conclusion was reached that NO release is partially dependent on sympathetic activity. Conversely, other in vivo studies measuring blood pressure before and
after systemic NOS inhibition, as well as arterial nor-
epinephrine and epinephrine levels in sympathecto-
mized, ganglion-blocked, and normal rats, found NO-
dependent vasodilation preserved in spite of varying 
sympathetic and humoral adrenergic stimuli between 
the groups (20). We found that the combination of local 
neurogenic and NOS inhibition with topically applied 
TTX + L-NNA had no effect on baseline NMVC other 
than that caused by topical L-NNA alone. Thus, at the 
microcirculatory level, basal NO release is not depend-
ent on neurogenic activity in the rat sciatic nerve.

Using SIN-1 as a pharmacological probe to deter-
mine the level of functional activity of the NO-cGMP 
cascade, we found that inhibition of NOS with topical 
L-NNA (1 mM) increased the NMVC increment caused 
by SIN-1 more than fourfold from 35.3% in the pres-
ence of Ringer solution to 160.8% in the presence of 
L-NNA. This corresponds well with previous studies, in 
which NOS inhibition in vivo resulted in a supersensit-
vitiveness to nitrovasodilators, e.g., the hypotensive re-
sponse to NO donors was enhanced after systemic NOS 
inhibition (17) and nitroglycerin-induced vasodilation of 
the iliac artery was potentiated by local NOS inhibi-
bition (23). By using topically applied L-NNA and 
SIN-1 and measuring blood flow responses in periph-
eral nerve tissue, we have shown that this potentiation 
found systemically and in conduit arteries also occurs 
at the microcirculatory level.

Furthermore, we found that this L-NNA-enhanced 
NMVC increment to SIN-1 was abolished after subse-
quent treatment with the specific sGC inhibitor ODQ. 
These results are in good agreement with earlier stud-
ies using the mouse cortex, in which ODQ inhibited 
blood flow increments caused by NO donors by 80–90% 
(26). Because the vasodilatory action of the NO donor 
SIN-1 is solely dependent on sGC (19), this response 
pattern to SIN-1 could indicate that NOS inhibition 
potentiated the functional activity of this enzyme. Us-
ing homogenates of rat sciatic nerve and cerebellum, 
we then examined the effects of NOS inhibition with 
L-NNA and sGC inhibition with ODQ on the bio-
chemical activity of sGC. In both tissues, the SIN-1-stimu-
ulated activity of sGC was significantly inhibited by 
ODQ, whereas the basal activity of sGC was not. These 
results are in good agreement with previous studies 
that showed that stimulated activity of the enzyme was 
more easily inhibited than basal activity (30). How-
ever, we were not able to demonstrate that L-NNA, at 
concentrations of either 10 μM or 100 μM, enhanced 
basal or SIN-1-stimulated activity of sGC in vitro. In 
contrast, an earlier study using aortic rings did dem-
onstrate enhanced sGC activity during NOS inhibition 
(17). Because this earlier study did not examine the 
activity of the enzyme when saturated with substrate, 
its findings may have reflected other factors, such as 
substrate availability. Therefore, we speculate that the 
mechanism by which L-NNA potentiates the NMVC 
response to SIN-1 and the mechanism by which ODQ 
abolishes it are not the same, i.e., L-NNA may poten-
tiate the NO-cGMP cascade downstream of sGC.

Inhibition of NOS and cyclooxygenase with L-NNA + 
indomethacin unmasked two phases of ACh-induced 
NMVC response, i.e., an initial, transient vasodilation 
peaking at 23 ± 6 s and lasting 145 ± 69 s followed by 
a second phase that exhibited no vasodilation at all, 
lasting for the duration of the exposure to ACh. The 
time-to-peak interval of the first phase (from 0 to 23 s) 
found in the presence of L-NNA + indomethacin corre-
sponded to the initial rise in NBF induced by ACh in 
the presence of Ringer solution, whereas the second 
phase corresponded to a period of sustained vasodila-
tion to ACh, found in the presence of Ringer solution. 
These findings are in good agreement with recent stud-
ies using isolated arterioles from the skeletal muscle 
and cheek pouch, in which ACh-induced vasodilation 
was shown to consist of a NO-independent component 
that initiated vasodilation peaking between 10 and 
35 s and lasting ~2 min followed by a NO-dependent 
component that maintained vasodilation for the dura-
tion of the stimulus (1, 3). Thus the initial and steady-
state components of ACh-induced vasodilation seen in 
vitro correlate timewise to the first and second phases 
found in vivo in the present study.

Regarding ACh-induced, sustained vasodilation 
found in the presence of Ringer solution, our present 
data showed a complete lack of attenuation of this 
response during NOS inhibition alone. Likewise, no 
statistically significant effect on this response was seen 
after subsequent addition of ODQ. Assuming that both 
ACh and SIN-1 use the NO-cGMP cascade to induce 
vasodilation in the microcirculation of peripheral 
nerve, any potentiation of the cascade at or beyond 
the level of sGC would be expected to affect the blood flow 
responses to both substances equally. Because topical 
L-NNA caused the NMVC increment to SIN-1 to in-
crease more than fourfold without affecting the NMVC 
response to ACh, the conclusion could be drawn that 
this response pattern represents a 75% inhibition of 
NOS. However, a supramaximal concentration of L-
NNA of 1 mM was chosen to ensure total NOS inhibi-
tion (4), whereas ODQ was shown to entirely inhibit 
sGC, as reported above. Therefore, at these concentra-
tions of L-NNA and ODQ, NO-dependent vasodilation 
was practically abolished. Despite combined treatment 
with L-NNA and ODQ, a substantial NMVC response 
to ACh during the sustained phase was seen. Thus, 
although the steady-state component of the in vitro 
response to ACh was NO dependent (1, 3), the corre-
sponding sustained phase of ACh-induced vasodilation 
in the sciatic nerve appeared to be NO resistant in vivo.

Examining this phenomenon, we hypothesized that 
this NO-resistant component of the sustained NMVC 
response to ACh could be of neurogenic origin, because 
previous in vitro studies have shown that ACh can 
produce vasodilation independently of the endothelium 
by inhibiting sympathetic vasoconstriction (13;27). Us-
ing topical TTX to block neurogenic activity, we found, 
however, no effect of TTX on second-phase NMVC 
responses to ACh. These results are in good agreement 
with those found in hamster skeletal muscle, in which 
maximal vasodilation induced by microiontophoresi-
cally applied ACh was not influenced by either TTX or l-NNa (24). Similarly, in another study, denervated and innervated rat skeletal muscle responded equally to ACh (6). Thus our studies indicate that sustained ACh-induced vasodilation in the microcirculation of peripheral nerve is independent of neurogenic activity.

As mentioned above, we found the sustained phase of the ACh-induced NMVC increment entirely abolished in the presence of l-NNa + indomethacin. This in vivo finding is in good agreement with a recent in vitro study using spinal resistance arteries, in which combined treatment with l-NAME and indomethacin completely reduced ACh-induced vasodilation (31). Because treatment with l-NNa alone had no effect on this response in the rat sciatic nerve, the second phase of ACh-induced vasodilation appears to be mediated by a vasodilatory prostanoid.

In contrast, the NMVC increment measured during the time-to-peak interval of the ACh response was not attenuated by NOS and cyclooxygenase inhibition. However, nonspecific K+ channel blockade with TEA together with l- NNa + indomethacin halved this phase of ACh-induced vasodilation. ACh-induced vasodilation, occurring in the presence of simultaneous NOS and cyclooxygenase inhibition and dependent on K+ channel activity, has been attributed to EDHF (29). Thus we found that the time-to-peak phase of ACh-induced vasodilation is mediated to a large extent by EDHF, which is supported by the in vitro findings of Bakker and Sipkema (1). We also found that sustained ACh-induced vasodilation appears to be NO resistant and mediated to a large extent by a vasodilatory prostanooid. This is in contrast to the in vitro studies, in which steady-state vasodilation has been shown to be highly inhibitable by NO (1, 3). The reason for this difference may lie in the types of tissue examined in these studies, because Yashiro and Ohhashi (31) found in their in vitro study using neural tissue from the spinal cord that ACh-induced vasodilation was mediated by prostanoids as well as NO.

In conclusion, we found that the basal release of local NO, which accounts for ~50% of baseline NMVC, was not dependent on neurogenic activity. EDHF was also found to contribute to the regulation of baseline NMVC, although to a smaller degree. NOS inhibition with l-NNa potentiated the functional activity of the NO-cGMP cascade in the microcirculation of peripheral nerve in vivo without potentiating the biochemical activity of sGC, indicating that the site of potentiation lies distal to cGMP. Most importantly, ACh-induced vasodilation in vivo in rat sciatic microcirculation appears to consist of two phases, a transient phase initiating vasodilation mediated partially by EDHF followed by a second phase of sustained vasodilation that was not affected by NO alone but was abolished by NOS and cyclooxygenase inhibition, suggesting mediation by a vasodilatory prostanooid.

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