

# Rise in endothelium-derived NO after stimulation of rat perivascular sympathetic mesenteric nerves

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**Boric, Mauricio P., Xavier F. Figueroa, M. Verónica Donoso, Alfonso Paredes, Inés Poblete, and J. Pablo Huidobro-Toro.** Rise in endothelium-derived NO after stimulation of rat perivascular sympathetic mesenteric nerves. *Am. J. Physiol.* 277 (*Heart Circ. Physiol.* 46): H1027–H1035, 1999.—To evaluate whether sympathetic activity induces nitric oxide (NO) production, we perfused the rat arterial mesenteric bed and measured luminally accessible norepinephrine (NE), NO, and cGMP before, during, and after stimulation of perivascular nerves. Electrical stimulation (1 min, 30 Hz) raised perfusion pressure by  $97 \pm 7$  mmHg, accompanied by peaks of  $23 \pm 3$  pmol NE,  $445 \pm 48$  pmol NO, and 1 pmol cGMP. Likewise, perfusion with 10  $\mu$ M NE induced vasoconstriction coupled to increased NO and cGMP release. Electrically elicited NO release depended on stimulus frequency and duration. Endothelium denudation with saponin abolished the NO peak without changing NE release. Inhibition of NO synthase with 100  $\mu$ M *N*<sup>o</sup>-nitro-L-arginine reduced basal NO and cGMP release and blocked the electrically stimulated and exogenous NE-stimulated NO peak while enhancing vasoconstriction. Blocking either sympathetic exocytosis with 1  $\mu$ M guanethidine or  $\alpha_1$ -adrenoceptors with 30 nM prazosin abolished the electrically evoked vasoconstriction and NO release.  $\alpha_2$ -Adrenoceptor blockade with 1  $\mu$ M yohimbine reduced both vasoconstriction and NO peak while increasing NE release. In summary, sympathetically released NE induces vasoconstriction, which triggers a secondary release of endothelial NO coupled to cGMP production.

nitric oxide chemiluminescence; norepinephrine; guanosine 3',5'-cyclic monophosphate release; sympathetic neurotransmission; rat arterial mesenteric bed; endothelial activation; adrenoceptor blockade

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THE HOMEOSTASIS of the vascular wall is complex and involves a growing number of humoral, neuronal, and endothelial factors (19, 41). The importance of the vascular endothelium was highlighted by the finding that nitric oxide (NO) plays a pivotal role in the minute-to-minute regulation of blood pressure (27, 29, 36). The best support for this notion derives from studies using drugs that act as selective blockers of NO synthase. Numerous reports demonstrate that guanidino-substituted analogs of L-arginine act as selective inhibitors of NO synthase (21). Administration of NO synthase blockers evokes an increase in local or systemic blood pressure in experimental subjects, including humans (36, 38, 39). The assessment of the direct

role of NO in vascular wall homeostasis has been precluded by the technical difficulties in accurate determination of NO in body fluids. Such limitations hindered our understanding of the physiological and pathophysiological relevance of NO levels in the control of vascular tone.

Because physiological regulation mechanisms usually involve opposing signals, we assessed whether the vasoconstriction associated with electrical stimulation leads to the release of NO as an opposing vasodilating agent. We reasoned that a meaningful physiological role of the NO eventually released was its coupling to the synthesis of cGMP by the vascular smooth muscles. We anticipated, therefore, that if NO is released after a vasoconstrictor response, it should cause an opposing vasodilation dependent on cGMP.

To assess this working hypothesis, we conducted experiments in the arterial mesenteric bed of the rat, in which we earlier studied the physiology and pharmacology of the perivascular sympathetic fibers (14). We demonstrated that the electrical stimulation of these nerve fibers elicits vasomotor responses that are mediated by the release of norepinephrine (NE), neuropeptide Y (NPY) and, also likely, ATP, the sympathetic triad (14, 16, 34). In the present work, we investigated the correlation between changes in perfusion pressure elicited by either transmural electrical depolarization or exogenous NE and the release of NO and cGMP. The results suggest that NE-driven vasoconstriction is coupled to endothelial release of NO and associated with a cGMP surge.

## MATERIALS AND METHODS

### *Perfusion of Rat Arterial Mesenteric Bed*

Rats were anesthetized with 40 mg/kg pentobarbital sodium, and the abdomen was opened by a midline incision. The superior mesenteric artery was cannulated and perfused with warm (37°C) Krebs-Ringer buffer solution, equilibrated with 95% O<sub>2</sub>-5% CO<sub>2</sub>, at a constant rate of 2 ml/min. The attachment of the mesentery to the intestinal wall was severed (28), and the isolated arterial mesenteric bed was transferred to a warm chamber. A pressure transducer was connected at the entrance of the mesenteric artery to monitor changes in the perfusion pressure in a recording polygraph. Platinum electrodes were placed surrounding the artery and were connected to a Grass S44 stimulator. A 30-min buffer perfusion period was allowed for equilibration. The perfusate was collected once every minute in test tubes for determination of the luminally accessible pool of NO and/or NE and cGMP in the tissue perfusate.

### *Electrical Depolarization of Nerves Surrounding Mesenteric Artery*

The basic protocol consisted of transmural depolarization of the perivascular mesenteric nerves with a train of 30 Hz (50 V, 1 ms) for 1 min (14, 16). Perfusion pressure and NE, NO, and cGMP content of perfusate were determined before, during, and after the stimulus. Other protocols were performed to assess the threshold of the frequency of stimulation required to evoke NO release (2.5, 5, 10, 20, and 30 Hz over 1 min). Additional experiments were aimed at determining the influence of the duration of the 30-Hz pulses on the recovery of NO in the mesenteric perfusate, giving train pulses of 30 Hz applied for 0.5, 1, 2, and 4 min.

### *Source of NO and Influence of Adrenergic Mechanism on Release of NO*

Six separate experimental protocols were performed to investigate the source of NO and to assess how the electrical depolarization of the sympathetic mesenteric nerve terminals evoked the release of NO to the perfusion media. In all these protocols, two 30-Hz, 1-min electrical stimuli were delivered. The first stimulus served as the control pretest value, and the second was performed after 30 min of perfusion with the test drug. Perfusate samples were collected before, during, and after each electrical stimulus. Drugs were dissolved in the perfusion buffer and maintained until completion of the second electrical stimulation protocol. Perfusion pressure was monitored over the whole experiment. In some experiments, NE release during the second stimulus was also measured.

*Krebs-Ringer controls.* To determine whether in an intact preparation the second stimulus evoked an equivalent amount of NO release compared with the first stimulus, six arterial mesenteric beds were perfused with buffer between the first and second stimuli. This series served as the control for the second stimulus.

*Saponin-induced endothelial shedding.* To assess whether NO measured in the perfusion medium derived from the mesenteric endothelium, four preparations were perfused with 0.1% saponin for 55 s, and the second stimulus was delivered 30 min later, once the perfusion pressure returned to basal levels, as previously characterized (15, 33).

*Blockade of NO synthase.* To assess whether the NO recovered in the perfusate was sensitive to blockade of NO synthase, in a series of six experiments, the second electrical stimulus was performed 30 min after perfusion with 100  $\mu$ M *N*<sup>o</sup>-nitro-L-arginine to inhibit NO synthase (1, 11, 23). Control measurements were performed to assess whether this concentration of *N*<sup>o</sup>-nitro-L-arginine interfered with NO determination.

*Blockade of sympathetic exocytosis.* In four preparations, the second stimulus was performed during and after perfusion with 1  $\mu$ M guanethidine.

*Blockade of  $\alpha_1$ -adrenoceptors.* The influence of  $\alpha_1$ -adrenoceptors was studied in four mesenteries perfused with 30 nM prazosin.

*Blockade of  $\alpha_2$ -adrenoceptors.* Five mesenteries were used to assess the effect of perfusion with 1  $\mu$ M yohimbine, a well-characterized  $\alpha_2$ -adrenoceptor antagonist.

### *Perfusion With Exogenous NE*

The cause-effect relationship between NE-induced vasoconstriction and NO and cGMP release was further studied in the following protocols. First, in six mesenteries the electrical stimulus was mimicked by perfusion for 1 min with buffer containing 10  $\mu$ M NE, and the time course of perfusion

pressure and luminal NO release was measured. To check whether NE-induced NO release was caused by NO synthase stimulation, four additional mesenteries were submitted to an identical protocol in the presence of 100  $\mu$ M *N*<sup>o</sup>-nitro-L-arginine. Furthermore, to clarify the results obtained with the electrical stimulation during adrenergic blockade, a similar 1-min exogenous NE stimulation was used with 1  $\mu$ M guanethidine ( $n = 3$ ) and 1  $\mu$ M yohimbine ( $n = 6$ ).

Second, to get a better quantitative assessment of cGMP release, six mesenteries were perfused for 10 min with 10  $\mu$ M NE and perfusate was collected in 10-ml samples just before, and at the end of, the NE perfusion period. The total cGMP content of these samples was determined. In addition, to assess whether cGMP release was caused by NO stimulation of guanylyl cyclase, four mesenteries were submitted to an identical protocol during NO synthase blockade by perfusion with 100  $\mu$ M *N*<sup>o</sup>-nitro-L-arginine.

### *Analytic Techniques*

*NE quantification.* Each perfusate sample (2 ml) was collected in prechilled 5-ml collection tubes containing 100  $\mu$ l of 1 M perchloric acid and 15  $\mu$ l of 5% sodium metabisulfite. The NE in the samples was concentrated and purified using activated alumina (2). One hundred microliters of each purified sample, including an added internal standard of dihydroxybenzyl amine, were injected into an HPLC system, using the Merck L-6200A intelligent pump as described by Donoso et al. (14). The NE was quantified using a 656 DE Metrohm electrochemical detection system (6). Results are expressed as either the time course of the lumenally accessible NE recovered (pmol/ml) or as the total integrated, recovered release elicited by the electrical depolarization (pmol). When simultaneous measurements were performed, a 200- $\mu$ l perfusate sample was used for NO determination.

*Quantification of NO by chemiluminescence.* Freshly obtained triple-distilled water was used to prepare all buffer and drug solutions. Samples were collected in test tubes and immediately sealed with Parafilm to avoid contamination from room air. The sample content of NO was quantified using a Sievers 280 NO analyzer within 1 h after the experiment was terminated (3). The reaction chamber of the equipment was filled with 8 ml of glacial acetic acid containing 100 mg of potassium iodide at room temperature to reduce nitrites to NO (30, 32). A 50- $\mu$ l perfusate sample was injected into the reaction chamber, and a nitrogen stream carried the resulting NO gas to a cell in which the specific chemiluminescence generated by the NO-ozone reaction was detected by a photomultiplier. Calibration of the equipment was performed daily using standards of 10–1,000 nM sodium nitrite. The sensitivity of the equipment allows for a detection threshold of 0.5–1.0 pmol NO (10–20 pmol/ml). Background buffer readings were subtracted to determine mesentery NO release. Results are expressed either as the time course of lumenally accessible NO recovered before, during, and after the stimulus (pmol/ml) or as the integrated NO recovered above basal values elicited by the electrical pulse (pmol). We chose to integrate NO release for 4 min because this period accounted for >90% of the NO peak.

*cGMP determinations.* The cyclic nucleotide was quantified with an RIA for acetylated cGMP. The sensitivity limit of this assay was 10 fmol. The procedure outlined by Boric and Croxatto (7) was followed. As radioactive tracer we used 2'-O-monosuccinylguanosine 3',5'-cyclic monophosphate tyrosyl methyl ester that was labeled locally with <sup>125</sup>I. In a few electrical stimulus protocols, cGMP was measured directly from 100- to 200- $\mu$ l perfusate samples. In the experiments with exogenous NE perfusion, 10-ml samples were concen-

trated by passage through C-18 Sep-Pak columns (Merck), eluted with 2 ml of methanol, evaporated, and resuspended in 1 ml of RIA buffer for determination of cGMP (31).

#### Animal and Drug Sources

Male Sprague-Dawley rats (250–300 g) were bred in the animal facilities of our faculty. Experiments were conducted in accordance with the Helsinki declaration on research involving animals and human beings. Protocols complied with the guiding principles in the care and use of laboratory animals endorsed by the American Physiological Society and were approved by the Internal Animal Care and Use Committee of the Pontifical Catholic University of Chile. Saponin, *N*<sup>ω</sup>-nitro-L-arginine, guanethidine sulfate, NE, prazosin, and yohimbine hydrochloride were purchased from Sigma (St. Louis, MO).

#### Data Analysis

Time-course experiments were analyzed using two-way ANOVA. Paired or unpaired Student's *t*-test and regression analysis were used to compare differences between groups. Dunnett tables for multiple comparisons with a common control were used when appropriate. Significance was set at a probability of  $P < 0.05$ .

## RESULTS

### Release of NE, NO, and cGMP After Electrical Depolarization of Perivascular Mesenteric Nerves

Baseline levels of 0.1 pmol/ml NE, 40–80 pmol/ml NO, and 80–150 fmol/ml cGMP were detected in the tissue perfusate; basal perfusion pressure was 20–25 mmHg. The time course of a representative experiment showing lumenally accessible NE, NO, and cGMP in the perfusate is depicted in Fig. 1. A 30-Hz stimulus for 1 min evoked an abrupt and sustained increase in the perfusion pressure, which returned to baseline immediately after the stimulation was ended. The NE peak was sharp, reaching its maximum during the stimulus, and faded to baseline mainly within the next 2 min. There was a NO surge that rose for at least 2 min, and its maximum was usually detected the minute after the stimulation. The cGMP peak was slightly more prolonged compared with NO, suggesting that these mediators are released in sequence (Fig. 1).

The accuracy and reproducibility of the measurements of the NO release in response to electrical stimulation were confirmed when the values obtained in all the experimental protocols were compared. The time course of NO release is shown in Fig. 2. The NO surge ended 5 min after the stimulus ( $P < 0.07$  vs. average baseline, paired *t*-test,  $n = 28$ ). The average integrated release of NE and NO is shown in Table 1; the associated increase in cGMP was  $\sim 1$  pmol ( $n = 3$ ). In controls, the second electrical stimulus induced a vasomotor response and peaks of NE and NO release similar to those attained during the first stimulation, indicating the reliability of the preparations over time (Table 1).

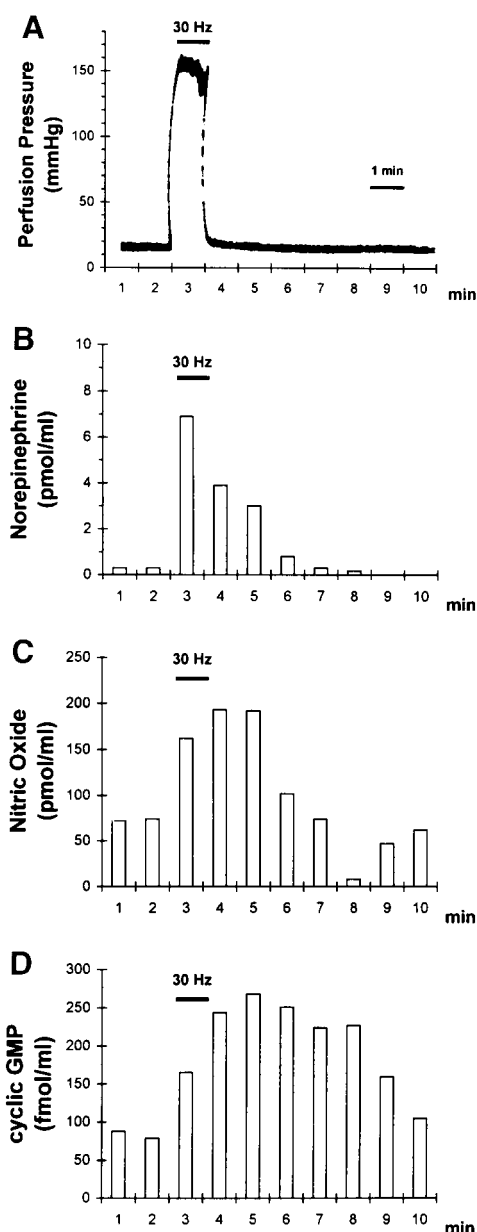


Fig. 1. Representative experiments show that electrical depolarization of perivascular mesenteric arterial nerve terminals increases perfusion pressure and lumenally accessible release of norepinephrine (NE), nitric oxide (NO), and cGMP. A 1-min train of 30-Hz (50 V, 1 ms) electrical pulses (horizontal bar) caused an abrupt increase in perfusion pressure that was sustained during stimulation (A). B, C, and D: concomitant changes in NE, NO, and cGMP, respectively, recovered in perfusate derived from a single perfused rat mesentery.

### Influence of Frequency and Duration of Electrical Nerve Stimulation

The electrically evoked increase in perfusion pressure and the associated NO surge were proportional to the frequency of nerve stimulation. One-minute trains of 5-, 10-, 20-, and 30-Hz stimuli caused a graded increase in perfusion pressure of  $5 \pm 0$ ,  $20 \pm 3$ ,  $61 \pm 18$ , and  $90 \pm 16$  mmHg, respectively, with a parallel rise of the integrated NO release recovered in the perfusate (Fig. 3). A 1-min, 2.5-Hz train did not change perfusion



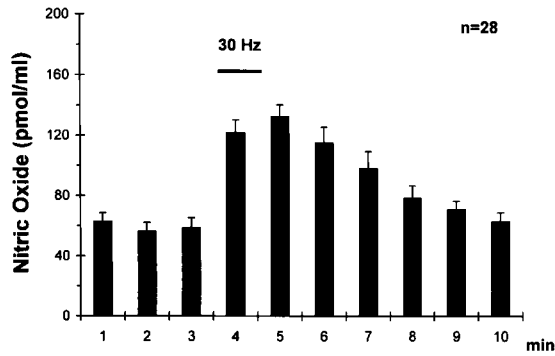


Fig. 2. Time course of NO release. Luminally recovered NO obtained in 28 separate experiments in rat arterial mesenteric bed electrically stimulated 1 min with 30 Hz (50-V, 1-ms pulses, horizontal bar). Values are means  $\pm$  SE. Time-course changes in NO release were highly significant according to 2-way ANOVA ( $F_{(9,243)} = 20.8, P < 10^{-25}$ ).

pressure or basal NO. A significant correlation was observed between the increase in perfusion pressure and the integrated peak of NO released ( $r = 0.73, P < 0.01$ ).

A 0.5-min, 30-Hz stimulation resulted in almost undetectable NO release compared with the 1-min stimulus that attained the maximum release. This stimulus induced a brief peak of  $74 \pm 13$  mmHg rise in perfusion pressure. Interestingly, increasing the duration of the stimulus to 2 or 4 min did not increase the total NO recovered (Fig. 3) or change the maximal rise in perfusion pressure ( $107 \pm 14$  and  $100 \pm 6$  mmHg, respectively).

*Role of Endothelial Cell Layer*

Removal of the endothelium with saponin resulted in a significant increase in the baseline NE released to the lumen ( $P < 0.05$ , paired *t*-test; Fig. 4). However, NE release evoked by the electrical stimulus was comparable, both in time course and the integrated total, to that attained in the controls (Fig. 4, Table 1). Although baseline NO release showed a tendency to decrease, the

Table 1. Change in perfusion pressure and total release of luminally accessible NO and NE after 30-Hz, 1-min electrical stimulation of perivascular arterial mesenteric nerves

Treatment	$\Delta$ Pressure, mmHg	NO, pmol	<i>n</i>	NE, pmol	<i>n</i>
First stimulus	$97 \pm 7$	$444.8 \pm 47.6$	28	$23.2 \pm 3.4$	8
Krebs-Ringer controls	$113 \pm 26$	$425.8 \pm 189.0$	6	$26.9 \pm 3.3$	6
Saponin	$90 \pm 18$	$152.8 \pm 76.4^*$	4	$26.8 \pm 3.4$	4
<i>N</i> <sup>ω</sup> -nitro-L-arginine (100 $\mu$ M)	$110 \pm 13$	$168.2 \pm 43.4^*$	6		
Guanethidine (1 $\mu$ M)	$1 \pm 0$	$44.4 \pm 24.8^\dagger$	4	ND	4
Prazosin (30 nM)	$2 \pm 0$	$-73.8 \pm 18.2^\dagger$	4		
Yohimbine (1 $\mu$ M)	$36 \pm 11$	$193.0 \pm 48.4^\dagger$	5		

Values are means  $\pm$  SE; *n* = no. of preparations. NO, nitric oxide; NE, norepinephrine; ND, not detectable. \* $P < 0.05$ ,  $^\dagger P < 0.01$  compared with saline-treated control preparations (Student's *t*-test with Dunnett tables for multiple comparisons with a single control).

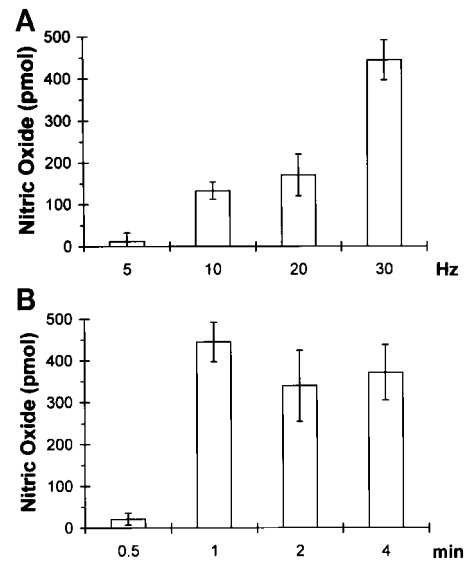


Fig. 3. Effect of frequency and time of electrical stimulation on NO release. A: separate preparations were electrically stimulated during 1 min with trains of 5, 10, 20, and 30 Hz (50-V, 1-ms pulses). Bars show integrated, total NO released above baseline. Five rats were used per determination, except for 30-Hz stimulus, which represents 28 determinations. B: influence of duration of 30-Hz stimuli on perfusate NO release. Five rats were utilized per experimental condition, except for 1-min protocol ( $n = 28$ ). Values are means  $\pm$  SE.

integrated peak of NO elicited by the stimulus was abolished (Fig. 4, Table 1). The rise in perfusion pressure was not altered (Table 1). Light microscopy revealed that saponin induced endothelial shedding without muscle edema or other alterations (not shown), in agreement with our previous report (15).

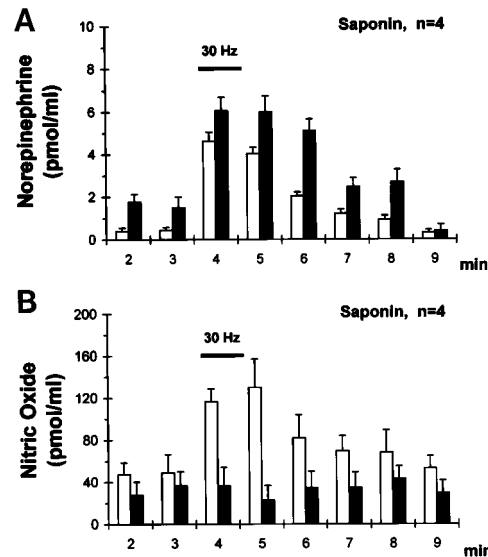


Fig. 4. Effect of endothelial denudation. Time course of luminally accessible NE (A) and NO (B) release before, during, and after a 1-min, 30-Hz stimulus (horizontal bar). Open bars, values obtained in control conditions; filled bars, values obtained 30 min after tissue perfusion with 0.1% saponin for 55 s. Values are means  $\pm$  SE. Two-way ANOVA indicated that time-course changes in NE release were highly significant in both conditions ( $P < 0.005$ ). NO release changed significantly in control ( $F_{(9,27)} = 4.19, P < 0.002$ ) but not after saponin [ $F_{(9,27)} = 0.49$ , not significant (NS)].

### Inhibition of NO Synthase

In the electrical stimulation experiment, treatment with 100  $\mu\text{M}$   $N^{\omega}$ -nitro-L-arginine tended to reduce baseline NO release and blunted the evoked luminal NO release (Fig. 5, Table 1). In parallel trials, a 1-min electrical stimulation of buffer containing 100  $\mu\text{M}$   $N^{\omega}$ -nitro-L-arginine produced a large artifact signal, which was not detected in the control buffer. This interference fully accounts for the residual NO peak elicited by the stimulus (Fig. 5).

The inhibitor did not change baseline perfusion pressure or the electrically evoked vasoconstriction (Table 1). However, in the course of the 4-min, 30-Hz stimulation protocols, we noticed that perfusion pressure was not maintained. To assess the influence of NO release in this effect, we conducted similar experiments during NO synthase blockade. As shown in Fig. 5, the pressor response was prolonged in the presence of 100  $\mu\text{M}$   $N^{\omega}$ -nitro-L-arginine. More evident effects of NO synthase inhibition on perfusion pressure and NO release were observed in the experiments using exogenous NE.

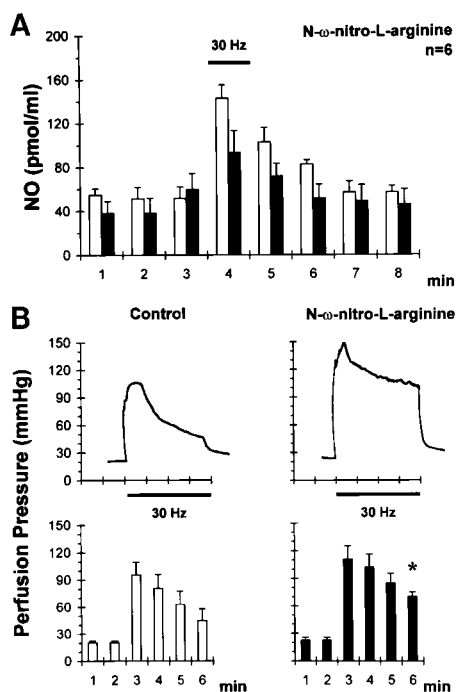


Fig. 5. Influence of  $N^{\omega}$ -nitro-L-arginine on release of NO. *A*: luminal NO release induced by a 1-min, 30-Hz electrical stimulation (horizontal bar) before (open bars) and after mesentery perfusion with 100  $\mu\text{M}$   $N^{\omega}$ -nitro-L-arginine (filled bars). Values are means  $\pm$  SE. Time course of NO release changed significantly in control ( $F_{(9,45)} = 12.3$ ,  $P < 10^{-8}$ ) and after NO synthase inhibition ( $F_{(9,45)} = 5.31$ ,  $P < 10^{-4}$ , 2-way ANOVA). *B*: changes in perfusion pressure elicited by a 4-min, 30-Hz stimulation in control and 100  $\mu\text{M}$   $N^{\omega}$ -nitro-L-arginine-treated mesenteries. For each condition, a representative polygraph tracing and 1-min average of 6 separate experiments are shown. Although in control condition pressor response fades to 31% of its maximum, in presence of inhibitor, perfusion pressure faded only to 54%. At end of stimulation period, perfusion pressure was significantly higher in  $N^{\omega}$ -nitro-L-arginine-treated mesenteries ( $*P < 0.05$ , unpaired Student's *t*-test).

Table 2. Change in perfusion pressure and total release of lumenally accessible NO induced by 1-min perfusion with 10  $\mu\text{M}$  NE

Treatment	$\Delta$ Pressure, mmHg	NO, pmol	<i>n</i>
Control	78.3 $\pm$ 10.5	350.7 $\pm$ 84.1	6
$N^{\omega}$ -nitro-L-arginine (100 $\mu\text{M}$ )	121.3 $\pm$ 10.7*	18.1 $\pm$ 61.3*	4
Guanethidine (1 $\mu\text{M}$ )	90.0 $\pm$ 5.1	295.3 $\pm$ 92.5	3
Yohimbine (1 $\mu\text{M}$ )	25.0 $\pm$ 6.5†	141.0 $\pm$ 34.1*	6

Values are means  $\pm$  SE; *n* = no. of preparations. \* $P < 0.05$ , † $P < 0.01$  compared with control (unpaired Student's *t*-test).

### Effect of NE

Mimicking the effect of the electrical stimulus, a 1-min perfusion with 10  $\mu\text{M}$  NE produced a sustained rise in perfusion pressure associated with an increase in NO release (Table 2). The time course of pressure and NO changes was similar to that attained during 1-min electrical stimulation (Fig. 6).

Perfusion with 100  $\mu\text{M}$   $N^{\omega}$ -nitro-L-arginine resulted in a significant reduction of baseline NO ( $57.6 \pm 8.1$  vs.  $88.3 \pm 3.2$  pmol/ml,  $P < 0.02$ ). In addition, the NE-induced contractile response was significantly enhanced, whereas the associated NO peak was completely suppressed (Fig. 6, Table 2).

Similar to the electrical stimulation, exogenous NE also increased luminal release of cGMP, the second messenger of NO. Perfusion with 10  $\mu\text{M}$  NE for 10 min produced a sustained pressure increase and doubled cGMP release (Table 3). NO synthase inhibition resulted in a significant reduction of baseline cGMP release. Moreover, the cGMP rise induced by 10  $\mu\text{M}$  NE was abolished, whereas the vasomotor response elicited by NE was significantly increased (Table 3).

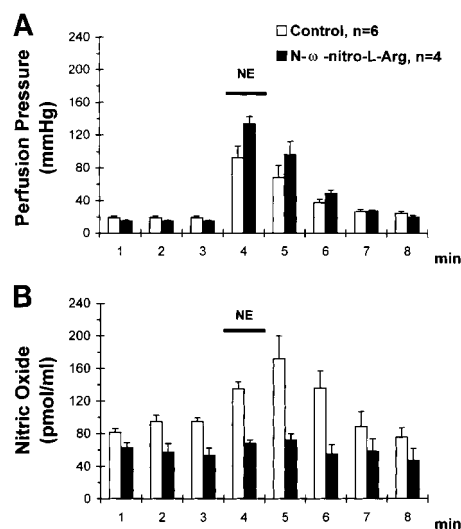


Fig. 6. Effect of exogenous NE. Time-course changes in perfusion pressure (*A*) and NO release (*B*) induced by 1-min perfusion with 10  $\mu\text{M}$  NE (horizontal bar). Open bars, control mesenteries; filled bars, tissues perfused with 100  $\mu\text{M}$   $N^{\omega}$ -nitro-L-arginine. Changes in perfusion pressure were highly significant in control ( $F_{(7,35)} = 17.8$ ,  $P < 10^{-9}$ ) and during NO synthase inhibition ( $F_{(7,21)} = 49.2$ ,  $P < 10^{-10}$ ). Changes in NO release were significant only in control ( $F_{(7,35)} = 5.95$ ,  $P < 0.0002$ ).

**Table 3.** Effect of NE and *N*<sup>ω</sup>-nitro-L-arginine on cGMP release and perfusion pressure in rat mesenteric arterial bed

	<i>n</i>	cGMP, fmol/ml		Perfusion Pressure, mmHg	
		Baseline	10 μM NE	Baseline	10 μM NE
Control	6	119.2 ± 8.2	275.7 ± 30.3*	22.9 ± 3.1	77.1 ± 10.5*
<i>N</i> <sup>ω</sup> -nitro-L-arginine	4	54.2 ± 7.6‡	52.8 ± 10.9‡	25.3 ± 1.0	187.5 ± 7.2‡‡

Values are means ± SE; *n* = no. of preparations. Significant differences from baseline: \**P* < 0.005, †*P* < 0.0001 (paired Student's *t*-test); ‡significant differences from respective control without *N*<sup>ω</sup>-nitro-L-arginine (*P* < 0.005, unpaired Student's *t*-test).

### Guanethidine-Induced Acute Chemical Sympathectomy

Luminal NE recovery during perfusion with guanethidine was undetectable (Table 1), confirming our previous report (14). Consequently, the elevation in perfusion pressure evoked by electrical stimulation was obliterated (Table 1). Baseline NO release was unchanged, but the NO surge after electrical stimulation was abolished (Fig. 7 and Table 1). In contrast, guanethidine did not affect vasoconstriction or luminal NO release induced by 1-min perfusion with 10 μM NE (Table 2), indicating that this drug does not interfere with NO synthase activity.

### α<sub>1</sub>-Adrenoceptor Blockade

Prazosin did not affect the baseline release of NO; however, it obliterated the increase in perfusion pressure and the surge of the accessible NO elicited by the electrical stimulus (Fig. 7, Table 1).

### α<sub>2</sub>-Adrenoceptor Blockade

Yohimbine did not alter baseline NO release but significantly reduced the electrically evoked peak of luminal NO and the associated increase in perfusion pressure by 65 to 80% (Fig. 7, Table 1). Individual assessment of NO variation revealed a significant increase, which amounted to 37 ± 20% above baseline, the minute after the stimulus (*P* < 0.05 vs. baseline, paired *t*-test). In a separate series of experiments, 1 μM yohimbine significantly increased the NE release elicited by a 1-min, 20-Hz electrical stimulus (25.1 ± 5.6 vs. 6.23 ± 0.35 pmol, *n* = 5). Exactly as with the electrical stimulation, 1 μM yohimbine blunted in the same proportions the vasocontractile response and NO release induced by exogenous NE (Table 2).

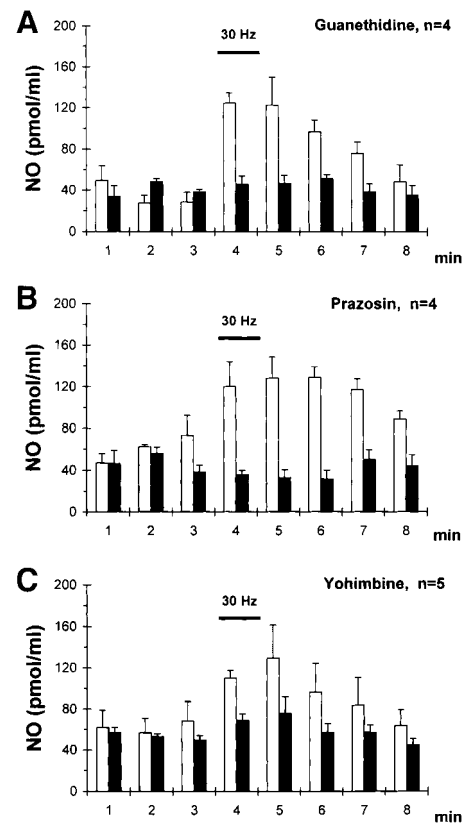
## DISCUSSION

The main finding of this investigation is that electrical stimulation of the perivascular arterial mesenteric nerves of the rat results in endothelial NO release followed by a time-integrated increment in cGMP release. The temporal sequence of the changes in perfusion pressure and NE, NO, and cGMP release allows us to invoke a cause-effect cascade of chemical signals that are triggered by the electrical stimulus. We conclude that the NO release is most likely a NE-driven, vasocon-

striction-mediated response, because the increases in perfusion pressure and NO release are elicited by exogenous NE and suppressed by sympathetic blockade.

### Technical Considerations

We chose to investigate the possible relations between nerve stimulation and NE, NO, and cGMP release in the isolated arterial mesenteric bed because this preparation allows for monitoring vascular tone and direct access to chemicals released to the vascular lumen (14, 16). In addition, neurotransmitter release in response to electrical stimulation of sympathetic nerves has been characterized previously (14, 25). The term lumenally accessible NE, NO, and cGMP was used to indicate that our measurements account for only a fraction of the total mediators released by the vascular wall to the lumen. The recovered fraction represents the sum of mediators released directly into the lumen plus diffusion from the interstitial space to the lumen.



**Fig. 7.** Blockade of sympathetic mechanisms reduces NO release elicited by electrical stimulation. Two trains of 1-min, 30-Hz electrical stimuli (horizontal bars) were delivered before (control, open bars) and in presence of 1 μM guanethidine (A), 30 nM prazosin (B), or 1 μM yohimbine (C) (filled bars). Each drug was perfused 30 min before second stimulus, and it was maintained in buffer until completion of protocol. Values are means ± SE. Two-way ANOVA indicated that electrical stimulus produced a significant rise in NO release in all control series (*P* < 0.0001). Although in presence of guanethidine and prazosin release of NO was abolished ( $F_{(9,27)} = 1.10$  and  $F_{(9,27)} = 1.11$ , respectively, *P* = NS), in the presence of yohimbine the residual increase in NO release was significant ( $F_{(9,36)} = 2.72$ , *P* < 0.02).

Measurements of NO are burdened with multiple technical problems. To date, chemiluminescence is the most specific, sensitive, and accurate method available for NO detection (3). However, the short half-life of NO in oxygen-rich biological fluids requires the measurement of NO oxidized products, nitrites and nitrates. To minimize background noise, we opted to use mild reducing conditions (KI/CH<sub>3</sub>COOH) that only reduce nitrites to NO (30, 32). In our hands, the use of strong reducing conditions (12) resulted in a low signal-to-background ratio because of the presence of chemicals containing amino groups that interfered with the measurement. Therefore, our measurements underestimate the total amount of NO released, because we did not detect the NO fraction further oxidized to nitrate.

The reproducibility of our measurements is highlighted by the small standard error obtained in baseline and stimulus-evoked NO determined in a large series of separate preparations. This indicates that our measurements accurately account for the changes of NO generated by the vascular wall within a minute-to-minute temporal resolution. Furthermore, the basal and stimulated luminal NO values we report in this study seem to be physiologically meaningful, because endothelium-dependent vasodilators induce NO increases of similar magnitude (18). Pilot experiments using authentic NO dissolved in the perfusing medium show that threshold arterial relaxation can be observed when 10–20 pmol/ml of NO are infused.

Perfusate cGMP levels were near detection threshold, making it difficult to regularly assess the minute-to-minute changes in the nucleotide simultaneously with NO. However, an equivalent cGMP production rate was detected after we extracted 10-ml perfusate samples. Thus our cGMP measurements likely correspond to the outflow fraction of this intracellular nucleotide produced before, during, and after activation of the vascular wall soluble guanylyl cyclase by NO (5).

NE recovery is influenced by the endothelial diffusion barrier and by specific cellular uptake mechanisms. Bitran and Tapia (6) recently showed that in the sympathetic neurons of the vas deferens, close to 80% of the released NE is recaptured by the sympathetic varicosities. In the present experiments, we avoided the use of drugs that interfere with the mechanism of NE recapture, in an effort to study the sympathetic nerves under their most physiological conditions.

#### *Source and Mechanisms for Electrically Elicited NO Release*

Our experiments with endothelium denudation after saponin treatment support the endothelial origin of the NO surge after transmural depolarization. This experimental maneuver abolished the NO increase elicited by electrical stimulation while maintaining muscular reactivity. The finding that the total amount of NE release elicited by electrical stimulation was unchanged by saponin demonstrates that the detergent did not alter the nerve terminals or the mechanism of the NE exocytosis. On the other hand, the increase in baseline luminal NE recovery may be explained by a faster

transit of the neurotransmitter into the arterial lumen in absence of the major diffusion barrier posed by the endothelium, altering the kinetics of cellular NE recapture. In addition, we demonstrated that the NO surge depends on NO synthase activation, because inhibition of this enzyme with N<sup>ω</sup>-nitro-L-arginine abolished NO release evoked by exogenous NE. In the case of electrical stimulation, an artifact signal attributable to the electrolytic formation of a nitritelike chemical is responsible for the apparent rise in NO release.

The mechanisms underlying the basal levels of luminal NO remain only partially known. Endothelium removal and NO synthase inhibition reduced basal NO release, although not always significantly. However, neither maneuver abolished baseline NO release, possibly reflecting a contribution of nitrites and nitrosothiols not originating from NO (3). The present results do not allow us to fully discard a possible contribution of remnant functional endothelial cells, residual NO synthase activity, or the presence of resident macrophages within the mesenteric smooth muscles. We deem unlikely any significant expression of inducible NO synthase occurring in the 60–90 min lapsing between setting the preparation and the experimental maneuvers. Whatever the source of the remnant NO measured, it is most likely that the fraction sensitive to saponin treatment and/or NO synthase inhibition is the physiologically relevant fraction.

Chemical sympathectomy and  $\alpha$ -adrenoceptor blockade experiments argue against the possibility that NO is derived from sympathetic nerves or from the vascular smooth muscle cells. As expected, neither chemical sympathectomy nor  $\alpha$ -adrenoceptor blockade affected basal NO release. Guanethidine abolished the vasopressor activity and NE release evoked by electrical stimulation, confirming previous reports in the rat mesenteric arterial bed (14). We now demonstrate that guanethidine also abolishes the increase in NO release after transmural depolarization. Guanethidine blocks exocytosis of sympathetic vesicles (10), but it would not prevent a rise in cytosolic calcium or other depolarization-induced changes that may affect neuronal NO synthase in the sympathetic terminals, perivascular nonadrenergic-noncholinergic nerve fibers (22), or nitrergic nerves (35). Furthermore, it is well established that this concentration of guanethidine does not modify smooth muscle contractility (14, 16). The finding that guanethidine did not affect either the motor response or the NO release induced by exogenous NE rules out any interference of this drug on NO synthase. Therefore, the negligible NE exocytosis and lack of vasoconstriction explain the lack of NO release after transmural depolarization in the presence of guanethidine.

A more detailed assessment of the mechanisms involved in endothelium release of NO can be derived from the results with  $\alpha$ -adrenoceptor blocking drugs. Prazosin, an  $\alpha_1$ -adrenoceptor antagonist, abolished electrically evoked vasoconstriction and NO release. Prazosin does not preclude the release of NE or other sympathetic transmitters or the putative release of NO from any type of nervous terminals. Therefore, the



result with prazosin supports the concept that electrically evoked NO release is secondary to vasoconstriction and renders it unlike any possible direct effect of electrically released NE on endothelial  $\alpha_2$ -adrenoceptors (40). This conclusion is also supported by the finding that 1  $\mu$ M yohimbine, an  $\alpha_2$ -adrenoceptor blocker, reduced in the same proportion vasoconstriction and NO release elicited either by electrical stimulation or exogenous NE. The efficacy of this concentration of antagonist on presynaptic sympathetic  $\alpha_2$ -adrenoceptors was confirmed by the increased NE released on electrical depolarization. However, because it reduced the vasoconstriction, this concentration of yohimbine probably also partially blocked  $\alpha_1$ -adrenoceptors present on the rat mesenteric smooth muscle (4), as well as postjunctional  $\alpha_2$ -adrenoceptors that may contribute to vasoconstriction in this tissue (C. Meynard and J. P. Huidobro-Toro, unpublished observations).

### Physiological Implications

We interpret the NO release as an endothelial response secondary to vasoconstriction. In chronological sequence, electrical stimulation elicits NE release that activates adrenergic receptors, leading to smooth muscle contraction and increased shear stress at the endothelial surface. Under constant flow, the elevation in shear stress may serve as the efficacious stimulus for endothelial NO synthase activation and NO release (12). Alternatively, smooth muscle activation after adrenergic stimulation may result in a rise of cytosolic calcium in endothelial cells through gap junction intercellular communication between both cell types (17). Our experiments support the notion that arteriolar NO release is sensitive to changes rather than to sustained levels of shear stress and/or vascular tone. With 1-min electrical stimulation, a clear, graded, frequency-dependent NO release proportional to the rise in perfusion pressure was observed. However, when the duration of the stimulus was changed, an apparent all-or-none NO release was evidenced after a 0.5-min threshold.

Whatever the direct stimulus for endothelial NO synthase activation, the subsequently produced NO induces an increase in cGMP. The physiological response is a vasodilatory signal that counteracts the vasoconstriction elicited by NE. Evidence in favor of this counteracting mechanism is provided by the results observed in NO synthase-inhibited mesenteries, which showed prolonged vasoconstriction during 4-min electrical stimulation and a notable enhancement of the vasoconstriction induced by exogenous NE. These findings confirm what has been repeatedly shown in endothelium-denuded, isolated vascular rings that respond more vigorously to exogenous NE (13, 20, 26, 29). In intact mesenteries, the reduction in perfusion pressure and shear stress may contribute to the lack of further increase in NO release observed during 2- and 4-min stimulation.

We should also consider that ATP and NPY are coreleased with NE. ATP is well known as an endothelium-dependent vasodilator, which putatively acts through release of NO (9, 24, 25, 37). Therefore, it is

possible that in vivo, in addition to the increased shear stress, NE and its cotransmitters may participate as a driving mechanism for endothelial NO production during sympathetic stimulation. For instance, we have observed a NO synthase-dependent hyperemia after NE-induced vasoconstriction in the hamster microcirculation, which is enhanced by NPY (8). Nevertheless, our results with  $\alpha$ -adrenoceptor blockers rule out any significant vasoconstriction-independent participation of these sympathetic cotransmitters on electrically elicited NO release in the isolated, perfused mesentery.

In summary, our measurements of NE, NO, and cGMP release in the mesenteric arterial bed give a quantitative assessment of how sympathetically induced vasoconstriction elicits a secondary endothelial response that buffers the contractile efficacy of the neurotransmitters.

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