Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 2166–2170, March 1991
Physiology/Pharmacology

Development and mechanism of a specific supersensitivity to nitrovasodilators after inhibition of vascular nitric oxide synthesis *in vivo*

(vessel wall/guanylate cyclase)

S. MONCADA*, D. D. REES, R. SCHULZ, AND R. M. J. PALMER

Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS, United Kingdom

Communicated by Robert F. Furchgott, December 12, 1990 (received for review July 4, 1990)

ABSTRACT **The mechanism of the increased sensitivity to nitrovasodilators after removal of endothelial nitric oxide (NO) was investigated *in vitro* and *in vivo*. The vasoconstrictor potency of phenylephrine and the force of contraction of rat isolated aortic rings were significantly enhanced after endothelium removal or treatment with inhibitors of endothelial NO synthase. Furthermore, these procedures led to a significant decrease in the basal levels of cGMP in the vascular rings. Moreover, the potency of glyceryl trinitrate (n_3Gro) and sodium nitroprusside (SNP) as relaxing agents and the ability of SNP to induce increases in cGMP in aortic rings were significantly enhanced in those rings denuded of endothelium or treated with the inhibitors. These procedures did not affect the vasodilator actions of isoprenaline or 8-bromo-cGMP. In the anesthetized rat, treatment with the inhibitors enhanced significantly the hypotensive responses to n_3Gro without affecting those to isoprenaline. These data indicate that the removal of the basal NO-mediated vasodilator tone in the cardiovascular system leads, at the level of the soluble guanylate cyclase, to a specific supersensitivity to nitrovasodilators *in vivo*. The existence of such a phenomenon has important implications for understanding the local physiological control of blood flow, its pathological disturbances, and the mechanism of action of nitrovasodilators.**

The vascular endothelium synthesizes nitric oxide (NO) from L-arginine (1, 2). This NO accounts for the actions of endothelium-derived relaxing factor (3) and acts via the stimulation of the soluble guanylate cyclase in the vascular smooth muscle (4). The soluble guanylate cyclase may be considered the intracellular receptor of NO (5). Inhibition of the synthesis of NO by N^G-monomethyl-L-arginine (L-NMMA; refs. 6 and 7) and other arginine analogues (8, 9) induces endothelium-dependent contraction of vascular tissue *in vitro* and a dose-related hypertensive response (for review, see ref. 10). Furthermore, L-NMMA decreases conductance in several vascular beds in animals (11) and reduces blood flow in the arterial circulation of humans (12). These observations show that the basal release of endothelium-derived relaxing factor/NO (7, 13) is responsible for maintaining a vasodilator tone in the cardiovascular system (14). The removal of a basal mediator tone in other tissues, most notably innervated structures, frequently leads to a specific supersensitivity to the exogenous application of that mediator (15, 16), a phenomenon recognized over 100 years ago (17). Based on this, it is possible to hypothesize that the removal of NO in the vasculature and the consequent up-regulation of its receptor might lead to an increase in the sensitivity to those vasodilators that act by stimulating the soluble guanylate cyclase.

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Increased sensitivity to sodium nitroprusside (SNP), glyceryl trinitrate (n_3Gro), or 3-morpholinosydnonimine (SIN-1) has been described *in vitro* in deendothelialized vascular tissue (18) or after treatment with nonspecific (19) or specific inhibitors of NO synthesis (20–22). At present, there is no clear explanation for this increased sensitivity. It has been suggested that basally released NO reduces the sensitivity to NO derived from SIN-1 (20) or down-regulates the increase in cGMP in the smooth muscle at a site distal to the formation of cGMP (21). Another suggestion is that the increased sensitivity is due to the inactivation of the NO derived from SIN-1 by oxygen radicals formed by the endothelium, together with an unknown inhibitory interaction between NO and SIN-1 (22).

In view of all this, we have investigated the mechanism of this increased sensitivity *in vitro* in aortic rings of the rat and *in vivo* in anesthetized rats and have shown that the absence of NO in the vasculature *in vivo* leads to a specific supersensitivity to nitrovasodilators related to an up-regulation of the soluble guanylate cyclase.

METHODS

Organ Bath Studies. Male Wistar rats (250–300 g) were killed by a blow on the head and exsanguination. Rings of the thoracic aorta, with and without endothelium, were prepared and their tone was studied in the presence of indomethacin (5 μM) as described (9). Experiments were always carried out on two sets of paired rings. Each set consisted of one ring with the endothelium intact and one denuded of endothelium. One of the sets was pretreated for 15 min with one of three inhibitors of NO synthase—namely, L-NMMA, *N*-iminoethyl-L-ornithine (L-NIO), or N^G-nitro-L-arginine methyl ester (L-NAME).

For studies in which the contractile activity of phenylephrine was investigated, a cumulative concentration–response curve was carried out in all rings. In rings treated with an inhibitor or in rings denuded of endothelium, 10–40 nM phenylephrine induced a similar submaximal tension (≈3 g) to that induced by 750 nM phenylephrine in untreated rings with intact endothelium (see Fig. 1). Because of this, the studies on the relaxations induced by SNP, n_3Gro, isoprenaline, and 8-bromo-cGMP were carried out in the presence of 10–40 nM phenylephrine for rings treated with the inhibitor or denuded of endothelium and 750 nM phenylephrine for untreated rings with intact endothelium.

***In Vivo* Studies.** Male Wistar rats (250–300 g) were anesthetized with sodium thiobutabarbital (120 mg·kg^{-1}; i.p.) and

Abbreviations: L-NMMA, N^G-monomethyl-L-arginine; L-NIO, *N*-iminoethyl-L-ornithine; L-NAME, N^G-nitro-L-arginine methyl ester; ACh, acetylcholine; SNP, sodium nitroprusside; n_3Gro, glyceryl trinitrate.

*To whom reprint requests should be addressed.

prepared for intravenous administration of drugs as described (9). Phenylephrine (150–300 $\mu g \cdot kg^{-1} \cdot hr^{-1}$) was used to raise the blood pressure to a level similar to that induced by L-NMMA, L-NIO, and L-NAME (all at 100 $mg \cdot kg^{-1}$). The hypotension induced by bolus injections of n_3Gro (3–30 $\mu g \cdot kg^{-1}$) and isoprenaline (0.01–0.1 $\mu g \cdot kg^{-1}$) was measured as the area comprising the fall and duration of the systolic pressure and was determined by computerized planimetry.

cGMP Measurements. Aortic rings were isolated as described above and allowed to equilibrate in microcentrifuge tubes for 2 hr in Krebs buffer gassed with 95% O_2/5% CO_2 at 37°C containing indomethacin (5 μM). The buffer was replaced with fresh Krebs buffer containing the specific cGMP phosphodiesterase inhibitor M&B22948 (3 μM; ref. 23) and saline or L-NMMA, L-NIO, or L-NAME (100 μM) was added. After a further 40 min, SNP, acetylcholine (ACh), or Krebs buffer was added for 1 min and the tissue was rapidly frozen in liquid nitrogen. The frozen tissues were homogenized in a stainless steel pestle and mortar, which was cooled in dry ice. The homogenate was added to ice-cold perchloric acid (0.5 M) and centrifuged for 1 min at 10,000 $\times g$ at least 1 hr later. The supernatant was removed and neutralized with 1.08 M K_3PO_4 and centrifuged as described above; cGMP levels were determined, in duplicate, by specific radioimmunoassay. The protein content of the homogenate pellet was measured after boiling for 10 min in 2 M NaOH with BCA protein reagent and bovine serum albumin used as a standard.

Chemicals. Isoprenaline HCl, 8-bromo-cGMP, SNP, ACh, indomethacin, phenylephrine, L-NAME (all from Sigma), sodium thiobutabarbital (Inactin, Byk-Guiden Pharmazeutika), n_3Gro (Tridil, American Critical Care, Puerto Rico), BCA protein reagent (Pierce), and M&B22948 (May & Baker, Dagenham, U.K.) were obtained as indicated. L-NMMA and L-NIO were synthesized as described (24, 25).

Statistics. Results are expressed as means ± SEM for n separate experiments. The EC_{50} values for each experiment were obtained from sigmoid logistic curves. Student's paired or unpaired t test or analysis of variance followed by Fisher's least significant difference test to compare individual means were used as appropriate. $P < 0.05$ was considered statistically significant.

RESULTS

Organ Bath Studies. The vasoconstrictor potency of phenylephrine and the force of contraction of rings were significantly enhanced after removal of the endothelium (Fig. 1). L-NMMA, L-NIO, and L-NAME (100 μM), which inhibit endothelium-dependent relaxation by >80% (9), induced a small, but significant, endothelium-dependent contraction of the rings to 5.2% ± 0.4%, 12.4% ± 1.2%, and 4.3% ± 0.5%, respectively (n = 9–12) of that induced in these rings by phenylephrine (750 nM). The vasoconstrictor potency and the force of contraction of rings were increased significantly by L-NIO and L-NAME to the same extent as that observed after endothelium removal (Fig. 1; Table 1). The potency of phenylephrine, but not the force of contraction, was also similarly enhanced by L-NMMA (Fig. 1, Table 1). None of these compounds affected these parameters in rings denuded of endothelium (n = 3 for each; data not shown).

In rings contracted to a similar level of tension with phenylephrine, the relaxations induced by n_3Gro and SNP were significantly enhanced in those without endothelium compared to those with intact endothelium (Fig. 2). The potency of n_3Gro and SNP in endothelium-denuded rings and in endothelium-intact rings treated with L-NMMA, L-NIO, or L-NAME was similar (Fig. 2; Table 2). The relaxations induced by isoprenaline and 8-bromo-cGMP were also enhanced, but to a lesser extent, by removal of the endothelium but were not significantly affected by treatment with

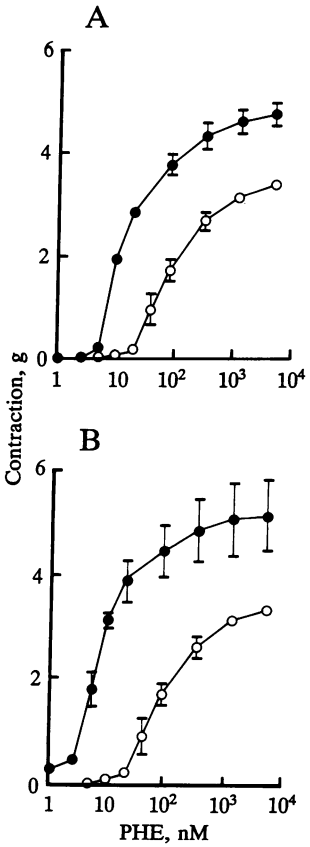

FIG. 1. Effect of endothelium removal (*A*) and L-NIO (100 μM) (*B*) on the contraction of rings of rat aorta induced by phenylephrine (PHE). ○, Responses to phenylephrine in untreated rings with endothelium. Both removal of endothelium and L-NIO increase the potency of phenylephrine and the force of contraction of the arterial rings. Each point is the mean ± SEM of three separate determinations. Similar results were obtained with L-NMMA and L-NAME (n = 3 for each; data not shown).

L-NMMA, L-NIO, or L-NAME (Table 2). Furthermore, none of these inhibitors affected the relaxations induced by n_3Gro, SNP, isoprenaline, or 8-bromo-cGMP in rings denuded of endothelium (n = 3 for each; data not shown).

***In Vivo* Studies.** L-NMMA, L-NIO, and L-NAME (100 $mg \cdot kg^{-1}$; i.v.) caused an increase in mean arterial blood pressure of 42.0 ± 3.0 (n = 7), 45.1 ± 5.1 (n = 8), and 43.6 ± 3.3 mmHg (n = 6), respectively. When the resting mean arterial pressure in untreated animals was raised to a similar level with phenylephrine (150–300 $\mu g \cdot kg^{-1} \cdot hr^{-1}$), these compounds increased significantly the hypotensive response to

Table 1. Effect of endothelium removal or treatment with L-NMMA, L-NIO, or L-NAME (100 μM each) on the potency and force of contraction of phenylephrine

Conditions	Phenylephrine EC_{50}, nM	Maximum tension, g
+ endothelium	104.0 ± 32.3	3.4 ± 0.1
− endothelium	12.2 ± 1.1*	4.8 ± 0.2*
+ endothelium		
+ L-NMMA	10.0 ± 0.9*	4.2 ± 0.5
+ L-NIO	7.9 ± 1.6*	5.2 ± 0.7*
+ L-NAME	10.9 ± 0.4*	4.1 ± 0.3*

The potency of phenylephrine is expressed as the EC_{50} (the concentration that produces 50% of the maximum response to phenylephrine). Representative data for the effect of endothelium removal and of L-NIO (100 μM) are shown in Fig. 1. Each value is the mean ± SEM of three separate determinations.
*$P < 0.05$ versus endothelium intact control.

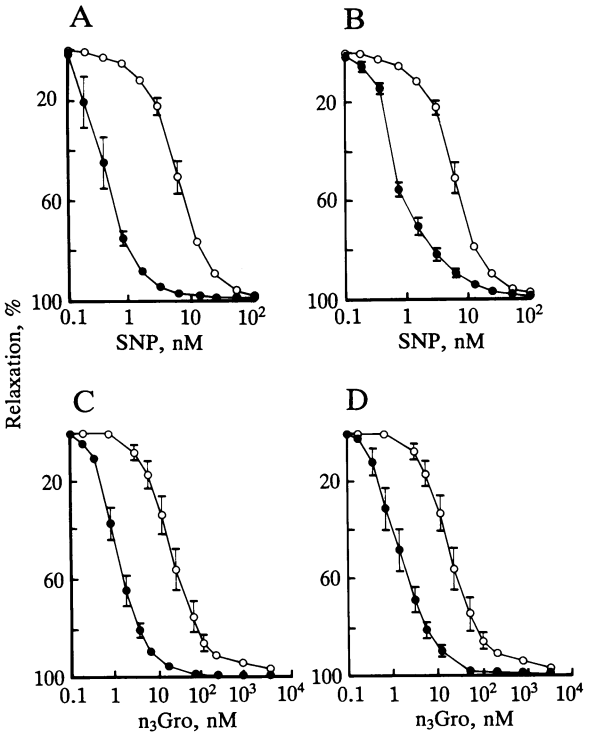

FIG. 2. Effect of endothelium removal (*A*) and L-NMMA (100 μM) (*B*) on the relaxation of rings of rat aorta induced by SNP, and effect of endothelium removal (*C*) and L-NAME (100 μM) (*D*) on the relaxation of rings of rat aorta induced by n_3Gro. ○, Responses to SNP or n_3Gro in untreated rings with endothelium. Each point is the mean ± SEM of three to six separate determinations.

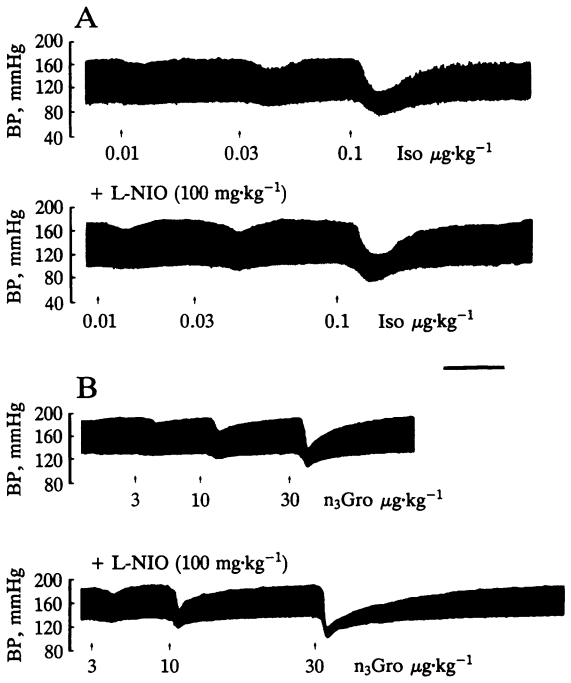

FIG. 3. Effect of L-NIO (100 mg·kg^{-1}) on the hypotension induced by isoprenaline (Iso; 0.01–0.1 μg·kg^{-1}) (*A*) and n_3Gro (3–30 μg·kg^{-1}) (*B*). The hypotensive responses to these compounds were compared under conditions where initial mean arterial pressure was similarly elevated by phenylephrine (control) or by L-NIO. Trace representative of three to five experiments and of a similar number with L-NMMA and L-NAME (both at 100 mg·kg^{-1}). Bar indicates 30 s. BP, blood pressure.

n_3Gro (Figs. 3 and 4) but not to isoprenaline (Fig. 3). This effect was observed within 10 min after administration of the compound. The absolute fall in mean arterial blood pressure induced by n_3Gro (30 μg·kg^{-1}) and isoprenaline (0.1 μg·kg^{-1}) was 40.11 ± 6.2 (n = 14) and 42.8 ± 2.8 (n = 19) mmHg, respectively. When measured as the area comprising the fall and duration of the systolic pressure, these values were 309.6 ± 35.8 and 285.3 ± 25.7 mm^2, respectively. These control values were taken as 100%.

cGMP Measurements. M&B22948 (3 μM) increased basal levels of cGMP in rings of rat aorta with intact endothelium from 4.7 ± 0.4 to 16.8 ± 2.0 pmol per mg of protein (n = 5). In the presence of M&B22948, the basal level of cGMP in aortic rings was significantly lower in those treated with L-NIO, L-NMMA, and L-NAME (100 μM) or denuded of endothelium. ACh (1 μM) enhanced significantly the level of cGMP in endothelium intact rings, without having any significant effect on denuded rings or endothelium intact rings treated with the inhibitors (Table 3).

SNP induced a concentration-dependent increase in cGMP that was significantly potentiated in endothelium-denuded rings (Table 4) The increase in cGMP induced by SNP was also significantly greater in endothelium intact rings treated with L-NMMA, L-NIO, and L-NAME (Table 4). L-NIO (100 μM) did not affect basal levels in rings denuded of endothelium and had no effect on the increase in cGMP in endothelium-denuded rings stimulated with 1 μM SNP (n = 5 for each; data not shown).

DISCUSSION

Removal of the endothelium from vascular rings led to an increase in the vasoconstrictor potency of phenylephrine, an increase in the force of contraction of the arterial rings, and a decrease in the basal levels of cGMP. These results are in agreement with previous findings (4, 26, 27) and are consistent with the removal of a basal release of NO that produces a vasodilator tone. This conclusion is supported by the

Table 2. Effect of endothelium removal or treatment with L-NMMA, L-NIO, or L-NAME (100 μM each) on the potency of n_3Gro, SNP, isoprenaline (Iso), and 8-bromo-cGMP (8-Br)

Conditions	n_3Gro, nM (n = 6)	SNP, nM (n = 3)	Iso, nM (n = 3)	8-Br, μM (n = 3–6)
+ endothelium	23.5 ± 5.8	6.5 ± 0.7	28.9 ± 3.8	12.3 ± 1.8
− endothelium	1.2 ± 0.1*	0.4 ± 0.2*	11.5 ± 2.9*	6.7 ± 0.5*
+ endothelium				
+ L-NMMA	3.3 ± 1.2*	0.7 ± 0.1*	31.3 ± 15.0	10.0 ± 1.7
+ L-NIO	2.2 ± 0.5*	0.8 ± 0.1*	36.9 ± 7.5	10.1 ± 1.3
+ L-NAME	1.7 ± 0.4*	0.8 ± 0.1*	29.0 ± 4.4	9.7 ± 1.2

The potency of n_3Gro, SNP, isoprenaline, and 8-bromo-cGMP is expressed as the EC_{50} (the concentration that produces 50% of the maximum response to each agonist). Representative data for the effect of endothelium removal, L-NAME, and L-NMMA on the relaxations induced by n_3Gro and SNP are shown in Fig. 2. Each value is the mean ± SEM of three to six separate determinations.
*$P < 0.05$ versus endothelium intact control.

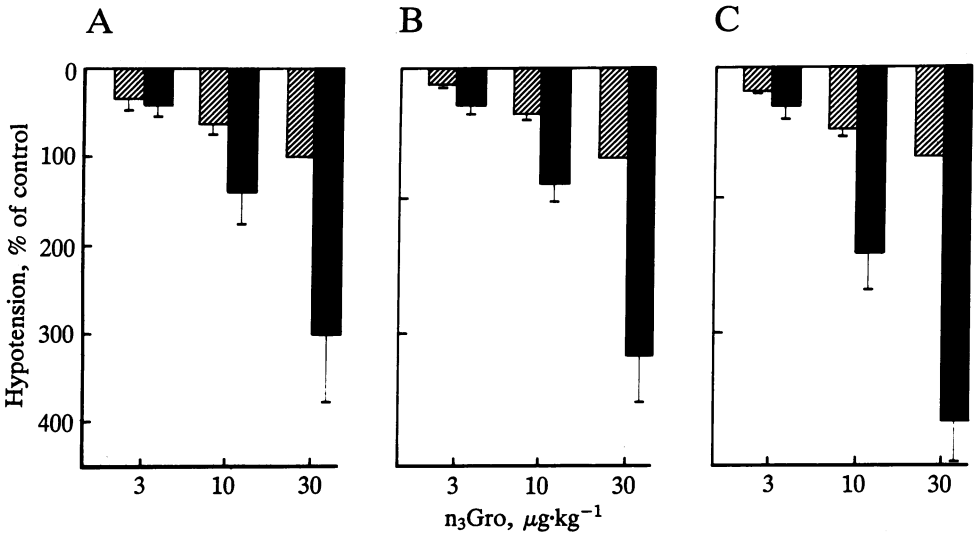

FIG. 4. Effect of L-NMMA (*A*), L-NIO (*B*), and L-NAME (*C*) (100 mg·kg^{-1} for each) on the hypotension induced by n_3Gro (▨; 3–30 μg·kg^{-1}). Each compound (■) enhanced significantly the hypotensive response to both 10 and 30 μg·kg^{-1} of n_3Gro. Results are expressed as percentage of the response to n_3Gro (30 μg·kg^{-1}) in the presence of phenylephrine (150–300 μg·kg^{-1}·hr^{-1}). Each value is the mean ± SEM of four or five separate determinations.

finding that treatment of the vascular rings with inhibitors of NO synthase also caused a similar increase in the potency of phenylephrine. The force of contraction induced by phenylephrine was also significantly increased in the presence of L-NIO and L-NAME, but not of L-NMMA. The reason for this difference is not clear, but it may be a reflection of the fact that the concentration of L-NMMA used in these experiments was not maximally effective, unlike that for L-NIO and L-NAME (9).

Since the existence of this vasodilator tone will not only affect the response of the rings to vasoconstrictors but also to vasodilators, we compensated for this by studying vasodilator effects in rings contracted to a similar tension with appropriate concentrations of phenylephrine. Under these conditions, treatment with the inhibitors enhanced the relaxation responses to the nitrovasodilators SNP and n_3Gro without affecting those to isoprenaline or 8-bromo-cGMP. A similar enhancement of the responses to SNP and n_3Gro was observed after removal of the endothelium. The relatively small increase in the potency of isoprenaline and 8-bromo-cGMP observed after removal of the endothelium, compared with that of n_3Gro and SNP, might indicate that the endothelium acts as a diffusion barrier for these compounds.

Treatment of endothelium intact rings with the inhibitors of NO synthase also reduced the level of cGMP, providing further evidence for the basal release of NO. The increase in cGMP induced by ACh was prevented by removal of the endothelium or treatment with the inhibitors. In addition, the increase in cGMP induced by SNP was significantly potentiated by removal of the endothelium or by treatment of endothelium intact rings with inhibitors of NO synthase, albeit to a lesser extent. These results, together with the finding that responses to 8-bromo-cGMP were not potentiated by treatment with the inhibitors of the NO synthase, indicate that the specific supersensitivity to nitrovasodilators, which follows removal of the basal release of NO, occurs at the level of their receptor, the soluble guanylate cyclase.

Table 3. Effect of endothelium removal or treatment with L-NMMA, L-NIO, or L-NAME (100 μM each) on the basal and ACh-stimulated levels of cGMP in rat aortic rings

Conditions	cGMP, pmol per mg of protein	
	Basal	ACh (1 μM)
+ endothelium	16.8 ± 2.0 (5)	94.4 ± 8.1 (12)
− endothelium	10.9 ± 1.5 (5)*	9.0 ± 1.8 (5)*
+ endothelium		
+ L-NMMA	5.2 ± 0.7 (4)*	13.9 ± 2.7 (4)*
+ L-NIO	5.1 ± 0.9 (8)*	6.5 ± 1.0 (8)*
+ L-NAME	6.5 ± 1.7 (4)*	5.6 ± 0.6 (4)*

Results are absolute cGMP levels from individual rings from (*n*) animals expressed as means ± SEM.
*$P < 0.05$ versus endothelium intact rings in the same column.

Treatment of rats with the inhibitors not only raised the blood pressure, as described (9, 14), but also caused a rapid enhancement of the hypotensive responses to n_3Gro without affecting those to isoprenaline. These results clearly show that the observations in the isolated rings are applicable to the *in vivo* situation. Furthermore, they strongly support our conclusion about the development of a specific supersensitivity to nitrovasodilators after the removal of endogenous NO.

The demonstration of this phenomenon *in vitro* and *in vivo* therefore indicates that removal of an NO-dependent vasodilator tone in the cardiovascular system gives rise to a phenomenon akin to postjunctional supersensitivity of denervated tissues (15). Whether after several days of treatment with an inhibitor of the NO synthase a more chronic type of supersensitivity develops as a consequence of *de novo* synthesis of soluble guanylate cyclase remains to be established. If this does occur, then the supersensitivity to exogenous NO would resemble denervation supersensitivity in both its acute and chronic characteristics. In this context, it is interesting that venous tissue (28) and the venous circulation of humans *in vivo* (29) seem to have a lower basal release of NO and an increased sensitivity to nitrovasodilators when compared to the arterial side of the circulation. It is possible that the difference in sensitivities between the venous and arterial sides of the circulation is due to a more effective metabolism of nitrovasodilators by venous tissues (30). However, an explanation consistent with our hypothesis would be that this increased venous sensitivity is either the result of a soluble guanylate cyclase that is avid for the stimulation by exogenous NO or that the venous smooth muscle contains more soluble guanylate cyclase.

The concept of altered vascular reactivity following changes in the basal NO tone may also explain some aspects of the tolerance to nitrovasodilators, which has been recog-

Table 4. Effect of endothelium removal or treatment with L-NMMA, L-NIO, or L-NAME (100 μM each) on the SNP-stimulated levels of cGMP in rat aortic rings

Conditions	cGMP, pmol per mg of protein		
	SNP (10 nM)	SNP (100 nM)	SNP (1 μM)
+ endothelium	−0.1 ± 0.5 (5)	7.8 ± 0.8 (7)	184.9 ± 21.4 (7)
− endothelium	3.8 ± 1.5 (3)*	27.4 ± 4.2 (5)*	427.2 ± 34.9 (5)*
+ endothelium			
+ L-NMMA	2.4 ± 0.4 (4)*	18.7 ± 2.3 (4)*	288.8± 14.6 (4)*
+ L-NIO	2.3 ± 0.4 (7)*	15.4 ± 1.6 (8)*	260.9 ± 25.3 (9)*
+ L-NAME	2.1 ± 1.2 (4)*	15.3 ± 1.2 (4)*	355.8 ± 34.9 (4)*

Results are the absolute increase in cGMP induced by SNP after subtraction of the basal cGMP level in a similarly treated ring from the same animal but not stimulated with SNP. Data are expressed as means ± SEM from (*n*) animals.
*$P < 0.05$ versus endothelium intact rings in the same column.

nized since the last century (31). Although tolerance to n_3Gro is due in part to an impairment in its metabolism to NO (32, 33), a more general form of tolerance to nitrovasodilators is due to desensitization of the soluble guanylate cyclase to the action of NO (34, 35). Indeed, this tolerance is the opposite of supersensitivity and is therefore similar to the postjunctional subsensitivity, which follows an increase in the neurotransmitter (36). As a result of this, it is possible that long-term treatment with nitrovasodilators may down-regulate the soluble guanylate cyclase or even the synthesis of NO.

In summary, the related phenomena of supersensitivity and tolerance to NO are important because they demonstrate the relevance of the state of tonic vasodilatation in the vasculature. It is likely that this tone is entirely locally regulated since its main triggering stimuli seem to be shear stress (37), pulsatile flow (38, 39), and, therefore, any conditions that modify these parameters. If this is the case, then it is probably one of the simplest and yet most fundamental adaptive mechanisms in the cardiovascular system.

We wish to thank Annie Higgs and Gillian Henderson for help in the preparation of the manuscript. R.S. is a Fellow of the Medical Reserch Council of Canada and the Alberta Heritage Foundation for Medical Research.

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