*Proc. Natl. Acad. Sci. USA*
Vol. 86, pp. 3375–3378, May 1989
Medical Sciences

# Role of endothelium-derived nitric oxide in the regulation of blood pressure

(L-arginine/hypertension/vascular endothelium/endothelium-derived relaxing factor)

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

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

*Communicated by George H. Hitchings, January 27, 1989*

ABSTRACT **The role of endothelium-derived nitric oxide in the regulation of blood pressure in the anesthetized rabbit was studied with $N^{\omega}$-monomethyl-L-arginine (L-NMMA), a specific inhibitor of its formation from L-arginine. L-NMMA (3–100 mg·kg$^{-1}$), but not its D-enantiomer, induced a dose-dependent long-lasting (15–90 min) increase in mean systemic arterial blood pressure. L-NMMA (100 mg·kg$^{-1}$) also inhibited significantly the hypotensive action of acetylcholine, without affecting that of glyceryl trinitrate. Both these actions of L-NMMA were reversed by L-arginine (300 mg·kg$^{-1}$), but not by D-arginine (300 mg·kg$^{-1}$), indomethacin (1 mg·kg$^{-1}$), prazosin (0.3 mg·kg$^{-1}$), or by vagotomy. The effects of L-NMMA *in vivo* were associated with a significant inhibition of the release of nitric oxide from perfused aortic segments *ex vivo*. This inhibition was reversed by infusing L-arginine through the aortic segments. These results indicate that nitric oxide formation from L-arginine by the vascular endothelium plays a role in the regulation of blood pressure and in the hypotensive actions of acetylcholine.**

Although endothelium-dependent vascular relaxation and the release of endothelium-derived relaxing factor *in vitro* has been clearly established (1–4), there is only circumstantial evidence to indicate their occurrence *in vivo*. Alterations in vessel diameter that follow changes in blood flow are endothelium dependent (5). Furthermore, damage to the endothelium (6, 7) or treatment with methylene blue (8) or gossypol (9), two nonspecific inhibitors of endothelium-dependent relaxation, all abolish the response to endothelium-dependent vasodilators *in vivo* without affecting the response to the endothelium-independent vasodilators, sodium nitroprusside or glyceryl trinitrate ($n_3$Gro).

Nitric oxide (NO) accounts for the biological actions of endothelium-derived relaxing factor (10–15) and is formed by vascular endothelial cells from the terminal guanido nitrogen atom(s) of the amino acid L-arginine (16, 17). This biosynthetic process, the endothelium-dependent relaxation of vascular rings, and the vasodilatation induced by acetylcholine (ACh) in the coronary circulation of the rabbit heart are inhibited by the L-arginine analogue, $N^{\omega}$-monomethyl-L-arginine (L-NMMA; refs. 4 and 18–20). These results indicate that L-arginine is the physiological precursor for NO synthesis by the vascular endothelium.

We have now used L-NMMA to investigate the role of NO in the regulation of blood pressure in the anesthetized rabbit.

## MATERIALS AND METHODS

**Methods.** Male New Zealand White rabbits (2.0–2.2 kg) were anesthetized with sodium pentobarbitone (40–50 mg·kg$^{-1}$). Anesthesia was then maintained by a continuous infusion of sodium pentobarbitone (15 mg·kg$^{-1}$·hr$^{-1}$) via the left marginal ear vein and the rabbits were ventilated with room air via a tracheotomy tube.

Mean arterial blood pressure was monitored with a pressure transducer (Bell & Howell, Ashford, U.K.) connected, via a cannula containing heparinized (10 units·ml$^{-1}$) saline, to the right carotid artery. Phenylephrine was administered as a continuous infusion into the right marginal ear vein. All other drugs were administered as 15-sec infusions via a cannula in the right femoral vein. Blood pressure and heart rate were monitored continuously on a four-channel polygraph (Grass).

For *ex vivo* experiments, L-NMMA (100 mg·kg$^{-1}$; i.v.) was administered and the animals were sacrificed 10 min later. The thoracic aorta was rapidly removed and perfused intraluminally with Krebs' buffer within 30 min of treatment. The release of NO induced by ACh was determined by cascade bioassay and chemiluminescence (4).

**Materials.** NO (>99.98% pure, British Oxygen, Guildford, U.K.) solutions were prepared as described (11).

$n_3$Gro (Wellcome), phenylephrine, ACh, atropine sulfate, indomethacin, L- and D-arginine (Sigma), U46619 (9,11-dideoxy-9$\alpha$,11$\alpha$-methanoepoxy prostaglandin $F_{2\alpha}$) (Cayman Chemicals, Ann Arbor, MI), sodium pentobarbitone (May & Baker, Dagenham, U.K.), and prazosin (Pfizer Diagnostics) were obtained as indicated. L-NMMA and $N^{\omega}$-monomethyl-D-arginine (D-NMMA) were synthesized as described (21). All drugs used were administered in saline.

**Statistical Evaluation.** Results, expressed as means ± SEM of $n$ experiments, were analyzed statistically by Student's $t$ test for paired data. A value of $P < 0.05$ was considered statistically significant.

## RESULTS

L-NMMA (3–100 mg·kg$^{-1}$), but not D-NMMA (100 mg·kg$^{-1}$), caused a dose-dependent increase in mean arterial blood pressure (Fig. 1). The hypertension induced by L-NMMA was rapid in onset, reaching a plateau within 5 min (Fig. 2*A*). Its duration was also dose dependent, so that the hypertension induced by 3 mg·kg$^{-1}$ and 100 mg·kg$^{-1}$ of L-NMMA lasted 10–15 and 60–90 min, respectively ($n$ = 5). The increase in blood pressure induced by L-NMMA (100 mg·kg$^{-1}$) was always accompanied by a slight bradycardia (Fig. 2; $n$ = 5). Administration of L-arginine (300 mg·kg$^{-1}$), which had no direct effect on either mean arterial blood pressure or on heart rate, reversed the hypertension and the decrease in heart rate induced by L-NMMA within 10 min (Fig. 2*B*).

ACh (0.1–3.0 $\mu$g·kg$^{-1}$) and $n_3$Gro (1–30 $\mu$g·kg$^{-1}$) caused a dose-dependent fall in mean arterial blood pressure ($ED_{50}$ 0.20 ± 0.04 and 2.4 ± 0.3 $\mu$g·kg$^{-1}$, respectively; $n$ = 3) without affecting the heart rate significantly. Phenylephrine

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: L-NMMA, $N^{\omega}$-monomethyl-L-arginine; D-NMMA, $N^{\omega}$-monomethyl-D-arginine; $n_3$Gro, glyceryl trinitrate; ACh, acetylcholine.

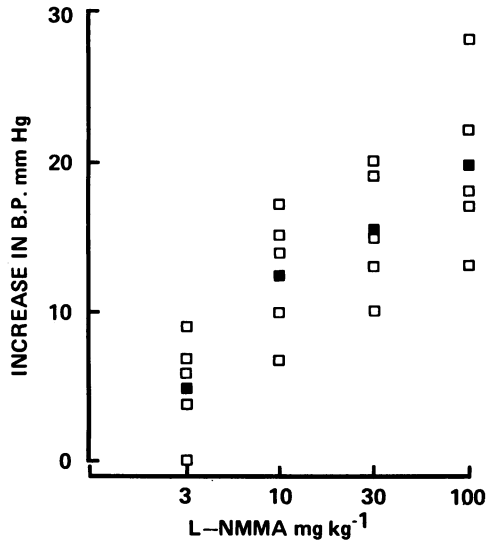


FIG. 1. Effect of L-NMMA (3–100 mg·kg$^{-1}$; i.v.) on the mean arterial blood pressure (B.P.) of the rabbit. □, Increase in blood pressure in each animal; ■, mean. Measurements were made 5 min after administration of L-NMMA. Resting blood pressure was 68 ± 3 mmHg ($n$ = 5).

(300 μg·kg$^{-1}$·hr$^{-1}$) raised the blood pressure to a level similar to that induced by L-NMMA (100 mg·kg$^{-1}$) and significantly enhanced the hypotensive actions of ACh and $n_3$Gro. When the enhanced responses to ACh and $n_3$Gro in phenylephrine-treated animals were taken as controls, then L-NMMA (100 mg·kg$^{-1}$), but not D-NMMA (100 mg·kg$^{-1}$), caused a significant inhibition of the ACh-induced hypotension, without affecting that induced by $n_3$Gro (Table 1). Administration of L-arginine (300 mg·kg$^{-1}$), but not D-arginine (300 mg·kg$^{-1}$),

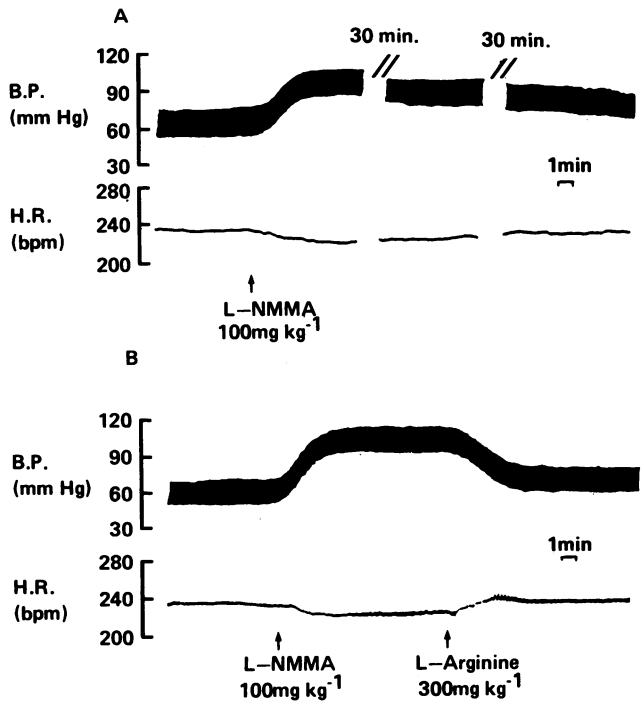


FIG. 2. (A) Effect of L-NMMA (100 mg·kg$^{-1}$; i.v.) on blood pressure (B.P.) and heart rate (H.R.). Trace is representative of three experiments, in which the duration of these effects of L-NMMA was between 60 and 90 min. (B) Reversal of the effect of L-NMMA (100 mg·kg$^{-1}$; i.v.) on blood pressure and heart rate by L-arginine (300 mg·kg$^{-1}$; i.v.). Trace is representative of three experiments.

Table 1. Effect of L-NMMA on the hypotensive responses induced by ACh and $n_3$Gro

| Vasodilator | Fall in blood pressure, mmHg (mean ± SEM) | |
|---|---|---|
| | Phenylephrine | L-NMMA |
| ACh, μg·kg$^{-1}$ | | |
| 0.1 | 13.3 ± 2.8 | 2.6 ± 1.7* |
| 0.3 | 23.3 ± 2.7 | 10.1 ± 2.0* |
| 1 | 33.1 ± 2.6 | 22.6 ± 1.9* |
| 3 | 37.4 ± 3.0 | 28.5 ± 1.1* |
| $n_3$Gro, μg·kg$^{-1}$ | | |
| 1 | 6.1 ± 1.5 | 6.8 ± 1.9 |
| 3 | 16.7 ± 4.7 | 16.4 ± 3.1 |
| 10 | 30.0 ± 3.9 | 30.8 ± 3.0 |
| 30 | 37.0 ± 5.2 | 37.3 ± 5.0 |

The vasodilator effects of ACh and $n_3$Gro were compared in animals treated with L-NMMA and in animals whose blood pressure had been elevated to a comparable level with phenylephrine. The blood pressure in the L-NMMA (100 mg·kg$^{-1}$)- and phenylephrine (300 μg·kg$^{-1}$·hr$^{-1}$)-treated animals was 84.6 ± 3.2 and 88.6 ± 7 mmHg, respectively. L-NMMA inhibited the vasodilator effects of ACh but not those of $n_3$Gro.
*$P < 0.05$.

abolished within 15 min this inhibition by L-NMMA (100 mg·kg$^{-1}$; $n$ = 3).

When the blood pressure was raised by phenylephrine, the hypotensive actions of similarly effective doses of ACh (0.3 μg·kg$^{-1}$) and $n_3$Gro (3 μg·kg$^{-1}$) were accompanied by an increase in the heart rate of 11 ± 2 and 10 ± 2 beats per min, respectively ($n$ = 3). These doses of ACh and $n_3$Gro caused a similar increase in heart rate when the blood pressure was raised by L-NMMA (9 ± 1 and 12 ± 2 beats per min, respectively; $n$ = 3).

The blood pressure was not affected by indomethacin (1 mg·kg$^{-1}$) but was slightly reduced by prazosin (0.3 mg·kg$^{-1}$). Bilateral vagotomy caused a small increase in mean arterial blood pressure. None of these interventions affected the actions of L-NMMA ($n$ = 3 for each).

Infusion of ACh (0.1–3.0 μM) for 1 min through the excised aortae from untreated animals induced a concentration-dependent release of NO detected by bioassay (Fig. 3A) or by chemiluminescence (Fig. 4A). In contrast, the release of NO induced by ACh (0.1–3.0 μM) from aortae obtained from animals treated with L-NMMA (100 mg·kg$^{-1}$) was inhibited significantly when measured by bioassay (Fig. 3B) or chemiluminescence (Fig. 4B). Infusion of L-arginine (100 μM) through the aortae from control animals did not affect significantly the release of NO induced by ACh but restored fully the release observed in aortae from L-NMMA-treated animals ($n$ = 3).

## DISCUSSION

A physiological role for endothelium-derived NO in the control of vascular tone *in vivo* has not been clearly demonstrated. This has mainly been due to the difficulties associated with removal of the endothelium *in vivo*, the extremely labile nature of NO, and the absence of a specific inhibitor of its synthesis. There is, however, some evidence to support the proposal that the endothelium-dependent vasodilator responses observed in conduit arteries *in vitro* also occur *in vivo* (5–9).

The formation of NO in the coronary circulation *in vitro* accounts for the vasodilatation induced by ACh in the rabbit heart (22) and by bradykinin in the guinea pig heart (23), indicating that NO also plays a role in regulating the tone of resistance arteries. The demonstration that L-arginine is the physiological precursor for NO synthesis by the vascular

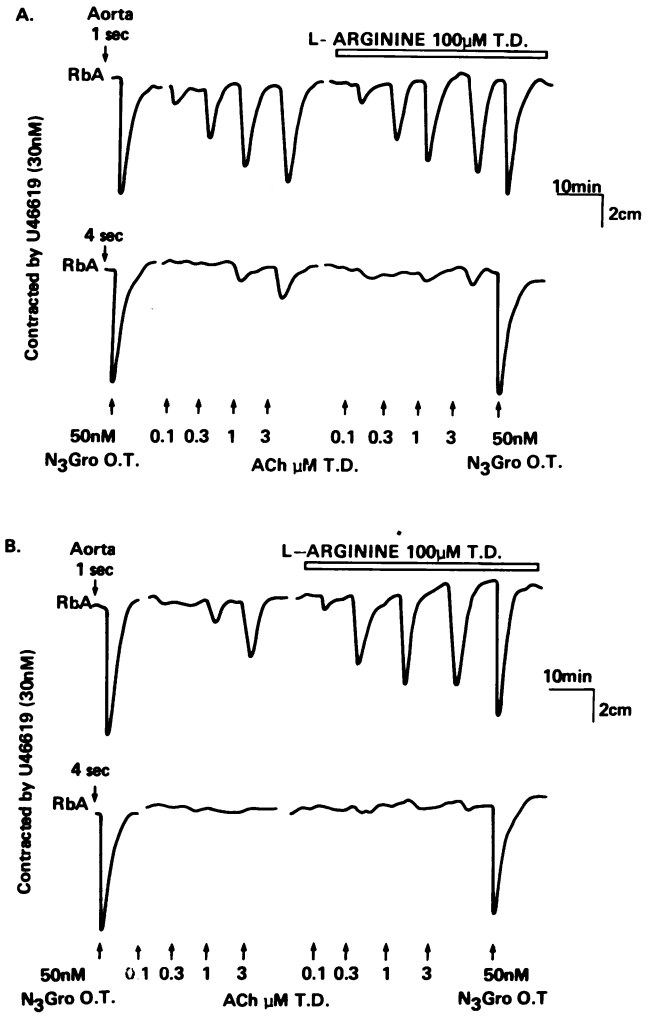


FIG. 3. Effect of L-NMMA on the *ex vivo* release of NO induced by ACh. A segment (6 cm) of the thoracic (donor) aorta was removed 10 min after treatment with L-NMMA (100 mg·kg$^{-1}$; i.v.), placed in a perspex chamber, and perfused at 5 ml·min$^{-1}$ with Krebs' buffer. The effluent was used to superfuse in a cascade two spiral strips of rabbit aorta denuded of endothelium (RbA) and submaximally contracted with U46619 (30 nM). Atropine (200 nM) was infused over the tissues (O.T.) to inhibit the direct effects of ACh administered as 1-min infusions. (*A*) Control, untreated rabbit. ACh (0.1–3.0 μM) infused through the donor aorta (T.D.) induced the release of NO as shown by the relaxations of the bioassay tissues. This release was not significantly affected by a continuous infusion of L-arginine (100 μM) through the donor aorta. (*B*) Rabbit treated with L-NMMA (100 mg·kg$^{-1}$; i.v.). The release of NO by ACh (0.1–3.0 μM) infused through the donor aorta was greatly reduced and was restored by a continuous infusion of L-arginine (100 μM) through the donor aorta. Trace is representative of three experiments.

endothelium and the identification of L-NMMA as a specific inhibitor of this pathway (4, 18, 19) has allowed the investigation of the role of NO in the regulation of blood pressure.

Intravenous administration of L-NMMA induced a dose-dependent enantiomer-specific hypertension and partially inhibited the hypotensive action of ACh, but not that of $n_3$Gro. Whether L-NMMA affects the uptake or the utilization of arginine by cells is not yet known; however, the present data are quantitatively and qualitatively similar to those obtained *in vitro*. Indeed, in vascular strips (4, 18, 19), and in the isolated perfused heart of the rabbit (20), L-NMMA induces an increase in vascular tone and an inhibition of endothelium-dependent responses. These actions are due to the inhibition of the release of NO, suggesting that the results obtained *in vivo* are also attributable to the same mechanism.

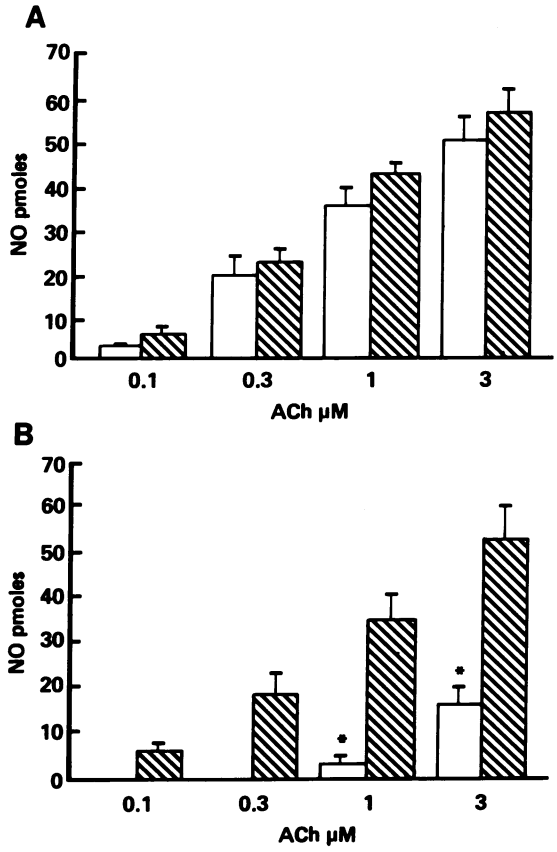


FIG. 4. Release of NO from the perfused rabbit aorta detected by chemiluminescence. The effluent of the donor aorta was infused continuously into a reaction vessel containing 75 ml of 1% sodium iodide in glacial acetic acid under reflex. NO was removed under reduced pressure in a stream of $N_2$ and mixed with ozone; the chemiluminescent product was quantified by reference to NO standards. (*A*) Control, untreated rabbit. Release of NO by ACh (0.1–3.0 μM; 1-min infusions) in the absence (□) and presence (▨) of a continuous infusion of L-arginine (100 μM). (*B*) Rabbit treated with L-NMMA (100 mg·kg$^{-1}$; i.v.) 10 min prior to removal of the aorta. Release of NO by ACh (0.1–3.0 μM) in the absence (□) and presence (▨) of a continuous infusion of L-arginine (100 μM). Each value is the mean ± SEM of three separate experiments. *, $P < 0.05$.

The effect of L-NMMA *in vivo* was slow to disappear unless accelerated by a 3-fold molar excess of exogenous L-arginine, reinforcing our suggestion (4, 18) that L-NMMA is a competitive inhibitor of the NO-forming enzyme(s). Furthermore, this effect could also be demonstrated *ex vivo* where the inhibition of the formation of NO observed after treatment with L-NMMA was reversed by an infusion of L-arginine. These findings suggest that there is a continuous utilization of L-arginine for the enzymic formation of NO by resistance arteries and provide the first evidence that NO formation contributes to the regulation of blood pressure. The failure of L-arginine to affect blood pressure directly is also consistent with observations in conduit and in resistance vessels *in vitro* (4, 20, 24) and endothelial cells in culture (16), which suggest that under normal conditions there is sufficient endogenous L-arginine to saturate the NO-forming enzyme.

More L-NMMA was necessary to inhibit the hypotension induced by ACh than to increase the blood pressure, suggesting that during stimulation with ACh there is an increased mobilization of L-arginine, the antagonism of which requires more L-NMMA. These results are consistent with those obtained in vascular rings *in vitro*, where more L-NMMA was required to inhibit ACh-induced relaxation than to increase basal tone (4).

The hypertension induced by L-NMMA was accompanied by bradycardia and the hypotension induced by ACh and $n_3$Gro, in the presence of L-NMMA, was accompanied by tachycardia. These effects were small and were also observed when the blood pressure was raised by phenylephrine and were not affected by the $\alpha_1$-antagonist prazosin or by vagotomy, suggesting that they are reflex in nature rather than a direct effect of L-NMMA on heart rate.

Our results indicate that the vascular endothelium, by synthesizing NO from L-arginine, plays a significant role in the control of blood pressure. The precise regional hemodynamic changes that occur as a result of preventing the synthesis of NO by the vascular endothelium require elucidation.

A decrease in endothelium-dependent relaxation has been observed in vessels from hypertensive animals (25–29), although the precise mechanism has not been established. In view of our data, it is tempting to speculate that changes in the synthesis or the actions of NO in the vasculature may either be involved in some forms of hypertension or be one of the mechanisms involved in the genesis of hypertension in general.

Whether administration of arginine would be beneficial for the treatment of hypertension and whether populations consuming a low arginine diet exhibit an increased incidence of hypertension should also be investigated. Further work is also needed to determine the long-term pathophysiological consequences of this way of changing vascular reactivity and inducing experimental hypertension.

We are indebted to Dr. Harold Hodson and Richard Beams for the synthesis of L- and D-NMMA.

1. Furchgott, R. F. & Zawadzki, J. V. (1980) *Nature (London)* **288,** 373–376.
2. Furchgott, R. F. (1984) *Annu. Rev. Pharmacol. Toxicol.* **24,** 175–197.
3. Griffith, T. M., Edwards, D. H., Lewis, M. J., Newby, A. C. & Henderson, A. H. (1984) *Nature (London)* **308,** 645–647.
4. Rees, D. D., Palmer, R. M. J., Hodson, H. F. & Moncada, S. (1989) *Br. J. Pharmacol.* **96,** 418–424.
5. Holtz, J., Forstermann, U., Pohl, U., Giesler, M. & Bassenge, E. (1984) *J. Cardiovasc. Pharmacol.* **6,** 1161–1169.
6. Angus, J. A., Campbell, G. R., Cocks, T. M. & Manderson, J. A. (1983) *J. Physiol. (London)* **344,** 209–222.
7. Rosenblum, W. I., Nelson, G. H. & Povlishock, J. T. (1987) *Circ. Res.* **60,** 169–176.
8. Sobey, C. G., Woodman, O. L. & Dusting, G. J. (1988) *Clin. Exp. Physiol. Pharmacol.* **15,** 401–410.
9. Dudel, C. & Forstermann, U. (1988) *Eur. J. Pharmacol.* **145,** 217–221.
10. Palmer, R. M. J., Ferrige, A. G. & Moncada, S. (1987) *Nature (London)* **327,** 524–526.
11. Radomski, M. W., Palmer, R. M. J. & Moncada, S. (1987) *Br. J. Pharmacol.* **92,** 181–187.
12. Radomski, M. W., Palmer, R. M. J. & Moncada, S. (1987) *Biochem. Biophys. Res. Commun.* **148,** 1482–1489.
13. Ignarro, L. J., Buga, G. M., Wood, K. S., Byrns, R. E. & Chaudhuri, G. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 9265–9269.
14. Furchgott, R. F. (1988) in *Mechanisms of Vasodilatation,* ed. Vanhoutte, P. M. (Raven, New York), pp. 401–414.
15. Kelm, M., Feelisch, M., Spahr, R., Piper, H.-M., Noack, E. & Schrader, J. (1988) *Biochem. Biophys. Res. Commun.* **154,** 236–244.
16. Palmer, R. M. J., Ashton, D. S. & Moncada, S. (1988) *Nature (London)* **333,** 664–666.
17. Schmidt, H. H. H. W., Nau, H., Wittfoht, W., Gerlach, J., Prescher, K.-E., Klein, M. M., Niroomand, F. & Bohme, E. (1988) *Eur. J. Pharmacol.* **154,** 213–216.
18. Palmer, R. M. J., Rees, D. D., Ashton, D. S. & Moncada, S. (1988) *Biochem. Biophys. Res. Commun.* **153,** 1251–1256.
19. Sakuma, I., Stuehr, D., Gross, S. S., Nathan, C. & Levi, R. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 8664–8667.
20. Amezcua, J. L., De Souza, B. M., Palmer, R. M. J. & Moncada, S. (1989) *Br. J. Pharmacol.*, in press.
21. Patthy, A., Bajusz, S. & Patthy, L. (1977) *Acta Biochim. Biophys. Acad. Sci. Hung.* **12,** 191–196.
22. Amezcua, J. L., Dusting, G. J., Palmer, R. M. J. & Moncada, S. (1988) *Br. J. Pharmacol.* **95,** 830–834.
23. Kelm, M. & Schrader, J. (1988) *Eur. J. Pharmacol.* **155,** 313–316.
24. Thomas, G., Mostaghim, R. & Ramwell, P. W. (1986) *Biochem. Biophys. Res. Commun.* **141,** 446–451.
25. Luscher, T. F., Diederick, D., Weber, E., Vanhoutte, P. M. & Buhler, F. R. (1988) *Hypertension* **11,** 573–578.
26. Winquist, R. J., Bunting, P. B., Baskin, E. P. & Wallace, A. A. (1984) *J. Hypertens.* **2,** 541–545.
27. De Mey, J. G. & Gray, S. D. (1985) *Prog. Appl. Microcirc.* **8,** 181–187.
28. Luscher, T. F., Raij, L. & Vanhoutte, P. M. (1987) *J. Hypertens.* **5,** Suppl. 5, S153–S155.
29. Otsuka, U., DiPiero, A., Hirt, B., Brennaman, B. & Lockette, W. (1988) *Am. J. Physiol.* **254,** H163–H169.