Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 3913–3916, May 1992
Neurobiology

Does nitric oxide mediate the increases in cerebral blood flow elicited by hypercapnia?

(endothelium-derived relaxing factor/cerebral circulation/laser–Doppler flowmetry/rat)

COSTANTINO IADECOLA

Department of Neurology, University of Minnesota Medical School, Minneapolis, MN 55455

Communicated by William H. Oldendorf, January 22, 1992 (received for review November 7, 1991)

ABSTRACT **The endothelium-derived relaxing factor (EDRF), probably nitric oxide (NO) or a closely related compound (EDRF/NO), is a potent vasodilator that appears to regulate vascular tone in several vascular beds. I have investigated whether EDRF/NO is also involved in the regulation of the cerebral circulation—in particular, whether EDRF/NO participates in the increases in cerebral blood flow elicited by hypercapnia. Rats were anesthetized with halothane, 1–2% (vol/vol), paralyzed, and artificially ventilated. Arterial pressure was monitored and blood gases were controlled. Cerebral blood flow was continuously monitored through a cranial window over the sensory cortex by a laser–Doppler probe. The window was superfused with Ringer's solution (pH 7.3–7.4 at 37°C). During superfusion with Ringer's solution, hypercapnia (P_{CO_2} = 55.8 ± 0.8 mmHg) increased cerebral blood flow by 121 ± 6% (n = 27; $P < 0.001$; analysis of variance). Topical superfusion with the NO synthase inhibitors N^{ω}-nitro-L-arginine (1 mM) attenuated the cerebrovasodilation by 93 ± 6% (n = 8). In contrast, the vasodilation elicited by topical papaverine (1 mM) was not affected by N^{ω}-nitro-L-arginine (n = 10). Application of N^{ω}-nitro-D-arginine (1 mM) did not affect the cerebrovasodilation elicited by hypercapnia ($P > 0.05$; n = 8). N^{ω}-Methyl-L-arginine (1 mM) attenuated the cerebrovasodilation elicited by hypercapnia by 44 ± 4% (n = 8; $P < 0.001$), an effect completely reversed by coapplication of L-arginine (10 mM; $P > 0.05$; n = 13). These findings indicate that the powerful effects of CO_2 on the cerebral circulation are mediated by arginine-derived EDRF/NO. EDRF/NO is an important molecular signal whose actions may also include the regulation cerebral circulation.**

The endothelium-derived relaxing factor (EDRF) is a potent vasodilator originally described in endothelial cells (1). EDRF appears to have the pharmacological and chemical properties of nitric oxide (NO) and is involved in a wide variety of biological actions (2), including the modulation of vascular tone in the systemic circulation (3, 4). In the cerebral circulation, EDRF/NO is produced by endothelial cells and mediates the vascular relaxation elicited by topical application of several endogenous vasodilators, most notably acetylcholine and bradykinin (5, 6). EDRF/NO is also produced by activated neurons (7–10), astrocytes (11), and, probably, perivascular nerves (12). Collectively, these observations suggest that this agent may be an important modulator of cerebral blood flow (CBF). However, whether EDRF/NO plays a physiologically relevant role in the regulation of the cerebral circulation has not been established.

One of the most distinctive features of the physiology of the cerebral circulation is its powerful reactivity to carbon dioxide (13). During ongoing studies on the role of EDRF/NO in the neurogenic regulation of the cerebral circulation (14), it was observed that the inhibitor of EDRF/NO synthesis N^{ω}-nitro-L-arginine (L-NA) nearly abolished the increases in CBF elicited by hypercapnia. This finding is surprising as the cerebrovascular responses elicited by hypercapnia are thought to result from a direct vascular action of H^+ resulting in smooth muscle hyperpolarization (13). The present study was, therefore, designed to investigate in greater detail whether EDRF/NO participates in the cerebrovasodilation elicited by hypercapnia.

METHODS

The methods used in this study have been described in detail (15–18) and are briefly summarized below. Studies were performed on 30 male Sprague–Dawley rats (290–380 g) anesthetized with halothane (5%, induction; 2%, during surgery; 1%, maintenance) in 100% oxygen. Catheters were inserted in both femoral arteries and in the right femoral vein, and the trachea was cannulated. Animals were then placed on a stereotaxic frame (Kopf, Tujunga, CA), paralyzed with tubocurarine (2 mg/kg, i.m.), and artificially ventilated by a mechanical ventilator (Rodent respirator, Harvard Apparatus). Body temperature was maintained at 37 ± 0.5°C using a thermostatically controlled heating lamp. Arterial pressure and heart rate were continuously recorded. Arterial P_{CO_2}, P_{O_2}, and pH were measured on 100 μl of blood using a blood gas analyzer (model 178, Corning) (Table 1).

CBF was continuously monitored over the sensory cortex by a laser–Doppler flowmeter (BPM 403, Vasamedic, Minneapolis) as described (16, 17). A 3 × 3 mm cranial window was drilled at a site 2–3 mm lateral and 1–2 mm caudal to bregma. The dura was carefully removed and the craniotomy site was continuously superfused at a rate of 0.33 ml/min with Ringer's solution warmed at 37°C and aerated with 95% O_2/5% CO_2 (pH = 7.3–7.4). The laser–Doppler probe (tip diameter, 0.8 mm) was mounted on a micromanipulator (Kopf) and positioned 0.5 mm above the pial surface. Once a suitable placement was obtained, the probe was left at that site for the duration of the experiment. At the end of the experiment, the heart was stopped by an i.v. bolus injection of saturated KCl and the zero level for CBF was recorded.

The arginine analogues L-NA (Sigma), N^{ω}-nitro-D-arginine (D-NA; Serva), and N^{ω}-methyl-L-arginine monoacetate (L-NMA; Calbiochem) were topically superfused on the exposed cortex. L- and D-NA (1 mM) were dissolved in Ringer's solution acidified by bubbling with CO_2 for 15–20 min. Solutions were applied to the brain after their pH rose to the range of pH 7.3–7.4. CO_2 reactivity was tested after 45–60 min of superfusion. Hypercapnia (P_{CO_2}, 50–60 mmHg) was produced by mixing the inspired oxygen with 5% CO_2. Readings were taken when stable values of P_{CO_2} and CBF were reached. In some experiments papaverine hydrochlo-

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: CBF, cerebral blood flow; D-NA, N^{ω}-nitro-D-arginine; L-NA, N^{ω}-nitro-L-arginine; L-NMA, N^{ω}-methyl-L-arginine; NO, nitric oxide; EDRF, endothelium-derived relaxing factor.

Table 1. Arterial pressure and P_{CO_2} in the groups of animals studied

Treatment	Normocapnia		Hypercapnia		
	P_{CO_2}, mmHg	AP, mmHg	P_{CO_2}†, mmHg	AP, mmHg	n
Ringer	34.9 ± 0.3	118 ± 2	55.8 ± 0.8	121 ± 2	27
L-NA	34.8 ± 0.8	106 ± 2	53.5 ± 1.2	108 ± 2	8
D-NA	34.9 ± 0.9	114 ± 4	54.0 ± 3.1	119 ± 2	8
L-NMA	35.5 ± 0.6	112 ± 3	56.0 ± 1.5	116 ± 2	8
L-NMA + L-arginine	35.0 ± 0.5	103 ± 3	56.0 ± 0.7	113 ± 2*	13
Indomethacin	34.7 ± 0.5	111 ± 2	58.5 ± 2.5	114 ± 2*	12
Indomethacin + L-NA	35.6 ± 0.6	96 ± 2	56.7 ± 1.1	99 ± 3	8

Values are the mean ± SEM. AP, arterial pressure. *, $P < 0.05$ from the corresponding arterial pressure at normocapnia, paired t test; †, $P < 0.001$ from normocapnia, paired t test.

ride (1 mM; Research Biochemicals, Natick, MA) was topically superfused for 3–5 min.

Changes in CBF were calculated as percent of the baseline value. Data are expressed as the mean ± SEM. Comparisons among multiple groups were statistically evaluated by the analysis of variance and the Tukey's test as a post hoc procedure (Systat, Evanston, IL). Two-group comparisons were evaluated by the Student's t test.

RESULTS

During superfusion with Ringer's solution, induction of hypercapnia (P_{CO_2} = 55.8 ± 0.8 mmHg) increased CBF by 121 ± 6% (n = 27) (13). Topical application of L-NA (1 mM), a potent inhibitor of EDRF/NO synthesis, did not affect resting arterial pressure nor CBF ($P > 0.05$; paired t test; Table 1). However, this agent reduced the elevations in CBF elicited by hypercapnia (P_{CO_2} = 53.5 ± 1.2) by 93 ± 6% (n = 8; $P < 0.001$; analysis of variance and Tukey's test; Figs. 1 and 2*A*). In contrast to hypercapnia, the increases in CBF elicited by topical superfusion with papaverine (1 mM) were not affected by L-NA. During superfusion with Ringer's solution papaverine increased CBF by 128 ± 21% (n = 10) and with L-NA superfusion this drug increased CBF by 144 ± 24% (n = 10; $P > 0.05$ from Ringer's solution). Superfusion with D-NA (1 mM) did not influence the CBF response to hypercapnia (n = 8; $P > 0.05$; Figs. 1 and 2*A*). Topical application of L-NMA, the less potent inhibitor of EDRF/NO synthesis, reduced the cerebrovasodilation elicited by hypercapnia (−44 ± 4%; n = 8; $P < 0.001$). This inhibition was less pronounced than that elicited by L-NA ($P < 0.001$) and was completely reversed by superfusion with L-arginine (10 mM; $P > 0.05$ from Ringer's solution; n = 13; Fig. 2*A*).

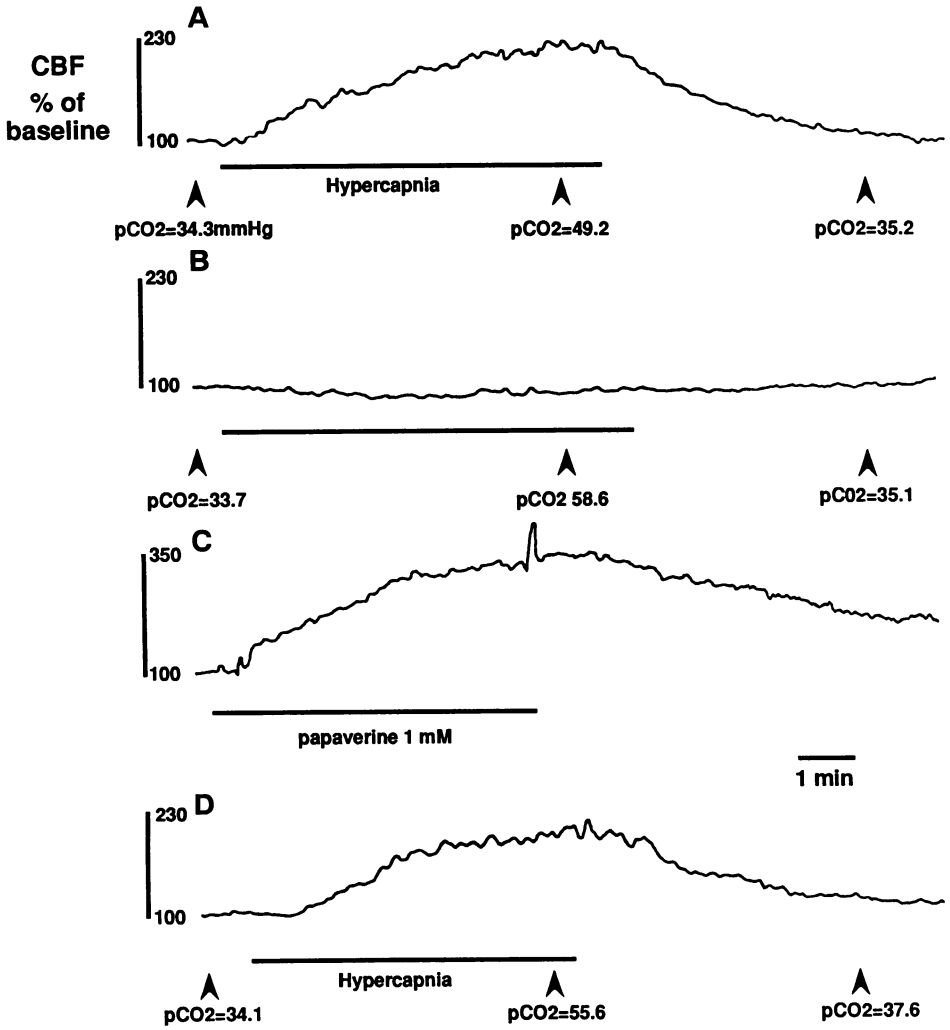

FIG. 1. Effect of arginine analogues on the increases in cortical CBF elicited by hypercapnia. (*A*) Elevation in CBF elicited by hypercapnia during superfusion with Ringer's solution. (*B*) Effect of L-NA (1 mM) superfusion on the increases in CBF elicited by hypercapnia. Note that the increase in CBF is abolished. (*C*) In contrast to hypercapnia, the increase in CBF elicited by papaverine (1 mM) is not affected by L-NA. (*D*) D-NA does not influence the cerebrovasodilation elicited by hypercapnia.

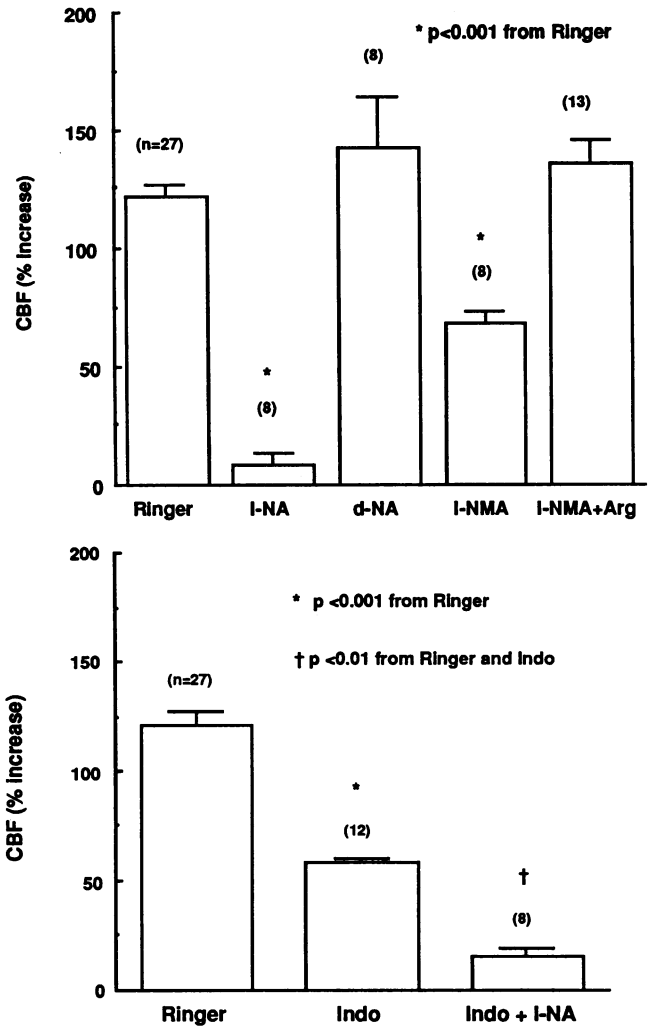

FIG. 2. (*Upper*) Effect of L-NA, D-NA, L-NMA, and L-NMA plus L-arginine (L-NMA + Arg) on the increase in CBF elicited by hypercapnia (P_{CO_2}, 50–60 mmHg; see text for exact values). Note that superfusion with L-NA (1 mM) but not D-NA (1 mM) virtually abolished the increase in CBF elicited by hypercapnia. Similarly, superfusion with L-NMA (1 mM) substantially reduced the elevations in CBF, an effect that was completely reversed by coapplication of L-arginine (10 mM). (*Lower*) Effect of indomethacin (Indo) and L-NA on the increases in CBF elicited by hypercapnia (P_{CO_2}, 50–60 mmHg; see text for values). Indomethacin (5 mg/kg, i.v.) substantially reduced the CBF elevations. However, after indomethacin, topical superfusion with L-NA (1 mM) reduced the CBF elevations further.

Inhibition of prostaglandin synthesis has been reported to reduce the CBF response to hypercapnia in rat (19). To explore the possibility that the effect of L-NA is mediated through cyclooxygenase products, the effect of indomethacin and L-NA on the cerebrovasodilation elicited by hypercapnia was compared. Indomethacin was administered at a dose (5 mg/kg, i.v.) that virtually abolishes prostaglandin synthesis in the rat brain (19). Indomethacin reduced the CBF elevations elicited by hypercapnia by 52 ± 3% ($n = 12$; $P < 0.01$; Fig. 1*B*). Superfusion with L-NA after administration of indomethacin reduced the elevations in CBF elicited by hypercapnia by 87 ± 5%, an effect greater than that observed with indomethacin ($P < 0.01$; Fig. 2*B*).

DISCUSSION

This study sought to establish whether EDRF/NO participates in the cerebrovasodilation elicited by hypercapnia. EDRF/NO is synthesized from L-arginine by the enzyme NO synthase (20–22). N^{ω}-substituted arginine analogues, including L-NA and L-NMA, compete with L-arginine for the active site of NO synthase thereby inhibiting EDRF/NO production (23, 24). It has been shown here that L-NA virtually abolishes the cerebrovasodilation elicited by hypercapnia, an effect that is stereospecific. The less-potent NO synthase inhibitor L-NMA also attenuates the CO_2 response and its effect is reversed by coapplication of L-arginine, the substrate from which EDRF/NO is synthesized. The results of these experiments indicate that the cerebrovasodilation elicited by hypercapnia depends upon arginine-derived EDRF/NO production.

The inhibitory effect of L-NA and L-NMA on the CO_2 reactivity of the cerebral circulation is not a consequence of differences in arterial pressure (25) or level of hypercapnia as these variables were closely monitored and did not differ among treated and untreated groups. Similarly, the inhibition of the CBF response to hypercapnia cannot be the result of a nonspecific action of these drugs resulting in vasoparalysis: L-NA did not affect the vasodilation elicited by topical application of papaverine. Finally, the action of L-NA is unlikely to be the consequence of a local depression of cerebral metabolism resulting in reduced responsivity to CO_2 (26). L-NA did not reduce resting CBF, suggesting that cerebral metabolism, a variable closely linked to CBF in most states (27), was also not affected.

In several animal species, cyclooxygenase products, probably prostaglandins, contribute substantially to the cerebrovasodilation elicited by hypercapnia (19). Therefore, EDRF/NO could participate in the cerebrovasodilation by promoting the production and/or enhancing the action of cyclooxygenase products. However, it has been shown here that the effect of indomethacin is less marked than that produced by inhibition of EDRF/NO synthesis. Furthermore, inhibition of EDRF/NO synthesis after indomethacin administration attenuates the response further. Thus, the mechanism by which EDRF/NO mediates the cerebrovasodilation elicited by hypercapnia is unlikely to depend entirely on cyclooxygenase products.

A fundamental question concerns the site of EDRF/NO production in hypercapnia. In brain EDRF/NO can be generated by endothelial cells (5, 6), central and peripheral neurons and their processes (7–10, 12), and astrocytes (11). It is unlikely that EDRF/NO is produced in local cortical neurons or in terminals of input pathways as the cerebrovasodilation elicited by hypercapnia is not affected by selective destruction of local neurons or of major thalamic and extrathalamic input pathways (15, 18, 28, 29). Alternatively, EDRF/NO could be produced in perivascular nerves innervating cerebral blood vessels (13). In support of this hypothesis are (*i*) the presence of NO synthase in the adventitial nerve plexus of large cerebral arteries (9) and (*ii*) the observation that activation of perivascular nerve endings by transmural electrical stimulation of isolated cerebral arteries releases EDRF/NO (12). In apparent contrast with this hypothesis, however, is the observation that the cerebrovasodilation elicited by hypercapnia is not affected by ablation of some of the perivascular nerves (13).

Other possible sources of EDRF/NO in hypercapnia are vascular cells or perivascular glia (5, 6, 11). In isolated dog cerebral arteries, removal of the endothelium did not affect the relaxation elicited by hypercapnia (30), a finding that raises the possibility that the endothelium may not be the site of EDRF/NO production. This evidence is, however, far from conclusive since, at the high partial pressures (110 mmHg) used in this study (30), CO_2 may have produced vasodilatation by a direct effect on smooth muscle cells (31). The cellular source of EDRF/NO in hypercapnia remains, therefore, unknown. However, the fact that EDRF/NO has a very short biological half-life (2) indicates that its site of

production must be close to its site of action. It is therefore likely that EDRF/NO is produced either at the vascular level or perivascularly.

Another question concerns the segment of the cerebral vasculature on which these arginine analogues act to inhibit EDRF/NO production. The increases in CBF elicited by hypercapnia result from dilatation of both large cerebral arteries as well as of pial arteries and arterioles (32, 33). Thus, for the CBF response to CO_2 to be nearly abolished, L-NA and L-NMA must act not only on the smaller arteries located at the site of drug application but also on distant larger arteries. It is therefore likely that these agents diffuse in the subarachnoid space and involve larger arteries whose terminal branches supply the area of the neocortex from which CBF is recorded.

Whether the cerebrovasodilation elicited by hypercapnia is the result of increased EDRF/NO production or reduced inactivation of EDRF/NO basally released cannot be determined from the present study. However, the observation that inhibition of NO synthesis by L-NA and L-NMA does not substantially reduce resting CBF argues against a significant basal production of EDRF/NO. Thus hypercapnia may enhance EDRF/NO production. The molecular mechanisms by which CO_2 and/or H^+ would stimulate NO synthase remain to be determined.

In summary, this study provides evidence that arginine-derived EDRF/NO mediates the cerebrovasodilation elicited by hypercapnia. These preliminary observations support the hypothesis that EDRF/NO is an important molecular signal with widespread actions that may also include the regulation cerebral circulation.

Note Added in Proof. Subsequent experiments, in which CBF was measured using the quantitative [^{14}C]iodoantipyrine technique with autoradiography, have revealed that superfusion of the cerebral cortex with L-NA reduces resting CBF at the site of application by 26%. These experiments have also demonstrated that L-NA reduces the local increases in CBF elicited by hypercapnia by 88%.

This work was supported by grants-in-aid from the American Heart Association (Minnesota), the Minnesota Medical Foundation, and the Graduate School of the University of Minnesota.

1. Furchgott, R. F. & Zawadzky, J. V. (1980) *Nature (London)* **228,** 373–376.
2. Moncada, S., Palmer, R. M. J. & Higgs, E. A. (1991) *Pharmacol. Rev.* **43,** 109–142.
3. Rees, D. D., Palmer, R. M. J. & Moncada, S. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 3375–3378.
4. Persson, M. G., Wiklund, N. P. & Gustafsson, L. E. (1991) *Acta Physiol. Scand.* **141,** 49–56.
5. Kontos, H. A., Wei, H. P. & Marshall, J. (1988) *Am. J. Physiol.* **255,** H1259–H1262.
6. Rosenblum, W. I. (1986) *Stroke* **17,** 494–497.
7. Garthwaite, J. (1991) *Trends Neurosci.* **14,** 60–67.
8. Garthwaite, J., Charles, S. L. & Chess-Williams, R. (1988) *Nature (London)* **336,** 385–388.
9. Bredt, D. S., Hwang, P. M. & Snyder, S. H. (1990) *Nature (London)* **347,** 768–770.
10. Knowles, R. G., Palacios, M. R., Palmer, M. J. & Moncada, S. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 5159–5162.
11. Murphy, S., Minor, R. L. Jr., Welk, G. & Harrison, D. G. (1990) *J. Neurochem.* **55,** 349–351.
12. Toda, N. & Okamura, T. (1990) *Am. J. Physiol.* **259,** H1511–H1517.
13. Heistad, D. D. & Kontos, H. (1983) in *Handbook of Physiology: The Cardiovascular System*, eds. Abboud, F. & Shephard, J. (Am. Physiol. Soc., Bethesda, MD), pp. 137–182.
14. Iadecola, C. (1991) *Soc. Neurosci. Abstr.* **17,** 27.
15. Iadecola, C., Arneric, S., Baker, H., Tucker, L. & Reis, D. (1987) *Am. J. Physiol.* **252,** R1082–R1091.
16. Iadecola, C. & Reis, D. J. (1990) *J. Cereb. Blood Flow Metab.* **10,** 608–617.
17. Iadecola, C. & Kraig, R. P. (1991) *Brain Res.* **563,** 273–277.
18. Iadecola, C., Mraovitch, S., Meeley, M. & Reis, D. J. (1983) *Brain Res.* **279,** 41–52.
19. Pickard, J. D. (1981) *J. Cereb. Blood Flow Metab.* **1,** 361–384.
20. Bredt, D. S., Hwang, P. M., Glatt, C. E., Lowenstein, C., Reed, R. R. & Snyder, S. H. (1991) *Nature (London)* **351,** 714–718.
21. Palmer, R., Ashton, D. & Moncada, S. (1988) *Nature (London)* **333,** 664–666.
22. Snyder, S. H. & Bredt, D. S. (1991) *Trends Pharmacol. Sci.* **12,** 125–128.
23. Dwyer, M. A., Bredt, D. S. & Snyder, S. H. (1991) *Biochem. Biophys. Res. Commun.* **176,** 1136–1141.
24. Sakuma, I., Stuehr, D. J., Gross, S. S., Nathan, C. & Levi, R. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 8664–8667.
25. Lifson, J. D., Rubinstein, E. H., Scremin, O. U. & Sonnenschein, R. (1985) *Experientia* **41,** 467–468.
26. Fujishima, M., Scheinberg, P., Busto, R. & Reinmuth, O. (1971) *Stroke* **2,** 251–257.
27. Lou, H. C., Edvinsson, L. & MacKenzie, E. T. (1987) *Ann. Neurol.* **22,** 289–297.
28. Harik, S. I., Prado, R., Busto, R. & Ginsberg, M. D. (1986) *Stroke* **17,** 1235–1238.
29. Ishitsuka, T., Iadecola, C., Underwood, M. & Reis, D. (1986) *Am. J. Physiol.* **251,** H269–H281.
30. Toda, N., Hatano, Y. & Mori, K. (1989) *Am. J. Physiol.* **257,** H141–H146.
31. Aubineau, P., Pearce, W., Cuevas, J. & Issertial, O. (1989) *J. Cereb. Blood Flow Metab.* **9,** S107 (abstr.).
32. Wei, E. P., Kontos, H. A. & Patterson, J. L. (1980) *Am. J. Physiol.* **238,** H697–H703.
33. Heistad, D. D., Marcus, M. L. & Abboud, F. M. (1978) *J. Clin. Invest.* **62,** 761–768.