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VASCULAR SMOOTH MUSCLE-DERIVED RELAXING FACTOR (MDRF) AND ITS CLOSE SIMILARITY TO NITRIC OXIDE

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SUMMARY: The principal finding in this study is that vascular smooth muscle generates a labile relaxing factor that possesses pharmacological and chemical properties that are similar to those of authentic nitric oxide. MDRF was generated by perfusion of endothelium-denuded bovine pulmonary artery as assessed by bioassay. In addition, endothelium-denuded arterial rings that were incubated at 37°C for 24 hr to lower endogenous L-arginine levels relaxed in response to L-arginine but not D-arginine. Freshly mounted, endothelium-denuded arterial rings were not relaxed by L-arginine but did relax in response to the dipeptide L-arginyl-L-alanine. Relaxant responses were accompanied by increases in smooth muscle levels of cyclic GMP and nitrite, and were inhibited by oxyhemoglobin, methylene blue, and N^G-nitro-L-arginine. N^G-Nitro-L-arginine also caused endothelium-independent contractile responses. Thus, a relaxing factor with the properties of nitric oxide can be generated from vascular smooth muscle.

Endothelium-derived relaxing factor (EDRF) was first described as a chemical substance that was generated from the vascular endothelium, which then interacted with underlying smooth muscle cells to cause relaxation (1,2). Various endogenous and exogenous substances were found to elicit endothelium-dependent relaxation by this mechanism (3). In each instance, the endothelium was found to be obligatory for the relaxant response and the belief has been that only the endothelial cells are responsible for EDRF generation. The extracellular receptors that interact with endothelium-dependent relaxants and/or the biochemical pathways that couple receptor occupancy to EDRF synthesis appear to reside in the endothelial rather than smooth muscle cells.

Much progress has been made in our understanding of the biosynthesis, metabolism, and actions of EDRF (4,5). EDRF appears to be nitric oxide (NO) or a labile nitroso compound that is synthesized from endogenous L-arginine or an L-arginine-containing substance within vascular endothelial cells (6-9). NO biosynthesis is not confined, however, to endothelial cells but occurs also in macrophages (10-12), neutrophils (13,14), cerebellar cells (15,16), and other cell types. Vascular smooth muscle has not been excluded from this expanding list, and evidence is presented in this communication for the formation and

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action of a vascular smooth muscle-derived relaxing factor with pharmacological and chemical properties that are similar to those of NO.

MATERIALS AND METHODS

Chemicals and solutions: L-Arginine, D-arginine, L-arginyl-L-alanine, indomethacin, phenylephrine HCl, acetylcholine chloride, bradykinin triacetate, methylene blue, hemoglobin (human), superoxide dismutase (bovine liver), N^G-nitro-L-arginine, and the reagents for the diazotization reaction were purchased from Sigma Chemical Co. (St. Louis, MO). Glyceryl trinitrate (10% w/w triturated mixture in lactose) was a gift from ICI Americas, Wilmington, DE. NO and oxyhemoglobin solutions were prepared as described (7,8). The water soluble crystalline HCl salt of authentic N^G-nitro-L-arginine was prepared by standard chemical procedures and used in this study. Krebs-bicarbonate solution consisted of (millimolar): NaCl, 118; KCl, 4.7; $CaCl_2$, 1.5; $NaHCO_3$, 25; $MgSO_4$, 1.2; KH_2PO_4, 1.2; glucose, 11; and disodium ethylenediaminetetraacetic acid, 0.023.

Arterial smooth muscle relaxation and bioassay cascade: Bovine intrapulmonary arterial rings (4-6 mm) were isolated and carefully but totally denuded of endothelium as recently described (17). The intimal surface was examined periodically by en face silver staining or scanning electron microscopy (18). Arterial rings were mounted, equilibrated under optimal tension, and precontracted as described (19). Changes in isometric force were measured and length-tension relationships were determined initially for each arterial ring (19), and optimal tensions were 4-5 g. The 24 hr incubations of arterial rings were conducted in oxygenated (95% O_2 - 5% CO_2) Krebs-bicarbonate solution at 37°C with the arterial rings mounted under tension (20). The bioassay cascade procedure was described in detail (17,21). Briefly, in the present study, a 6-8 cm length of pulmonary artery was denuded of endothelium by several alternating cycles of flushing the lumen with distilled water followed by mechanically rubbing the intimal lining gently with a cotton swab for 5 min. Pulmonary artery denuded in this manner failed to generate any relaxing factors in response to bradykinin (10 - 100 nM) or A23187 (1 μM). The perfusate from pulmonary artery was superfused over 3 strips of endothelium-denuded artery arranged in a cascade (17,21). Indomethacin (10 μM) was present in both the perfusion and superfusion media bathing all tissues in order to prevent formation of vasorelaxant prostaglandins. Flow of Krebs-bicarbonate solution at 37°C through the pulmonary artery was maintained at 5 ml/min. Glyceryl trinitrate was superfused over the strips to standardize the preparations (21).

Cyclic GMP determination: Cyclic GMP levels were measured in arterial rings that had been equilibrated under tension and subjected to precontraction as was done for all other rings, and tone was monitored until the time of quick-freezing with the aid of brass clamps precooled in liquid nitrogen. Preparation of tissues and cyclic GMP radioimmunoassay procedures were described (17).

Inorganic nitrite determination: Nitrite levels were measured in arterial rings that had been equilibrated under tension, precontracted, and quick frozen as described above. Each ring was homogenized in 0.5 ml of ice-cold absolute methanol, allowed to stand at 4°C for 18-20 hr, and samples were centrifuged at 10,000 x g for 10 min. Aliquots (300 μl) of clear supernatant were assayed for nitrite by a modification of procedures described previously (7). Briefly, 400 μl of 1% w/v sulfanilic acid in 4 N HCl were added to each sample. Samples were mixed and 100 μl of conc. HCl were added to clarify samples that had turned slightly cloudy. After 10 min, 300 μl of 1% w/v N-(1-naphthyl)-ethylenediamine in methanol were added and optical densities were measured at 548 nM.

RESULTS

Release of MDRF with the properties of NO from perfused pulmonary artery: Perfusion of endothelium-denuded bovine pulmonary artery caused the release of a labile vascular smooth muscle relaxing factor (Fig. 1). As assessed by bioassay, MDRF had a half-life of 3-4 sec and its biological activity was markedly enhanced by superoxide dismutase and abolished by oxyhemoglobin. Bradykinin, an endothelium-dependent relaxant, failed to generate any relaxing factor, even in the presence of superoxide dismutase, thus attesting to the absence of functional endothelial cells in the perfused artery. Morphological examination (en face silver staining) confirmed the absence of endothelium.

L-Arginine causes endothelium-independent arterial relaxation that is inhibited by oxyhemoglobin, methylene blue, and N^G-nitro-L-arginine: As reported previously (20), incubation of arterial rings at 37°C under tension for 24 hr results in a 3- to 5- fold lowering of tissue arginine levels. Endothelium-intact arterial rings incubated under such conditions became completely refractory to endothelium-dependent relaxation, but relaxation was restored upon addition of L-arginine to the bathing medium. Endothelium-denuded rings were not studied. Fig. 2 illustrates that endothelium-denuded arterial rings

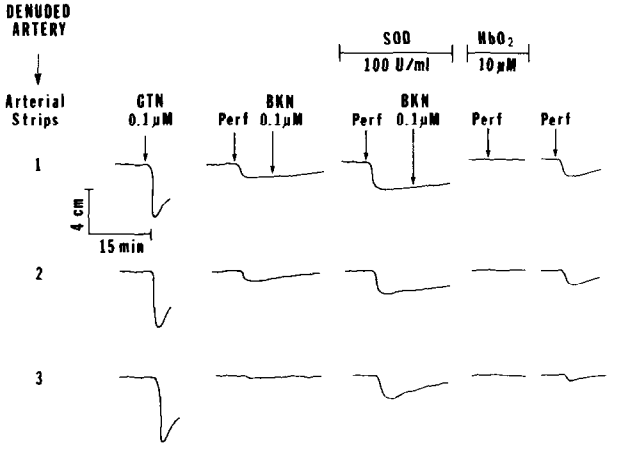

Fig. 1: Release of MDRF with the properties of NO from perfused pulmonary artery. A segment of endothelium-denuded pulmonary artery was perfused and the perfusate (Perf) was allowed to superfuse, where indicated, three strips (designated 1, 2 and 3) of endothelium-denuded pulmonary artery arranged in a cascade. Arterial strips were precontracted by 10 μM phenylephrine delivered by superfusion. Glyceryl trinitrate (GTN) was superfused over the strips for 1 min in order to check the responsiveness of the preparations. Bradykinin (BKN) was perfused through the artery for 3 min as indicated. Superoxide dismutase (SOD) was perfused through the artery during the time interval shown. Oxyhemoglobin (HbO_2) was superfused over the strips during the time interval shown. Tracings represent 1 of 4 separate experiments and responses varied by less than 25% among all experiments.

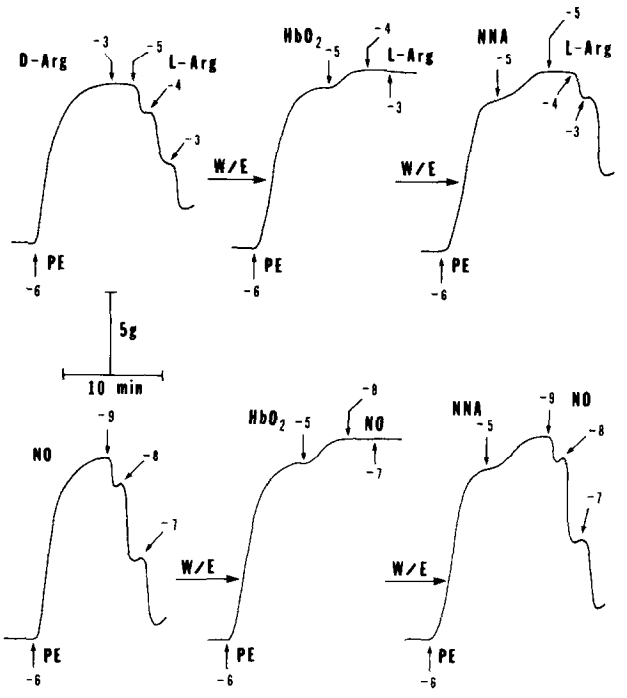

Fig. 2: L-Arginine-elicited relaxation of endothelium-denuded pulmonary arterial rings. Isolated rings were precontracted by phenylephrine (PE) and challenged with D-arginine (D-Arg), L-arginine (L-Arg), oxyhemoglobin (HbO_2), N^G-nitro-L-arginine (NNA), and NO as indicated. Indomethacin (10 μM) was present in the bathing media. W/E signifies washing and equilibration of rings for 45 min. Concentrations are expressed as exponents to the base power 10 and are final bath concentrations. Tracings represent 1 of 3-5 separate experiments and responses varied by less than 20% among all experiments.

incubated for 24 hr also relaxed in response to L-arginine in a concentration-dependent manner. Although endothelium-intact rings were somewhat more responsive to L-arginine (35 ± 5 percent relaxation at 10 μM; 65 ± 8 percent relaxation at 100 μM; n = 8) than were endothelium-denuded rings (12 ± 3 percent relaxation at 10 μM; 36 ± 5 percent relaxation at 100 μM; n = 8), the endothelium-independent responses to L-arginine were still highly significant (Fig. 2). Such relaxant responses were abolished by oxyhemoglobin and partially inhibited by N^G-nitro-L-arginine (Fig. 2) and methylene blue (not shown). Endothelium-denuded, 24 hr incubated arterial rings relaxed also in response to the methyl and ethyl esters of L-arginine as well as to L-homoarginine, and these responses were inhibited by oxyhemoglobin, methylene blue, and N^G-nitro-L-arginine (not shown). The 24 hr incubated, L-arginine-depleted arterial rings relaxed in response to authentic NO and responses were abolished by oxyhemoglobin but unaffected by N^G-nitro-L-arginine, indicating that incubated rings respond as do freshly mounted rings to NO. Complete removal of endothelium was verified morphologically by scanning electron microscopy or en face silver staining.

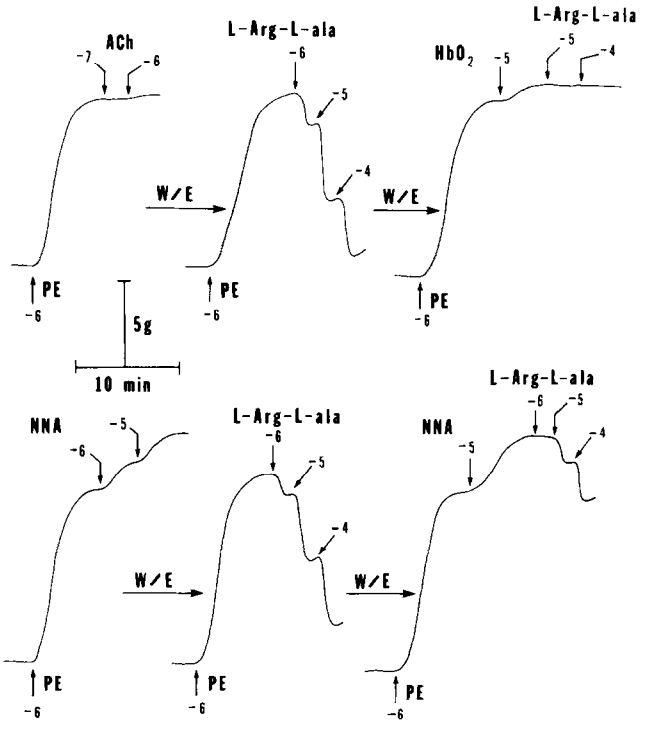

Fig. 3: L-Arginyl-L-alanine-elicited relaxation of endothelium-denuded pulmonary arterial rings. Isolated rings were precontracted by phenylephrine (PE) and challenged with acetylcholine (ACh), L-arginyl-L-alanine (L-Arg-L-ala), oxyhemoglobin (HbO_2), and N^G-nitro-L-arginine (NNA) as indicated. Indomethacin (10 μM) was present in the bathing media. W/E signifies washing and equilibration of rings for 45 min. Concentrations (final in bath) are expressed as exponents to the base power 10. Tracings represent 1 of 4-5 separate experiments and responses varied by less than 20% among all experiments.

L-Arginyl-L-alanine causes endothelium-independent arterial relaxation that is inhibited by oxyhemoglobin, methylene blue, and N^G-nitro-L-arginine: Freshly mounted, endothelium-denuded arterial rings failed to relax in response to acetylcholine or L-arginine (20) but relaxed in response to L-arginyl-L-alanine in a concentration-dependent manner (Fig. 3). L-Arginyl-L-alanine relaxed also 24 hr incubated arterial rings (not shown). Relaxant responses were abolished by oxyhemoglobin and partially inhibited by N^G-nitro-L-arginine (Fig. 3) and methylene blue (not shown). Endothelium-independent contractile responses were elicited by N^G-nitro-L-arginine (Fig. 3). Such contractions were somewhat greater in endothelium-intact rings (24 ± 3 percent contraction at 1 μM; 41 ± 8 percent contraction at 10 μM; n = 6) than in endothelium-denuded rings (16 ± 3 percent contraction at 1 μM; 28 ± 5 percent contraction at 10 μM; n = 6).

L-Arginine and L-arginyl-L-alanine stimulate formation of cyclic GMP and nitrite in endothelium-denuded arterial rings: Basal levels of nitrite were 3- fold higher in endothelium-intact (10 ± 2.1 μM; n = 14) than denuded freshly mounted

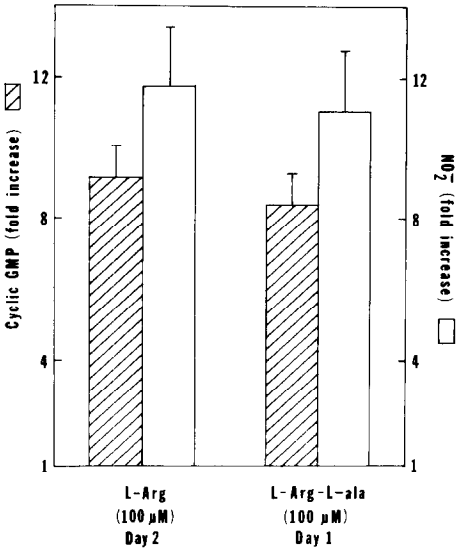

Fig. 4: L-Arginine and L-arginyl-L-alanine stimulate cyclic GMP and nitrite accumulation in endothelium-denuded pulmonary arterial rings. Isolated rings were precontracted by 1 μM phenylephrine and at peak contractile tension L-arginine (L-Arg) or L-arginyl-L-alanine (L-Arg-L-ala) was added to 24 hr incubated rings (Day 2) or freshly mounted rings (Day 1), respectively. Arterial rings were quick frozen at 180 sec after addition of L-Arg or L-Arg-L-ala. Values represent the mean ± S.E.M. of 24 individual arterial rings studied in 2 separate experiments. Basal cyclic GMP levels in Day 2 and Day 1 arterial rings were 4.1 ± 0.3 pmol/g (n = 12 rings) and 12 ± 2 pmol/g (n = 12 rings), respectively. Basal nitrite levels in Day 2 and Day 1 arterial rings were 2.0 ± 0.2 μM (n = 12 rings) and 3.3 ± 0.5 μM (n = 12 rings), respectively.

arterial rings (3.3 ± 0.5 μM; n = 12). Similarly, basal levels of nitrite were 5- fold lower in 24 hr incubated, endothelium-intact (2.1 ± 0.3 μM; n = 12) or denuded (2.0 ± 0.2 μM; n = 12) arterial rings than in freshly mounted rings. In 24 hr incubated, endothelium-denuded rings the addition of 100 μM L-arginine caused 9- to 12- fold increases in both cyclic GMP and nitrite levels (Fig. 4). In freshly mounted, endothelium-denuded rings the addition of 100 μM L-arginyl-L-alanine caused 8- to 11- fold increases in both cyclic GMP and nitrite levels (Fig. 4). Oxyhemoglobin (10 μM) and methylene blue (10 μM) inhibited both cyclic GMP and nitrite accumulation in response to 100 μM L-arginine or L-arginyl-L-alanine by 70 - 100 percent (n = 4 for each).

DISCUSSION

The principal observations in this communication are: 1, flow of fluid through or increased shear forces on endothelium-denuded pulmonary artery caused the generation of a labile relaxing factor with a half-life of less than 5 sec whose biological activity was enhanced by superoxide dismutase and abolished by oxyhemoglobin; 2, L-arginine caused endothelium-independent relaxation, cyclic

GMP formation and nitrite formation in L-arginine-depleted arterial rings, and these responses were inhibited by oxyhemoglobin, methylene blue, and N^G-nitro-L-arginine; 3, L-arginyl-L-alanine caused endothelium-independent relaxation, cyclic GMP formation and nitrite formation in freshly mounted arterial rings, and these responses were inhibited by oxyhemoglobin, methylene blue, and N^G-nitro-L-arginine. NO formation was assessed by determination of inorganic nitrite, the spontaneous oxidation product of NO. This study demonstrates that endothelium-denuded bovine pulmonary artery possesses the capacity to generate a labile relaxing factor (MDRF) with the pharmacological and chemical properties of NO or a nitroso compound.

The properties of MDRF resemble most closely the properties of authentic NO or a labile nitroso compound that decomposes spontaneously to liberate NO (6-8). L-Arginine, which is an endogenous substrate for the NO synthetase system (9-12,20,22), causes relaxation of 24 hr incubated, L-arginine-depleted arteries that is accompanied by cyclic GMP and nitrite accumulation and is inhibited by oxyhemoglobin, methylene blue, and N^G-nitro-L-arginine, a newly described potent inhibitor of NO synthetase (23). As was found previously with endothelium-intact rings (20), freshly mounted, endothelium-denuded rings failed to relax in response to L-arginine, whereas 24 hr incubated rings showed relaxation. The failure of freshly mounted rings to relax to added L-arginine is attributed to the already high endogenous levels of L-arginine (0.3 mM) in pulmonary artery, which decline 3- to 5- fold after 24 hr incubation (20). Thus, the conversion of L-arginine to NO in fresh tissue may already be at or near maximal velocity in the absence of added enzyme activators.

The present findings explain previous observations from this laboratory that the guanylate cyclase inhibitor methylene blue lowered basal cyclic GMP levels and increased intrinsic tone, whereas the cyclic GMP phosphodiesterase inhibitor M&B 22,948 elevated basal cyclic GMP levels and decreased intrinsic tone in endothelium-denuded artery and vein (19). Such findings are consistent with the present observations that an NO-like chemical factor that activates cytosolic guanylate cyclase is continuously generated by endothelium-denuded arterial tissue. Thus, modulation of the continual formation of cyclic GMP in smooth muscle can lead to alterations in intrinsic tone.

The most likely cells in the endothelium-denuded arterial rings that are responsible for the generation of the relaxing factor are the vascular smooth muscle cells. There were no morphologically identifiable or functional endothelial cells present in the denuded preparations. Moreover, it is unlikely that adventitial or other connective tissue is responsible for the formation of the relaxing factor, although this remote possibility has not been ruled out experimentally. Preliminary experiments with cultured bovine and rat thoracic aortic smooth muscle cells indicate that such cells contain both a constitutive and an inducible NO synthetase pathway. The former is involved in the immediate

formation of NO and nitrite in response to L-arginine, whereas the latter is involved in the 6 hr delayed formation of NO and nitrite in response to lipopolysaccharide, interleukin-1, and gamma interferon. After completion of this communication an abstract appeared on the induction of NO formation and cyclic GMP accumulation in rat vascular smooth muscle cells elicited by interleukin-1 and endotoxin (24).

L-Arginyl-L-alanine caused endothelium-independent relaxation and cyclic GMP and nitrite accumulation in freshly mounted arterial rings. These responses appear to be mediated by an NO-like factor because they were abolished by oxyhemoglobin and partially inhibited by methylene blue and N^G-nitro-L-arginine. Whether L-arginyl-L-alanine elicits its effects by acting as a precursor to NO or by activating NO synthetase is unknown. If L-arginyl-L-alanine does serve as a substrate for NO synthetase, the question arises as to whether the enzyme system involved is the same as that which utilizes L-arginine as substrate. There is evidence that multiple forms of NO synthetase exist in different cell types (25-27). This may also be possible in vascular smooth muscle.

The physiological or pathophysiological significance of these observations is presently unclear. The capacity of vascular smooth muscle to elaborate MDRF continuously could explain why endothelial damage *in vivo* rarely causes severe vasoconstriction or vasospasm.

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REFERENCES

1. Furchgott, R.F. and Zawadzki, J.V. (1980) *Nature (Lond.)* **288:** 373-376.
2. Cherry, P.D., Furchgott, R.F., Zawadzki, J.V. and Jothianandan, D. (1982) *Proc. Natl. Acad. Sci. USA* **72:** 2106-2110.
3. Furchgott, R.F. (1983) *Circ. Res.* **53:** 557-573.
4. Moncada, S., Radomski, M.W. and Palmer, R.M.J. (1988) *Biochem. Pharmacol.* **37:** 2495-2501.
5. Ignarro, L.J. (1989) *Circ. Res.* **65:** 1-21.
6. Palmer, R.M.J., Ferrige, A.G. and Moncada, S. (1987) *Nature (Lond.)* **327:** 524-526.
7. Ignarro, L.J., Buga, G.M., Wood, K.S., Byrns, R.E. and Chaudhuri, G. (1987) *Proc. Natl. Acad. Sci. USA* **84:** 9265-9269.
8. Ignarro, L.J., Byrns, R.E., Buga, G.M. and Wood, K.S. (1987) *Circ. Res.* **61:** 866-879.
9. Palmer, R.M.J., Ashton, D.S. and Moncada, S. (1988) *Nature (Lond.)* **333:** 664-666.
10. Hibbs, J.B., Taintor, R.R., Vavrin, Z. and Rachlin, E.M. (1988) *Biochem. Biophys. Res. Commun.* **157:** 87-94.
11. Stuehr, D.J., Gross, S.S., Sakuma, I., Levi, R. and Nathan, C.F. (1989) *J. Exp. Med.* **169:** 1011-1020.
12. Marletta, M.A., Yoon, P.S., Iyengar, R., Leaf, C.D. and Wishnok, J.S. (1988) *Biochemistry* **27:** 8706-8711.

13. Wright, C.D., Mulsch, A., Busse, R. and Osswald, H. (1989) Biochem. Biophys. Res. Commun. **160:** 813-819.
14. McCall, T.B., Boughton-Smith, N.K., Palmer, R.M.J., Whittle, B.J.R. and Moncada, S. (1989) Biochem. J. **261:** 293-296.
15. Knowles, R.G., Palacios, M., Palmer, R.M.J. and Moncada, S. (1989) Proc. Natl. Acad. Sci. USA **86:** 5159-5162.
16. Bredt, D.S. and Snyder, S.H. (1989) Proc Natl. Acad. Sci. USA **86:** 9030-9034.
17. Gold, M.E., Buga, G.M., Wood, K.S., Byrns, R.E., Chaudhuri, G. and Ignarro, L.J. (1990) Circ. Res. **66:** 355-366.
18. Ignarro, L.J., Gold, M.E., Buga, G.M., Byrns, R.E., Wood, K.S., Chaudhuri, G. and Frank, G. (1989) Circ. Res. **64:** 315-329.
19. Ignarro, L.J., Byrns, R.E. and Wood, K.S. (1987) Circ. Res. **60:** 82-92.
20. Gold, M.E., Bush, P.A. and Ignarro, L.J. (1989) Biochem. Biophys. Res. Commun. **164:** 714-721.
21. Ignarro, L.J., Buga, G.M. and Chaudhuri, G. (1988) Eur. J. Pharmacol. **149:** 79-88.
22. Sakuma, I., Stuehr, D., Gross, S.S., Nathan, C. and Levi, R. (1988) Proc. Natl. Acad. Sci. USA **85**: 8664-8667.
23. Moore, P.K., al-Swayeh, O.A., Chong, N.W.S., Evans, R.A. and Gibson, A. (1990) Br. J. Pharmacol. **99:** 408-412.
24. Beasley, D., Brenner, B.M. and Schwartz, J.H. (1990) FASEB J. **4:** A685.
25. Kwon, N.S., Nathan, C.F. and Stuehr, D.J. (1989) J. Biol. Chem. **264:** 20496-20501.
26. Tayeh, M.A. and Marletta, M.A. (1989) J. Biol. Chem. **264:** 19654-19658.
27. Bredt, D.S. and Snyder, S.H. (1990) Proc Natl. Acad. Sci. USA **87:** 682-685.