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Synthesis of Nitric Oxide by the Haemocytes of the American Horseshoe Crab (Limulus polyphemus)

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Synthesis of nitric oxide by the haemocytes of the American horseshoe crab (*Limulus polyphemus*)

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SUMMARY

Nitric oxide (NO) synthase, the enzyme responsible for the production of NO from L-arginine, is present in haemocytes of the American horseshoe crab (*Limulus polyphemus*). The synthesis of NO results in down-regulation of the aggregatory function of these cells in a manner similar to that previously described for mammalian platelets. These data indicate that formation of NO from L-arginine is a pathway of early evolutionary origin.

1. INTRODUCTION

In mammalian cells, nitric oxide (NO) is synthesized from the amino acid L-arginine (L-Arg) by constitutive or inducible NO synthases. The NO thus formed represents, respectively, the transduction mechanism for the soluble guanylate cyclase which regulates cell function and communication, or a cytotoxic mechanism that protects the host from invading micro-organisms or tumour cells (Moncada *et al.* 1989; Hibbs *et al.* 1990; Moncada & Palmer 1990). We now show that NO is also synthesized from L-arginine by haemocytes: the multicompetent cells in the haemolymph of an arthropod, the American horseshoe crab (*Limulus polyphemus*), a phylogenetic relic whose unchanged existence spans over 500 million years of evolution (Moore *et al.* 1952).

2. METHODS

Limulus crabs, measuring 20–35 cm across the carapace, were obtained from the Institute of Marine Biology, Cape Cod, Massachusetts, U.S.A. They were kept in an aquarium containing natural sea water maintained at 15–20 °C. The effect of L-Arg, an inhibitor of NO synthase (N^G-monomethyl-L-arginine; L-NMMA; Palmer *et al.* 1988*a*) and its inactive enantiomer (D-NMMA) on aggregation of *Limulus* crab haemocytes was studied *ex vivo* after systemic administration of the compounds to the animals. In addition, the effect of prostacyclin and S-nitroso-acetylpenicillamine (SNAP) on aggregation of haemocytes *in vitro* was investigated.

The flexure between the animal's prosoma and opisthoma was exposed, wiped with 70% ethanol, and L-Arg (300 mg kg^{-1}) or L(or D)-NMMA (100 mg kg^{-1}) was administered intracardially in 0.5 ml of Tris-buffered saline (0.51 M NaCl mixed with 0.05 M Tris-HCl, 9:1 by volume). After 10 min, a sample of haemolymph (0.2 ml, 1100–2100 cells per microlitre) was taken by cardiac puncture and added 30 s later to 0.8 ml of Tris-buffered saline in an aggregometer cuvette and stirred at 300 r.p.m. Spontaneous aggregation was then measured (Kenney *et al.* 1972) for 150 s in a platelet-ionized calcium aggregometer (Chronolog). All aggregations were done at 37 °C, at which temperature the haemocyte aggregation was similar to that observed at room temperature (*ca.* 22 °C; Kenney *et al.* 1972). The maximal light transmission was calibrated using a mixture of Tris-

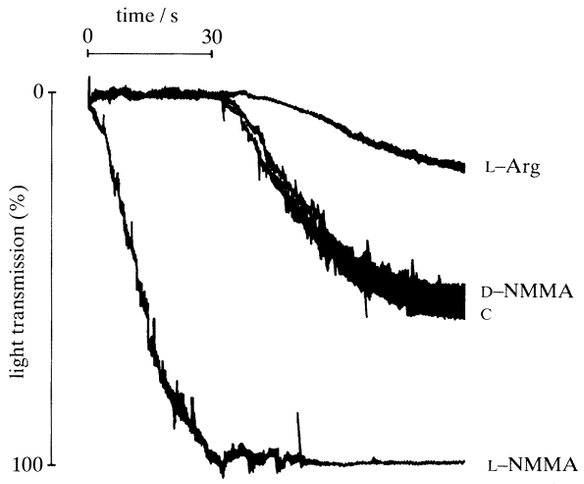

Figure 1. The effect of L-Arg and L-NMMA on the *ex vivo* aggregation of haemocytes. The aggregation of control haemocytes (C) occurred after a lag-phase of *ca.* 30 s and reached 72 ± 11% of the maximal light transmission (mean ± s.e.m., $n = 3$). This was not significantly different in samples from crabs treated with D-NMMA (66 ± 12%, $p > 0.05$, Student's t test, $n = 3$). However, aggregation of haemocytes from crabs treated with L-NMMA occurred immediately and was maximal ($n = 3$). In contrast, haemocytes from crabs treated with L-Arg showed a reduced aggregation (19 ± 11%, $p < 0.05$, $n = 3$). The tracings are representative of three similar experiments.

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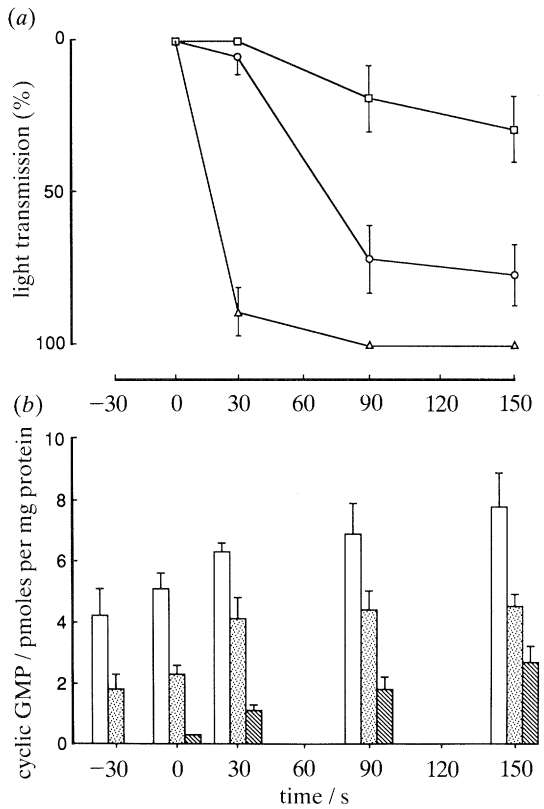

Figure 2. Timecourse of spontaneous aggregation of haemocytes and corresponding changes in intracellular cyclic GMP levels. The aggregation (*a*) and the levels of intracellular cyclic GMP (*b*) of haemocytes obtained from control animals (circles), and those treated with L-Arg (squares) or with L-NMMA (triangles) were measured by light aggregometry and RIA respectively. The cyclic GMP levels in control haemocytes (stippled bars) measured at the time of collection (−30 s) were significantly lower ($p < 0.05$) than those measured from haemocytes obtained after treatment with L-Arg (empty bars; $p < 0.05$, $n = 3$). Cyclic GMP was not detectable (less than 0.1 pmoles per mg protein, $n = 3$) in haemocytes obtained after treatment with L-NMMA (dashed bars). The aggregation of these cells resulted in a time-dependent increase in cyclic GMP levels in all treatment groups but the value of each treatment group was significantly different from the others ($p < 0.05$, $n = 3$).

buffered saline and cell-free haemolymph (4:1 by volume) and aggregation was expressed as a percentage of this value. In some experiments, cyclic GMP and cyclic AMP levels in samples containing haemocytes were measured by radioimmunoassay (RIA, Amersham; Radomski *et al.* 1990*a*).

The presence and the activity of NO synthase was determined in the cytosol of haemocytes prepared by homogenization and ultracentrifugation as described previously (Radomski *et al.* 1990*b*). For this, haemolymph (60 ml) was collected by cardiac puncture and centrifuged for 5 min at 600 *g* at room temperature to sediment the haemocytes. The pellet was washed once (600 *g*, 3 min, room temperature) with Tris-buffered saline, resuspended in 3 ml of homogenization buffer, homogenized and then centrifuged at 100000 *g* for 30 min at 4 °C. The supernatant was passed through a 2 ml column of cation-exchange resin (AG-50W-X8, Bio-Rad) to remove endogenous arginine. In some experiments the supernatant was passed through a 2 ml column of Sephadex G-25 to remove low molecular mass components.

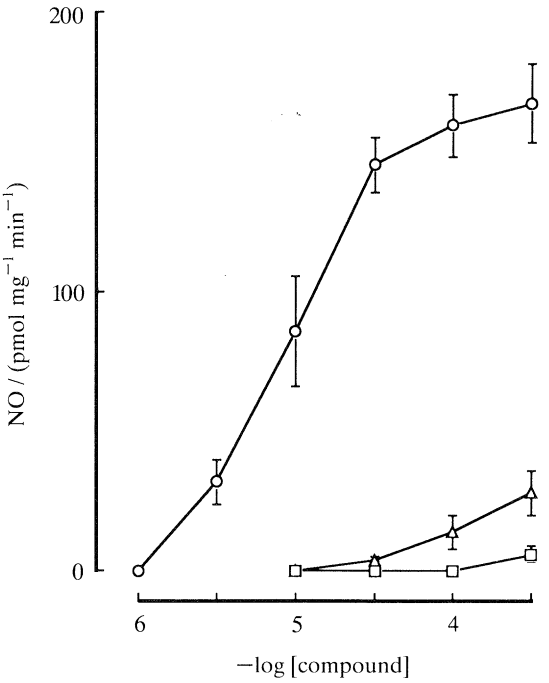

Figure 3. L-arginine-dependent formation of NO in the cytosol of *Limulus* crab haemocytes. Addition of L-Arg (circles) but not D-Arg (squares, 3–300 μM) to the incubate depleted of L-Arg resulted in a concentration-dependent increase in the rate of NO formation to a maximum of 168 ± 14 pmoles per mg protein per min ($n = 3$). A small but significant increase in the formation of NO was also seen with L-citrulline (triangles) at concentrations of 100 and 300 μM ($n = 3$). The rate of NO synthesis was monitored for 10 min and was linear over this time.

Nitric oxide synthesis was measured spectrophotometrically (Feelisch & Noack 1987; Radomski *et al.* 1990*b*) or by bioassay as inhibition of platelet aggregation. For the platelet assay, haemocyte cytosol was incubated for 1 min at 37 °C in the presence of indomethacin (10 μM) and the effect of incubates (10–100 μl) on collagen (4 μg ml^{-1})-induced aggregation of human washed platelets (Radomski & Moncada 1983; Radomski *et al.* 1987*a*) was studied.

Results are expressed as mean ± s.e.m. for (n) separate experiments. Dose-response curves were compared by analysis of variance and the significance of differences between mean values was estimated by *t*-test. A p value of less than 0.05 was considered to be significant.

3. RESULTS

Incubation of haemocytes from control animals resulted in spontaneous aggregation which occurred after a lag period of 30 s (figures 1 and 2*a*). This aggregation was not significantly changed in haemocytes obtained from crabs treated with D-NMMA; however, the aggregation was significantly enhanced by pretreatment with L-NMMA and was inhibited by pretreatment with L-Arg (figures 1 and 2*a*). The cyclic GMP levels in haemocytes obtained from control animals increased with the time of aggregation (figure

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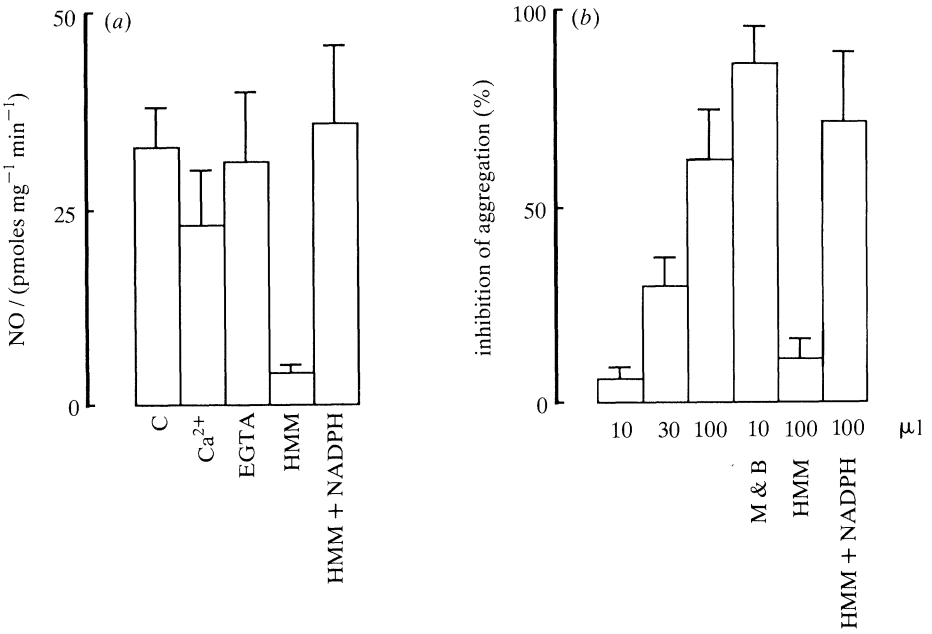

Figure 4. Characterization of NO synthase activity in cytosol of haemocytes. The basal rate of NO formation (C) was 33 ± 5 pmoles per mg protein per min, $n = 3$. This was independent of the presence of Ca^{2+}, because neither the addition of Ca^{2+} (200 µM) nor EGTA (1 mM) significantly affected the formation of NO (*a*). Nitric oxide could also be detected by bioassay as haemocyte cytosol inhibited platelet aggregation in a volume-dependent manner (*b*) and this inhibitory effect was potentiated by M&B22948 (1 µM, M & B), a selective inhibitor of platelet cyclic GMP phosphodiesterase (Radomski *et al.* 1987*a*). The formation of NO was NADPH-dependent because it was greatly reduced in cytosol which was depleted of low molecular mass components (HMM), but could be restored to the control levels by the addition of NADPH (300 µM, HMM + NADPH).

2*b*). Pretreatment of crabs with L-Arg significantly enhanced, whereas L-NMMA inhibited, the formation of cyclic GMP in the haemocytes (figure 2*b*). Cyclic AMP was not detected (< 0.1 pmol per mg protein; $n = 3$) in these incubates. The aggregation of haemocytes obtained from control animals was not inhibited by prostacyclin (< 100 nM, $n = 3$), however, SNAP (0.3–30 µM; Feelisch 1991), which releases NO, caused concentration-dependent inhibition of aggregation with an $IC_{50} = 5.6 \pm 2.0$ µM, $n = 3$.

The cytosol obtained from haemocytes synthesized NO from L-Arg but not from D-Arg (figure 3). Incubation of cytosol with high concentrations of L-citrulline also resulted in an increase in the synthesis of NO (figure 3). The NO synthesis was Ca^{2+}-independent but required NADPH (figure 4*a*). Moreover, the amounts of NO produced by the cytosol were sufficient to inhibit aggregation of human platelets (figure 4*b*). L-arginine-induced synthesis of NO was inhibited in a concentration-dependent manner by L-canavanine, L-NMMA, *N*-iminoethyl-L-ornithine (L-NIO) and by N^G-nitro-L-arginine methyl ester (L-NAME) (figure 5).

4. DISCUSSION

Our results show that the haemocytes of *Limulus polyphemus* contain an NO synthase. L-Arginine and L-citrulline were substrates for the formation of NO but L-citrulline was 16–20 times less potent than L-Arg, suggesting that the conversion of L-citrulline to L-Arg may be occurring before its metabolism to NO (Palmer *et al.* 1988*b*). Nitric oxide synthase in the haemocyte cytosol was Ca^{2+}-independent, required NADPH and its activity was inhibited by L-canavanine, L-NMMA and other L-arginine analogues, with maximal inhibition ranging from 46 to 73%. Interestingly, the activities of NO synthases from mammalian macrophages and platelets can be completely inhibited by L-NMMA (Hibbs *et al.* 1990; Radomski *et al.* 1990*a*) suggesting that arthropodous and mammalian NO synthases may differ from each other.

When L-NMMA was administered *in vivo* it caused a significant enhancement of haemocyte aggregation and a concomitant decrease in guanylate cyclase activity. In contrast, administration of L-Arg resulted in inhibition of aggregation and enhancement of cyclic GMP levels. Thus, haemocytes of *Limulus* crabs contain NO synthase and guanylate cyclase which act to down-regulate the haemostatic function of these cells in a way similar to that described for human platelets (Radomski *et al.* 1990*a*, *c*). In the haemocytes we could not measure any changes in cyclic AMP levels during aggregation. It is likely, therefore, that in these cells, increases in cyclic GMP resulting from the formation of NO is the only autocrine nucleotide system which regulates aggregation.

Whether NO is also produced by the vessels of the crab and, if so, whether it may influence haemocyte aggregation as a paracrine system remains to be investigated. In this context we have shown that SNAP inhibited haemocyte aggregation, indicating that haemocytes can respond to exogenously applied NO. An inhibitory effect on haemocytes of high doses of

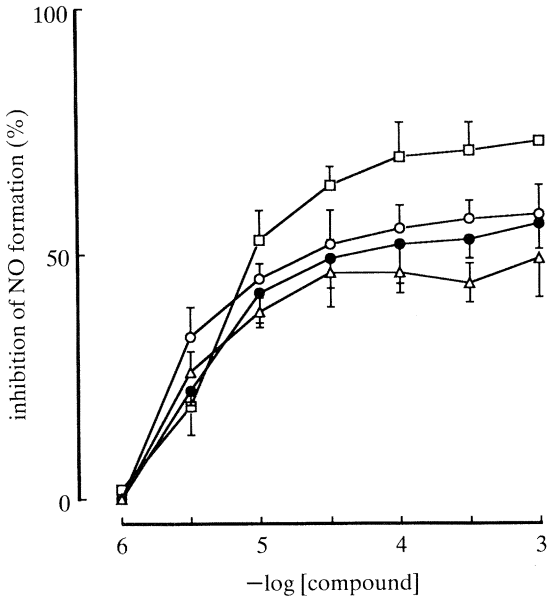

Figure 5. Inhibition by L-canavanine, L-NMMA, L-NIO and L-NAME of the NO synthase activity in cytosol from haemocytes. The cytosol was depleted of endogenous L-Arg. L-canavanine (open squares), L-NMMA (open circles), L-NIO (closed circles) and L-NAME (open triangles, 1–1000 μM) were incubated for 3 min before stimulation of NO synthesis by L-Arg (30 μM) and NADPH (300 μM). L-canavanine (Iyengar *et al.* 1987) and other inhibitors of NO synthase (Radomski *et al.* 1990*a*; Rees *et al.* 1990) inhibited, in a concentration-dependent manner, the rate of NO formation. L-canavanine was the most effective inhibitor, resulting in $73 \pm 3\%$ inhibition at 1000 μM ($n = 3$). The efficacies of the remaining inhibitors were significantly less than that of L-canavanine but did not differ from each other ($p > 0.05$, $n = 3$). However, the potencies of all four inhibitors were similar ($p > 0.05$, $n = 3$).

prostacyclin, in combination with the phosphodiesterase inhibitor theophylline, has been reported and ascribed to the formation of cyclic AMP (Armstrong & Rickles 1982). However, it is more likely that these effects are due to an indirect effect on the guanylate cyclase, as has been described for human platelets (Radomski *et al.* 1987*b*).

The formation of NO from L-Arg in mammalian macrophages is a potent cytotoxic mechanism that plays an important role in host defence (Hibbs *et al.* 1988, 1990). *Limulus* haemocytes not only act as haemostatic cells but also as phagocytic cells comparable with the macrophage (Levin 1988). It is possible that the NO generated by haemocytes is both an autocrine regulator of their own aggregability and a molecule cytotoxic for invading microorganisms. Evolution has resulted in the separation of these functions with the development of specialized blood elements such as the mammalian platelet and the macrophage. Whether bone marrow stem cells, from which both these cell types derive, contain both NO synthases and also a guanylate cyclase deserves investigation. Nitric oxide synthase in *Limulus* haemocytes is Ca^{2+} independent, like the enzyme induced in mammalian cells by bacterial endotoxin and cytokines (Radomski *et al.* 1990*b*; McCall *et al.* 1991). This enzyme may be constitutive in the *Limulus* crab, or it may be in a state of continuous induction due to endotoxin to which the *Limulus* haemocytes are exposed as a result of their open cardiovascular system (Bang 1956; Levin 1985; Miyata *et al.* 1989).

Because the *Limulus* crab is a species of early evolutionary origin it is likely that the L-arginine to NO pathway ranks among the oldest regulatory systems that have contributed to the development of animal life.

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REFERENCES

Armstrong, P. B. & Rickles, F. B. 1982 Endotoxin-induced degranulation of the *Limulus* amebocyte. *Expl Cell Res.* **140**, 15–24.

Bang, F. B. 1956 A bacterial disease of *Limulus polyphemus*. *Bull. Johns Hopkins Hosp.* **98**, 325–351.

Feelisch, M. 1991 The biochemical pathways of nitric oxide formation from nitrovasodilators: appropriate choice of exogenous NO-donors and aspects of preparation and handling of aqueous NO solutions. *J. Cardiovasc. Pharmacol.* **17** (Suppl. 3), S25–S33.

Feelisch, M. & Noack, E. A. 1987 Correlation between nitric oxide formation during degradation of organic nitrates and activation of guanylate cyclase. *Eur. J. Pharmacol.* **139**, 19–30.

Hibbs, J. B., Jr., Taintor, R. R., Vavrin, Z. & Rachlin, E. M. 1988 Nitric oxide: a cytotoxic activated macrophage molecule. *Biochem. biophys. Res. Commun.* **157**, 87–94.

Hibbs, J. B., Jr., Taintor, R. R., Vavrin, Z., Granger, D. L., Drapier, J. C., Amber, I. J. & Lancaster, J. R. Jr 1990 Synthesis of nitric oxide from a terminal guanidino nitrogen atom of L-arginine: a molecular mechanism regulating cellular proliferation that targets intracellular ion. In *Nitric oxide from L-arginine: a bioregulatory system* (ed. S. Moncada & E. A. Higgs), pp. 189–223. Amsterdam: Elsevier.

Iyengar, R., Stuehr, D. & Marletta, M. A. 1987 Macrophage synthesis of nitrite, nitrate and N-nitrosamines: precursors and role of the respiratory burst. *Proc. natn. Acad. Sci. U.S.A.* **84**, 6369–6373.

Kenney, D. M., Belamarich, F. A. & Shepro, D. 1972 Aggregation of horseshoe crab (*Limulus polyphemus*) amebocytes and reversible inhibition of aggregation by EDTA. *Biol. Bull.* **143**, 548–567.

Levin, J. 1985 The history of the development of the *Limulus* amebocyte lysate test. In *Bacterial endotoxins: structure, biomedical significance, and detection with* Limulus *amebocyte lysate test*, pp. 3–28. New York: Alan R. Liss, Inc.

Levin, J. 1988 The horseshoe crab: a model for Gram-negative sepsis in marine organisms and humans. In *Bacterial endotoxins: pathophysiological effects, clinical significance, and pharmacological control*, pp. 3–15. Alan R. Liss, Inc.

McCall, T. B., Feelisch, M., Palmer, R. M. J. & Moncada, S. 1990 Identification of N-iminoethyl-L-ornithine as an irreversible inhibitor of nitric oxide synthase in phagocytic cells. *Br. J. Pharmacol.* **102**, 234–238.

Miyata, T., Tokunaga, F., Yoneya, T., Yoshikawa, K., Iwanaga, S., Niwa, M., Takao, T. & Shimonishi, Y. 1989 Antimicrobial peptides, isolated from crab hemocytes,

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

tachyplesin II, and polyphemusins I and II: chemical structures and biological activity. *J. Biochem.* **106**, 663–668.

Moncada, S. & Palmer, R. M. J. 1990 The L-arginine: nitric oxide pathway in the vessel wall. In *Nitric oxide from L-arginine: a bioregulatory system* (ed. S. Moncada & E. A. Higgs), pp. 19–33. Amsterdam: Elsevier.

Moncada, S., Palmer, R. M. J. & Higgs, E. A. 1989 Biosynthesis of nitric oxide from L-arginine: a pathway for regulation of cell function and communication. *Biochem. Pharmacol.* **38**, 1709–1715.

Moore, R. C. Lalicker, C. G. & Fischer, A. G. 1952 *Invertebrate fossils*. New York: McGraw-Hill.

Palmer, R. M. J., Rees, D. D., Ashton, D. S. & Moncada, S. 1988*a* L-arginine is the physiological precursor for the formation of nitric oxide in endothelium-dependent relaxation. *Biochem. Biophys. Res. Commun.*, **153**, 1251–1256.

Palmer, R. M. J., Ashton, D. S. & Moncada, S. 1988*b* Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature, Lond.* **333**, 664–666.

Radomski, M. & Moncada, S. 1983 An improved method for washing of platelets with prostacyclin. *Thromb. Res.* **30**, 383–389.

Radomski, M. W., Palmer, R. M. J. & Moncada, S. 1987*a* Comparative pharmacology of endothelium-derived relaxing factor, nitric oxide and prostacyclin in platelets. *Br. J. Pharmacol.* **92**, 181–187.

Radomski, M. W., Palmer, R. M. J. & Moncada, S. 1987*b* The role of nitric oxide and cGMP in platelet adhesion to vascular endothelium. *Biochem. biophys. Res. Commun.* **148**, 1482–1489.

Radomski, M. W., Palmer, R. M. J. & Moncada, S. 1990*a* An L-arginine/nitric oxide pathway present in human platelets regulates aggregation. *Proc. natn. Acad. Sci. U.S.A.* **87**, 5193–5197.

Radomski, M. W., Palmer, R. M. J. & Moncada, S. 1990*b* Glucocorticoids inhibit the expression of an inducible, but not the constitutive nitric oxide synthase in vascular endothelial cells. *Proc. natn. Acad. Sci. U.S.A.* **87**, 10043–10047.

Radomski, M. W., Palmer, R. M. J. & Moncada, S. 1990*c* Characterization of the L-arginine: nitric oxide pathway in human platelets. *Br. J. Pharmacol.* **101**, 325–328.

Rees, D. D., Palmer, R. M. J., Schulz, R., Hodson, H. F. & Moncada, S. 1990 Characterization of three inhibitors of endothelial nitric oxide synthase in vitro and in vivo. *Br. J. Pharmacol.* **101**, 746–752.

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