Characterization of the L-arginine:nitric oxide pathway in human platelets

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1 The activation of the L-arginine:nitric oxide (NO) pathway during aggregation of human platelets by adenosine 5'-diphosphate (ADP), arachidonic acid, thrombin and the calcium ionophore A23187 and its inhibition by N^G-monomethyl-L-arginine (L-NMMA), N^G-nitro-L-arginine methyl ester (L-NAME) and N-iminoethyl-L-ornithine (L-NIO) were studied. The inhibition of the cytosolic platelet NO synthase by these compounds was also examined.

2 Platelet aggregation induced by ADP (1–10 μ M) and arachidonic acid (0.1–10 μ M), but not that induced by thrombin (1–30 μ M) or A23187 (1–10 nM), was inhibited by L-, but not D-arginine (1–30 μ M). However, in the presence of a subthreshold concentration of prostacyclin (0.1 nM) or of M & B 22948 (1 μ M), a selective inhibitor of guanosine 3':5'-cyclic monophosphate (cyclic GMP) phosphodiesterase, L-arginine caused concentration-dependent inhibition of aggregation induced by all of these aggregating agents.

3 L-NMMA, L-NAME and L-NIO (all at 1–30 μ M), but not their D-enantiomers, enhanced to the same extent platelet aggregation induced by ADP, arachidonic acid and thrombin without affecting that induced by A23187.

4 In the presence of 300 μ M L-arginine, the NO synthase in platelet cytosol was inhibited by L-NMMA, L-NAME and L-NIO with IC₅₀s of 74 ± 9 , 79 ± 8 and 8.5 ± 1.5 μ M ($n = 3$), respectively.

5 These results indicate that the L-arginine:NO pathway in human platelets plays a role in the modulation of platelet aggregation.

Introduction

Synthesis of nitric oxide (NO) from L-arginine is now known to occur in vascular endothelial cells (Palmer *et al.*, 1988a), macrophages (Hibbs *et al.*, 1988; Marletta *et al.*, 1988; Kwon *et al.*, 1989), neutrophils (McCall *et al.*, 1989; Salvemini *et al.*, 1989), brain synaptosomes (Knowles *et al.*, 1989), adrenal glands (Palacios *et al.*, 1989) and a number of other tissues (for review see Moncada *et al.*, 1989). This pathway plays a role in the regulation of vascular tone and blood pressure (Rees *et al.*, 1989; Aisaka *et al.*, 1989) and in neurotransmission in the central nervous system (Garthwaite *et al.*, 1989; Bredt & Snyder 1989), in peripheral non-adrenergic, non-cholinergic neurones (Gillespie *et al.*, 1989; Gibson *et al.*, 1990) and possibly in the regulation of the entry of sensory stimuli into the peripheral nerve endings (Duarte *et al.*, 1990). In addition, NO released by phagocytic cells mediates some of their cytotoxic functions (Hibbs *et al.*, 1990). These findings led us to propose that the L-arginine:NO pathway is a widespread regulatory mechanism and the endogenous activator of the soluble guanylate cyclase (Moncada *et al.*, 1989).

Nitric oxide also inhibits platelet aggregation (Mellion *et al.*, 1981; Radomski *et al.*, 1987a). This effect is mediated via activation of the soluble guanylate cyclase and is potentiated by a selective inhibitor of the guanosine 3':5'-cyclic monophosphate (cyclic GMP) phosphodiesterase, 2-O-propoxyphenyl-8-azapurin-6-one (M&B 22948, Lugnier *et al.*, 1986) and by a subthreshold concentration of prostacyclin (Radomski *et al.*, 1987a). Furthermore, we have recently shown that when human platelets are stimulated with collagen, they synthesize NO from L-arginine. This NO activates the soluble guanylate cyclase and down-regulates the response of platelets to collagen (Radomski *et al.*, 1990).

There are at least two distinct NO synthases in different tissues; the endothelial/brain enzyme (Palmer & Moncada,

1989; Meyer *et al.*, 1989; Knowles *et al.*, 1989) and the phagocytic cell enzyme (Marletta *et al.*, 1988; McCall *et al.*, 1989). The former is NADPH- and Ca²⁺-dependent and not affected by L-canavanine (Palmer & Moncada 1989; Meyer *et al.*, 1989; Knowles *et al.*, 1989) and the latter is not Ca²⁺-dependent, is inhibited by L-canavanine and requires tetrahydrobiopterin in addition to NADPH (Marletta *et al.*, 1988; Kwon *et al.*, 1989). Our previous findings indicate that the platelet NO synthase is of the endothelial/brain type since it is Ca²⁺-dependent (Radomski *et al.*, 1990).

N^G-monomethyl-L-arginine (L-NMMA) was originally described as an inhibitor of NO₂⁻ and NO₃⁻ synthesis from L-arginine by macrophages (Hibbs *et al.*, 1987) and of NO synthesis in vascular endothelial cells (Palmer *et al.*, 1988b). Furthermore, L-NMMA inhibits NO synthesis in platelets (Radomski *et al.*, 1990) and in a number of other tissues (for review see Moncada *et al.*, 1989). More recently, N^G-nitro-L-arginine or its methyl ester (L-NAME, Palacios *et al.*, 1989; Rees *et al.*, 1990; Moore *et al.*, 1990; Mulsch & Busse 1990) and N^G-iminoethyl-L-ornithine (L-NIO, Palacios *et al.*, 1989; Rees *et al.*, 1990; Knowles *et al.*, 1990) have also been shown to inhibit NO synthesis in adrenal glands, endothelial cells and brain synaptosomes.

In the present study we have examined the activation of the L-arginine:NO pathway in platelets stimulated with adenosine 5'-diphosphate (ADP), arachidonic acid, thrombin and A23187 and have characterized the activity of these L-arginine analogues as inhibitors of platelet NO synthase.

Methods

Platelet aggregation

Blood was collected from healthy volunteers into trisodium citrate (0.315%) and platelets were isolated and washed using prostacyclin as described previously (Radomski & Moncada, 1983). The platelets were finally resuspended in Tyrode solution at a concentration of 2×10^8 platelets ml⁻¹. Platelet aggregation induced by ADP, arachidonic acid, thrombin and

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A23187 was measured in an aggregometer (Payton Associates) by the method of Born (1962). Test compounds were added to the platelet suspension in the aggregometer 3 min before the addition of the aggregating agent.

NO synthase assay

Washed platelets ($7-8 \times 10^{10}$) in 3 ml Tyrode solution were sonicated twice for 5 s and the homogenate centrifuged at $150,000g$ for 30 min at 4°C . The supernatant was then depleted of endogenous L-arginine by incubation with 500 mg AG50X8 (Dowex) cation exchange resin for 10 min at 4°C . Following incubation, the supernatant was removed and used as the source of cytosolic NO synthase. The activity of NO synthase was determined, following incubation for 20 min in the presence of NADPH ($300 \mu\text{M}$) and L-arginine ($300 \mu\text{M}$), by measuring the formation of cyclic GMP as described previously (Knowles *et al.*, 1989; Radomski *et al.*, 1990).

Reagents

Arachidonic acid sodium salt, ADP, L(D)-arginine, L-canavanine, N^G -nitro-L-arginine methyl ester (L-NAME, Sigma), A23187 (Calbiochem), M&B 22948 (May & Baker), L-NMMA, D-NMMA, L-NIO, prostacyclin sodium salt (Wellcome) and thrombin (Ortho Diagnostics) were obtained as indicated.

Statistics

All values are means $\pm$ s.e.mean of n experiments. Values were compared by use of Student's t test for paired or unpaired samples as appropriate and $P < 0.05$ was considered as statistically significant.

Results

Platelet aggregation

Platelet aggregation induced by ADP ($10 \mu\text{M}$) or arachidonic acid ($3 \mu\text{M}$) was inhibited in a concentration-dependent manner by L-arginine (Table 1) but not D-arginine ($100 \mu\text{M}$, $n = 3$). In contrast, platelet aggregation induced by thrombin (30 mU ml^{-1}) or A23187 (10 nM) was not inhibited by L-arginine ($30 \mu\text{M}$). Preincubation of platelets with a subthreshold concentration of prostacyclin (0.1 nM) enhanced the activity of L-arginine such that the IC_{50} against arachidonic acid and ADP was significantly reduced and its action against thrombin- or A23187-induced aggregation was evident (Table 1). The inhibition by L-arginine of platelet aggregation induced by thrombin or A23187 was also evident in the presence of M&B 22948 (Table 1).

Table 1 The effect of L-arginine on platelet aggregation induced by ADP, arachidonic acid, thrombin and A23187

	L-Arginine IC_{50} (μM , $n = 3-5$)		
	L-Arginine	L-Arginine + PGI_2	L-Arginine + M&B 22948
ADP	9.2 ± 1.4	3.8 ± 1.2	n.a.
Arachidonic acid	7.8 ± 1.6	2.6 ± 0.7	n.a.
Thrombin*	>100	16.5 ± 3.2	27.2 ± 5.2
A23187**	>100	28.3 ± 4.0	34.2 ± 5.8

* $22 \pm 4\%$ inhibition at $100 \mu\text{M}$.

** $14 \pm 3\%$ inhibition at $100 \mu\text{M}$.

n.a. not assayed. PGI_2 = prostacyclin

Figure 1 Enhancement of arachidonic acid-induced platelet aggregation by N^G -monomethyl-L-arginine (L-NMMA). Aggregation was induced by a submaximal concentration of arachidonic acid (AA, $1 \mu\text{M}$). L-NMMA dose-dependently enhanced the effect of AA with a maximal effect at $30 \mu\text{M}$. The maximal aggregation (C) was obtained with $10 \mu\text{M}$ AA. Representative tracings of 5 similar experiments.

Platelet aggregation induced by arachidonic acid was enhanced in a concentration-dependent manner by L-NMMA (Figure 1). L-NAME and L-NIO (both at $30 \mu\text{M}$) were equipotent at enhancing platelet aggregation ($n = 3-5$). The maximum enhancement of arachidonic acid-induced aggregation by all these compounds was also similar (Figure 2). L-NMMA, L-NAME and L-NIO (all at $30 \mu\text{M}$) enhanced platelet aggregation induced by ADP and thrombin to a similar

Figure 2 Enhancement of arachidonic acid-induced platelet aggregation by N^G -monomethyl-L-arginine (L-NMMA), N^G -nitro-L-arginine methyl ester (L-NAME) or N -iminoethyl-L-ornithine (L-NIO). Platelet aggregation induced by arachidonic acid ($\bullet$) was maximally enhanced to the similar extent by L-NMMA ($\circ$), L-NAME ($\square$) or L-NIO (∇) all at $30 \mu\text{M}$. The results are mean of 3-5 experiments; vertical lines show s.e.mean.

Table 2 The effect of N^G-monomethyl-L-arginine (L-NMMA), N^G-nitro-L-arginine methyl ester (L-NAME) and N-iminoethyl-L-ornithine (L-NIO) on platelet aggregation

	Aggregating agent		
	ADP (μ M)	Thrombin (μ g ml ⁻¹)	A23187 (nM)
Control	3.5 $\pm$ 0.5	16.0 $\pm$ 3.0	4.5 $\pm$ 1.0
L-NMMA	1.4 $\pm$ 0.3	9.0 $\pm$ 2.0	4.3 $\pm$ 1.2
L-NAME	1.0 $\pm$ 0.2	7.0 $\pm$ 1.0	3.9 $\pm$ 0.9
L-NIO	1.2 $\pm$ 0.2	8.0 $\pm$ 2.0	4.6 $\pm$ 1.1

Data shown are mean EC₅₀ values $\pm$ s.e.mean ($n = 3-5$).

extent ($n = 3-5$), but did not have an effect on the aggregation induced by A23187 (Table 2). In this last respect there were no significant differences between the compounds. L-Canavanine, D-NMMA, D-NAME and D-NIO (100 μ M, $n = 3$) did not affect platelet aggregation induced by ADP, arachidonic acid, thrombin or A23187 ($n = 3$).

The effect of L-NMMA, L-NAME and L-NIO on platelet NO synthase

Platelet NO synthase activity in the presence of L-arginine and NADPH (both at 300 μ M) was inhibited in a concentration-dependent manner by L-NMMA, L-NAME and L-NIO (Figure 3). L-NIO was significantly more potent than either L-NMMA or L-NAME, which were not significantly different from each other (Figure 3). D-NMMA, D-NAME, D-NIO and L-canavanine (all at 300 μ M, $n = 3$) did not affect the activity of platelet NO synthase.

Discussion

We have previously demonstrated that platelets contain an NO synthase which, when activated during platelet aggregation induced by collagen, synthesizes NO from L-arginine (Radomski *et al.*, 1990). This NO stimulates the soluble guanylate cyclase, increases cyclic GMP and down-regulates

platelet aggregation. We have now demonstrated that other aggregating agents, such as ADP and arachidonic acid, activate the synthesis of NO since platelet aggregation induced by these agents was also inhibited by L-, but not D-arginine. Moreover, the inhibitors of NO synthase L-NMMA, L-NAME and L-NIO (Palmer & Moncada, 1989; Palacios *et al.*, 1989; Knowles *et al.*, 1990) enhanced aggregation induced by these agents.

Interestingly, although L-arginine also inhibited platelet aggregation induced by thrombin and A23187, this was only evident under conditions in which the effect of NO is potentiated, such as in the presence of M&B 22948 or a subthreshold concentration of prostacyclin (Radomski *et al.*, 1987b). Furthermore, the inhibitors of NO synthase were similarly effective in potentiating aggregation induced by arachidonic acid and ADP, but were less so for thrombin and ineffective against aggregation induced by A23187. This profile of modulation of platelet aggregation by endogenous NO correlates well with that observed for the effect of exogenous NO on the aggregating action of these agents, for the order of potency of NO against these agents is arachidonic acid = ADP > thrombin > A23187 (Radomski *et al.*, 1987b).

These results show that aggregating agents stimulate a common pathway which leads to the synthesis of NO from L-arginine. The stimulation of platelets with aggregating agents results in an approximately 100 fold increase in the resting (100 nM) $[Ca^{2+}]_i$ (Ware *et al.*, 1986). Since the platelet NO synthase is Ca^{2+} -dependent (Radomski *et al.*, 1990), it is likely that this sharp increase in $[Ca^{2+}]_i$ activates the enzyme and triggers the synthesis of NO. The formation of NO in platelets is accompanied by stimulation of soluble guanylate cyclase and an increase in intra-platelet cyclic GMP levels (Radomski *et al.*, 1990). The mechanism by which elevated cyclic GMP levels attenuate platelet aggregation is not known. However, the increases in cyclic GMP cause Ca^{2+} sequestration (Busse *et al.*, 1987), leaving less free Ca^{2+} available for aggregation. The net biological effect after stimulation of the platelets therefore will depend on the amounts of free Ca^{2+} available for aggregation, for it appears that even when NO synthase is fully active, agents which are more powerful elevators of $[Ca^{2+}]_i$, such as A23187, overcome the inhibitory effect of intra-platelet NO.

The L-arginine analogues L-NMMA, L-NAME and L-NIO potentiated platelet aggregation to similar extents and with similar potency. However, L-NIO was approximately 8 times more potent than L-NMMA and L-NAME as an inhibitor of NO synthase in platelet cytosol. This is similar to the order of potency of these compounds against endothelial NO synthase (Moncada & Palmer, 1990), although a different rank order was observed in intact endothelial cells (Rees *et al.*, 1990). The reason for the difference in rank order of these compounds in whole platelets and platelet cytosol is not known, but may indicate differences in the uptake, distribution or metabolism of these compounds in the platelets. Similar differences between the activity of these compounds on neutrophils and neutrophil NO synthase have also been observed (McCall *et al.*, 1990). The activity of these inhibitors and the finding that L-canavanine does not affect platelet aggregation support our proposal that the platelet NO synthase is of the brain/endothelial type (Radomski *et al.*, 1990).

Platelet aggregation *in vivo* is likely to be regulated by intraplatelet NO, as well as by NO and prostacyclin released from vascular endothelium. The combined action of these two mediators could result in an enhanced inhibition of $[Ca^{2+}]_i$ elevation and subsequent inhibition of platelet aggregation (Radomski *et al.*, 1987a). The results of these experiments reinforce our original suggestions that the combined use of agents which increase activity of the adenylate and guanylate cyclase offers an interesting strategy for development of effective antiplatelet drugs (Radomski *et al.*, 1987a; Moncada *et al.*, 1988). They also point to the need for examining the pharmacological action of dietary L-arginine in respect to its anti-platelet potential.

Figure 3 Inhibition of nitric oxide (NO) synthase activity in platelet cytosol by N^G-monomethyl-L-arginine (L-NMMA), N^G-nitro-L-arginine methyl ester (L-NAME) or N-iminoethyl-L-ornithine (L-NIO). NO synthase activity in the presence of L-arginine and NADPH, both at 300 μ M, was inhibited by L-NIO (●), L-NMMA (○) and L-NAME (□). L-NIO was the most potent inhibitor and L-NMMA and L-NAME were not significantly different from each other. The results are mean of 3-5 experiments; vertical lines show s.e.mean.

References

- AISAKA, K., GROSS, S.S., GRIFFITH, O.W. & LEVI, R. (1989). N^G-methylarginine, an inhibitor of endothelium-derived nitric oxide synthesis is a potent pressor agent in the guinea-pig: does nitric oxide regulate blood pressure in vivo? *Biochem. Biophys. Res. Commun.*, **160**, 881–886.
- BORN, G.V.R. (1962). Aggregation of blood platelets by adenosine diphosphate and its reversal. *Nature*, **194**, 927–929.
- BREDT, P.S. & SNYDER, S.A. (1989). Nitric oxide mediates glutamate-linked enhancement of cGMP levels in the cerebellum *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 9030–9033.
- BUSSE, R., LUCKHOFF, A. & BASSENGE, E. (1987). Endothelium-derived relaxant factor inhibits platelet activation. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **336**, 566–571.
- DUARTE, I.D.G., LORENZETTI, B.B. & FERREIRA, S.H. (1990). Acetylcholine induces peripheral analgesia by the release of nitric oxide. In *Nitric Oxide from L-Arginine: a Bioregulatory System*, ed. Moncada, S. & Higgs, E.A. pp. 159–164. North Holland: Elsevier.
- GARTHWAITE, J., GARTHWAITE, G., PALMER, R.M.J. & MONCADA, S. (1989). NMDA receptor activation induces nitric oxide synthesis from arginine in rat brain slices. *Eur. J. Pharmacol.*, **172**, 413–416.
- GIBSON, A., MIRZAZADEH, S., HOBBS, A.J. & MOORE, P.K. (1990). L-N^G-monomethyl arginine and L-N^G-nitroarginine inhibit non-adrenergic, non-cholinergic relaxation of the mouse anococcygeus muscle. *Br. J. Pharmacol.*, **99**, 602–606.
- GILLESPIE, J.S., LIU, X. & MARTIN, W. (1989). The effects of L-arginine and N^G-monomethyl L-arginine on the response of the rat anococcygeus muscle to NANC nerve stimulation. *Br. J. Pharmacol.*, **98**, 1080–1082.
- HIBBS, J.B. Jr, TAINTOR, R.R., VAVRIN, Z. & RACHLIN, E.M. (1988). Nitric oxide: a cytotoxic activated macrophage effector 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. (1990). Synthesis of nitric oxide from a terminal guanidino nitrogen atom of L-arginine: a molecular mechanism regulating cellular proliferation that targets intracellular iron. In *Nitric Oxide From L-Arginine: a Bioregulatory System*, ed. Moncada, S. & Higgs, E.A. pp. 183–216. North Holland: Elsevier.
- HIBBS, J.B. Jr, VAVRIN, Z. & TAINTOR, R.R. (1987). L-Arginine is required for expression of the activated macrophage effector mechanism causing selective metabolic inhibition in target cells. *J. Immunol.*, **138**, 550–565.
- KNOWLES, R.G., PALACIOS, M., PALMER, R.M.J. & MONCADA, S. (1989). Formation of nitric oxide from L-arginine: a transduction mechanism for stimulation of the soluble guanylate cyclase. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 5159–5162.
- KNOWLES, R.G., PALACIOS, M., PALMER, R.M.J. & MONCADA, S. (1990). Kinetic characteristics of nitric oxide synthase from rat brain. *Biochem. J.*, **269**, 207–210.
- KWON, N.S., NATHAN, C.F. & STUEHR, D.J. (1989). Reduced biopterin as a cofactor in the generation of nitrogen oxides by murine macrophages. *J. Biol. Chem.*, **264**, 20496–20501.
- LUGNIER, C., SCHOEFFTER, P., LE BEC, A., STROUTHOU, E. & STOCLET, J.C. (1986). Selective inhibition of cyclic nucleotide phosphodiesterases of human, bovine and rat aorta. *Biochem. Pharmacol.*, **35**, 1743–1751.
- MARLETTA, M.A., YOON, P.S., IYENGAR, R., LEAF, C.D. & WISHNOK, J.S. (1988). Macrophage oxidation of L-arginine to nitrite and nitrate: nitric oxide is an intermediate. *Biochemistry*, **27**, 8706–8711.
- MCCALL, T.B., BOUGHTON-SMITH, N.K., PALMER, R.M.J., WHITTLE, B.J.R. & MONCADA, S. (1989). Synthesis of nitric oxide from L-arginine by neutrophils. Release and interaction with superoxide anion. *Biochem. J.*, **261**, 293–296.
- MCCALL, T.B., PALMER, R.M.J., BOUGHTON-SMITH, N.K., WHITTLE, B.J.R. & MONCADA, S. (1990). The L-arginine:nitric oxide pathway in neutrophils. In *Nitric Oxide From L-Arginine: A Bioregulatory System*, ed. Moncada, S. & Higgs, E.A. pp. 249–257. North Holland: Elsevier.
- MELLION, B.T., IGNARRO, L.J., OHLSTEIN, E.H., PONTECORVO, E.G., HYMAN, A.L. & KADOWITZ, P.J. (1981). Evidence for the inhibitory role of guanosine 3',5'-monophosphate in ADP-induced human aggregation in the presence of nitric oxide and related vasodilators. *Blood*, **57**, 946–955.
- MEYER, B., SCHMIDT, K., HUMBERT, R. & BOHME, E. (1989). Biosynthesis of endothelium-derived relaxing factor, a cytosolic enzyme in porcine aortic endothelial cells Ca²⁺-dependently converts L-arginine into an activator of guanylate cyclase. *Biochem. Biophys. Res. Commun.*, **164**, 678–685.
- 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. Moncada, S. & Higgs, E.A. pp. 17–31. North Holland: Elsevier.
- MONCADA, S., PALMER, R.M.J. & HIGGS, E.A. (1989). Biosynthesis of nitric oxide from L-arginine: a pathway for the regulation of cell function and communication. *Biochem. Pharmacol.*, **38**, 1709–1715.
- MONCADA, S., RADOMSKI, M.W. & PALMER, R.M.J. (1988). Endothelium-derived relaxing factor: identification as nitric oxide and role in the control of vascular tone and platelet function. *Biochem. Pharmacol.*, **37**, 2495–2501.
- MOORE, P.K., AL-SWAYEH, O.A., CHONG, N.W.S., EVANS, R.A. & GIBSON, A. (1990). L-N^G-nitro-arginine (L-NOARG), a novel, L-arginine reversible inhibitor of endothelium-dependent vasodilatation *in vitro*. *Br. J. Pharmacol.*, **99**, 408–412.
- MULSCH, A. & BUSSE, R. (1990). N^G-nitro-L-arginine (N^G-[imino(nitroamino)methyl]-L-ornithine) impairs endothelium-dependent dilatations by inhibitory cytosolic nitric oxide synthesis from L-arginine. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **341**, 143–147.
- PALACIOS, M., KNOWLES, R.G., PALMER, R.M.J. & MONCADA, S. (1989). Nitric oxide from L-arginine stimulates the soluble guanylate cyclase in adrenal glands. *Biochem. Biophys. Res. Commun.*, **165**, 802–809.
- PALMER, R.M.J. & MONCADA, S. (1989). A novel citrulline-forming enzyme implicated in the formation of nitric oxide by vascular endothelial cells. *Biochem. Biophys. Res. Commun.*, **158**, 348–352.
- PALMER, R.M.J., ASHTON, D.S. & MONCADA, S. (1988a). Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature*, **333**, 664–666.
- PALMER, R.M.J., REES, D.D., ASHTON, D.S. & MONCADA, S. (1988b). L-Arginine is the physiological precursor for the formation of nitric oxide in endothelium-dependent relaxation. *Biochem. Biophys. Res. Commun.*, **153**, 1251–1256.
- RADOMSKI, M.W. & MONCADA, S. (1983). An improved method for washing platelets with prostacyclin. *Thromb. Res.*, **30**, 383–389.
- RADOMSKI, M.W., PALMER, R.M.J. & MONCADA, S. (1987a). The anti-aggregating properties of vascular endothelium. Interactions between prostacyclin and nitric oxide. *Br. J. Pharmacol.*, **92**, 629–636.
- RADOMSKI, M.W., PALMER, R.M.J. & MONCADA, S. (1987b). 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. (1990). An L-arginine to nitric oxide pathway in human platelets regulates aggregation. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 5193–5197.
- REES, D.D., PALMER, R.M.J. & MONCADA, S. (1989). Role of endothelium-derived nitric oxide in the regulation of blood pressure. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 3375–3378.
- REES, D.D., SCHULZ, R., HODSON, H.F., PALMER, R.M.J. & MONCADA, S. (1990). Identification of some novel inhibitors of the vascular nitric oxide synthase *in vivo* and *in vitro*. In *Nitric Oxide from L-Arginine: A Bioregulatory System*, ed. Moncada, S. & Higgs, E.A. pp. 473–475. North Holland: Elsevier.
- SALVEMINI, D., DE NUCCI, G., GRYGLEWSKI, R.J. & VANE, J.R. (1989). Human neutrophils and mononuclear cells inhibit platelet aggregation by releasing a nitric oxide-like factor. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 6328–6332.
- WARE, J.A., JOHNSON, P.C., SMITH, M. & SALZMAN, E.W. (1986). The effect of common agonists on cytoplasmic ionized calcium concentration in platelets: measurement with quin2 and aequorin. *J. Clin. Invest.*, **77**, 878–886.

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