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A specific inhibitor of nitric oxide formation from L-arginine attenuates endothelium-dependent relaxation

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1 The role of L-arginine in the basal and stimulated generation of nitric oxide (NO) for endothelium-dependent relaxation was studied by use of N^G-monomethyl L-arginine (L-NMMA), a specific inhibitor of this pathway.

2 L-Arginine (10–100 μM), but not D-arginine (100 μM), induced small but significant endothelium-dependent relaxations of rings of rabbit aorta. In contrast, L-NMMA (1–300 μM) produced small, endothelium-dependent contractions, while its enantiomer N^G-monomethyl-D-arginine (D-NMMA; 100 μM) had no effect.

3 L-NMMA (1–300 μM) inhibited endothelium-dependent relaxations induced by acetylcholine (ACh), the calcium ionophore A23187, substance P or L-arginine without affecting the endothelium-independent relaxations induced by glyceryl trinitrate or sodium nitroprusside.

4 The inhibition of endothelium-dependent relaxation by L-NMMA (30 μM) was reversed by L-arginine (3–300 μM) but not by D-arginine (300 μM) or a number of close analogues (100 μM).

5 The release of NO induced by ACh from perfused segments of rabbit aorta was also inhibited by L-NMMA (3–300 μM), but not by D-NMMA (100 μM) and this effect of L-NMMA was reversed by L-arginine (3–300 μM).

6 These results support the proposal that L-arginine is the physiological precursor for the basal and stimulated generation of NO for endothelium-dependent relaxation.

Introduction

The release of nitric oxide (NO) by endothelial cells in culture accounts for the relaxation of vascular strips (Palmer *et al.*, 1987) and for the inhibition of platelet aggregation and adhesion (Radomski *et al.*, 1987a,b) attributed to endothelium-derived relaxing factor. Nitric oxide is also released from the perfused rabbit aorta and in the rabbit coronary circulation in amounts sufficient to account for the relaxation of aortic rings and the fall in coronary perfusion pressure induced by acetylcholine (ACh; Chen *et al.*, 1989; Amezcua *et al.*, 1988). These and other data (Moncada *et al.*, 1988) have established that NO mediates endothelium-dependent relaxation (Furchgott & Zawadzki, 1980).

We have recently demonstrated that aortic endothelial cells of the pig in culture synthesize NO from the terminal guanidino nitrogen atom(s) of the amino acid L-arginine (Palmer *et al.*, 1988a). In addition, the L-arginine analogue, N^G-monomethyl-L-arginine (L-NMMA), inhibits both the release of NO from these cells and the endothelium-dependent relaxation of rings of rabbit aorta (Palmer *et al.*, 1988b). As a result, we have proposed that L-arginine is the physiological precursor for NO synthesis by vascular endothelial cells.

In order to investigate this NO-generating system further and to characterize its substrate specificity, we have now examined the actions of L-arginine, L-NMMA and some other arginine analogues on the endothelium-dependent relaxations of rings of rabbit aorta. Furthermore, we have studied the effects of L-arginine and L-NMMA on the release of NO from perfused segments of this tissue.

Methods

Organ bath studies

Endothelium-dependent relaxation of rings of rabbit aorta was studied as described previously (Rees *et al.*, 1988). Briefly, male New Zealand White rabbits (2.0–2.3 kg) were killed with an overdose of sodium

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pentobarbitone. The thoracic aorta was removed, trimmed free of adhering fat and connective tissue and cut into 4 mm rings. The endothelium was removed from some rings by gently rubbing the intimal surface with a pipe cleaner. The failure of ACh (1 μM) to induce relaxation of these rings was taken as an indication of endothelium removal. This was confirmed in some experiments by scanning electron microscopy.

The rings were mounted under 2.5 g resting tension on stainless steel hooks in 20 ml organ baths containing Krebs buffer at 37°C, gassed with 95% O_2 and 5% CO_2. Tension was recorded with Grass FT03 isometric transducers on a 6-channel multipen recorder (Rikadenki). The tissues were allowed to equilibrate for 40 min before being contracted submaximally by addition of phenylephrine (750 nM). Cumulative relaxation curves to ACh, substance P, A23187, glyceryl trinitrate (GTN) or sodium nitroprusside were obtained in each ring. After washout, L-arginine or its analogues were added to the organ bath 10 min before a second cumulative relaxation curve was obtained. After further washout, a third curve was obtained to demonstrate the reversibility of any effects observed.

Cascade bioassay

A segment (6 cm) of the rabbit thoracic aorta (donor aorta) was removed, cleaned and placed in a perspex chamber and perfused intraluminally with Krebs buffer (5 ml min^{-1}). For the bioassay of NO, the effluent from this aorta was used to superfuse 3 spiral strips of rabbit aorta denuded of endothelium (bioassay tissues) in a cascade (Gryglewski *et al.*, 1986a; Palmer *et al.*, 1987). The bioassay tissues were contracted submaximally with either 9,11-dideoxy-9α, 11α methanoepoxy-prostaglandin $F_{1\alpha}$ (U46619; 30 nM) or phenylephrine (750 nM). Atropine sulphate (0.2 μM) was infused over the bioassay tissues in order to block any direct action of ACh. The amplification of the recorder was adjusted so that similar relaxations to GTN (50 nM) were observed in each tissue.

Chemiluminescence

The release of NO from the perfused rabbit aorta was also detected by chemiluminescence (Palmer *et al.*, 1987). Briefly, the effluent of a perfused donor aorta was infused continuously into a reaction vessel containing 75 ml 1% sodium iodide in glacial acetic acid under reflux. Nitric oxide was removed under reduced pressure in a stream of N_2, mixed with ozone and the chemiluminescent product quantified by reference to NO standards.

Chemicals

Nitric oxide (>99.98% pure, British Oxygen Corporation) solutions were prepared as described previously (Palmer *et al.*, 1987). Glyceryl trinitrate (Wellcome), phenylephrine, ACh, substance P, L- and D-arginine, L-homoarginine, L-α-amino-γ-guanidinobutyric acid, L-α-amino-β-guanidinopropionic acid, L-canavanine, L-argininamide, L-citrulline, urea, L-arginine methyl ester, L-arginyl-aspartate, sodium nitroprusside, atropine sulphate (Sigma), agmatine sulphate (Aldrich), the calcium ionophore A23187 (Calbiochem), U46619 (Cayman Chemicals) and sodium pentobarbitone (May and Baker) were obtained as indicated. L-NMMA, D-NMMA and N^G, N^{1G}-dimethyl-L-arginine were synthesized as described previously (Patthy *et al.*, 1977). 5-Guanidino valeric acid was synthesized according to the published method (Kobayashi, 1947).

Statistics

Results are expressed as mean $\pm$ s.e.mean for n separate experiments. Student's unpaired t test was used to determine the significance of differences between means and $P < 0.05$ was taken as statistically significant.

Results

Endothelium-dependent relaxation

L-Arginine (10–100 μM) caused small but significant endothelium-dependent relaxations of rings of rabbit aorta pre-contracted with 750 nM phenylephrine (maximum relaxation = 9 $\pm$ 1% of the contraction induced by phenylephrine, $P < 0.05$, $n = 3$). These relaxations were concentration-dependent (ED_{50} = 28 $\pm$ 5 μM) and reversible. In contrast, L-NMMA (1–300 μM) caused small but significant endothelium-dependent contractions (maximum 17 $\pm$ 2% of the contraction induced by phenylephrine; $P < 0.05$, $n = 3$) which were also concentration-dependent (ED_{50} = 4 $\pm$ 2 μM) and reversible (Figure 1). The relaxations induced by L-arginine (100 μM) were completely reversed by L-NMMA (30 μM) and the contractions induced by L-NMMA (30 μM) were completely reversed by L-arginine (100 μM; $n = 3$). The tone of the rings and the effects of L-arginine or L-NMMA ($n = 3$) were not affected by D-arginine or D-NMMA (both at 100 μM).

Acetylcholine (1 μM), substance P (10 nM) and A23187 (0.1 μM) caused endothelium-dependent relaxation of rings of rabbit aorta which were

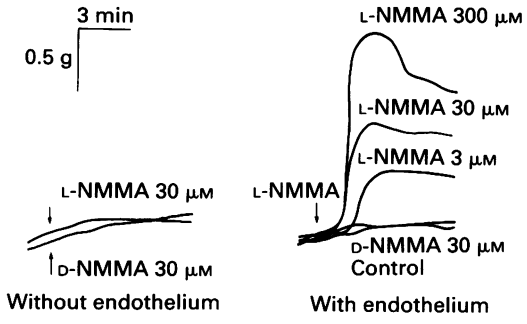

Figure 1 Effect of N^G-monomethyl-L-arginine (L-NMMA) and N^G-monomethyl-D-arginine (D-NMMA) on the basal tone of rabbit aortic rings pre-contracted with phenylephrine (750 nM), with and without endothelium. Trace representative of three experiments.

76 ± 3, 47 ± 9 and $67 \pm 5\%$ ($n = 3$) respectively of the contraction induced by phenylephrine. The relaxations induced by ACh (1 μM) were marginally increased ($9 \pm 5\%$; $n = 3$; $P > 0.05$) by L-arginine (100 μM). The relaxations induced by ACh, substance P and A23187 were inhibited in a concentration-dependent manner by L-NMMA (1–300 μM) but not D-NMMA (100 μM; $n = 3$; see Figure 2). The IC_{50}s were 10 ± 2, 5 ± 3 and 12 ± 2 μM ($n = 3$) for ACh, substance P and A23187 respectively. The maximum degree of inhibition by L-NMMA (300 μM) of these relaxations was 64 ± 1, 55 ± 5 and $76 \pm 5\%$ ($n = 3$ for each compound). The endothelium-independent relaxations induced by GTN or sodium nitroprusside were not affected by L-NMMA (300 μM, $n = 3$).

The inhibition by L-NMMA (1–300 μM) of endothelium-dependent relaxation induced by ACh was fully reversible after washing (90 min). Concomitant addition of L-arginine (3–300 μM), but not D-arginine (300 μM), overcame, in a concentration-dependent manner, the inhibition of ACh-induced endothelium-dependent relaxation produced by L-NMMA (30 μM; Figure 3). The ED_{50} against L-NMMA (30 μM) was 25 ± 3 μM ($n = 3$) and approximately three times more L-arginine was required to abolish the effect of a given concentration of L-NMMA.

The tone of contracted aortic rings was not affected by N^G, N^{1G}-dimethyl-L-arginine, L-homoarginine, L-α-amino-γ-guanidinobutyric acid, L-α-amino-β-guanidinopropionic acid, L-canavanine, L-argininamide, agmatine, 5-guanidino valeric acid, L-citrulline, urea, L-arginine methyl ester and L-arginyl-aspartate (all at 100 μM; $n = 3$ for each). Of these compounds, only L-arginine methyl ester (100 μM) caused a partial ($37 \pm 3\%$) reversal of the

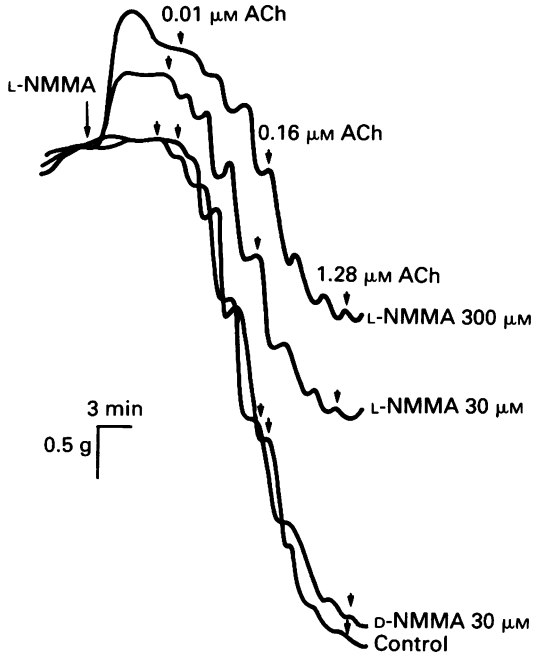

Figure 2 Inhibition by N^G-monomethyl-L-arginine (L-NMMA), but not by N^G-monomethyl-D-arginine (D-NMMA), of ACh-induced endothelium-dependent relaxation. Cumulative relaxation curves were obtained by addition of ACh (0.01–1.28 μM) to the organ bath; the concentration of ACh doubled at each addition. The points of addition of 0.01, 0.16 and 0.128 μM ACh are indicated by the arrows; those of intermediate concentrations of ACh are not shown. Trace representative of three experiments.

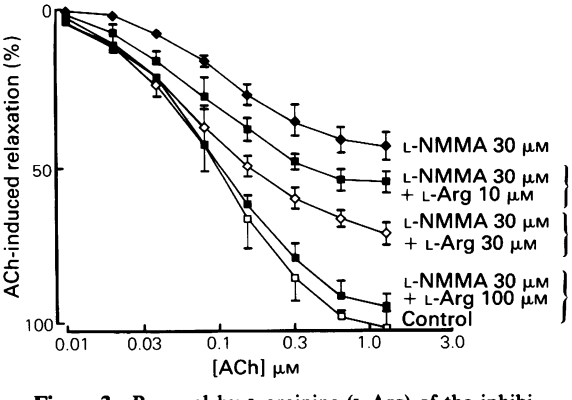

Figure 3 Reversal by L-arginine (L-Arg) of the inhibitory effect of N^G-monomethyl-L-arginine (L-NMMA) on ACh-induced endothelium-dependent relaxation. Each point is the mean of 3 experiments; vertical bars show s.e.mean.

inhibition by L-NMMA (30 μM) of ACh-induced endothelium-dependent relaxation.

Cascade bioassay

The tone of the bioassay tissues in the cascade and their response to NO was not affected by infusions over the tissues of ACh (30 μM), L- and D-arginine (300 μM) or L-NMMA (300 μM; $n = 3$ for each). However, when ACh (0.1–30.0 μM) was infused for 1 min through the donor aorta, it induced a concentration-dependent release of NO detected by bioassay ($ED_{50} = 0.6 \pm 0.2$ μM, $n = 3$).

Nitric oxide release induced by ACh (1 μM) was enhanced by L-arginine (100 μM) to a small ($21 \pm 3\%$, $n = 3$) but significant ($P < 0.05$) extent and was inhibited in a concentration-dependent manner ($IC_{50} = 16 \pm 5$ μM, $n = 3$) by L-NMMA (3–300 μM; Figure 4), but not by D-NMMA (100 μM; TD, $n = 3$). The inhibition by L-NMMA (30 μM) of NO release induced by ACh was slowly reversible over a period of 90 min. The response to ACh could also be fully restored by a 10 min infusion of L-arginine (100 μM) following termination of the infusion of L-NMMA (Figure 4). The inhibition of NO release by L-NMMA (30 μM) was reversed by L-arginine (3–300 μM), but not by D-arginine (300 μM; $n = 3$), in a concentration-dependent manner ($ED_{50} = 28 \pm 3$ μM, $n = 3$).

Chemiluminescence

Infusion of ACh (0.1–30.0 μM) through the donor aorta for 1 min induced a concentration-dependent release of NO (4 ± 2–93 ± 15 pmol) detected by chemiluminescence ($ED_{50} = 2 \pm 1$ μM, $n = 3$). The release of NO (41 ± 5 pmol) induced by ACh (1 μM), was inhibited in a concentration-dependent manner by L-NMMA (3–300 μM) with an IC_{50} of 15 ± 3 μM ($n = 3$), but not by D-NMMA (100 μM; $n = 3$). The effect of L-NMMA was reversed by L-arginine (3–300 μM; Figure 5) but not by D-arginine (300 μM) and L-arginine caused a small ($18 \pm 5\%$, $n = 3$) but significant ($P < 0.05$) potentiation of the release of NO induced by ACh (1 μM; Figure 5).

Discussion

Vascular endothelial cells synthesize NO from the terminal guanidino nitrogen atom(s) of L-arginine by an enzymic process which exhibits strict substrate specificity (Palmer *et al.*, 1988a). Furthermore, we have suggested that NO release from endothelial

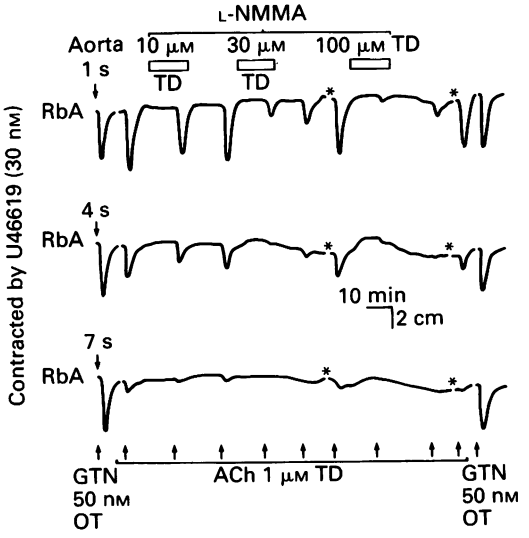

Figure 4 Effect of N^G-monomethyl-L-arginine (L-NMMA; 10–100 μM), infused through the donor rabbit aorta (TD), on the relaxation of the bioassay tissues in the cascade by ACh- (1 μM, TD) induced NO release. Asterisks denote L-arginine infusion (100 μM; TD) for 10 min followed by washout (10 min). The responses of the tissues were standardized by an infusion of glyceryl trinitrate administered over the tissues (GTN; 50 nM, OT). Trace representative of three experiments.

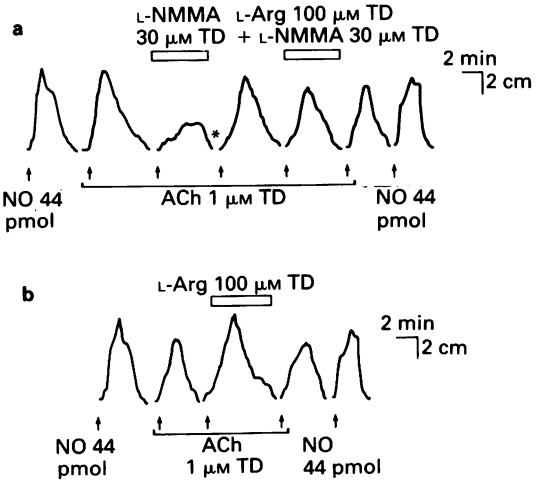

Figure 5 (a) Reversal by L-arginine (L-Arg) of the inhibition by N^G-monomethyl-L-arginine (L-NMMA) of ACh-induced release of NO and (b) potentiation of ACh-induced release of NO by L-arginine. Asterisk denotes L-arginine infusion (100 μM; TD) for 10 min followed by washout (10 min). The release of NO, determined by chemiluminescence, was quantified as the area under the peak compared with those of NO standards. Traces representative of three experiments.

cells is a two-step process involving mobilisation of endogenous substrate and subsequent formation of NO. In addition, we have shown that the formation of NO from L-arginine and the endothelium-dependent relaxation induced by ACh is inhibited by the arginine analogue L-NMMA, but not by its D-enantiomer (Palmer *et al.*, 1988b).

We now show that L-arginine but not D-arginine, marginally enhances NO release induced by ACh from segments of rabbit aorta and induces concentration-related, endothelium-dependent relaxation of vascular rings without significantly affecting endothelium-dependent relaxation induced by ACh. Furthermore, L-arginine does not affect endothelium-independent relaxation induced by GTN or sodium nitroprusside. These results are consistent with the synthesis of NO from L-arginine; however, the enhancement of NO release and the relaxations induced by L-arginine are small. This suggests that exogenous L-arginine is unable to compete with endogenous L-arginine which may be presented to the enzyme more efficiently. In addition, it is possible that during stimulation there is activation of the NO-forming enzyme and sufficient mobilisation of substrate to saturate the enzyme.

The release of NO from fresh vascular tissue and the endothelium-dependent relaxation induced by different agonists were inhibited by L-NMMA, but not by its D-enantiomer. Furthermore, L-NMMA induces an endothelium-dependent contraction. All the effects of L-NMMA can be antagonized by L- but not by D-arginine. These data indicate that endogenous L-arginine is utilized for the generation of NO during endothelium-dependent relaxation. Moreover, they show that there is continuous use of L-arginine for the basal release of endothelium-derived relaxing factor (NO) which has been demonstrated in fresh vascular tissue (Martin *et al.*, 1985) and vascular endothelial cells in culture (Gryglewski *et al.*, 1986b).

Our data also suggest a competitive interaction between L-NMMA and L-arginine, for equimolar concentrations of L-arginine are required to reverse the effects of L-NMMA by 50%. Furthermore, the concentration of L-NMMA which produces 50% contraction of the rings (4 μM) is approximately 6 times lower than that required to inhibit ACh-induced relaxation by 50%. This, together with the fact that the IC_{50} of L-NMMA against substance P is lower than that required to inhibit the greater relaxations induced by ACh or A23187 by 50%, is consistent with an enhanced mobilisation of substrate during activation of the endothelial cell which may vary according to the degree of stimulation.

Unlike L-arginine, the effect of L-NMMA on NO release is slow to disappear unless accelerated by exogenous L-arginine. Whether the 90 min required for the complete reversal of the effect of L-NMMA in the absence of exogenous L-arginine reflects simple dissociation or is the result of a gradual displacement by small amounts of endogenous L-arginine should be investigated.

The NO-forming enzyme system has a strict structural requirement for the substrate, as indicated by the inability of arginine analogues to reverse or mimic the effects of L-NMMA and to affect endothelium-dependent relaxation. None of the analogues studied altered the tone of aortic rings and only L-arginine methyl ester, which may contain some unesterified L-arginine, reversed the effects of L-NMMA. The studies with these analogues show that alterations in the guanidino or the amino acid function of L-arginine or in the length of the methylene chain of the molecule are sufficient to modify the interaction with the enzyme site profoundly. The fact that L-citrulline does not induce endothelium-dependent relaxation or compete with L-NMMA is consistent with the possibility that this compound needs to be converted to L-arginine prior to formation of NO as has been suggested to occur in endothelial cells in culture (Palmer *et al.*, 1988a).

The maximum degree of inhibition by L-NMMA of endothelium-dependent relaxation was 55–76% with the three agonists studied. When determined by bioassay or chemiluminescence, L-NMMA causes complete inhibition of NO release. Endothelium-dependent relaxation detects the abluminal release of NO and is more sensitive than the cascade bioassay or the chemiluminescence methods (Chen *et al.*, 1989) which detect luminal release of NO. It is, therefore, possible that there is a site for the abluminal synthesis of NO which is inaccessible to L-NMMA. Alternatively, there may be factors other than NO involved in endothelium-dependent relaxation (Feletou & Vanhoutte, 1988). The existence of such factors will be determined with the help of L-NMMA in perfusion experiments where biologically active materials are transferred from donor to detector systems.

Our finding that L-canavanine does not affect endothelium-dependent relaxation is at variance with a recent observation that this compound inhibited endothelium-dependent relaxation in rings of the rat aorta (Schmidt *et al.*, 1988). Whether the effect reported is due to endothelial damage by the very large concentrations of L-canavanine used or is due to a specific effect on the NO-generating system, remains to be established.

The oxidation products of NO, NO_2^- and NO_3^-, are formed from the terminal guanidino nitrogen atom(s) of L-arginine by activated macrophages (Hibbs *et al.*, 1987a). There seem to be some differences between the NO-generating system in the endothelium and in the activated macrophage. For

example, L-homoarginine, L-argininamide and L-arginyl-aspartate are substrates and L-canavanine is an inhibitor of the generation of NO_2^- and NO_3^- by macrophages (Iyengar *et al.*, 1987; Hibbs *et al.*, 1987b) and yet are inactive in the endothelium.

The vascular endothelial cell has cytotoxic and antitumour properties that have been ascribed to the generation of superoxide ions (Ryan & Vann, 1987). It is possible that, as in the macrophage (Granger *et al.* 1988; Hibbs *et al.*, 1987b), some of these effects are associated with the generation of NO. If this is indeed the case, the generation of NO by the vascular endothelium may have biological consequences beyond endothelium-dependent relaxation.

A relaxing factor, likely to be NO, is released by rat peritoneal neutrophils (Rimele *et al.*, 1988). In addition, EMT-6 adenocarcinoma cells (Amber *et al.*, 1988) and haemopoietic cells (Schneider *et al.*, 1988) form L-citrulline directly from L-arginine, a process probably associated with NO generation. It is likely that other cells will be shown to have the ability to generate NO. The significance of the generation of NO in different biological systems remains to be established. For this purpose, L-NMMA, a specific inhibitor of NO_2^- and NO_3^- release in the macrophage (Hibbs *et al.*, 1987a) and of NO generation in the vascular endothelium may prove invaluable.

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