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Nitric oxide synthesized from L-arginine regulates vascular tone in the coronary circulation of the rabbit

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1 The role of nitric oxide (NO) in the regulation of the vascular tone of the coronary circulation of the Langendorff-perfused rabbit heart was investigated.

2 N^G-monomethyl-L-arginine (L-NMMA; 10–100 μM), a specific inhibitor of NO formation from L-arginine (L-Arg), but not its D-enantiomer (D-NMMA; 100 μM) produced a dose-related, sustained increase in the coronary perfusion pressure (CPP). In addition, L-NMMA inhibited the vasodilator responses of acetylcholine (ACh), unmasking in some instances its direct vasoconstrictor effect. These effects of L-NMMA were attenuated by L-Arg.

3 L-NMMA (10 and 30 μM), but not D-NMMA (30 μM), caused a long-lasting inhibition of NO formation which was reversed by L-Arg (30 and 100 μM), but not by D-Arg (100 μM).

4 This study indicates that the formation of NO from L-Arg in the coronary circulation of the rabbit plays a role both as a regulator of vascular tone and as a mediator of the vasodilatation induced by ACh.

Introduction

The role of endothelium-derived relaxing factor (EDRF; Furchgott & Zawadzki, 1980) in the microcirculation has not yet been established. This is mainly due to its intrinsic instability (Griffith *et al.*, 1984), which makes its detection difficult, and to the problems inherent in obtaining 'endothelium-free' vascular beds. However, the identification of EDRF as nitric oxide (NO; Palmer *et al.*, 1987) and the elucidation of the metabolic pathway to its synthesis from L-arginine (L-Arg; Palmer *et al.*, 1988a; Schmidt *et al.*, 1988) have allowed a fresh approach to the study of the role of NO in the microcirculation.

Nitric oxide is released in the coronary circulation of the isolated perfused rabbit heart and this release accounts for the vasodilatation induced by acetylcholine (ACh; Amezcua *et al.*, 1988) and bradykinin (Kelm & Schrader, 1988). The synthesis of NO by vascular endothelial cells in culture or by fresh vascular tissue is inhibited in a dose-dependent and enantiomerically-specific manner by N^G-monomethyl-L-arginine (L-NMMA; Palmer *et al.*, 1988b; Rees *et al.*, 1989a). This compound also inhibits endothelium-dependent relaxation in rings of rabbit aorta (Rees *et al.*, 1989a) and guinea-pig pulmonary artery (Sakuma *et al.*, 1988).

In view of this, we have now assessed the role of NO synthesized from L-Arg in the coronary circulation of the rabbit isolated heart by studying the effects of L-Arg and L-NMMA on the coronary perfusion pressure (CPP) and on the responses of the coronary circulation to ACh.

Methods

Modified Langendorff-perfused hearts

Male New Zealand White rabbits (1.8 to 2.4 kg) were given heparin (100 u kg^{-1}) and anaesthetized with sodium pentobarbitone (40–50 mg kg^{-1}) via an ear

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vein. The thorax was rapidly opened and the aorta cannulated retrogradely. The heart was removed, flushed with Krebs buffer gassed with 5% CO_2 and 95% O_2 at room temperature and perfused at constant flow with Krebs buffer at 37°C containing indomethacin (5 μM). The Krebs buffer consisted of (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.17, $CaCl_2$ 2.5, $NaHCO_3$ 25 and glucose 8.4. The rate of perfusion was either 15 ml min^{-1} for bioassay and chemiluminescence experiments or 25 ml min^{-1} for measurement of CPP.

The hearts were paced at 180 beats min^{-1} via two platinum pin electrodes inserted into the left ventricular wall and connected to a Grass Stimulator S9 (square wave pulses, 6V, 3 ms). In some experiments the CPP was measured with a pressure transducer connected to a side arm of the perfusion line and was displayed as the average signal on a Gould recorder. The average CPP was raised from a basal level of 15–20 mmHg to 35–70 mmHg by a continuous infusion of 9,11-dideoxy-9α,11α methano epoxyprostaglandin $F_{2\alpha}$ (U46619, 30–150 nM). All experiments were completed within 2 h of starting the perfusion of the heart.

Drugs were administered through an injection port immediately before the aortic cannula. Prostacyclin and ACh were delivered as 1 min infusions, NO as bolus injections of 3 to 10 μl of the appropriate solution and all other drugs, unless otherwise specified, as 20 min infusions starting 10 min before administration to the heart of ACh or prostacyclin. The falls in CPP induced by ACh, prostacyclin and NO were measured as the areas under the curves. The vasoconstrictor responses were expressed as the percentage increase of the CPP above the resting value.

Detection of nitric oxide

NO formation by the heart was measured by chemiluminescence as described previously (Palmer *et al.*, 1987; Amezcua *et al.*, 1988). Briefly, the cardiac effluent (15 ml min^{-1}) was collected into the stem of a funnel closed at the bottom. An aliquot of the effluent (3.5 ml min^{-1}) was continuously transferred into the reaction vessel of the chemiluminescence apparatus while the volume of the sampling pool was kept constant by continuous removal of the excess. The delay between the collecting funnel and the reflux vessel, which contained 75 ml of 1% sodium iodide in glacial acetic acid under reflux, was 15 s. NO was removed under reduced pressure in a stream of N_2, mixed with ozone and the chemiluminescent product was quantified by reference to NO standards.

In some experiments the cardiac effluent (15 ml min^{-1}) was used to superfuse a cascade of three spirally cut strips of rabbit thoracic aorta denuded of endothelium with a pipe cleaner as described previously (Gryglewski *et al.*, 1986) and contracted either with U46619 (150 to 750 nM) or with phenylephrine (0.75 to 2.5 μM). There was a delay of 7 s between the port of injection of drugs into the heart and the uppermost bioassay tissue and a delay of 1 s between this tissue and the heart. The subsequent tissues were separated from each other by 3 s delays.

We have observed previously that superfusion of the aortic strips with the cardiac effluent caused a pronounced relaxation of the tissues which caused a decreased sensitivity to NO. As a result of this, the release of EDRF/NO activity from the heart could not be detected (Amezcua *et al.*, 1988). We have now modified our method so that the rate of infusion of the contracting agent (U46619 or phenylephrine) is increased to restore the tone of the bioassay tissues to the level prior to superfusion with the heart effluent.

Chemicals

Nitric oxide (>99.98% pure, British Oxygen Corporation) solutions were prepared as described previously (Palmer *et al.*, 1987). Glyceryl trinitrate and prostacyclin (Wellcome), phenylephrine, ACh, L- and D-Arg (Sigma), U46619 (Cayman Chemicals), sodium pentobarbitone (May and Baker) and heparin (Abbott) were obtained as indicated. L-NMMA and D-NMMA were synthesized as described previously (Patthy *et al.*, 1977).

Statistics

Differences between means were assessed with Student's t test for paired or unpaired samples as appropriate and considered significant when $P < 0.05$. The results are presented as the mean ± s.e.mean of (n) experiments.

Some of these data were presented at a meeting of The British Pharmacological Society in December, 1988.

Results

Effects on the coronary perfusion pressure

L-NMMA (10, 30, 100 μM) induced a dose-dependent increase in the resting CPP (Figure 1). This increase was long-lasting, usually persisting for the duration of the experiment (1.5 h, $n = 11$).

The vasodilator responses to ACh (0.03 to 3.0 μM) were also inhibited in a dose-related manner by

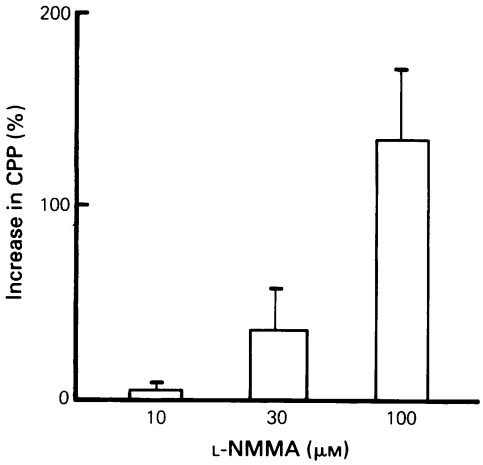

Figure 1 Effect of N^G-monomethyl-L-arginine (L-NMMA) (20 min infusions) on the mean coronary perfusion pressure (CPP) of the Langendorff-perfused rabbit heart. The results are expressed as percentage change from the basal CPP ($n = 4$).

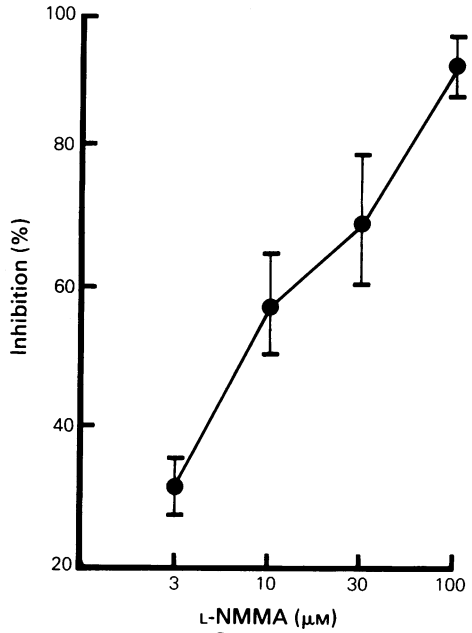

Figure 2 Effect of N^G-monomethyl-L-arginine (L-NMMA) (20 min infusions) on the fall in coronary perfusion pressure (CPP) induced by acetylcholine (ACh) (0.3 μM). The results are expressed as percentage inhibition of the control response to ACh ($n = 4$–6).

L-NMMA (IC_{50} against 0.3 μM ACh was 8.4 ± 1.4 μM, $n = 11$; Figure 2). In addition, L-NMMA (30 and 100 μM) frequently revealed the vasoconstrictor property of ACh (0.3 μM; Figure 3) so that in seven out of eleven experiments the fall in CPP induced by ACh (0.3 μM) was converted into a vasoconstrictor response by L-NMMA. The rise in CPP induced by ACh under these conditions lasted for 3 to 5 min, ranged from 25 to 120% of the resting value and was occasionally preceded by a short-lasting (< 1 min) reduction in CPP. In contrast, the increase in CPP induced by L-NMMA (100 μM) enhanced the vasodilator responses to prostacyclin (1 μM; $n = 3$) and exogenous NO (450 pmol; $n = 4$) to $184 \pm 27\%$ and $208 \pm 36\%$ respectively ($P < 0.05$). The actions of L-NMMA were enantiomerically specific, since D-NMMA (100 μM; $n = 4$) did not exhibit any of the effects described above.

Neither L- nor D-Arg (100 μM) had any effect on resting CPP, nor did they affect the responses to ACh in the absence of L-NMMA. Infusion of L-Arg (100 μM) after administration of L-NMMA (30 μM) partially reversed the increase in CPP induced by L-NMMA and reduced significantly the inhibition of the ACh-induced vasodilatation from 69 ± 9 to $41 \pm 4\%$ ($n = 4$; $P < 0.05$). When L-Arg was infused from the beginning of the experiment it prevented the rise in CPP induced by L-NMMA and reduced significantly (from 69 ± 9 to $34 \pm 12\%$; $n = 4$, $P < 0.05$) the inhibition of the vasodilator response to ACh. The ACh-induced vasodilatation was never

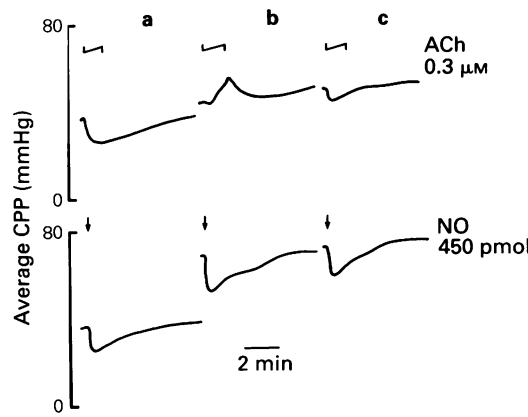

Figure 3 Effects of acetylcholine (ACh, 0.3 μM; 1 min infusions, upper panel) and nitric oxide (NO, 450 pmol; bolus injections, lower panel) on the coronary perfusion pressure (CPP) of the Langendorff-perfused rabbit heart. (a) Control responses; (b) Responses obtained during a 20 min infusion of N^G-monomethyl-L-arginine (L-NMMA, 30 μM); and (c) responses obtained after a 20 min washout with L-Arg (100 μM). Note the elevation in CPP after infusion of L-NMMA. Trace representative of 3 experiments.

converted into the vasoconstriction in the presence of L-Arg ($n = 4$).

Chemiluminescence

Acetylcholine (1 and 3 μM) produced a dose-dependent release of NO, detected by chemiluminescence, which was matched by exogenous NO (450 to 1340 pmol) administered through the heart. The release of NO by ACh (1 μM) was enhanced significantly from 499 $\pm$ 180 pmol to 1031 $\pm$ 270 pmol by L-Arg (30 μM, $n = 4$, $P < 0.05$; Figure 4). L-NMMA (10 μM) inhibited the release of NO induced by a higher concentration of ACh (3 μM) from 1025 $\pm$ 256 to 367 $\pm$ 51 pmol ($n = 3$; $P < 0.05$). This inhibition persisted for more than 1 h but could be reversed by an infusion of L-Arg (30 μM; Figure 5). The ACh-induced release of NO was not affected by either D-Arg (100 μM, $n = 3$) or D-NMMA (30 μM, $n = 3$).

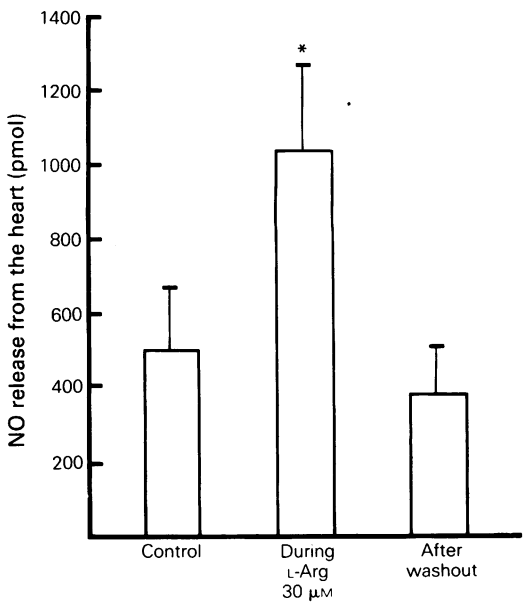

Figure 4 Nitric oxide (NO) formation by the coronary circulation in response to three consecutive challenges with acetylcholine (ACh, 1 μM; 1 min infusions) in the same preparation. The second challenge was given at the midpoint of a 20 min infusion of L-arginine (L-Arg, 30 μM) and the third 20 min after the end of the L-Arg infusion. The amount of NO detected by chemiluminescence was calculated by reference to authentic NO standards injected through the heart ($n = 4$). * Indicates that the values differ significantly ($P < 0.05$) from the control in a paired t test.

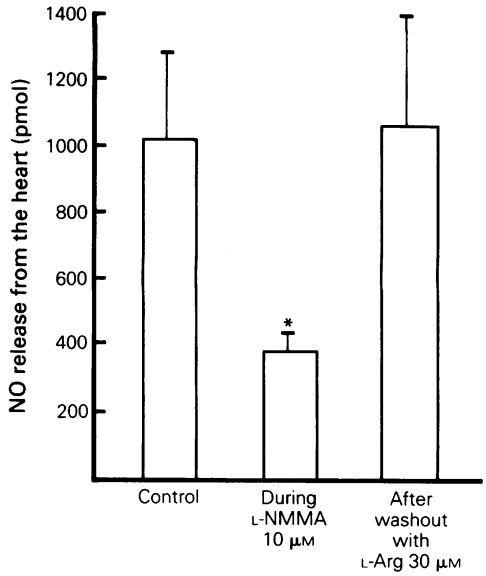

Figure 5 Nitric oxide (NO) formation by the coronary circulation of the Langendorff-perfused rabbit heart in response to three consecutive challenges with acetylcholine (ACh, 3 μM; 1 min infusion) in the same preparation. The second challenge was given at the midpoint of a 20 min infusion of N^G-monomethyl-L-arginine (L-NMMA, 10 μM). The third challenge was given immediately after a 20 min infusion of L-arginine (L-Arg, 30 μM). The amounts of NO detected by chemiluminescence were calculated by reference to authentic NO standards injected through the heart. * Indicates that the values differ significantly ($P < 0.05$) from the control in a paired t test ($n = 3$).

Bioassay

Acetylcholine (3 μM) induced the release of a relaxing factor from the heart which was identified as NO for it decayed during passage down the bioassay cascade at the same rate as NO (45 to 134 pmol) applied over the tissues (OT). Furthermore, its activity was inhibited by a concomitant infusion of haemoglobin (100 nM, OT, $n = 6$, $P < 0.05$) and enhanced by an infusion of superoxide dismutase (15 u ml^{-1}, $n = 6$, $P < 0.05$) administered through the heart.

The release of NO induced by ACh (3 μM) was increased to 175 $\pm$ 15% of control ($n = 6$, $P < 0.05$) by L-Arg (100 μM; Figure 6) and was inhibited significantly by 61 $\pm$ 5% by L-NMMA (30 μM; $n = 3$, $P < 0.05$). This inhibition could be reversed by a 20 min infusion of L-Arg (100 μM, $n = 3$, $P < 0.05$). None of these effects were observed with the D-enantiomers of these compounds ($n = 3$). Neither L-Arg (100 μM) nor L-NMMA (30 μM) had any effect

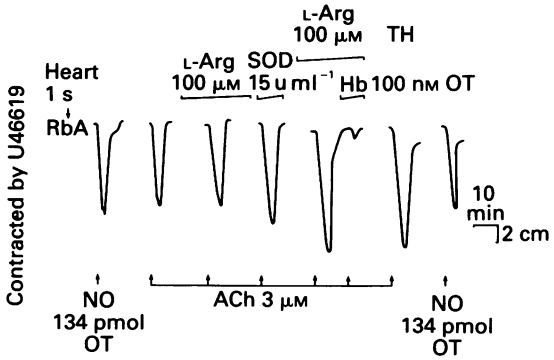

Figure 6 Effect of L-arginine (L-Arg, 100 μM; 30 min infusions), administered over the tissues (OT) and through the heart (TH), on the relaxation of a rabbit aortic strip (RbA) caused by acetylcholine (ACh, 3 μM; 1 min infusion; TH)-induced release of nitric oxide (NO). The effects of superoxide dismutase (SOD; 15 u ml; 10 min infusion; OT) and haemoglobin (Hb, 100 nM; 10 min infusion; OT) are also shown. NO was administered as a bolus injection (134 pmol; OT). The tissue was not affected by ACh (3 μM; OT; not shown). The trace is representative of 6 experiments.

on the bioassay tissues or on their response to exogenous NO.

Discussion

We have shown that L-NMMA, a specific inhibitor of NO formation from L-Arg (Palmer *et al.*, 1988b; Rees *et al.*, 1989a) induces a dose-dependent, long-lasting increase in the CPP. In addition, L-NMMA inhibited the vasodilatation induced by ACh and these effects were enantiomerically specific. These results indicate that NO is formed from L-Arg in the coronary microcirculation to regulate CPP and to mediate the effects of endothelium-dependent vasodilators.

In rabbit aortic rings we have previously shown that L-NMMA induces a small increase ($\approx$20%) in vascular tone ($EC_{50} \approx 4\,\mu M$) and inhibits ACh-induced relaxation. Furthermore, L-NMMA was more potent at increasing basal tone than as an inhibitor of ACh-induced relaxation (Rees *et al.*, 1989a). This is in contrast to our present findings in which lower doses of L-NMMA were required to inhibit the ACh-induced fall in CPP than were required to increase basal CPP. This most probably indicates that in resistance vessels there is a high basal level of NO formation which plays an important role in the regulation of tone in these vessels. This suggestion is supported by the observation of Kelm & Schrader (1988) who have recently reported that basal release of NO is high in the coronary circulation of the guinea-pig heart when compared to that induced by bradykinin.

Exogenous L-Arg had no direct effect on CPP or on the fall in CPP induced by ACh. This is in agreement with previous observations in vascular tissue (Sakuma *et al.*, 1988; Rees *et al.*, 1989a) and is consistent with our suggestion that under basal conditions the amounts of endogenous substrate are sufficient to provide for the basal formation of NO.

Exogenous L-Arg partially reversed the effect of L-NMMA on CPP and on ACh-induced fall in CPP. This is in contrast to the effects in large conduit arteries where a complete reversal of the effects of L-NMMA by L-Arg (Palmer *et al.*, 1988b; Sakuma *et al.*, 1988; Rees *et al.*, 1989a) has been observed. This difference could be due to mechanical or pharmacokinetic changes in the microcirculation caused by the oedema which develops during the experiment and which is enhanced by elevation of CPP. The competitive interaction between L-Arg and L-NMMA could be more clearly seen in experiments in which L-Arg was infused from the beginning of the experiment. Under these circumstances the effects of L-NMMA were greatly reduced.

Acetylcholine releases NO from the coronary circulation, although this decays rapidly to NO_2^- which is biologically inactive (Amezcua *et al.*, 1988). In the present studies we could also detect the release of biologically active NO from the heart and this was inhibited by L-NMMA. L-Arg not only reversed the effects of L-NMMA but, interestingly, enhanced the release of NO induced by a submaximal dose of ACh. The fact that this was not associated with potentiation of the ACh-induced fall in CPP in the absence of L-NMMA may be due to differences between luminal and abluminal release of NO (Bassenge *et al.*, 1987) or to the high doses of ACh required to elicit detectable NO release compared to those that lower CPP.

In summary, our results indicate a major role for EDRF/NO in the regulation of vascular tone and reactivity in the coronary circulation. The speculation that altered responses to endothelium-dependent vasodilators may play a role in the pathogenesis of coronary vasospasm has so far been supported by observations made in conditions associated with gross morphological damage of the endothelium. It is possible that a specific biochemical defect of the endothelium, with or without morphological damage, also plays a role in the development of coronary vasospasm. L-NMMA, which produces what could be considered a selective reversible 'biochemical denudation' of the endothe-

lium, offers the possibility of studying the consequences of removing the synthesis of NO by the vascular endothelium. Indeed, we have recently observed that L-NMMA induces a rise in blood pressure in the anaesthetized rabbit (Rees *et al.*, 1989b).

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