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Acetylcholine induces vasodilatation in the rabbit isolated heart through the release of nitric oxide, the endogenous nitrovasodilator

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1 Acetylcholine (ACh, 0.03–3.0 μM) induced a dose-dependent vasodilatation in the isolated Langendorff-perfused heart of the rabbit. The vasodilatation was mimicked by exogenous nitric oxide (NO, 0.045–4.5 nmol).

2 There was no detectable vascular relaxing activity in the cardiac effluent when these concentrations of ACh or NO were injected through the heart, even in the presence of an infusion of superoxide dismutase (SOD).

3 Acetylcholine (0.03–3.0 μM), however, induced the release into the cardiac effluent of a material which produced a chemiluminescent signal when reacted with ozone, a response which could be mimicked with exogenous NO (0.045–4.5 nmol) injected through the heart.

4 The effects of ACh, but not those of NO, were antagonized by atropine (2 μM). Prostacyclin (1 μM) injected through the heart induced vasodilatation without the release of a biologically active or chemiluminescent material.

5 During passage through the heart, >99% of the biological activity of exogenous NO disappeared, whereas there was approximately 50% reduction of its chemiluminescent response. This indicates complete transformation into a mixture containing approximately 50% NO_2^- and 50% of other non-chemiluminescent material(s), presumably NO_3^-.

6 This study suggests that ACh induces endothelium-dependent vasodilatation in the coronary circulation through the release of the endogenous nitrovasodilator, NO, which is rapidly converted to NO_2^- and NO_3^-.

Introduction

The relaxation of strips or rings of vascular smooth muscle induced by a wide variety of agents depends on the presence of the endothelium (Furchgott, 1984). Endothelium-dependent relaxation involves the release of a labile humoral mediator, endothelium-derived relaxing factor (EDRF; Furchgott & Zawadzki, 1980). Although the release of EDRF has been demonstrated from large conduit vessels *in vitro* (Furchgott & Zawadzki, 1980; Griffith *et al.*, 1984), its release *in vivo* or from whole organs *in vitro* has not been shown. Palmer *et al.* (1987) have recently established the chemical identity of EDRF as nitric oxide (NO). Indeed, the release of NO by the vascular endothelium accounts not only for the vascular relaxation induced by EDRF, but also for its effects on platelet aggregation (Radomski *et al.*, 1987a) and platelet adhesion (Radomski *et al.*, 1987b).

We have developed a method for the detection of NO in biological samples by chemiluminescence. This method detects NO released as such from endothelial cell cultures (Palmer *et al.*, 1987) or from fresh vascular tissue (Chen, Palmer & Moncada, unpublished observations, 1988). It also detects NO_2^- following its conversion to NO by acidification in the chemiluminescence apparatus (Radomski *et al.*, 1987b). Using this method in combination with a superfusion cascade bioassay, which detects NO but not NO_2^- (Palmer *et al.*, 1987), we now demonstrate that acetylcholine (ACh) induces vasodilatation in the isolated Langendorff-perfused heart of the rabbit through the release of NO. This is rapidly converted into biologically inactive NO_2^-, and possibly NO_3^-, which appear in the cardiac effluent. These results

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provide the first evidence for the release of an endogenous nitrovasodilator in an isolated organ *in vitro*.

Methods

Langendorff-perfused hearts

Male New Zealand White rabbits (1.8–2.4 kg) were given heparin (1000 u kg^{-1}) and anaesthetized with pentobarbitone (40–50 mg kg^{-1}) via an ear vein. The thorax was opened rapidly and the aorta cannulated retrogradely. The heart was removed and flushed with oxygenated Krebs buffer from a 50 ml syringe and then perfused at 37°C with Krebs buffer (15 ml min^{-1}), gassed with 5% CO_2 and 95% O_2.

The ventricles were stimulated with two pin electrodes at 180 beats min^{-1} by use of a Grass Stimulator S9 (square wave pulses, 6V, 3 ms). In some experiments the coronary perfusion pressure (CPP) was measured by connecting a pressure transducer to a side arm of the perfusion line. The CPP was raised from a basal pressure of approximately 10 mmHg to 25–70 mmHg by a continuous infusion of 9,11-dideoxy-9α,11α methano epoxy-prostaglandin $F_{2\alpha}$ (U46619; 30–150 nM). The partial pressure of O_2 in the Krebs buffer was >300 mmHg, and was between 150 and 180 mmHg in the cardiac effluent.

Bioassay experiments

Indomethacin (5 μM) was added to the Krebs buffer perfusing the heart. The cardiac effluent was used to superfuse in cascade three or four spirally cut strips of rabbit thoracic aorta denuded of endothelium and contracted either with U46619 (50–150 nM) or with phenylephrine (750 nM) (Gryglewski *et al.*, 1986). A mixture of propranolol (7 μM), hyoscine (3 μM), mepyramine (1 μM) and methysergide (0.3 μM) was infused over the tissues to increase the specificity of the bioassay for NO. 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 the exit of the cardiac effluent and this tissue. The subsequent tissues were separated from each other by 3 s delays. Changes in the length of the bioassay tissues were detected by Harvard 386 transducers and recorded on a Watanabe WRT 281 pen recorder. The amplification of the recorder channels was adjusted to give the same response to standard doses of glyceryl trinitrate (50–100 nM) over the tissues.

Detection of NO and NO_2^- by chemiluminescence

The cardiac effluent was collected into the stem of a funnel closed at the bottom (see Figure 1). A portion (3.5 ml min^{-1}) was pumped continuously into the reflux vessel of the chemiluminescence apparatus (Palmer *et al.*, 1987), while the volume of the sampling pool (2.0 ml) was kept constant by continuous removal of the overflow. The delay between the collecting funnel and the reflux vessel was 15 s. The fate of NO and NO_2^- in this system was assessed by comparing the signals obtained when NO or NO_2^- were injected either into the funnel collecting the cardiac effluent or directly into the reflux vessel (Figure 1). These experiments showed that there was virtually no disappearance (9 ± 5%, $n = 3$) of NO_2^- during its transit through the collecting system. The extent of NO conversion into NO_2^- was 54 ± 2% ($n = 3$), indicating that the following reactions had occurred prior to the reflux vessel:

$$2NO + O_2 \rightarrow 2NO_2$$

$$2NO_2 + H_2O \rightarrow NO_2^- + NO_3^- + 2H^+$$

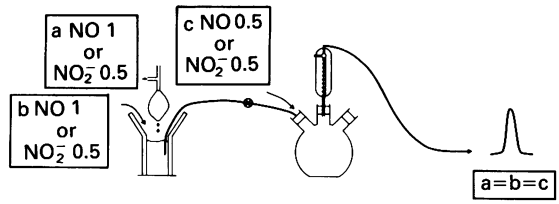

Figure 1 Diagram showing the system used to collect the cardiac effluent for detection of NO and NO_2^- by chemiluminescence. Cardiac effluent (15 ml min^{-1}) was collected into a closed funnel and a portion (3.5 ml min^{-1}) was pumped continuously into the reflux vessel of the chemiluminescence detector. The volume in the funnel (2 ml) was kept constant by continuous removal of the overflow. Drugs could be added at (a) through the heart, (b) into the funnel collecting the cardiac effluent or (c) into the reflux vessel. The delay between (a) and (b) was 7 s, that between (b) and (c) was 15 s. The chemiluminescent product of the reaction with ozone was detected with a photomultiplier and displayed on a pen recorder. The chemiluminescent signals obtained when 1 nmol NO was added at (a) or (b), 0.5 nmol of NO was added at (c) and when 0.5 nmol of NO_2^- was added at (a), (b) or (c) were all of the same magnitude after correction for the sampling fraction between (b) and (c).

Chemicals

$NaNO_2$ and $NaNO_3$ (analytical grade) were purchased from BDH Chemicals. Nitric oxide solutions in deoxygenated water were prepared as previously described (Palmer *et al.*, 1987). Prostacyclin sodium salt was obtained from Wellcome plc. All other drugs were obtained from Sigma and diluted in saline.

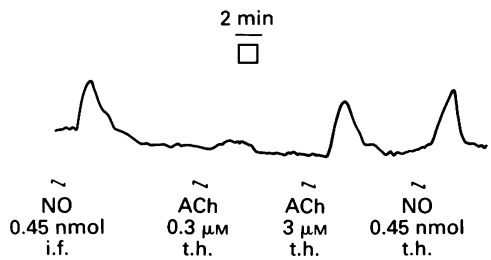

Figure 2 Chemiluminescent signals produced by authentic NO (0.45 nmol, administered as 1 min infusions) injected either into the funnel collecting the cardiac effluent (i.f.) or through the heart (t.h.). Acetylcholine (ACh; 0.3 and 3 μM, 1 min infusions, t.h.) released a chemiluminescent material in a dose-related manner. □ area equivalent to 0.22 nmol. Trace representative of 4 experiments.

Results

Effects of acetylcholine and prostacyclin

Acetylcholine (0.03–3 μM), administered as a 1 min infusion through the heart (t.h.), produced a concentration-dependent fall in the CPP ($n = 5$). The release of EDRF into the cardiac effluent induced by these concentrations of ACh was not detectable by bioassay ($n = 4$). A concomitant infusion of SOD (15 u ml^{-1}, t.h.) did not reveal the presence of EDRF ($n = 4$).

These concentrations of ACh (t.h.) caused a concentration-related release of a material which was detected by chemiluminescence (Figure 2). The release of chemiluminescent material induced by ACh was greatest at 3 μM and was equivalent to 1.45 ± 0.15 nmol NO (t.h.; $n = 7$). Atropine (2 μM t.h.), infused for 15 min prior to ACh, inhibited its effects both on the CPP and on the release of the chemiluminescent substance ($n = 3$). Prostacyclin (1 μM; 1 min infusion, t.h.) produced a fall in the CPP similar to that of ACh (3 μM t.h.) but did not release a substance detectable by bioassay or chemiluminescence ($n = 3$).

Effects of NO and $NaNO_2$

Nitric oxide (0.045–4.5 nmol; 1 min infusion t.h.) produced a dose-dependent fall in the CPP of a similar magnitude to that induced by the above concentrations of ACh (Figure 3). The biological activity of exogenous NO (0.045–4.5 nmol, t.h.) was not detectable on the bioassay tissues ($n = 5$) even in the presence of an infusion of SOD (15 u ml^{-1}, t.h., $n = 4$). Higher amounts of NO (134–450 nmol) were needed in order to detect any activity on the bioassay tissues

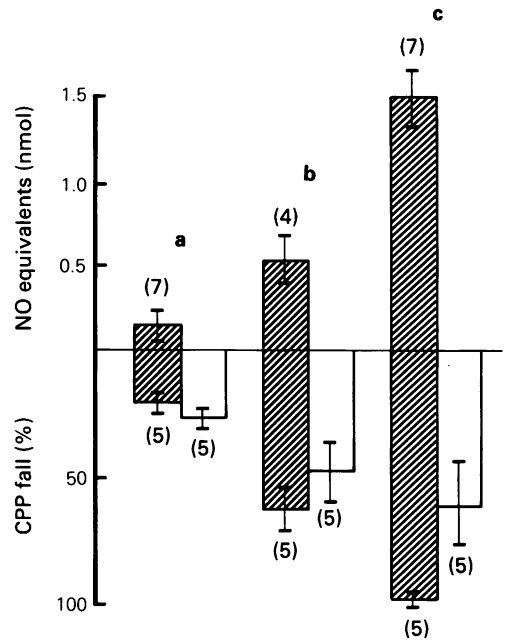

Figure 3 Release of the chemiluminescence-producing material and the vasodilatation induced by acetylcholine (ACh) and NO in the isolated heart. Upper panel: the chemiluminescent signals (expressed as NO equivalents released into the cardiac effluent) produced by ACh (a, 0.03; b, 0.3; c, 3.0 μM) injected through the heart (t.h.). Lower panel: the reductions in coronary perfusion pressure (CPP, expressed as % of the maximal response in each heart) induced by the above doses of ACh (hatched columns) and by exogenous NO (a, 0.13; b, 0.45; c, 1.3 nmol, open columns) given t.h. Each value is the mean of (n) experiments; vertical bars show s.e.mean.

(Figure 4). The threshold sensitivity of the bioassay tissues was 0.013–0.45 nmol. $NaNO_2$ (4.5 nmol) did not exhibit any biological activity either in the coronary circulation or on the assay tissues ($n = 3$).

The disappearance of 223 nmol of NO through the heart was 99.93 ± 0.01% ($n = 5$) as measured by bioassay and this was not changed by the presence of SOD (15 u ml^{-1}; 99.88 ± 0.04%; $n = 5$). In contrast, 50 ± 6% ($n = 4$) of the biological activity of NO (0.45 nmol) survived a delay of 40 s during passage through a polyethylene tube.

Nitric oxide (0.045–4.5 nmol) produced similar chemiluminescent signals when injected t.h. or into the cardiac effluent ($n = 9$). These signals, however, were 54 ± 2% ($n = 3$) of those produced by equivalent doses of NO injected directly into the reflux vessel (Figure 1). The chemiluminescent signals produced by $NaNO_2$ (0.45–4.5 nmol) were comparable

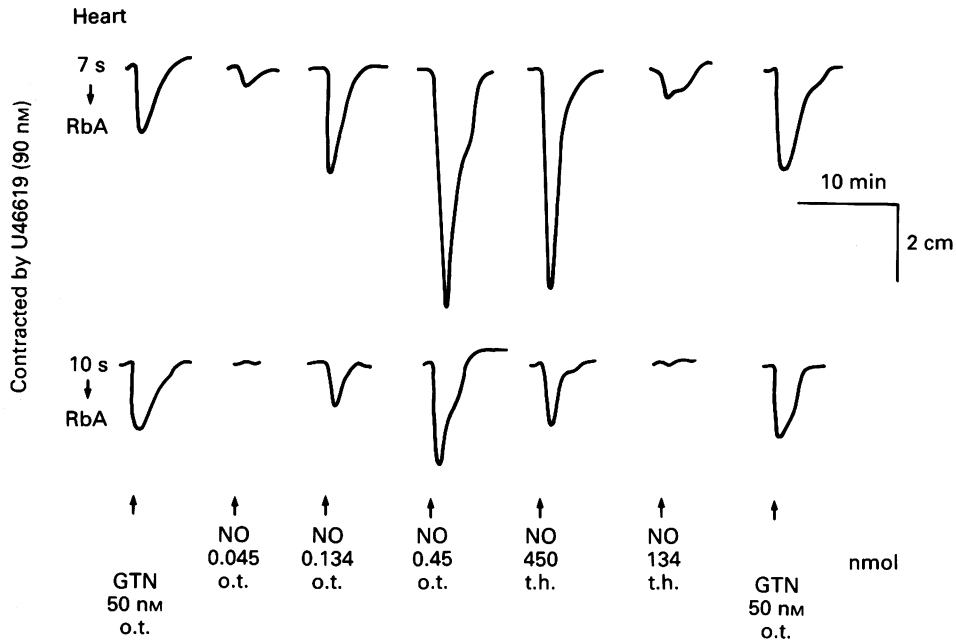

Figure 4 Relaxation of rabbit aortic strips (RbA) induced by nitric oxide (NO). The tissues were relaxed by low doses of NO infused directly over the tissues (o.t.) or by much higher doses administered through the heart (t.h.). The sensitivity of the tissues was standardized by use of glyceryl trinitrate (GTN, 50 nM, o.t.). The trace is representative of 5 experiments.

when given t.h., into the cardiac effluent or directly into the reflux vessel ($n = 7$). $NaNO_3$ (4.5 nmol, t.h.) was devoid of both biological and chemiluminescent activity ($n = 3$).

Prostacyclin, $NaNO_3$ and ACh did not induce chemiluminescent signals when injected into the cardiac effluent or directly into the reflux vessel ($n = 3$).

Discussion

This study provides the first evidence that the endogenous nitrovasodilator, NO, is formed by the vasculature of an intact organ.

The release of NO in the coronary circulation induced by ACh is the result of a specific pharmacological interaction at muscarinic receptors, as indicated by its inhibition by atropine. Furthermore, prostacyclin, which is an endothelium-independent vasodilator, did not release any chemiluminescent material, showing that the release by ACh is specifically related to endothelium-dependent vasodilatation.

Vasodilatation elicited by ACh in the heart can be attributed to NO formation for it could be matched by exogenous NO, but not by NO_2^- or NO_3^-. Furthermore, the magnitude of the chemiluminescent signals which accompanied the vasodilator responses was also matched by the signals obtained with these doses of exogenous NO injected through the heart. In addition, neither endogenously released nor exogenous NO survived passage through the heart. The disappearance of exogenous NO, but not NO_2^-, during passage through the heart was almost complete, and could not be modified with SOD. This explains why ACh-induced coronary vasodilatation is not accompanied by the release of EDRF into the cardiac effluent even in the presence of SOD.

This study supports our previous suggestion that of all the known 'local hormones' NO is the one with the least possibility of acting as a circulating substance (Moncada *et al.*, 1987). Indeed, a direct transfer between cells is more likely to provide the optimal conditions for such an ephemeral substance to act.

Exogenous NO injected t.h. gives rise to a chemiluminescent material in the effluent which is approximately 50% of the NO injected. Since concentrations of ACh that match the vasodilatation induced by NO also produce equivalent amounts of the chemiluminescent material, it is reasonable to

assume that endogenously released and exogenously added NO are subjected to the same chemical degradation process.

The time required for the disappearance of 50% of the biological activity of NO in a polyethylene tube (40 s) is much longer than that needed for its complete disappearance in the heart (7 s). This implies the existence of a very active pathway for oxidation of NO in the heart. This is unlike the situation in a segment of rabbit aorta where ACh induces the release of NO into the effluent of this tissue (Chen, Palmer & Moncada, unpublished observations) and suggests that the microcirculation may be the site of the greatest conversion of NO.

It is interesting to note that in order to induce vasodilatation exogenous NO has to cross the endothelium to activate the soluble guanylate cyclase in the vascular smooth muscle (Waldman & Murad, 1987). At present it is impossible to know how much NO crosses the endothelium and how much is actually necessary to elicit the biological response. It is possible that only a fraction of the NO released by ACh or added exogenously is required to achieve this.

It is also tempting to speculate that the main site of inactivation of NO is the vascular smooth muscle itself, either during or immediately after a rapid activation of the soluble guanylate cyclase. The NO generated from nitrovasodilators (Feelisch & Noack, 1987) in vascular smooth muscle may be metabolized in the same way as the endogenous NO generated by vascular endothelium. It would indeed be fascinating if the intracellular metabolites in vascular smooth muscle, resulting from its stimulation either with exogenous nitrovasodilators or with endothelium-dependent vasodilators, were the same.

Whether vagally mediated vasodilator responses in the heart (Hackett *et al.*, 1972) are also dependent on the release of NO remains to be investigated. If this is so, it will be interesting to elucidate the way in which ACh liberated in the adventitial surface of the vessels reaches the endothelium to release NO.

Our demonstration of NO formation by the coronary circulation of the rabbit heart indicates that the role of this endogenous nitrovasodilator in the physiological and the pathophysiological regulation of the coronary blood flow can now be investigated.

Note added in proof

While this paper was in press we have refined our bioassay system so that small quantities of EDRF released from the heart can be detected. These results will be communicated elsewhere.

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References

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.

FURCHGOTT, R.F. (1984). The role of endothelium in the responses of vascular smooth muscle to drugs. *Ann. Rev. Pharmacol. Tox.*, **24**, 175–197.

FURCHGOTT, R.F. & ZAWADZKI, J.V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*, **288**, 373–376.

GRIFFITH, T.M., EDWARDS, D.H., LEWIS, M.J., NEWBY, A.C. & HENDERSON, A.H. (1984). The nature of endothelium-derived vascular relaxant factor. *Nature*, **308**, 645–647.

GRYGLEWSKI, R.J., MONCADA, S. & PALMER, R.M.J. (1986). Bioassay of prostacyclin and endothelium-derived relaxing factor (EDRF) from porcine aortic endothelial cells. *Br. J. Pharmacol.*, **87**, 685–694.

HACKETT, J.G., ABBOUD, F.M., MARKE, A.L., SCHMID, P.G. & HEISTAD, D.D. (1972). Coronary vascular responses to stimulation of chemoreceptors and baroreceptors. Evidence for reflex activation of vagal cholinergic innervation. *Circ. Res.*, **31**, 8–17.

MONCADA, S., PALMER, R.M.J. & HIGGS, E.A. (1987). Prostacyclin and endothelium-derived relaxing factor: biological interactions and significance. In *Thrombosis and Haemostasis*. ed. Verstraete, M., Vermylen, J., Lijnen, H.R. & Arnout, J. pp. 597–618. Leuven University Press.

PALMER, R.M.J., FERRIGE, A.G. & MONCADA, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature*, **327**, 524–526.

RADOMSKI, M.W., PALMER, R.M.J. & MONCADA, S. (1987a). 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. (1987b). The role of nitric oxide and cGMP in platelet adhesion to vascular endothelium. *Biochem. Biophys. Res. Commun.*, **148**, 1482–1489.

WALDMAN, S.A. & MURAD, F. (1987). Cyclic GMP synthesis and function. *Pharmacol. Rev.*, **39**, 163–196.

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