A comparison of basal and agonist-stimulated release of endothelium-derived relaxing factor from different arteries

M.I. Christie, *T.M. Griffith and ¹M.J. Lewis

Departments of Pharmacology & Therapeutics and *Radiology, University of Wales College of Medicine, Heath Park, Cardiff CF4 4XN

- 1 The release of endothelium-derived relaxing factor (EDRF) from rabbit aorta and pig coronary artery vessels in response to acetylcholine (ACh), substance P (SP) and the calcium ionophore A23187 has been studied by means of a bioassay cascade system.
- 2 A technique is described which allows the quantification of EDRF release rates from vessels of different sizes, perfused at different flow rates and with different donor-recipient transient times.
- 3 Rabbit aorta and pig coronary arteries, perfused at flow rates which equalise endothelial shear stress, released EDRF at a similar basal rate.
- 4 In response to ACh, rabbit aortic endothelium released EDRF at a significantly greater maximum rate than pig coronary artery endothelium.
- 5 In response to SP, both endothelium types released EDRF; SP was a significantly more potent agonist in pig coronary artery than in rabbit aorta, but maximum SP-induced EDRF release from rabbit aorta was twice that of pig coronary artery.
- 6 These data indicate that different endothelium types can release EDRF at widely different rates, according to the agonist used, and that the previously obtained lack of relaxant response to ACh in pig coronary artery was due to a lack of EDRF release rather than concomitant smooth muscle constriction.

Introduction

Endothelium-derived relaxing factor (EDRF) activity varies greatly in different arteries. For example, Griffith and colleagues (1984a) have shown large differences in basal EDRF activity between rabbit aorta and rabbit coronary preparations, with activity being greatest in coronary vessels. Similarly Collins and co workers (1986) have demonstrated that agonist-induced Ca^{2+} influx is inhibited more by basal EDRF activity in dog isolated coronary artery preparations than in rabbit aorta.

Receptor-activated EDRF responses also vary according to vessel type. Relaxant responses to substance P (SP) and noradrenaline in isolated preparations of dog large arteries vary in the order coronary > carotid > femoral $\gg$ renal = mesenteric (Angus *et al.*, 1986), and EDRF responses in perfused rat renal and mesenteric preparations differ widely according to the agonist used to release EDRF (Bhardwaj & Moore, 1988). These differences could

be due to variations in the amount of EDRF released by different arteries, or a variation in the response of the smooth muscle to EDRF. We have previously demonstrated that differences in EDRF activity between pig coronary artery and rabbit aorta can be explained in part by differences in their smooth muscle response to EDRF, with pig coronary artery being more responsive than rabbit aorta (Christie & Lewis, 1988).

Substance P is a potent endothelium-dependent relaxant in both pig coronary artery (Cocks & Angus, 1983) and rabbit aorta (Zawadzki *et al.*, 1981), and acetylcholine (ACh) is the standard EDRF-releasing agent in both endothelium-intact rings of rabbit aorta (Furchgott & Zawadzki, 1980) and in bioassay preparations (Griffith *et al.*, 1984b). In pig coronary artery preparations, however, endothelium-dependent relaxation cannot be elicited by ACh (Kalsner, 1985), and dose-response curves to the constrictor effects of ACh in this preparation are unaffected by the presence of an intact endothelium

¹ Author for correspondence.

(Graser *et al.*, 1986). This lack of ACh-mediated EDRF activity in pig coronary artery may be due to either a lack of EDRF release, or masking of the relaxant response to EDRF by increased activation of smooth muscle muscarinic receptors mediating constriction.

In this study the amount of EDRF released from each endothelial type both basally and in response to the endothelium-dependent relaxants SP and ACh has been quantified by use of cascade bioassay techniques. Direct comparisons between EDRF release from the pig coronary artery and rabbit aorta are complicated by differences in diameter (d) and flow rate (Q) as shear stress, which is proportional to Q/d^3 , enhances EDRF release in bioassay systems (Pohl *et al.*, 1986; Rubanyi *et al.*, 1986). To compensate for this effect, in the present study, pig coronary artery and rabbit aorta donors were perfused at flow rates which result in equivalent endothelial shear stresses. As differences in perfusion rate will influence both the dilution of EDRF released into the lumen of each donor, and the transit time (during which EDRF decays exponentially) between donor and recipient vessels, these factors were also taken into account before EDRF release rates from the two endothelium types were calculated and compared.

The results demonstrated that, although basal EDRF release was similar in the two vessel types, rabbit aortic endothelium releases EDRF at a higher rate in response to both SP and ACh. The relevance of these findings to results obtained from endothelium-intact vascular preparations is discussed.

Methods

Vessel preparations

Rabbit aorta Male New Zealand white rabbits (2–2.5 kg) were killed by a blow to the neck. The descending thoracic aorta was removed and transferred to pre-gassed (95% O_2 : 5% CO_2) Holman's solution of the following composition (mM): NaCl 120, KCl 5, NaH_2PO_4 1.3, $NaHCO_3$ 25, $CaCl_2$ 2.5, D-glucose 11, sucrose 10, containing indomethacin (10^{-5} M) as a cyclo-oxygenase inhibitor to prevent prostanoïd synthesis. A 3 cm length of rabbit aorta was carefully cleaned of connective tissue and mounted in the bioassay system as an EDRF donor.

Pig coronary artery Pig circumflex coronary arteries were collected from a local abattoir, cut into 2–3 mm wide rings, denuded of endothelium and mounted as recipients in the bioassay system as previously described (Christie & Lewis, 1988). Recipient rings were perfused initially at 2 ml min^{-1} with

gassed Holman's solution at 37°C , and tension responses recorded on a devices chart recorder. Rings were equilibrated for 60 min at a resting tension of 5 g before being tested for the successful removal of endothelium by precontraction with 5-hydroxytryptamine (5-HT) 10^{-5} M, and brief (1 min) exposure to A23187, 10^{-7} M. Any rings showing a relaxant response to A23187 were considered to have some functional endothelium and discarded. Pig coronary artery rings were then allowed to return to a resting tension of 5 g, and the tension was adjusted if basal tone was altered by the initial exposure to 5-HT.

A 3 cm length of pig coronary artery was prepared and mounted as an EDRF donor in the bioassay system in a manner similar to rabbit aorta.

Experimental protocol

Recipient pig coronary artery rings were precontracted with 5-HT, (3×10^{-6} M) infused post-donor (i.e. into the perfusate between donor and recipient) by means of an LKB (type 3132) peristaltic pump. Atropine (3×10^{-6} M) was also infused post-donor in all experiments to prevent stimulation of pig coronary artery muscarinic receptors. After constrictor responses had stabilised, the donor vessel (either rabbit aorta or pig coronary artery) was perfused with Holman's solution for 3 min before being allowed to perfuse the recipient rings. Basal EDRF release, and EDRF release in response to either cumulative doses of ACh (10^{-7} – 10^{-5} M) or 60 s pulses (to avoid tachyphylaxis) of SP (10^{-11} – 3×10^{-8} M) was measured. At the end of each experiment a supramaximal (10^{-6} M) dose of A23187 was added to cause maximal EDRF release. This ensured that recipient relaxation in response to EDRF was submaximal throughout the dose-response curve and served as a test for the presence of a functional endothelium on donor vessels. At the concentrations used, ACh, SP and A23187 were without direct effect on the pig coronary artery rings used in this study.

Calculation of donor dimensions and endothelial shear stress

At the end of each experiment the donor vessel was removed from the bioassay, and its central portion sectioned into four 3–4 mm wide rings with a fixed-blade ring cutter. Each ring was cut longitudinally and the resulting strip laid on graduated (1 mm square) paper. The length of each strip could then be estimated to the nearest 0.5 mm, and used to calculate the mean circumference (c) of the intact donor. The diameter (d) of each donor could then be calculated (as $d = c/\pi$). For all donors the tips of the infu-

sion cannulae were 20 mm apart, making the effective length (L) of each donor identical. The endothelial shear stress of each donor at a fixed flow rate $\dot{Q}$ was derived from the formula $32\mu\dot{Q}/\pi d^3$ dyne/cm², where the coefficient of viscosity, μ , for Holman's solution at 37°C was taken as 0.7 centipoise.

In a preliminary series of experiments the dimensions of a representative sample of pig coronary artery and rabbit aorta vessels were measured and used to calculate the flow rates that produced similar levels of endothelial shear stress in each donor/vessel. These were 1 ml min⁻¹ (0.017 cm³ s⁻¹) and 7 ml min⁻¹ (0.117 cm³ s⁻¹) in pig coronary artery and rabbit aorta respectively, and were used in all experiments. The higher flow rate was obtained by fitting wider bore tubing in the Watson-Marlow perfusion pump, so that the frequency of flow pulsation was the same for each donor vessel (1.2–1.7 Hz).

Quantification of relaxant responses to EDRF

Sodium nitroprusside (NaNP) has been shown to exert its relaxant action by spontaneous release of nitric oxide (NO) (Feelisch & Noake, 1987), and EDRF is either NO or a closely related species (Palmer *et al.*, 1987). EDRF responses were therefore quantified by reference to the linear part of a standard NaNP concentration-response curve, constructed by mounting endothelium-denuded pig coronary artery rings as recipients in the bioassay, preconstricting them with 5-HT (3×10^{-6} M), and exposing them to increasing cumulative concentrations of NaNP (3×10^{-9} – 3×10^{-7} M). Relaxant responses induced by EDRF were then expressed as units of EDRF activity, where 3×10^{-6} units EDRF causes the same percentage relaxation of pig, 5-HT-preconstricted coronary artery rings as 3×10^{-6} M NaNP.

Calculation of rate of EDRF decay post-donor

The short half-life of EDRF must be taken into account at different flow rates. In this bioassay system, the transit time of EDRF between donor and recipient was 6.5 s at a flow rate of 1 ml min⁻¹, and 1.5 s at a flow rate of 7 ml min⁻¹. EDRF activity decreases exponentially as a function of time during transit (Griffith *et al.*, 1984b) so that its decay can be described by the equation:-

$$C_R = C_D \cdot e^{-\lambda t} \quad (1)$$

Where C_R = concentration of EDRF at recipient (units), C_D = concentration of EDRF leaving donor (units), λ = decay constant for EDRF (units s⁻¹) and t = transit time between donor and recipient (s).

The decay constant for EDRF was calculated by increasing transit time of EDRF between donor and recipient as described previously (Griffith *et al.*, 1984b).

Calculation of rate of EDRF release into, and decay within donor

The concentration of EDRF at the distal end of a donor vessel is a combination of the rate of release of EDRF into the luminal perfusate, and the rate of decay of EDRF in the perfusate as it travels through the donor. The rate of EDRF release from endothelium in a donor vessel can be calculated from the equation:-

$$f = \frac{\lambda d C_D}{4 \cdot \left[1 - e^{\left(\frac{-\lambda v}{\dot{Q}} \right)} \right]} \quad (2) \text{ (see Appendix)}$$

Where f = rate of EDRF release per unit area of endothelium (units cm⁻² s⁻¹), C_D = concentration of EDRF leaving donor (units), λ = decay constant for EDRF (units s⁻¹), d = diameter of donor (cm), V = volume of donor (cm³) and $\dot{Q}$ = flow rate (cm³ s⁻¹).

The equation combines the rate of decay and rate of production of EDRF in each donor type, the resultant of which is the concentration of EDRF in the perfusate leaving the donor.

Each pig coronary artery relaxant response induced by EDRF was first expressed as a percentage of the initial 5-HT-induced constriction. This response was then converted to EDRF units by comparing it with the NaNP calibration curve to give a value for C_R (total concentration of EDRF at recipient). This value for C_R was inserted into equation 1 to give a value for C_D which was then inserted into equation 2 to give a value of f for each response. These f values are directly comparable for pig coronary artery and rabbit aorta donors, as they describe the rate of EDRF release from a unit area of endothelium at the same shear stress, and correct for differences in vessel dimensions, flow rates and donor recipient transit times.

Statistics

Percentage relaxation and absolute EDRF release concentration-response curve slopes, and EC₅₀ values for SP and ACh, were calculated from a line fitted to the linear part of the concentration-response curve by a least squares minimisation procedure. Absolute EDRF values, and decay constants for EDRF were also calculated from a line fitted by least

Figure 1 Typical responses of pig coronary artery rings to endothelium-derived relaxing factor (EDRF) released from pig coronary artery donors (PCA+) by 60s pulses of substance P (SP, a), or cumulative concentrations of acetylcholine (ACh, b) followed by a supramaximal concentration of A23187 (10^{-6} M). Points indicate a change in the concentration of the EDRF-releasing agonist (log M). Rings are precontracted with 5-hydroxytryptamine (3×10^{-6} M) in the presence of atropine (3×10^{-6} M). Note that pig coronary artery releases EDRF in response to SP but not ACh, and that A23187 induces supramaximal release of EDRF.

squares minimisation to the percentage relaxation vs $\log_{10}$ [NaNP], and EDRF units vs transit time data, respectively.

Results are presented as mean $\pm$ s.e.mean, compared by use of Student's *t* test for unpaired data, and considered significant when $P < 0.05$.

Drugs used

5-Hydroxytryptamine (creatinine sulphate complex), acetylcholine chloride, substance P (acetate salt), A23187 (calcium, magnesium mixed salts), sodium nitroprusside and indomethacin were purchased from the Sigma Chemical Co Ltd, Poole, UK. Atropine sulphate was purchased from BDH Ltd, Poole, UK. Stock solutions of 5-HT, ACh and NaNP

Figure 2 Mean percentage relaxation of endothelium denuded, 5-hydroxytryptamine-precontracted coronary artery rings of the pig in response to endothelium-derived relaxing factor (EDRF) released from endothelium-intact coronary artery donors of the pig basally (B), and in response to 60s pulses of substance P ($n = 5$) or cumulative concentrations of acetylcholine (ACh) ($n = 5$) (●), followed by a supramaximal concentration (10^{-6} M) of A23187 (○). Vertical lines show s.e.mean.

(10^{-2} M) were made up in distilled water. Indomethacin (10^{-2} M) and A23187 (5×10^{-3} M) stock solutions were made up in 99% ethanol and DMSO, respectively. Substance P was diluted in 0.1 M HCl immediately before use. The final concentrations of ethanol, DMSO and HCl used did not affect tissue responses in this system.

Results

Relaxant responses to EDRF released from donor vessels

Pig coronary artery Figure 1 shows typical tension recordings obtained from atropine-treated, endothelium-denuded, coronary artery rings of the pig exposed to EDRF released from a pig coronary

artery donor. Both rings were perfused at a flow rate of 1 ml min^{-1} and precontracted with 5-HT ($3 \times 10^{-6} \text{ M}$) infused post-donor. Atropine ($3 \times 10^{-6} \text{ M}$) was also infused post-donor. (a) Shows EDRF released basally, that in response to 60 s pulses of SP ($10^{-4} - 3 \times 10^{-8} \text{ M}$) or in response to a supramaximal concentration of A23187 (10^{-6} M). (b) Shows EDRF release basally, in response to cumulative concentrations of ACh ($10^{-7} - 10^{-5} \text{ M}$) and in response to A23187 (10^{-6} M). Pig coronary artery endothelium therefore releases EDRF basally and in response to SP, but not to ACh, although in both experiments A23187 induces maximal EDRF release.

Figure 2 shows the mean relaxant responses obtained in rubbed coronary rings of the pig precontracted with 5-HT, to EDRF released from pig coronary artery donors. The pig coronary artery rings used to detect SP- and ACh-induced EDRF release were precontracted to $7.6 \pm 1.2 \text{ g}$ and $8.4 \pm 1.1 \text{ g}$ tension by 5-HT and relaxed by $116 \pm 4\%$ and $118 \pm 4\%$, respectively, in response to maximal EDRF release induced by A23187. The pig coronary artery donors used for the SP experiments were perfused at a flow rate of 1 ml min^{-1} , which produced a mean shear stress of $0.16 \pm 0.02 \text{ dyne cm}^{-1}$, and basal EDRF release corresponding to $41 \pm 7\%$ relaxation. The concentration-response curve to SP had a slope of 14.1 ± 2.8 , an EC_{50} value of $7.4 \pm 2.7 \times 10^{-11} \text{ M}$, and an asymptote corresponding to $107 \pm 6\%$ relaxation. Pig coronary artery donors used for the ACh experiments experienced a mean shear stress of $0.17 \pm 0.02 \text{ dyne cm}^{-2}$, which produced basal EDRF release corresponding to $45 \pm 8\%$ relaxation. The concentration-response curve to ACh had a slope of 4.6 ± 1.3 , an EC_{50} value of $3.1 \pm 0.8 \times 10^{-7} \text{ M}$, and an asymptote corresponding to $59 \pm 8\%$.

Rabbit aorta Figure 3 shows typical tension recordings obtained from atropine-treated, endothelium-denuded coronary artery rings of the pig exposed to EDRF released from a rabbit aorta donor. Both rings were perfused at 7 ml min^{-1} and precontracted with 5-HT infused post-donor. Atropine was also infused post-donor. The upper trace shows EDRF released basally and in response to 60 s pulses of SP, and in response to a supramaximal concentration of A23187. The lower trace shows EDRF released basally and in response to cumulative concentrations of ACh, and in response to A23187. Note that, unlike pig coronary artery, both SP and ACh released EDRF from rabbit aortic endothelium.

Figure 4 shows the mean relaxant responses of rubbed coronary artery rings of the pig, precontracted with 5-HT, to EDRF released from rabbit aorta donors. The pig coronary artery rings used to detect SP- and ACh-induced EDRF release were pre-

Figure 3 Typical responses of pig coronary artery rings to endothelium-derived relaxing factor (EDRF) released from rabbit aorta donors (RA+) by 60 s pulses of substance P (SP, a) or cumulative concentrations of acetylcholine (ACh, b), followed by A23187 (10^{-6} M). Points indicate a change in the concentration of the EDRF-releasing agonist (log M). Rings are precontracted with 5-hydroxytryptamine ($3 \times 10^{-6} \text{ M}$) in the presence of atropine ($3 \times 10^{-6} \text{ M}$). Note that rabbit aorta releases EDRF in response to both SP and ACh, and that A23187-induced release is supramaximal.

stricted to $7.8 \pm 0.7 \text{ g}$ and $9.8 \pm 0.7 \text{ g}$ tension by 5-HT, and relaxed by $126 \pm 8\%$ and $99 \pm 5\%$ respectively, in response to maximal EDRF release induced by A23187. The rabbit aorta donors used for the SP experiments were perfused at a flow rate of 7 ml min^{-1} , which produced a mean shear stress of $0.22 \pm 0.03 \text{ dyne cm}^{-2}$, and basal EDRF release corresponding to $28 \pm 6\%$ relaxation. The concentration-response curve to SP had a slope of 18.5 ± 3.8 , an EC_{50} value of $3.2 \pm 0.9 \times 10^{-10} \text{ M}$, and an asymptote corresponding to $96 \pm 13\%$ relaxation. The rabbit aorta donors used for the ACh experiments experienced a mean shear stress of $0.19 \pm 0.02 \text{ dyne cm}^{-2}$, which produced basal EDRF release corresponding to $27 \pm 8\%$ relaxation. The concentration-response curve to ACh had a slope of 29.9 ± 5.6 , an EC_{50} value of $3.2 \pm 0.8 \times 10^{-7} \text{ M}$, and an asymptote corresponding to $85 \pm 7\%$ relaxation.

Figure 4 Mean percentage relaxation of endothelium-denuded, 5-hydroxytryptamine-precontracted coronary artery rings of the pig in response to endothelium-derived relaxing factor (EDRF) released from endothelium-intact rabbit aorta donors basally (B) and in response to 60s pulses of substance P (SP, $n = 5$) or cumulative doses of acetylcholine (ACh, $n = 6$) (■) followed by a supramaximal concentration (10^{-6} M) of A23187 (□). Vertical lines show s.e.mean.

Mean shear stress values for pig coronary artery and rabbit aorta donors were not significantly different.

Calibration curves

Figure 5 shows the standard curve used to convert EDRF-induced pig coronary artery relaxant responses to total EDRF units released from each donor (C_R). The standard curve was constructed by use of the technique described in the Methods section. A regression line fitted to the data had a slope of 49.2 ($r = 0.96$, $P < 0.001$).

In order to calculate a value for the EDRF decay constant, λ , endothelium-intact coronary artery of the pig and rabbit aorta vessels were mounted as donors in the bioassay, perfused at 2 ml min^{-1} , and EDRF released by A23187 (3×10^{-7} M) measured.

Figure 5 The calibration curve used to convert percentage relaxations of pig coronary artery rings in response to endothelium-derived relaxing factor (EDRF), to units of EDRF activity. It is a standard concentration-response curve of endothelium-denuded, 5-HT (3×10^{-6} M) precontracted coronary artery rings of the pig, to increasing cumulative concentrations of NaNP. A regression line fitted to the pooled data ($r = 0.96$) is also shown ($n = 12$).

Pig coronary artery rings, used to detect EDRF at various donor-recipient transit times from pig coronary artery and rabbit aorta donors, were precontracted with 5-HT (3×10^{-6} M) to 5.7 ± 0.6 g and 8.5 ± 0.4 g respectively. Results were then expressed as EDRF units by reference to the NaNP standard curve. An exponential curve fitted to the pig coronary artery EDRF data gave a decay constant $\lambda = -0.1072$ ($r = 0.646$, $P < 0.001$). An exponential curve fitted to the rabbit aorta EDRF data gave a decay constant $\lambda = -0.0975$ ($r = 0.690$, $P < 0.001$). These values for λ which were not significantly different were used in all calculations.

EDRF release from pig coronary artery and rabbit aorta endothelium in response to SP

Figure 6 shows EDRF release rates (normalised for vessel dimensions, flow rates and transit times) from pig coronary artery and rabbit aorta endothelium in response to 60s pulses of increasing concentrations of SP. Basal EDRF release from pig coronary artery and rabbit aorta endothelium was 0.73 ± 0.25 and

Figure 6 The mean rate of endothelium-derived relaxing factor (EDRF) release from pig coronary artery (●, $n = 5$) and rabbit aorta (■, $n = 5$) endothelium in response to 60 s pulses of substance P (closed symbols). Basal release (open symbols). Note that although basal EDRF release rates are similar, pig coronary artery endothelium has significantly lower EC_{50} value ($P < 0.01$) and rabbit aorta endothelium is capable of releasing EDRF at twice the maximum rate of that of pig coronary artery. Vertical lines show s.e.mean.

$0.90 \pm 0.33 \text{ nu cm}^{-2} \text{ s}^{-1}$, respectively. The pig coronary artery concentration-response curve had a slope of 3.4 ± 1.0 , EC_{50} value of $3.3 \pm 0.6 \times 10^{-10} \text{ M}$, and an asymptote of $11.40 \pm 2.90 \text{ nu cm}^{-2} \text{ s}^{-1}$. The rabbit aorta concentration-response curve had a slope of 7.4 ± 2.2 , EC_{50} value of $8.6 \pm 1.4 \times 10^{-10} \text{ M}$ and asymptote of $24.60 \pm 6.85 \text{ nu cm}^{-2} \text{ s}^{-1}$. The EC_{50} value for pig coronary artery endothelium was significantly less than that for rabbit aorta endothelium ($P < 0.01$).

EDRF release from pig coronary artery and rabbit aorta endothelium in response to ACh

Figure 7 shows normalised EDRF release rates from pig coronary artery and rabbit aorta endothelium in response to cumulative doses of ACh. Basal EDRF release from pig coronary artery and rabbit aorta

Figure 7 The mean rate of endothelium-derived relaxing factor (EDRF) release from pig coronary artery (●, $n = 5$) and rabbit aorta (■, $n = 6$) endothelium in response to cumulative concentrations of acetylcholine (ACh, closed symbols). Basal release (open symbols). Note that although basal EDRF release rates are similar, the concentration-response curve for rabbit aorta endothelium has a significantly steeper slope ($P < 0.05$) and a significantly higher asymptote ($P < 0.05$). Vertical lines show s.e.mean.

endothelium was 0.85 ± 0.25 and $1.18 \pm 0.55 \text{ nu cm}^{-2} \text{ s}^{-1}$, respectively. The pig coronary artery concentration-response curve had a slope of 0.3 ± 0.1 , an EC_{50} value of $8.1 \pm 4.4 \times 10^{-7} \text{ M}$, and an asymptote of $2.00 \pm 0.48 \text{ nu cm}^{-2} \text{ s}^{-1}$. The concentration-response curve in the rabbit aorta had a slope of 5.9 ± 1.8 , an EC_{50} value of $7.2 \pm 1.8 \times 10^{-7} \text{ M}$, and an asymptote of $10.23 \pm 2.83 \text{ nu cm}^{-2} \text{ s}^{-1}$. The slope and asymptote of the rabbit aortic endothelium concentration-response curve was significantly greater than that of the pig coronary artery ($P < 0.05$).

Discussion

The results demonstrate that the intraluminal release of EDRF from pig coronary artery donors occurs in

response to SP, but not ACh. Rabbit aortic endothelium however, released EDRF in response to both SP and ACh. Basal EDRF release from both pig coronary artery and rabbit aorta donors, at comparable levels of endothelial shear stress, was quantitatively similar.

The value of λ , the decay constant for EDRF, provided an indirect measure of the chemical half-life for EDRF and was not significantly different for EDRF released from the pig coronary artery and rabbit aorta. Previous estimates of EDRF half-lives are based on relaxation of recipient vessels with no reference to a relaxation standard e.g. NaNP. The calculation of such 'biological' half-lives for EDRF could thus be dependent on the sensitivity of the recipient tissue to EDRF, more sensitive recipients giving an apparently longer half-life. Differences in the experimental procedures used and the presence or absence of superoxide dismutase may also be relevant. One or more of these factors could account for the marked differences between the half-lives found by different workers (for review see Angus & Cocks 1989). The present data therefore support the view that the chemical structure of EDRF released from different vessels is similar, at least in pig and rabbit, and probably also in other vessels. The release of EDRF from pig coronary artery in response to pulses of SP confirms both the potency of this agent in this preparation (threshold dose < 10 pM), and the onset of desensitization at higher doses, as shown by the tendency of the concentration-response curve asymptote to fall at doses above 10 nM SP. Both these phenomena have previously been observed in endothelium-intact conduit arteries of the rabbit, dog, cat and pig (Furchgott *et al.*, 1983; Cocks & Angus, 1983) and also in resistance arteries of the rabbit (Griffith *et al.*, 1988).

The lack of EDRF release from pig coronary endothelium in response to ACh explains the previously observed lack of endothelium-dependent relaxation to this agent in this preparation (Kalsner, 1985; Graser *et al.*, 1986), despite the fact that ACh-induced constrictions in pig coronary arteries are inhibited by NaNP (Itoh *et al.*, 1981). The small rise in EDRF release demonstrated could have been secondary to increased endothelial shear, as a result of constriction of the smooth muscle of the donor wall in response to ACh. This potential artifact would be technically very difficult to prevent in this system, where diffusion of luminal ACh into the media is likely to occur, activating the (normally quiescent) smooth muscle. The lack of EDRF release from pig coronary endothelium in response to ACh could be due to either a lack of muscarinic receptors, or high levels of cholinesterase activity.

Gordon & Martin (1983a) have demonstrated endothelium-dependent relaxation of pig aortic rings

in response to ACh, which they later showed to be independent of prostacyclin synthesis (Gordon & Martin 1983b) and likely, therefore, to be due to release of EDRF. This suggests that either regional variations in the endothelial response to ACh can occur, or that the vascular response of neonates (Gordon & Martin routinely used 10–14 day old piglets) are not representative of the adult animal. Endothelium-dependent relaxation of aortic strips in response to ACh has not been tested in the pigs used for the present experiments.

The release of EDRF from rabbit aorta donors by pulses of SP occurred at threshold concentrations of 10 pM, and showed desensitization at high doses, as demonstrated by Zawadzki *et al.* (1981) who used endothelium-intact rabbit aortic rings. Comparison of the absolute amount of EDRF released from pig coronary artery and rabbit aorta endothelium in response to maximal doses of SP (Figure 7), showed that rabbit aortic endothelium is capable of releasing EDRF at a rate of approximately twice that of pig coronary artery endothelium.

The results of the present investigation together with those of a previous study by Christie & Lewis (1988), showing greater vascular smooth muscle sensitivity to EDRF in the pig coronary artery than rabbit aorta, provide a possible explanation for the previous findings of Collins and co-workers (1986), in which it was shown that basal EDRF 'activity' was greater in the dog coronary artery than rabbit aorta. If, as the present study shows for pig coronary artery, basal EDRF release in the dog coronary artery is similar to rabbit aorta, then the findings of Collins and colleagues are likely to be due to a greater sensitivity of the dog coronary to EDRF rather than greater release.

The release of EDRF from rabbit aorta donors in response to ACh directly confirms the results obtained by other workers, who have used endothelium-intact rabbit aortae as donors for EDRF in bioassay systems that employ widely different detectors of EDRF activity. EDRF released from rabbit aorta donors in this system had an EC_{50} of between 0.01 and 0.1 μ M ACh, as originally found in the intact ring studies of Furchgott & Zawadzki (1980). EDRF release in response to SP reached a higher maximal rate than release induced by ACh (98.4 and 40.9 $\text{nu cm}^{-2} \text{s}^{-1}$, respectively), which is probably the mechanism responsible for maximal SP-induced relaxations being greater than ACh-induced relaxations in intact rabbit aortic rings (Zawadzki *et al.*, 1981).

Release of EDRF from both pig coronary artery and rabbit aorta donors in response to 10^{-6} M A23187 occurred at a higher rate than receptor-stimulated release of EDRF in response to either ACh or SP. Ionophore-induced release also effec-

tively defines the upper limit of EDRF detection by a rubbed 5-HT-precontracted coronary artery ring of the pig, as A23187 normally induces the release of EDRF from pig coronary artery and rabbit aorta donors at such high rates that the relaxant response of the recipient ring becomes saturated (i.e. greater than 100%). The concentration-response curve to NaNP for rubbed, 5-HT-precontracted coronary artery rings of the pig begins to plateau at concentrations which induced greater than 100% relaxation i.e. about $3 \times 10^{-6} \text{ M}$ NaNP. This renders the calibration curve for EDRF non-linear at the high EDRF concentrations induced by A23187, so that absolute EDRF release rates in response to A23187 could not be accurately calculated. This ability of A23187 to stimulate supramaximally EDRF release was therefore used in all experiments simply as a test for the presence of a functional endothelium on donor vessels.

In conclusion, we have developed a technique for quantitation of EDRF release from different artery types in bioassay which is independent of vessel dimensions, flow rates and donor recipient transit times.

This work was supported by the British Heart Foundation.

Appendix

Consider an arterial segment of length L and cross-sectional area A acting as a source of EDRF in a bioassay experiment. Let $c(x)$ be the equilibrium concentration of EDRF at a distance x from the proximal end of the segment. Let f be the rate of release of EDRF into the lumen of the vessel per unit length of artery, d be its diameter and $\dot{Q}$ the mean flow rate. Let the concentration of EDRF emerging from the donor segment be C_D (see Figure 8).

Figure 8

If a small volume of perfusate ($\Delta L \cdot A$) moves a distance Δx in time Δt it will acquire $f \cdot \Delta L \cdot \Delta t$ mol of EDRF and its concentration of EDRF will increase by

$$\begin{aligned} & \frac{f \cdot \Delta L \cdot \Delta t}{\Delta L \cdot A} \\ &= \frac{f \cdot \Delta t}{A} \\ &= \frac{f}{A} \cdot \frac{A}{\dot{Q}} \Delta x = \frac{f}{\dot{Q}} \cdot \Delta x \end{aligned}$$

since the velocity of the small volume

$$\frac{\Delta x}{\Delta t} = \frac{\dot{Q}}{A}$$

If EDRF is assumed to decay exponentially with a half-life $\frac{\log_e 2}{\lambda}$ then the small volume under consideration will lose $-\lambda c(x) \cdot \Delta t = -\lambda c(x) \cdot \frac{A}{\dot{Q}} \cdot \Delta x$ mol of EDRF in a time Δt .

Thus,

$$c(x + \Delta x) = c(x) + \frac{f}{\dot{Q}} \Delta x - \lambda c(x) \frac{A}{\dot{Q}} \Delta x$$

so that

$$\frac{dc(x)}{dx} = \frac{c(x + \Delta x) - c(x)}{\Delta x} = \frac{f}{\dot{Q}} - \frac{\lambda A}{\dot{Q}} c(x)$$

Thus

$$\frac{dc(x)}{f - \lambda A c(x)} = \frac{1}{\dot{Q}} \cdot dx$$

Integrating we obtain

$$\frac{-1}{\lambda A} [\log_e (f - \lambda A c(x))]_0^{c(L)} = \frac{1}{\dot{Q}} [x]_0^L$$

so that

$$\log_e \left[\frac{f - \lambda A c(L)}{f} \right] = \frac{-\lambda A L}{\dot{Q}} = \frac{-\lambda V}{\dot{Q}}$$

where V is the volume of the segment and

$$C_D = \frac{f}{\lambda A} [1 - e^{(-\lambda V/\dot{Q})}]$$

Thus the rate of intraluminal release of EDRF per unit surface area = $f/\pi d$

$$= \frac{\lambda d C_D}{4[1 - e^{(-\lambda V/Q)}]}$$

References

- ANGUS, J.A. & COCKS, T.M. (1989). Endothelium-derived relaxing factor. *Pharmacol. Ther.*, (in press).
- ANGUS, J.A., COCKS, T.M. & SATOH, K. (1986). α_2 -Adrenoreceptors and endothelium-dependent relaxation in canine large arteries. *Br. J. Pharmacol.*, **88**, 767–777.
- BHARDWAJ, R. & MOORE, P.K. (1988). Endothelium-derived relaxing factor and the effects of acetylcholine and histamine on resistance blood vessels. *Br. J. Pharmacol.*, **95**, 835–843.
- CHRISTIE, M.I. & LEWIS, M.J. (1988). Vascular smooth muscle sensitivity to endothelium derived relaxing factor is different in different arteries. *Br. J. Pharmacol.*, **95**, 630–636.
- COCKS, T.M. & ANGUS, J.A. (1983). Endothelium-dependent relaxation of coronary arteries by noradrenaline and serotonin. *Nature*, **305**, 627–629.
- COLLINS, P., CHAPPELL, S.P., GRIFFITH, T.M., LEWIS, M.J. & HENDERSON, A.H. (1986). Differences in basal endothelium-derived relaxing factor activity in different artery types. *J. Cardiovasc. Pharmacol.*, **8**, 1138–1162.
- FEELISCH, M. & NOACKE, E. (1987). Nitric oxide (NO) formation from nitrovasodilators occurs independently of hemoglobin or non-heme iron. *Eur. J. Pharmacol.*, **142**, 465–469.
- 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.
- FURCHGOTT, R.F., CHERRY, P.D. & ZAWADZKI, J.V. (1983). Endothelium-dependent relaxation of arteries by acetylcholine, bradykinin and other agents. In *Vascular Neuroeffector Mechanisms: 4th International Symposium*. ed. Bevan, J., Fujiwara, M., Maxwell, R.A., Mohri, K., Shibata, S. & Toda, N. pp. 151–157. New York: Raven Press.
- GORDON, J.L. & MARTIN, W. (1983a). Endothelium-dependent relaxation of the pig aorta; relationship to stimulation of ^{86}Rb efflux from isolated endothelial cells. *Br. J. Pharmacol.*, **79**, 531–541.
- GORDON, J.L. & MARTIN, W. (1983b). Stimulation of endothelial prostacyclin production plays no role in endothelium-dependent relaxation of the pig aorta. *Br. J. Pharmacol.*, **80**, 179–186.
- GRASER, J., LEISNER, A. & TIEDT, N. (1986). Absence of role of endothelium in the response of isolated porcine coronary arteries to acetylcholine. *Cardiovasc. Res.*, **20**, 299–302.
- GRIFFITH, T.M., EDWARDS, D.H., LEWIS, M.J. & HENDERSON, A.H. (1984a). Isolated perfused rabbit coronary artery and aortic strip preparations; the role of endothelium-derived relaxing factor. *J. Physiol.*, **351**, 13–24.
- GRIFFITH, T.M., EDWARDS, D.H., LEWIS, M.J., NEWBY, A.C. & HENDERSON, A.H. (1984b). The nature of endothelium-derived vascular relaxant factor. *Nature*, **308**, 645–647.
- GRIFFITH, T.M., EDWARDS, D.H., DAVIES, R.L.I., HARRISON, T.J. & EVANS, K.T. (1988). Endothelium-derived relaxing factor (EDRF) and resistance vessels in an intact vascular bed: a microangiographic study of the rabbit isolated ear. *Br. J. Pharmacol.*, **93**, 654–662.
- ITO, T., KAJIWARA, M., KITAMURA, K. & KURIYAMA, H. (1981). Effects of vasodilator agents on smooth muscle cells of the coronary artery of the pig. *Br. J. Pharmacol.*, **74**, 455–468.
- KALSNER, S. (1985). Cholinergic mechanisms in human coronary artery preparations: implications of species differences. *J. Physiol.*, **358**, 509–526.
- KELM, M., FEELISCH, M., SPAHT, R., PIPER, H., NOACKE, E. & SCHNADER, J. (1988). Quantitative and kinetic characterisation of nitric oxide and EDRF released from cultured endothelial cells. *Biochem. Biophys. Res. Commun.*, **154**, 236–244.
- PALMER, R.M.J., FERRIGE, A.G. & MONCADA, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-relaxing factor. *Nature*, **327**, 524–526.
- POHL, A., BUSSE, R., KUON, E. & BASSENGE, E. (1986). Pulsatile perfusions stimulate the release of endothelial autocooids. *J. Appl. Cardiol.*, **1**, 215–235.
- RUBANYI, G., CARLOS ROMERO, J. & VANHOUTTE, P.M. (1986). Flow-induced release of endothelium-derived relaxing factor. *Am. J. Physiol.*, **250**, H1145–H1149.
- ZAWADZKI, J.V., FURCHGOTT, R.F. & CHERRY, P. (1981). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by substance P. *Fed. Proc.*, **40**, 689.

(Received February 9, 1989)

Revised May 22, 1989

Accepted June 1, 1989)