EDRF-mediated dilatation in the rat isolated perfused kidney: a microangiographic study

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1 X-ray microangiographic techniques were used to study the influence of endothelium-derived relaxing factor (EDRF) on vasomotion in the isolated, intact, buffer-perfused kidney of the rat. The main renal (R_0), segmental (R_1) and interlobar (R_2) arteries (control diameters ca. 600, 400 and 300 μm respectively) were studied quantitatively.

2 Inhibition of basal EDRF activity by haemoglobin (1 μM) did not elevate perfusion pressure or constrict R_0 , R_1 and R_2 in control preparations, implying a low level of spontaneous myogenic tone. In preparations preconstricted by 0.3 μM methoxamine, haemoglobin caused a further rise in perfusion pressure and amplified constrictor responses in R_1 and R_2 while also inducing 'paradoxical' dilatation of R_0 .

3 A spatially heterogeneous pattern of diameter responses (constriction of R_2 and R_1 with minimal dilatation of R_0) was observed with two concentrations of methoxamine (0.3 μM and 3 μM). The magnitude of these responses was, however, smaller with 3 μM than 0.3 μM methoxamine, even though it increased perfusion pressure to a greater extent (88 mmHg cf. 24 mmHg). This 'paradoxical' behaviour indicates more pronounced constriction of distal arteries (which could not be resolved quantitatively) with 3 μM methoxamine.

4 In contrast to the heterogeneity of constrictor responses induced by methoxamine, the dilator action of acetylcholine was spatially homogeneous: log IC_{50} values calculated from the diameter changes induced in R_0 , R_1 and R_2 were similar and, moreover, equivalent to that calculated from the corresponding alterations in perfusion pressure. The fall in perfusion pressure induced by an approximately median effective concentration of acetylcholine (0.3 μM) was completely reversed by haemoglobin, consistent with the involvement of EDRF, although, reversal of the acetylcholine-induced dilatation of R_0 , R_1 and R_2 was not observed.

5 The results are consistent with the idea that constriction of distal vessels can attenuate and even directionally reverse intrinsic constrictor responses in the proximal R_0 , R_1 and R_2 'feed' arteries by producing an overriding increase in 'upstream' pressure. This effect explains the paradoxical dilatation of R_0 induced by haemoglobin in the presence of 0.3 μM methoxamine, the smaller magnitude of the diameter changes induced in R_0 , R_1 and R_2 by 3 μM as compared to 0.3 μM methoxamine, and the failure of haemoglobin to reverse the acetylcholine-induced dilatation of R_0 , R_1 and R_2 .

Introduction

The endothelium-dependent relaxation of arterial smooth muscle which can be induced by a wide variety of compounds such as acetylcholine (ACh), is mediated by stimulated release of endothelium-derived relaxing factor (EDRF). EDRF is an unstable endogenous vasodilator (Furchgott &

Zawadski, 1980; Griffith *et al.*, 1984), identical with or closely related to nitric oxide (Ignarro *et al.*, 1987; Palmer *et al.*, 1987; Furchgott, 1988), and is avidly bound by haemoglobin (Kelm *et al.*, 1988). Endothelium-dependent relaxation has been demonstrated widely in large arteries but the role of EDRF in intact organs remains largely uninvestigated.

We have recently shown that haemoglobin inhibits ACh-induced relaxation in resistance vessels of the rabbit buffer-perfused isolated ear *in situ* (Griffith

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et al., 1988). Similar conclusions have been reached by other workers on rabbit heart (Stewart *et al.*, 1987) and rat mesenteric (Furchgott *et al.*, 1987) preparations. This suggests that the dilator action of ACh in resistance vessels, as in large vessels, is mediated by stimulated release of EDRF. Indeed, when endothelium is removed mechanically from isolated segments of resistance arteries (De Mey & Gray, 1985; Owen & Bevan, 1985) the dilator response to ACh is abolished. Basal release of EDRF (i.e. spontaneous release in the absence of any exogenous pharmacological agent) also exerts a major effect on pressure-flow and diameter-flow relationships in the vascular network of the rabbit ear (Griffith *et al.*, 1987; 1989) and the rat mesentery (Randall & Hiley, 1988).

In the present study we have investigated the influence of haemoglobin on baseline perfusion pressure, constrictor responses induced by the α_1 -adrenoceptor agonist methoxamine, and dilator responses induced by ACh in the rat isolated perfused kidney. Microangiographic techniques (Griffith *et al.*, 1987; 1988; 1989) were used to measure diameter changes in the three primary sets of arteries of the rat kidney (down to approximately 200 μm in size). In combination with simultaneous measurement of pressure changes, this information allows inferences to be made about the responses of more distal vessels which cannot be visualized directly in this preparation by microangiography.

Methods

Preparation of the isolated perfused kidney of the rat

Male Wistar rats (U.W.C.M., Cardiff) weighing between 250–300 g were anaesthetized with chloroform, the thorax was opened and heparin was injected into the left ventricle (0.3 ml, 20,000 units ml^{-1}), following which the animals were killed by exsanguination. The left kidney was then removed after cannulation of the renal artery, and perfused with Krebs-Henseleit solution (composition in mM: Na^+ 143, K^+ 5.9, Ca^{2+} 2.6, Mg^{2+} 1.2, Cl^- 128, H_2PO_4^- 2.2, HCO_3^- 24.9, SO_4^{2-} 1.2, glucose 10) oxygenated with 95% O_2 /5% CO_2 at a constant flow rate of 2.25 ml min^{-1} .

The kidneys were mounted in front of a 4 μm microfocal X-ray source in such a manner that the effluent from the renal vein would not flow over the surface of the kidney and thus obscure the radiographic images. Microangiographs were obtained with an exposure time of 8 to 14 s at a kilovoltage of between 30 and 55 kV. The radiographic contrast material used was Omnipaque (iohexol, 300 mg iodine ml^{-1}), which caused a small rise in perfusion

Figure 1 High contrast topographical 'map' of the arterial system of rat isolated kidney obtained following injection of a BaSO_4 suspension (Micropaque). Diameter measurements were made of the main renal artery R_0 , the segmental, R_1 and the interlobar arteries, R_2 . The calibrated grid had an external diameter of 3 mm.

pressure attributable to its greater viscosity than Krebs-Henseleit solution. However, these rises were less than 5% of mean perfusion pressure during the X-ray exposure. Iohexol was introduced into the kidneys for 8 s before the radiographic exposure to ensure optimal opacification of arteries with minimal filling of veins and thus reduce problems due to vessel overlap on the radiographic images. At the conclusion of each experiment, Micropaque (BaSO_4 suspension) was injected into each kidney to provide a high-contrast topographical 'map' of its arterial system (Figure 1).

Quantitation

The microangiographs were analysed by a semi-interactive image analysis system as previously described (Griffith *et al.*, 1987; 1988). A calibrated gold grid was placed on the surface of each kidney to determine magnification factors (approximately $\times 3$) accurately. The radiographs were further magnified electronically by the image analysis system. The diameter of each vessel studied was measured 6 times and the results averaged. The accuracy of this protocol has been shown to be better than 5% (Griffith *et al.*, 1988). Quantitation of diameters was possible only for the main renal (R_0), segmental (R_1) and interlobar (R_2) arteries. In the case of the R_1 and R_2 arteries, three or four vessels were measured in each kidney and the results averaged to give a single representative value. Although the arcuate arteries could be visualised in some experiments, accurate measurement was not generally possible. As previously shown in the rabbit ear, intermittent expo-

sure to radiographic contrast medium did not impair endothelium-dependent relaxation or constrictor responsiveness (Griffith *et al.*, 1988).

Experimental protocol

Following equilibration, changes in the diameters of R_0 , R_1 and R_2 were investigated after administration of $1 \mu\text{M}$ haemoglobin to (1) resting preparations, (2) preparations constricted by the α_1 -adrenoceptor agonist methoxamine ($0.3 \mu\text{M}$) and (3) preparations dilated by $0.3 \mu\text{M}$ ACh after precontraction by methoxamine ($3 \mu\text{M}$). In addition, diameter changes induced by cumulative addition of ACh (0.03 – $30 \mu\text{M}$) to methoxamine ($3 \mu\text{M}$)-constricted preparations were studied. In all cases microangiographs were obtained following stabilization of perfusion pressure after each pharmacological intervention.

Drugs

Acetylcholine chloride was obtained from Sigma Chemical Co., Poole, UK. Methoxamine hydrochloride was a gift from the Wellcome Foundation Ltd, UK, iothexol was obtained from Nyegaard, UK, and Micropaque was obtained from Nicholas, UK. Haemoglobin, which is a specific inhibitor of EDRF activity in peripheral arteries (Martin *et al.*, 1985; Edwards *et al.*, 1986; Nishiye *et al.*, 1989), was prepared following lysis of red blood cells and purified by gel filtration on a Sephadex G200 column as previously described (Edwards *et al.*, 1986).

Statistical analysis

Data are given as mean $\pm$ s.e.mean. Logarithmic concentration-response curves to ACh were plotted in each preparation where response was (a) the fall in pressure across the whole kidney and (b) the increase in diameter of each of the three generations of arteries R_0 , R_1 and R_2 . IC_{50} values were determined graphically in each case for the individual preparations and geometric mean IC_{50} values of the various data groups calculated and compared by one-way analysis of variance. The effect of haemoglobin on basal perfusion pressure and constrictor or dilator responses in each generation of artery was assessed by paired Student's *t* test. $P < 0.05$ was taken as significant.

Results

Effects of haemoglobin in control preparations

Administration of $1 \mu\text{M}$ haemoglobin produced no significant change in baseline perfusion pressure

Figure 2 Steady-state diameters (in μm) of R_0 (○), R_1 (●) and R_2 (□) and corresponding perfusion pressures after administration of $1 \mu\text{M}$ haemoglobin (Hb) ($n = 9$, left), $0.3 \mu\text{M}$ methoxamine (M) followed by $1 \mu\text{M}$ haemoglobin ($n = 10$, centre) and $3 \mu\text{M}$ methoxamine followed by $0.3 \mu\text{M}$ acetylcholine (ACh) and then $1 \mu\text{M}$ haemoglobin ($n = 11$, right). Significant differences from control diameter are indicated (* $P < 0.05$; ** $P < 0.01$). Statistically, haemoglobin alone had no effect on the diameters of R_0 , R_1 and R_2 ; $0.3 \mu\text{M}$ methoxamine constricted R_1 and R_2 while $3 \mu\text{M}$ methoxamine constricted only R_2 . Methoxamine nevertheless increased steady-state perfusion pressure in a concentration-dependent fashion. When administered after $0.3 \mu\text{M}$ methoxamine, haemoglobin increased the constriction of R_1 and R_2 but, 'paradoxically', resulted in dilatation of R_0 (centre). Haemoglobin reversed the fall in perfusion pressure induced by $0.3 \mu\text{M}$ ACh but did not significantly alter the dilatation of R_0 , R_1 and R_2 induced by ACh (right).

(95 ± 8 cf. 95 ± 10 mmHg, $n = 9$). Consistently, there was no significant change in the diameters of the R_0 , R_1 and R_2 arteries (Figure 2, left).

Effects of methoxamine

Generations R_0 , R_1 and R_2 responded in a qualitatively similar way to the two concentrations of methoxamine studied. The diameter of the R_0 arteries increased slightly (although not significantly) in the presence of $0.3 \mu\text{M}$ methoxamine but significant constriction occurred in the R_1 and R_2 arteries ($P < 0.05$, $n = 10$) (Figure 2, centre). In the presence of higher concentrations of methoxamine ($3 \mu\text{M}$), the diameter of R_0 increased slightly (although again not significantly) while constriction was observed in R_1 and R_2 . However, this was significant only in the case of R_2 ($P < 0.05$, $n = 10$) (Figure 2, right). Quantitatively, therefore, the changes in diameter were less marked with the higher concentration of methoxamine.

By contrast, steady-state perfusion pressure varied in a concentration-dependent manner, increasing by 24% from 99 ± 7 resting to 123 ± 14 mmHg following administration of $0.3 \mu\text{M}$ methoxamine, and by

Figure 3 Averaged diameters of generations R_0 (○), R_1 (●) and R_2 (□) (in μm) as a function of acetylcholine (ACh) concentration ($0.3\text{--}30\ \mu\text{M}$) in preparations constricted by $3\ \mu\text{M}$ methoxamine (M) ($n = 14$). Microangiographs were taken following stabilization of perfusion pressure after each increase in the concentration of ACh. Error bars reflect both experimental factors and also biological variation in the size of the vessels selected for study. Diameters after addition of the highest concentration of ACh were in each case significantly larger than those obtained in the presence of methoxamine alone (* $P < 0.05$, ** $P < 0.01$).

73% from 119 ± 10 resting to 207 ± 10 mmHg following $3\ \mu\text{M}$ methoxamine (Figure 2).

Effects of haemoglobin in the presence of $0.3\ \mu\text{M}$ methoxamine

Following administration of $1\ \mu\text{M}$ haemoglobin in the presence of $0.3\ \mu\text{M}$ methoxamine, the diameters of R_1 and R_2 further decreased compared to control ($P < 0.01$, $n = 10$), while that of R_0 increased significantly above control ($P < 0.05$, $n = 10$) (Figure 2). Haemoglobin increased steady-state perfusion pressure in the presence of $0.3\ \mu\text{M}$ methoxamine by 51% from 123 ± 14 mmHg to 186 ± 21 mmHg.

Effect of haemoglobin on dilatation induced by an approximately median effective concentration of acetylcholine

Mean perfusion pressure in the presence of $3\ \mu\text{M}$ methoxamine was 207 ± 10 mmHg which fell to 126 ± 11 mmHg in the presence of $0.3\ \mu\text{M}$ ACh and

rose to 209 ± 11 mmHg in the presence of $1\ \mu\text{M}$ haemoglobin ($n = 10$) (Figure 2, right). Haemoglobin therefore completely reversed the dilatation induced by the addition of ACh in terms of perfusion pressure. The mean steady-state diameters of the constricted R_0 , R_1 and R_2 arteries were significantly increased by $0.3\ \mu\text{M}$ ACh ($P < 0.05$) (Figure 2, right), but in contrast to its effect on perfusion pressure, haemoglobin did not reverse the diameter increases induced by ACh in any of generations R_0 , R_1 and R_2 .

Concentration-response curves to acetylcholine

Concentration-dependent dilatation was induced by ACh over the range $0.03\text{--}30\ \mu\text{M}$ in preparations pre-constricted by $3\ \mu\text{M}$ methoxamine ($n = 14$). Log_{10} IC_{50} values calculated from the ACh-induced increases in diameter in R_0 , R_1 and R_2 (Figure 3), were statistically similar to each other and equivalent to that calculated from the ACh-induced falls in perfusion pressure (Figure 4) when compared by one-way analysis of variance (Table 1). The diameters of R_0 , R_1 and R_2 were increased by ca. 20% of their constricted value by ACh, so that the associated decreases in hydraulic resistance (proportional to $1/(\text{diameter})^4$ in Poiseuille flow) would be ca. 80%.

Discussion

We have used X-ray microangiography to study arterial responses in the rat isolated, perfused kidney. To obtain adequate spatial resolution with this technique an X-ray source size of the order of microns must be employed. This constraint necessarily limits the magnitude of the photon flux which can be generated by the microscope and consequently, in a 3-dimensional vascular bed such as the rat kidney, the size of vessel which can be studied quantitatively. The smallest generation of arteries studied here (R_2 , diameter ca. $200\ \mu\text{m}$) is, therefore, significantly larger than in the 2-dimensional bed of the rabbit ear in which vessels of ca. $70\ \mu\text{m}$ diameter can be adequately resolved (Griffith *et al.*, 1988).

Haemoglobin appears to be a specific inhibitor of EDRF activity at the concentration employed ($1\ \mu\text{M}$) (Martin *et al.*, 1985; Edwards *et al.*, 1986; Kelm *et al.*, 1988; Nishiye *et al.*, 1989). The finding that it failed to elevate baseline perfusion pressure or to constrict the main renal (R_0), segmental (R_1) and interlobar (R_2) arteries in uncontracted preparations could indicate, either that basal EDRF activity was negligible, or that spontaneous myogenic tone was low in these rat isolated preparations. The latter, in fact, seems to be the case as haemoglobin elevated perfusion pressure in preparations constricted by

Figure 4 Change in perfusion pressure (mmHg) in rat isolated kidneys constricted by 3 μ M methoxamine as a function of acetylcholine concentration (0.3–30 μ M) ($n = 14$). The $\log_{10}$ IC_{50} value derived from these data is given in Table 1.

0.3 μ M methoxamine, an α_1 -agonist which would not be expected to stimulate EDRF release. Although there are considerable species and regional differences in the ability of α_2 -adrenoceptors to mediate EDRF release (Cocks & Angus, 1983; Miller & Vanhoutte, 1985; Egleme *et al.*, 1984; Matsuda *et al.*, 1985), there is no evidence that α_1 -adrenoceptors can do so in any species or artery type. The lack of effect of haemoglobin in the absence of a constrictor agonist in this preparation differs from previous experiments with the rabbit isolated ear, in which inhibition of basal EDRF activity induced a small but significant rise in perfusion pressure in the absence of exogenous constrictor agents and in which haemoglobin therefore unmasked myogenic tone (Griffith *et al.*, 1987).

Table 1 Concentrations of acetylcholine resulting in half-maximal dilatation

Generation	$-\log_{10} (IC_{50})$
R ₀	6.5 $\pm$ 0.2
R ₁	6.2 $\pm$ 0.4
R ₂	6.3 $\pm$ 0.4
Mean value	6.3 $\pm$ 0.1
Pressure response	6.4 $\pm$ 0.3

Molar $\log_{10}$ IC_{50} values for inhibition of methoxamine (3 μ M)-induced vasoconstriction by acetylcholine. These were calculated separately for the diameter changes induced by acetylcholine in each of the three generations of arteries (i.e. R₀, R₁ and R₂) and for the corresponding falls in perfusion pressure of the intact preparations ($n = 14$). All $\log_{10}$ IC_{50} values were statistically similar.

The tendency of the main renal artery (R₀) to dilate after administration of either 0.3 or 3 μ M methoxamine (particularly in the presence of haemoglobin), contrasts with the observation that such vessels constrict in response to this agonist when studied as isolated ring preparations (Winqvist, 1988). The lobar (R₁) and segmental (R₂) arteries, by contrast, exhibited the expected constrictor response. Diameter changes induced by 3 μ M methoxamine, while being directionally similar, were smaller in magnitude than those induced by 0.3 μ M methoxamine, even though the converse was the case in terms of their effects on perfusion pressure. The findings suggest that a rise in 'upstream' intraluminal pressure, secondary to 'downstream' constriction in vessels not visualised by the microangiographic technique, can attenuate intrinsic constrictor responses in the proximal R₁ and R₂ arteries and even reverse those in R₀. The failure of haemoglobin to influence the dilatation of R₀, R₁ and R₂ induced by acetylcholine in preparations constricted by 3 μ M methoxamine can be explained by a similar mechanism. Analysis of responses in proximal R₀, R₁ and R₂ 'feed' vessels thus allows inferences to be made about the behaviour of the distal vasculature of these rat isolated kidney preparations.

When increasing concentrations of acetylcholine were administered after 3 μ M methoxamine, the R₀, R₁ and R₂ arteries dilated in a concentration-dependent fashion, IC_{50} values being similar in the three generations even though the effects of methoxamine were spatially heterogeneous. The fall in perfusion pressure induced by 0.3 μ M acetylcholine was completely reversed by haemoglobin, consistent with the involvement of EDRF. Spatial homogeneity in the sensitivity of these different sized vessels to acetylcholine is analogous to findings in the rabbit isolated ear, in which IC_{50} values for acetylcholine and substance P are identical in the central artery and its first three branch generations, in spite of spatial differences in agonist-induced constrictor responses (Griffith *et al.*, 1988). This contrasts with the behaviour of isolated resistance arteries in which the potency of acetylcholine and other endothelium-dependent agents has been shown to increase with diminishing vessel size (De Mey & Gray, 1985; Owen & Bevan, 1985).

In summary, the findings demonstrate that basal EDRF activity exerts a significant influence in this rat isolated kidney preparation only when tone is elevated exogenously. The results with methoxamine, acetylcholine and haemoglobin also indicate that, because of 'paradoxical' effects, net responses may differ between conventional arterial preparations and intact perfused organ preparations. This is a consequence of the hydraulic connection between vessels in an intact vascular network: the

response of any given artery to a pharmacological intervention will be determined both locally and by the behaviour of distant vessels.

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