Effects of hypoxia and metabolic inhibitors on production of prostacyclin and endothelium-derived relaxing factor by pig aortic endothelial cells

Joanna M. Richards, *Ian F. Gibson & *¹William Martin

Department of Cardiology, University of Wales College of Medicine, Heath Park, Cardiff CF4 4XN and *Department of Pharmacology, University of Glasgow, Glasgow G12 8QQ

- 1 The content of adenosine triphosphate (ATP) and basal and bradykinin-stimulated production of prostacyclin and endothelium-derived relaxing factor (EDRF) was measured in primary cultures of porcine aortic endothelial cells under normoxic (14.4% O₂) and hypoxic (2.8% O₂) conditions, and following treatment with rotenone and 2-deoxy glucose, which inhibit oxidative and glycolytic metabolism, respectively.
- 2 ATP content and basal and bradykinin-stimulated production of prostacyclin were similar under normoxic and hypoxic conditions. EDRF production, assessed as endothelial guanosine 3':5'-cyclic monophosphate (cyclic GMP) content, was also similar under both conditions.
- 3 Treatment with rotenone (0.3 µM) had no effect on ATP content or basal or bradykinin-stimulated production of prostacyclin or of EDRF, measured as endothelial cyclic GMP content. Elevation of cyclic GMP content by atriopeptin II was also unaffected.
- 4 Treatment with 2-deoxy glucose (20 mM) in glucose-free Krebs solution lowered ATP content, reduced bradykinin-stimulated production of prostacyclin and abolished the bradykinin-stimulated elevation of cyclic GMP content. Resting production of prostacyclin was unaffected but basal content of cyclic GMP was lowered in some experiments. Elevation of cyclic GMP content by atriopeptin II was abolished.
- 5 Combined treatment with rotenone (0.3 µM) and 2-deoxy glucose (20 mM) lowered ATP content more than with 2-deoxy glucose alone. Basal production of prostacyclin rose slightly and bradykinin-stimulated production was powerfully inhibited. Basal content of cyclic GMP was unaffected, but bradykinin-stimulated production was abolished. Elevation of cyclic GMP by atriopeptin II was also abolished.
- 6 Cascade bioassay experiments using endothelium-denuded rings of rabbit aorta as a detector system confirmed that bradykinin-stimulated production of EDRF was blocked by 2-deoxy glucose, but not by rotenone.
- 7 These data indicate that porcine aortic endothelial cells in culture operate under mainly glycolytic metabolism and this probably explains why production of prostacyclin and EDRF is unaffected under hypoxic conditions. They also indicate that glycolytic metabolism is required for agonist-stimulated production of prostacyclin and EDRF by these cells.

Introduction

Hypoxia has profound effects on tone of the vasculature *in vivo*, producing, in general, vasodilatation in the systemic circulation (Hilton & Eichholtz, 1925), and vasoconstriction in the pulmonary circulation (Fishman, 1976). The factors regulating these responses to hypoxia are complex, but include interference with respiratory chain function (Fay, 1971; Hellstrand *et al.*, 1977), and production of tissue-derived (Berne & Rubio, 1977) or blood vessel-derived vasoactive metabolites (Busse *et al.*, 1983; Rubanyi & Vanhoutte, 1985).

As the vascular endothelium is in intimate contact with the circulating blood, many studies have attempted to determine its role in regulating vascular responsiveness to hypoxia. For example, *in vitro* studies have shown that hypoxia-induced vasoconstriction or augmentation of vasoconstrictor tone is endothelium-dependent in porcine pulmonary artery (Holden & McCall, 1984), in canine femoral and cerebral artery (De Mey & Vanhoutte, 1983; Katusic & Vanhoutte, 1986), and in canine, porcine, bovine and ovine coronary artery (Rubanyi & Vanhoutte, 1985; Rubanyi & Paul, 1984; Kwan *et al.*, 1989). In contrast, in branches of canine femoral artery, and in rabbit aorta, the endothelium has been shown to mediate, at least in part, hypoxia-induced vasodilatation (Busse *et al.*, 1983; Coburn *et al.*, 1986; Bassenge *et al.*, 1988). These hypoxia-induced vasodilator responses may result from increased production of prostanoids (Kalsner, 1977), or a combination of

prostanoids and endothelium-derived relaxing factor (EDRF; Bassenge *et al.*, 1988). In other studies, mild or severe hypoxia has been shown to inhibit endothelium-dependent vasodilatation of rabbit aorta and pulmonary artery (Furchgott & Zawadzki, 1980; Johns *et al.*, 1989) and canine femoral and cerebral artery (De Mey & Vanhoutte, 1983; Katusic & Vanhoutte, 1986). Blockade of oxygen utilization by inhibitors of oxidative phosphorylation has, like hypoxia, been shown to inhibit agonist-induced endothelium-dependent vasodilatation in rabbit aorta (Griffith *et al.*, 1986; 1987). Furthermore, these studies showed that basal release of EDRF was unaffected by inhibition of oxidative metabolism.

It is clear, therefore, that hypoxia induces a complex array of endothelium-dependent changes in vascular responsiveness in different blood vessels, and in some, the effects of hypoxia can be mimicked by inhibition of oxidative metabolism. The above studies were conducted on isolated blood vessels containing a heterogeneous mixture of cell types, and where the responsiveness of the endothelium is often difficult to discern. Studies on endothelial cells in isolation from other vascular cells may provide a greater insight into the responsiveness of this cell type to hypoxia and metabolic inhibition. For example, it has recently been shown that hypoxia inhibits agonist-induced production of EDRF by bovine isolated pulmonary artery endothelium (Warren *et al.*, 1989), and this, rather than production of a constrictor factor may account for pulmonary hypoxic vasoconstriction. By examining the production of prostacyclin and EDRF by porcine cultured aortic endothelial cells, we hoped to determine how hypoxia and

¹ Author for correspondence.

metabolic inhibition regulate production of these two vasodilator substances in a systemic artery.

Methods

Endothelial cell culture

Endothelial cells were isolated from porcine aorta as previously described (Gordon & Martin, 1983). Briefly, after ligating the intercostal arteries, collagenase (0.2%, type II, Sigma) was introduced into the aortic lumen and the vessel incubated at 37°C for 20 min. The collagenase solution containing aortic endothelial cells was spun (50 g, 3 min, room temperature) and the cells resuspended in 60 ml of Medium E199 supplemented with: foetal bovine serum (10%); newborn bovine serum (10%); glutamine (4 mM); benzyl penicillin (100 units ml⁻¹); streptomycin (100 µg ml⁻¹) and kanamycin (100 ng ml⁻¹).

The endothelial cells were characterized by several criteria: they grew as a strict monolayer and in randomly selected cultures, no fewer than 98% of cells accumulated acetylated low-density lipoprotein labelled with a fluorescent marker (C.M.D. U.K. Ltd; Voyta *et al.*, 1984). This study shows also that prostacyclin and EDRF are produced by these cells.

For monolayer studies the cells from each aorta were seeded into 3 Linbro plates each containing 6 wells (9.6 cm²) and grown in an incubator at 37°C under an atmosphere of 5% CO₂ in air. The medium was changed every 2 days and the cells used within 3–7 days.

For experiments in which cells were used on microcarrier beads, the cells from each of four aortae were seeded into a T 75 Falcon flask. When confluent, cells were dispersed with a solution of trypsin (0.025%) in ethylenediamine tetra acetic acid (EDTA, 0.02%, disodium salt), spun (50 g, 3 min, room temperature), resuspended in 100 ml of Medium E199 supplemented as listed above, seeded onto 3 ml of Biosilon microcarrier beads (Nunc, 200 µm diameter) in a sterile siliconised Technic microcarrier flask, and grown at 37°C under an atmosphere of 5% CO₂ in air. The beads were stirred at 30 r.p.m. for 2.5 min every 30 min for 3–5 days during which time the cells grew to confluence, which was confirmed by microscopic examination after staining a sample of the cells with methyl violet (0.1%, B.D.H.).

All tissue culture media and supplements were obtained from Flow Laboratories.

Endothelial monolayer studies

The tissue culture medium was removed and the endothelial cells rinsed twice with 2 ml of Krebs solution containing (mM): NaCl 118, KCl 4.8, CaCl₂ 2.5, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 24 and glucose 11. The cells were then incubated in 2 ml of Krebs solution at 37°C under an atmosphere of 5% CO₂ in air for a minimum of 60 min.

In experiments in which the effects of oxygen tension were studied, cells were placed in an 'Atmos Bag' (Sigma) in an incubator at 37°C. The bag was then inflated with either 5% CO₂ in air (normoxic conditions) or with 95% N₂, 5% CO₂ (hypoxic conditions). Five minutes after inflation, the saturation of oxygen in the Krebs solution, measured with a Clark-type oxygen electrode, under normoxic and hypoxic conditions was 14.4 ± 0.4% and 2.8 ± 0.8% (*n* = 6), respectively and these values remained constant throughout the experiment. All subsequent procedures were carried out under these controlled atmospheres using gloves incorporated into the 'Atmos Bag'. After 30 min the Krebs solution bathing the cells was removed and retained for analysis of 6-keto-prostaglandin F_{1α} (6-keto-PGF_{1α}), and the cells were immediately extracted with 1 ml of ice-cold trichloroacetic acid (TCA, 6%) for analysis of guanosine 3':5'-cyclic monophosphate (cyclic GMP), ATP and DNA content.

All studies with metabolic inhibitors were performed under normoxic conditions. In these experiments rotenone (0.3 µM), 2-deoxyglucose (20 mM) or a combination of rotenone (0.3 µM) and 2-deoxyglucose (20 mM) was present throughout the 30 min incubation period. When 2-deoxyglucose was used, glucose was omitted from the Krebs solution.

Following extraction with TCA (6%), cells were scraped from the multiwells and transferred to Eppendorf tubes and spun at 13,000 r.p.m. for 6 min at room temperature. The DNA content of the pellets was measured by the fluorescence technique of Kissane & Robins (1958). The supernatants were neutralised (to pH 5.5–6) by adding 2 ml of 0.5 M tri-*n*-octylamine in freon (1, 1, 2 trichlorotrifluoroethane) and vortex mixing for 90 s. The aqueous layer (upper) was retained for cyclic GMP and ATP analysis.

Measurement of prostacyclin

The prostacyclin content of Krebs samples was determined by radioimmunoassay of the stable breakdown product 6-keto-PGF_{1α} using an antiserum kindly supplied by Dr A.C. Newby, Department of Cardiology, University of Wales College of Medicine. The cross-reactivity of the antiserum at 50% displacement with PGE₂, PGE₁ and PGF_{2α} was 5.0%, 1.3% and 1.4%, respectively. Prostacyclin production was expressed as ng 6-keto-PGF_{1α} µg⁻¹ DNA.

Measurement of cyclic GMP

The cyclic GMP content of neutralised cell extracts was measured by radioimmunoassay with New England Nuclear kits as previously described (Martin *et al.*, 1985). Cyclic GMP content was expressed as fmol µg⁻¹ DNA.

Measurement of ATP

The ATP content of neutralised cell extracts was measured in a luminometer (Products For Research Inc., U.S.A.) using the luciferin-luciferase reagent (Sigma). ATP content was expressed as pmol µg⁻¹ DNA.

Cascade bioassay

EDRF release from pig aortic endothelial cells grown on microcarrier beads and perfused in columns was detected by bioassay on an endothelium-denuded ring of rabbit aorta. The preparation of endothelial cell columns was as previously described (Gordon & Martin, 1983). Briefly, microcarrier beads containing endothelial cells were packed on top of a bed of glass wool in 1 ml syringes. The packed volumes of beads was 0.3–0.7 ml. After replacing the syringe plunger, through which a PP₃₀ polythene delivery tube had been inserted, the columns were perfused from the bottom upwards at a rate of 4 ml min⁻¹ at 37°C with Krebs solution containing superoxide dismutase (30 units ml⁻¹), to potentiate the actions of EDRF and gassed with 5% CO₂ in air. The perfusate was passed over an endothelium-denuded ring of rabbit aorta that had been suspended under 2 g resting tension and contracted sub-maximally with phenylephrine (0.1 µM). Where indicated in the Results, the aortic ring was perfused simultaneously by a second circuit that had no contact with the endothelial cells. Tension was recorded isometrically with Grass FTO3C transducers and displayed on a Grass recorder.

Drugs

Atriopeptin II (rat synthetic), bradykinin triacetate, 2-deoxy-D-glucose, haemoglobin (bovine Type 1), phenylephrine hydrochloride, rotenone and superoxide dismutase (bovine erythrocyte) were obtained from Sigma. Glyceryl trinitrate was obtained from Napp Laboratories. All drugs were dissolved in twice-distilled water except for rotenone, which was dissolved in ethanol.

Solutions of haemoglobin were reduced to the ferrous form with dithionite before use, as previously described (Martin *et al.*, 1985).

Statistical analysis

As the resting content of cyclic GMP and basal production of prostacyclin varied in batches of cells, all experiments were performed with their own internal controls. In the results, *n* represents the number of replicate dishes of cells randomly taken from different aortae. Results are expressed as the mean $\pm$ s.e.mean and comparisons were made by means of Student's *t* test. A probability of 0.05 or less was considered significant.

Results

Hypoxia

Under normoxic conditions (14.4% O₂) the resting production of 6-keto-PGF_{1 α} by primary cultures of pig aortic endothelial cells, measured over 30 min, was 0.24 ± 0.02 ng μ g⁻¹ DNA (*n* = 18). The resting content of cyclic GMP was 24.9 ± 3.4 fmol μ g⁻¹ DNA (*n* = 11), and the resting content of ATP was 500 ± 35 pmol μ g⁻¹ DNA (*n* = 8, Table 1). When placed under hypoxic conditions (2.8% O₂), resting endothelial production of 6-keto-PGF_{1 α} , and the resting content of cyclic GMP and ATP were unaffected (Table 1).

When bradykinin (0.1 μ M) was added to endothelial cells under normoxic conditions for the final 5 min of the 30 min incubation period, 10.1 fold (*n* = 16) and 44.4 fold (*n* = 7) increases in 6-keto-PGF_{1 α} production and cyclic GMP content were obtained, respectively. When placed under

hypoxic conditions, the bradykinin-induced increases in 6-keto-PGF_{1 α} production and cyclic GMP content were unaffected.

When oxyhaemoglobin (10 μ M), an EDRF blocking agent, was present throughout the 30 min incubation period, resting production of 6-keto-PGF_{1 α} was unaffected under both normoxic and hypoxic conditions (Table 2). The resting content of cyclic GMP was, however, lowered under both normoxic and hypoxic conditions (Table 2).

When superoxide dismutase (30 units ml⁻¹), an EDRF potentiating agent, was present throughout the 30 min incubation period, resting production of 6-keto-PGF_{1 α} was unaffected under either normoxic or hypoxic conditions (Table 2). However, the resting content of cyclic GMP was elevated under both normoxic and hypoxic conditions (Table 2).

Metabolic inhibitors

When rotenone (0.3 μ M), an inhibitor of oxidative phosphorylation, was present throughout the 30 min incubation period under normoxic conditions, resting and bradykinin (0.1 μ M)-stimulated production of 6-keto-PGF_{1 α} and cyclic GMP content were unaffected. Levels of ATP were also unaffected (Table 1).

When 2-deoxy glucose (20 mM), an inhibitor of glycolysis, was present throughout the 30 min incubation period, in glucose-free Krebs solution, resting production of 6-keto-PGF_{1 α} was unaffected, but bradykinin (0.1 μ M)-stimulated production was significantly inhibited (Figure 1). The bradykinin-stimulated rise in cyclic GMP content was abolished, and in some but not all experiments, the resting content fell (Figure 1, Table 3). The ability of superoxide dismutase (30 units ml⁻¹) to elevate cyclic GMP content was inhibited (Table 3) and levels of ATP fell by 60% (*n* = 6, Table 1). The bradykinin-stimulated rise in cyclic GMP content was unaffected when cells were incubated in glucose-free Krebs or glucose-containing Krebs solution in the presence of 2-deoxy glucose.

A combination of rotenone (0.3 μ M) and 2-deoxy glucose (20 mM) enhanced slightly resting production of 6-keto-PGF_{1 α} and blocked bradykinin (0.1 μ M)-stimulated production (Figure 2). Resting levels of cyclic GMP were unaffected, but the bradykinin-stimulated elevation was blocked and levels of ATP fell by 98% (Figure 2, Table 1).

Atriopeptin II

When a sub-maximal concentration of atriopeptin II (10 nM) was added to endothelial cells for the final 1.5 min of the 30 min incubation period under normoxic conditions, a 2.6 fold (*n* = 6) increase in cyclic GMP content was obtained (Figure 3). The atriopeptin II-induced elevation of cyclic GMP content was unaffected by treatment with rotenone (0.3 μ M), but was blocked following treatment with 2-deoxy

Table 1 Effects of hypoxia, rotenone, 2-deoxy glucose and a combination of rotenone and 2-deoxy glucose on the ATP content of pig aortic endothelial cells

Treatment	ATP content (pmol μ g ⁻¹ DNA)	n
Normoxia (control)	500 ± 35	8
Hypoxia	538 ± 27	8
Rotenone	555 ± 40	6
2-DOG	$200 \pm 6^{***}$	6
Rotenone and 2-DOG	$9 \pm 2^{***}$	6

The ATP content of pig aortic endothelial cells was determined following 30 min incubation in normoxic (14.4% O₂) and hypoxic conditions (2.8% O₂), and following treatment with rotenone (0.3 μ M), 2-deoxy glucose (2-DOG 20 mM) and a combination of rotenone and 2-deoxy glucose in normoxic conditions. Results are expressed as the mean $\pm$ s.e.mean. ****P* < 0.001 indicates a significant difference from control.

Table 2 Effects of haemoglobin and superoxide dismutase on prostacyclin production and cyclic GMP content of pig aortic endothelial cells in normoxic and hypoxic conditions

O ₂ tension	Treatment	6-keto-PGF _{1α} (ng μ g ⁻¹ DNA)	Cyclic GMP (fmol μ g ⁻¹ DNA)	n
Normoxia	None	0.24 ± 0.02	19.8 ± 3.5	12
Normoxia	Hb	0.25 ± 0.03	7.8 ± 1.5	
Hypoxia	None	0.32 ± 0.04	24.3 ± 2.6	11
Hypoxia	Hb	0.42 ± 0.07	14.1 ± 4.0	
Normoxia	None	0.25 ± 0.03	20.7 ± 3.3	6
Normoxia	SOD	0.26 ± 0.03	58.9 ± 8.4	
Hypoxia	None	0.26 ± 0.02	29.0 ± 6.2	6
Hypoxia	SOD	0.26 ± 0.03	94.2 ± 19.8	

The production of 6-keto-PGF_{1 α} and content of cyclic GMP was measured at the end of a 30 min incubation period in the presence of oxyhaemoglobin (Hb, 10 μ M) or superoxide dismutase (SOD, 30 units ml⁻¹) in normoxic (14.4% O₂) or hypoxic (2.8% O₂) conditions. Results are expressed as the mean $\pm$ s.e.mean. **P* < 0.05, ***P* < 0.005 indicates a significant difference between two groups joined by a bracket.

Figure 1 Production of 6-keto-PGF_{1α} (a) and content of cyclic GMP (b) under resting conditions (Control) and following stimulation with bradykinin (BK, 0.1 μM), measured both in normal Krebs and in glucose-free Krebs solution containing 2-deoxy glucose (2-DOG, 20 mM). Values indicate the mean of 6–12 observations and vertical bars indicate the s.e.mean. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, indicates a significant difference from control or between two groups joined by a bracket.

glucose (20 mM) or a combination of 2-deoxy glucose and rotenone (Figure 3).

Cascade bioassay

In cascade bioassay experiments, bradykinin (10 nM) infused into columns of pig aortic endothelial cells for 3 min periods, induced relaxation of phenylephrine (0.1 μM)-contracted, endothelium-denuded rings of rabbit aorta (Figure 4). When rotenone (0.3 μM) was added to the Krebs solution perfusing both the endothelial cells and bioassay tissue, bradykinin-induced relaxation was completely unaffected (Figure 4). In a separate series of experiments in which the bioassay tissues were perfused jointly by Krebs solution containing 2-deoxy glucose (20 mM) first passed into the endothelial column and by a separate circuit with normal glucose-containing Krebs, the relaxant effect of bradykinin (10 nM) was significantly reduced (Figure 4). However, the relaxant effect of a sub-maximal concentration of glyceryl trinitrate (10 nM) was completely unaffected (Figure 4).

Figure 2 Production of 6-keto-PGF_{1α} (a) and content of cyclic GMP (b) under resting conditions (Control) and following stimulation with bradykinin (BK, 0.1 μM), measured in the absence and presence of a combination of rotenone (Rot, 0.3 μM) and 2-deoxy glucose (2-DOG, 20 mM). Values indicate the mean of 6 observations and vertical bars indicate the s.e.mean. * $P < 0.05$, *** $P < 0.001$, indicates a significant difference from control or between two groups joined by a bracket.

Discussion

Our finding that ATP levels fell following treatment with 2-deoxy glucose in glucose-free Krebs but not with rotenone, suggest that porcine aortic endothelial cells in culture derive most of their energy from glycolytic metabolism. They probably derive some energy from oxidative phosphorylation, since a combination of rotenone and 2-deoxy glucose induced a greater fall in ATP levels than treatment with 2-deoxy glucose alone. These findings are consistent with a previous study on the metabolic properties of endothelial cells in culture (Dobrina & Rossi, 1983).

In rabbit aorta, agonist-induced production of EDRF is blocked by a variety of different inhibitors of oxidative metabolism, including rotenone, but 2-deoxy glucose is much less effective (Griffith *et al.*, 1986). If, as this study suggests, EDRF production is dependent upon the availability of ATP, it follows that in cells such as porcine aortic endothelial cells which derive most of their energy from glycolytic metabolism, EDRF production should be insensitive to inhibitors of oxida-

Table 3 Effects of 2-deoxy glucose and superoxide dismutase on prostacyclin production and cyclic GMP content of pig aortic endothelial cells

Stimulus	Pretreatment	6-keto-PGF _{1α} (ng μg ⁻¹ DNA)	Cyclic GMP (fmol μg ⁻¹ DNA)	n
None (control)	None	0.15 ± 0.02	31.6 ± 3.6	6
None	2-DOG	0.15 ± 0.02	20.9 ± 3.4	6
SOD	None	0.16 ± 0.02	63.0 ± 5.2***	6
SOD	2-DOG	0.20 ± 0.04	42.3 ± 6.2]*	6

The production of 6-keto-PGF_{1α} and content of cyclic GMP was measured at the end of a 30 min incubation in normal (control) or in glucose-free Krebs containing 2-deoxy glucose (2-DOG, 20 mM), with and without superoxide dismutase (SOD, 30 units ml⁻¹). Results are expressed as the mean ± s.e.mean. * $P < 0.05$, *** $P < 0.001$ indicates a significant difference from control or between two groups joined by a bracket.

Figure 3 Content of cyclic GMP under resting conditions (Control) and following stimulation with atriopeptin II (APII, 10 nM), measured in normal Krebs solution (Krebs) or following treatment with rotenone (0.3 μM), 2-deoxy glucose (2-DOG, 20 mM) or a combination of rotenone and 2-deoxy glucose. Values indicate the mean of 4–6 observations and vertical bars indicate the s.e.mean. ** $P < 0.01$, indicates a significant difference from control.

tive phosphorylation. Using endothelial cyclic GMP content as an index of EDRF production (Martin *et al.*, 1988; Boulanger *et al.*, 1990), we found this to be the case; rotenone had no effect on resting or bradykinin-stimulated content of cyclic GMP, indicating a lack of effect on spontaneous and agonist-induced production of EDRF. According to the same scheme, inhibition of glycolytic metabolism with 2-deoxy glucose should inhibit agonist-induced production of EDRF by porcine aortic endothelial cells. In keeping with this hypothesis, we found that treatment with 2-deoxy glucose in glucose-free Krebs solution abolished bradykinin-stimulated elevations of cyclic GMP content. Furthermore, in some but not all experiments, treatment with 2-deoxy glucose lowered resting levels of cyclic GMP and in experiments where the effect of spontaneously released EDRF was potentiated with superoxide dismutase, this too was inhibited. These data indicated a possible additional action of 2-deoxy glucose in inhibiting basal EDRF production. This was unlikely, however, since basal production of EDRF has been shown to be unaffected by metabolic inhibition (Griffith *et al.*, 1987). We therefore considered an alternative possibility that 2-deoxy glucose reduced endothelial cyclic GMP content not by inhibiting EDRF production, but by lowering the levels of the high energy phosphate, GTP, from which cyclic GMP is formed (Weir *et al.*, 1990). This suspicion was confirmed by our finding that atriopeptin II-induced elevations of endothelial cyclic GMP content, which occur independently of EDRF production (Martin *et al.*, 1988), were also inhibited following treatment with 2-deoxy glucose. At the concentration used (20 mM), 2-deoxy glucose is only effective in the absence of glucose (Griffith *et al.*, 1986). Using a cascade bioassay system

in which porcine aortic endothelial cells were perfused with glucose-free Krebs containing 2-deoxy glucose and the bioassay endothelium-denuded ring of rabbit aorta was perfused both with this and with a separate circuit of glucose-containing Krebs to protect it from metabolic inhibition, we found bradykinin-stimulated production of EDRF to be inhibited. Under these conditions, glyceryl trinitrate-induced vasodilatation was unaffected, indicating that soluble guanylate cyclase, the effector pathway for EDRF (Rapoport & Murad, 1983), was intact. Cascade bioassay experiments, like those in which endothelial cyclic GMP was measured, showed that rotenone had no effect on bradykinin-stimulated production of EDRF by porcine aortic endothelial cells. Thus, our data agree with those of Griffith *et al.* (1986) that ATP is required for agonist-induced production of EDRF, but shows, in contrast to rabbit aortic endothelium, that pig aortic endothelium derives this from glycolytic metabolism. The precise mechanism by which ATP contributes to agonist-induced EDRF production is unknown, but lowered levels of ATP might reduce the availability of NADPH, an important co-factor for the enzyme nitric oxide synthase which converts L-arginine to nitric oxide (Palacios *et al.*, 1989).

In common with EDRF, we found that bradykinin-stimulated production of prostacyclin was inhibited following treatment with 2-deoxy glucose but not rotenone. Inhibition was even more intensive following combined treatment with 2-deoxy glucose and rotenone. Agonist-induced production of prostacyclin, like EDRF, appears therefore to require metabolic energy, but the energy-dependent step is unknown at present.

As expected of cells operating mainly under glycolytic

Figure 4 Cascade bioassay experiments in which the ability of bradykinin (BK, 10 nM) to elicit EDRF production by porcine aortic endothelial cells grown on microcarrier beads and perfused in columns was measured on phenylephrine (0.1 μ M)-contracted, endothelium-denuded rings of rabbit aorta. (a) Rotenone (0.3 μ M, hatched column) infused through both the endothelial cells and bioassay tissue had no effect on the ability of bradykinin to elicit relaxation. Open column, control. (b) When the bioassay tissues were perfused jointly with glucose-free Krebs containing 2-deoxy glucose (2-DOG, 20 mM, hatched column) first passed into the endothelial column, and by a separate circuit with normal glucose-containing Krebs solution, the relaxant effect of bradykinin was inhibited but that of glyceryl trinitrate (GTN, 10 nM) was unaffected. Open column, control. Values are the mean of 4–6 observations and vertical bars indicate the s.e.mean. *** $P < 0.001$, indicates a significant difference from control.

metabolism, the ATP content of pig aortic endothelial cells did not fall when incubated under hypoxic conditions (2.8% O_2). Under these conditions both basal and bradykinin-stimulated production of prostacyclin was identical to the

obtained under normoxic conditions. Our data therefore differ from previous reports on bovine coronary artery and canine femoral artery where stimulation of prostanoid formation was obtained (Kalsner, 1977; Busse *et al.*, 1983), and on bovine cultured pulmonary artery endothelium where inhibition was obtained (Madden *et al.*, 1986). Furthermore, in contrast to the stimulation of EDRF production induced by hypoxia in canine femoral artery (Bassenge *et al.*, 1988), or the inhibition induced in bovine pulmonary artery (Warren *et al.*, 1989), we found no effect on bradykinin-stimulated production of EDRF, as assessed by measuring the cyclic GMP content of porcine aortic endothelial cells. Basal production of EDRF, indicated by haemoglobin-sensitive cyclic GMP content in unstimulated cells (Martin *et al.*, 1988), the action of which is potentiated following treatment with superoxide dismutase (Gryglewski *et al.*, 1986; Rubanyi & Vanhoutte, 1986), was also unaffected under hypoxic conditions. Porcine aortic endothelial cells in culture do not therefore appear to be a suitable model with which to study hypoxia-induced vasodilatation or inhibition of EDRF production.

Why endothelial cells from rabbit freshly isolated aorta should utilize mainly oxidative metabolism (Griffith *et al.*, 1986) while porcine cultured aortic endothelial cells utilize glycolytic metabolism is unclear. One possibility is that in culture, where the availability of oxygen is lower than in arterial blood, cells may switch from oxidative to glycolytic metabolism and this warrants further investigation. It is clear, however, that endothelial cells in culture sustain damage when grown in high PO_2 (Ody & Junod, 1985) due to increased production of oxygen-derived free radicals. Alternatively, since glutaminolysis is an important energy source in the endothelium (Leighton *et al.*, 1987), it is possible that removal from tissue culture medium containing 4 mM glutamine to Krebs solution containing none might induce a change in cellular metabolism.

In conclusion, our study supports the concept of Griffith *et al.* (1986) that ATP is required for agonist-induced production of EDRF. In contrast to rabbit isolated aorta which obtains ATP from oxidative metabolism (Griffith *et al.*, 1986), porcine aortic cells in culture derive this from glycolytic metabolism. This reliance on glycolytic rather than oxidative metabolism may explain why production of prostacyclin and EDRF by porcine aortic endothelial cells is insensitive to hypoxia.

This work was supported by the British Heart Foundation, the Medical Research Council, the Nuffield Foundation and the Medical Research Funds of the University of Glasgow.

References

- BASSENGE, E., BUSSE, R. & POHL, U. (1988). Release of endothelium-derived relaxing factor(s) by physicochemical stimuli. In *Relaxing and Contracting Factors*. ed. Vanhoutte, P.M. pp. 189–217. New Jersey: Humana.
- BERNE, R.M. & RUBIO, R. (1977). Circulatory effects of tissue oxygen tension sensors. In *Tissue Hypoxia and Ischaemia*. ed. Reivich, M., Coburn, R., Lahiri, S. & Chance, B. pp. 30–42. New York, London: Plenum.
- BOULANGER, C., SCHINI, V.B., HENDRICKSON, H. & VANHOUTTE, P.M. (1990). Chronic exposure of cultured endothelial cells to eicosapentaenoic acid potentiates the release of endothelium-derived relaxing factors. *Br. J. Pharmacol.*, **99**, 176–180.
- BUSSE, R., POHL, U., KELLNER, C. & KLEMM, U. (1983). Endothelial cells are involved in the vasodilatory response to hypoxia. *Pflügers Arch.*, **397**, 78–80.
- COBURN, R.F., EPPINGER, R. & SCOTT, D.P. (1986). Oxygen-dependent tension in vascular smooth muscle. Does the endothelium play a role? *Circ. Res.*, **58**, 341–347.
- DE MEY, J.G. & VANHOUTTE, P.M. (1983). Anoxia and endothelium-dependent reactivity of the canine femoral artery. *J. Physiol.*, **335**, 65–74.
- DOBRINA, A. & ROSSI, F. (1983). Metabolic properties of freshly isolated bovine endothelial cells. *Biochim. Biophys. Acta*, **762**, 295–301.
- FAY, F.S. (1971). Guinea pig ductus arteriosus. I. Cellular and metabolic basis for oxygen sensitivity. *Am. J. Physiol.*, **221**, 470–479.
- FISHMAN, A.P. (1976). Hypoxia on the pulmonary circulation. *Circ. Res.*, **38**, 221–231.
- 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.
- GORDON, J.L. & MARTIN, W. (1983). Stimulation of endothelial prostacyclin production plays no role in endothelium-dependent relaxation of the pig aorta. *Br. J. Pharmacol.*, **80**, 179–186.
- GRIFFITH, T.M., EDWARDS, D.H., NEWBY, A.C., LEWIS, M.J. & HENDERSON, A.H. (1986). Production of endothelium-derived relaxant

- factor is dependent of oxidative phosphorylation and extracellular calcium. *Cardiovasc. Res.*, **20**, 7–12.
- GRIFFITH, T.M., EDWARDS, D.H. & HENDERSON, A.H. (1987). Unstimulated release of endothelium derived relaxing factor is independent of mitochondrial ATP generation. *Cardiovasc. Res.*, **21**, 565–568.
- GRYGLEWSKI, R.J., PALMER, R.M.J. & MONCADA, S. (1986). Superoxide anion is involved in the breakdown of endothelium derived vascular relaxing factor. *Nature*, **320**, 454–456.
- HELLSTRAND, P., JOHANSSON, B. & NORBERG, K. (1977). Mechanical, electrical and biochemical effects of hypoxia and substrate removal on spontaneously active vascular smooth muscle. *Acta Physiol. Scand.*, **100**, 69–83.
- HILTON, R. & EICHHOLTZ, T. (1925). The influence of chemical factors on the coronary circulation. *J. Physiol.*, **59**, 413–425.
- HOLDEN, W.E. & MCCALL, E. (1984). Hypoxia-induced contractions of porcine pulmonary artery strips depend on intact endothelium. *Exp. Lung. Res.*, **7**, 101–112.
- JOHNS, R.A., LINDEN, J.M. & PEACH, M.J. (1989). Endothelium-dependent relaxation and cyclic GMP accumulation in rabbit pulmonary artery are selectively impaired by moderate hypoxia. *Circ. Res.*, **65**, 1508–1515.
- KALSNER, S. (1977). The effect of hypoxia on prostaglandin output and on tone in isolated coronary arteries. *Can. J. Physiol. Pharmacol.*, **55**, 882–887.
- KATUSIC, Z.S. & VANHOUTTE, P.M. (1986). Anoxic contractions in isolated canine cerebral arteries: contribution of endothelium-derived factors, metabolites of arachidonic acid, and calcium entry. *J. Cardiovasc. Pharmacol.*, **8**, S97–S101.
- KISSANE, J.M. & ROBINS, E. (1958). The fluorometric measurement of deoxyribonucleic acid in animal tissues with special reference to the central nervous system. *J. Biol. Chem.*, **233**, 184–188.
- KWAN, Y.W., WADSWORTH, R.M. & KANE, K.A. (1989). Hypoxia and endothelium-mediated changes in the pharmacological responsiveness of circumflex coronary artery rings from the sheep. *Br. J. Pharmacol.*, **96**, 857–863.
- LEIGHTON, B., CURT, R., HUSSEIN, A. & NEWSHOLME, E.A. (1987). Maximum activities of some key enzymes of glycolysis, glutaminolysis, Krebs cycle and fatty acid utilization in bovine pulmonary endothelial cells. *FEBS Lett.*, **225**, 93–96.
- MADDEN, M.C., VENDER, R.L. & FRIEDMAN, M. (1986). Effects of hypoxia on prostacyclin production in cultured pulmonary artery endothelium. *Prostaglandins*, **31**, 1049–1062.
- MARTIN, W., VILLANI, G.M., JOTHIANANDAN, D. & FURCHGOTT, R.F. (1985). Selective blockade of endothelium-dependent and glyceryl trinitrate-induced relaxation by hemoglobin and by methylene blue in the rabbit aorta. *J. Pharmacol. Exp. Ther.*, **232**, 708–716.
- MARTIN, W., WHITE, D.G. & HENDERSON, A.H. (1988). Endothelium-derived relaxing factor and atriopeptin II elevate cyclic GMP levels in pig aortic endothelial cells. *Br. J. Pharmacol.*, **93**, 229–239.
- ODY, C. & JUNOD, A.F. (1985). Direct toxic effects of paraquat and oxygen on cultured endothelial cells. *Lab. Invest.*, **52**, 77–84.
- PALACIOS, M., KNOWLES, R.G., PALMER, R.M.J. & MONCADA, S. (1989). Nitric oxide from L-arginine stimulates soluble guanylate cyclase in adrenal glands. *Biochem. Biophys. Res. Commun.*, **165**, 802–809.
- RAPOPORT, R.M. & MURAD, F. (1983). Agonist-induced endothelium-dependent relaxation in rat thoracic aorta may be mediated through cGMP. *Circ. Res.*, **52**, 352–357.
- RUBANYI, G. & PAUL, R.J. (1984). O₂-sensitivity of beta adrenergic responsiveness in isolated bovine and porcine coronary arteries. *J. Pharmacol. Exp. Ther.*, **230**, 692–703.
- RUBANYI, G.M. & VANHOUTTE, P.M. (1985). Hypoxia releases a vasoconstrictor substance from the canine vascular endothelium. *J. Physiol.*, **364**, 45–56.
- RUBANYI, G.M. & VANHOUTTE, P.M. (1986). Superoxide anions and hyperoxia inactivate endothelium-derived relaxing factor. *Am. J. Physiol.*, **250**, H822–H827.
- VOYTA, J.C., VIA, D.P., BUTTERFIELD, C.E. & ZETTER, B.R. (1984). Identification of endothelial cells based on their uptake of acetylated low-density lipoprotein. *J. Cell Biol.*, **99**, 2034–2040.
- WARREN, J.B., MALTBY, N.H., MACCORMACK, D. & BARNES, P.J. (1989). Pulmonary endothelium-derived relaxing factor is impaired in hypoxia. *Clin. Sci.*, **77**, 671–676.
- WEIR, C.J., GIBSON, I.F. & MARTIN, W. (1991). Effects of metabolic inhibitors on endothelium-dependent and endothelium-independent vasodilatation of rat and rabbit aorta. *Br. J. Pharmacol.*, **102**, 162–166.

(Received May 16, 1990

Revised July 27, 1990

Accepted August 17, 1990)