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Modulation of arterial endothelial permeability: studies on an *in vitro* model

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1 An *in vitro* model of the arterial endothelial barrier was established in which transfer of trypan blue-labelled albumin across confluent monolayers of pig aortic endothelial cells grown on polycarbonate membranes was measured.

2 A range of inflammatory mediators, i.e. histamine, bradykinin, platelet activating factor and thrombin, had no effect on the transfer of labelled albumin across aortic endothelial monolayers.

3 Calcium ionophore A23187 and the phorbol ester, phorbol myristate acetate (PMA), each induced concentration-dependent increases in transfer of labelled albumin. These increases were associated with changes in cell shape, consistent with endothelial contraction. Ionophore A23187 caused some detachment of cells.

4 The ability of PMA to increase transfer of labelled albumin probably results from activation of protein kinase C since it was not shared by the inactive analogue, 4α-phorbol 12,13-didecanoate.

5 Neither a combination of superoxide dismutase and catalase nor the cyclo-oxygenase inhibitor, flurbiprofen, affected resting or PMA-induced increases in albumin transfer. Oxygen-derived free radicals and prostaglandins appear not to be involved in the response to PMA.

6 Each of three procedures designed to elevate adenosine 3′:5′-cyclic monophosphate (cyclic AMP) levels, i.e. dibutyryl cyclic AMP, forskolin and (±)-isoprenaline, reduced the ability of PMA to promote increased transfer of labelled albumin but had no effect on resting transfer. The effect of (±)-isoprenaline was abolished by the β-adrenoceptor blocking agent, propranolol.

7 Elevation of cyclic GMP content by use of 8 bromo cyclic GMP or atriopeptin II had no effect on resting or PMA-induced transfer of labelled albumin.

8 Arterial endothelial barrier function can be compromised by agents that promote endothelial contraction. Agents that increase endothelial cyclic AMP levels, and so reduce entry of high molecular weight substances into the arterial wall, may warrant evaluation as potential anti-atherogenic drugs.

Introduction

The vascular endothelium plays a central role in regulating the exchange of fluid, solutes, and cells between the blood and the extravascular tissues. The exchange of fluid and solutes can be modulated either by trans-endothelial endocytosis (Palade, 1960), or, following the actions of inflammatory mediators such as histamine, by formation of inter-endothelial gaps (Majno & Palade, 1961). The occurrence of inter-endothelial gaps in response to inflammatory mediators is restricted to the post-capillary venule (Svensjo *et al.*, 1979), and is thought to be the result of active endothelial contraction (Majno *et al.*, 1967); a proposal supported by the discovery of contractile proteins, including actin and myosin (Becker & Nachmann, 1973; Drenckhahn, 1983) in endothelial cells.

The arterial endothelial barrier also is of critical importance since its impairment leads to increased entry of cholesterol-rich low-density lipoproteins into the arterial wall (Ross & Harker, 1976), with the subsequent development of atherosclerosis (Ross, 1986; Munro & Cotran, 1988). Factors which modulate the arterial endothelial permeability barrier are poorly understood: they may include endothelial contraction with the development of inter-endothelial gaps, or endothelial damage resulting

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from the effects of oxygen-derived free radicals or peroxidized lipids (Rosen & Freeman, 1984; Hennig & Chow, 1988). Ideally, studies of arterial endothelial barrier function should be performed *in vivo*, but under these circumstances concentrations of drugs and the possible involvement of cells other than endothelial cells are difficult to control. These disadvantages might be circumvented by establishing an *in vitro* model of arterial permeability. Rotrosen & Gallin (1986) devised a model of the venous endothelial permeability barrier in which transfer of labelled albumin across confluent monolayers of endothelial cells grown on polycarbonate membranes was measured. We wished to establish a similar model of the arterial endothelial permeability barrier in which pig aortic endothelial cells were grown on polycarbonate membranes. In particular, we wished to study the actions of the major intracellular transduction mechanisms involving calcium, cyclic nucleotides, and protein kinase C, on transfer of albumin across aortic endothelial monolayers.

Methods

Endothelial cell culture

Pig aortic endothelial cells were isolated and grown in 75 cm^2 tissue culture flasks as previously described (Martin *et al.*, 1988). When these primary cultures reached confluence (4–7 days) the cells from each flask was detached with trypsin (0.05%) and ethylenediamine tetraacetic acid (0.02%) and seeded into 48 Transwell (Costar) polycarbonate membrane assemblies (6.5 mm diameter, 3 μm pore size). The membrane assemblies were then placed in 1 ml of tissue culture medium in 24-well plates (Costar) and the cells allowed to grow for a further 2–4 days.

Albumin transfer experiments

Membrane assemblies with endothelial cells attached were washed twice by immersion in Krebs solution containing (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 2.4, glucose 11 and HEPES (N-2 hydroxyethylpiperazine-N′-2-ethanesulphonic acid) 5 at 37°C and pH 7.4, and then transferred to 24-well plates. Six hundred μl of Krebs solution was placed in each of the wells which formed the lower chamber and 100 μl of Krebs containing trypan blue-labelled albumin (4%) was placed above the endothelial monolayer. These volumes were chosen so that no hydrostatic gradient was created across the membrane. The preparations were then incubated at 37°C in air with mild, continuous agitation produced by an orbital mixer. After 5 min monolayers that demonstrated leakage of dye visible to the naked eye were discarded, and those remaining were used in transfer studies. Following incubation with drugs for the times indicated in the Results, albumin transfer across endothelial monolayers was quantified by measuring the absorbance of fluid from the lower chamber at 590 nm. The trypan blue-labelled albumin content of the lower chamber is expressed as a percentage of the maximum concentration that would have been achieved at equilibrium.

The trypan blue-labelled albumin complex was prepared by adding trypan blue (180 mg, Sigma) and bovine serum albumin (4 g, fraction V, Sigma) to 100 ml of Krebs solution. Precipitation with trichloroacetic acid (6%) showed that the trypan blue was >98% albumin bound.

Morphological studies

Transwell polycarbonate membranes are opaque which prevents the visualisation of cells by phase-contrast microscopy. Studies of the effects of drugs on cell morphology were performed with cells grown on glass coverslips where visualisation was possible.

Drugs

Atriopeptin II (rat synthetic), bradykinin triacetate, 8 bromo cyclic GMP, catalase (bovine liver), dibutyryl cyclic AMP, histamine dihydrochloride, ionophore A23187, (±)-isoprenaline hydrochloride, 4α-phorbol 12,13-didecanoate (4α-PDD), phorbol 12-myristate 13-acetate (PMA), superoxide dismutase (bovine erythrocyte) and thrombin (bovine plasma) were obtained from Sigma. Forskolin and platelet activating factor were obtained from Calbiochem and sodium flurbiprofen was a generous gift from Dr R.V. Holland, Boots Pure Drug Co. All drugs were dissolved in twice-distilled water except for ionophore A23187, 4α-PDD and PMA which were dissolved in ethanol, and forskolin which was dissolved in dimethyl sulphoxide (DMSO). Concentrations of ethanol or DMSO did not exceed 0.2% in experiments: concentrations of 1% and above were found to increase albumin transfer across monolayers.

Statistical analysis

Since the resting transfer of trypan blue-labelled albumin across endothelial monolayers varied from cell batch to batch, each experiment was performed with its own internal controls. Results are presented as the mean ± s.e.mean, and comparisons were made either with Student's *t* test or the Mann-Whitney test when there was unequal variance in samples. A probability of 0.05 or less was considered significant.

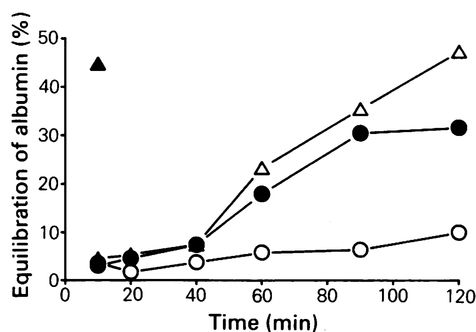

Figure 1 Time course showing the transfer of trypan blue-labelled albumin across confluent monolayers of pig aortic endothelial cells grown on polycarbonate membranes in the absence of drugs (○), and following treatment with ionophore A23187 (20 μM, △) or phorbol myristate acetate (6 μM, ●). The rapid transfer across membranes without cells is also shown (▲). Each point is the mean of 2–4 observations.

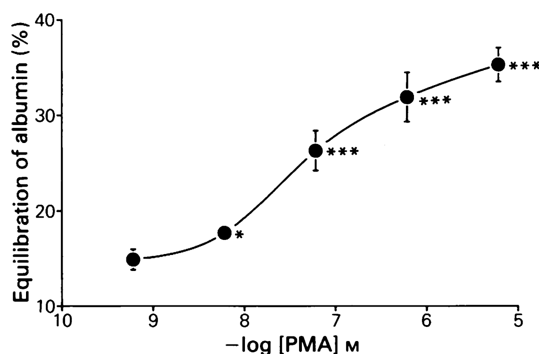

Figure 2 Concentration-response curve showing the ability of phorbol myristate acetate (PMA) to increase the transfer of trypan blue-labelled albumin across confluent monolayers of pig aortic endothelial cells during a 90 min incubation period. Each point is the mean of 5–6 observations; vertical bars show s.e.mean. * $P < 0.05$; *** $P < 0.001$, denotes significant difference from albumin transfer across untreated monolayers.

Results

Phorbol myristate acetate and A23187

Transfer of trypan blue-labelled albumin (4%) across polycarbonate membranes into the lower chamber was rapid: roughly 50% equilibration was achieved within 10 min and complete equilibrium within 60 min (Figure 1). When a confluent layer of pig aortic endothelial cells was present on membranes the transfer of labelled albumin was markedly restricted. Addition of PMA (6 μM) or ionophore A23187 (20 μM) increased the transfer of labelled albumin across endothelial monolayers in a time-dependent manner (Figure 1). The concentration-dependence of the increased transfer of labelled albumin induced by PMA and by ionophore A23187 during a 90 min incubation is shown in Figure 2 and Table 1, respectively. Combined exposure to sub-

Table 1 Effects of ionophore A23187, phorbol myristate acetate (PMA), forskolin and 8 bromo cyclic GMP (8 Br cGMP) on transfer of trypan blue-labelled albumin across monolayers of pig aortic endothelial cells

Stimulus	*Pretreatment*	*% equilibration of albumin*	n
None (control)‡	None	4.2 ± 0.2	4
A23187, 2 μM	None	9.8 ± 1.1***	4
A23187, 20 μM	None	24.2 ± 0.5***	4
None (control)†	None	11.6 ± 0.9	6
None	PMA, 60 nM	19.1 ± 2.1* ⎫	6
A23187, 2 μM	None	18.7 ± 2.6* ⎫* ⎬*	6
A23187, 2 μM	PMA, 60 nM	31.0 ± 3.6* ⎭ ⎭	6
None (control)‡	None	14.8 ± 1.1	
None	Forskolin, 20 μM	8.7 ± 3.0	4
A23187, 20 μM	None	47.6 ± 2.0***	4
A23187, 20 μM	Forskolin, 20 μM	46.1 ± 2.2***	4
None (control)‡	None	8.0 ± 1.1	4
None	8 Br cGMP, 30 μM	7.7 ± 0.9	4
A23187, 20 μM	None	44.9 ± 2.8***	4
A23187, 20 μM	8 Br cGMP, 30 μM	49.6 ± 4.1***	4

Following exposure of endothelial monolayers to ionophore A23187 for 60 min (†) or 90 min (‡) the % equilibration of trypan blue-labelled albumin in the lower chamber was measured spectrophotometrically. The effects of pretreatment with PMA, forskolin, or 8 bromo cyclic GMP, for 2 min before addition of A23187 are also shown. Results are expressed as the mean ± s.e.mean. * $P < 0.05$; *** $P < 0.001$, denotes significant difference from control or a difference between two groups joined by a bar.

Table 2 Effects of phorbol myristate acetate (PMA), 4α-phorbol 12,13-didecanoate (4α-PDD), superoxide dismutase (SOD) and catalase (Cat), and flurbiprofen (FBP) on transfer of trypan blue-labelled albumin across monolayers of pig aortic endothelial cells

Stimulus	*Pretreatment*	*% equilibration of albumin*	n
None (control)	None	9.2 ± 1.3	6
PMA, 0.6 μM	None	25.1 ± 0.6***	6
4α-PDD, 0.6 μM	None	9.3 ± 0.9	6
None (control)	None	9.1 ± 1.0	6
None	SOD, 30 u ml^{-1} and Cat, 30 u ml^{-1}	8.6 ± 0.3	6
PMA, 0.6 μM	None	22.7 ± 1.0***	6
PMA, 0.6 μM	SOD, 30 u ml^{-1} and Cat, 30 u ml^{-1}	23.2 ± 0.8***	6
None (control)	None	7.0 ± 0.3	13
None	FBP, 10 μM	6.9 ± 0.5	12
PMA, 0.6 μM	None	17.2 ± 1.3***	6
PMA, 0.6 μM	FBP, 10 μM	17.5 ± 1.2***	6

Following exposure of endothelial monolayers to PMA or 4α-PDD for 90 min the % equilibration of trypan blue-labelled albumin in the lower chamber was measured spectrophotometrically. The effects of pretreatment with a combination of superoxide dismutase and catalase or with flurbiprofen for 2 min before addition of PMA are also shown. Results are expressed as the mean ± s.e.mean. ***$P < 0.001$, denotes significant difference from control.

maximal concentrations of PMA (60 nM) and ionophore A23187 (2 μM) for 60 min showed an additive effect (Table 1).

The inactive phorbol ester, 4α-PDD (0.6 μM, 90 min), lacked the ability of PMA (0.6 μM, 90 min) to increase transfer of labelled albumin across endothelial monolayers (Table 2). Parallel studies in which phase-contrast microscopy was used showed that endothelial cells underwent a shape change in response to PMA (0.6 μM) consistent with cell contraction, but remained adherent to the substrate. A23187 (20 μM) also caused cells to change shape but definite detachment of cells was evident.

Modulation of phorbol myristate acetate

A combination of superoxide dismutase (30 u ml^{-1}) and catalase (30 u ml^{-1}) had no effect on resting or PMA (0.6 μM)-induced transfer of labelled albumin across endothelial monolayers during a 90 min incubation period (Table 2). The cyclo-oxygenase inhibitor, flurbiprofen (10 μM), also was without effect on resting or PMA (0.6 μM)-induced transfer of labelled albumin (Table 2).

Treatment with each of 3 agents designed to elevate endothelial cyclic AMP content, i.e. dibutyryl cyclic AMP (30 μM), forskolin (20 μM), and (±)-isoprenaline (20 μM), inhibited the PMA (0.6 μM)-induced increase in transfer of labelled albumin without affecting resting transfer (Table 3, Figure 3). Propranolol (20 μM) blocked the ability of (±)-isoprenaline (20 μM) to inhibit PMA (0.6 μM)-induced transfer of labelled albumin (Figure 3).

Treatment with 2 agents designed to elevate endothelial cyclic GMP content, i.e. 8-bromo cyclic GMP (30 μM) or atriopeptin II (20 nM), had no effect on resting or PMA (0.6 μM)-induced transfer of labelled albumin (Table 3).

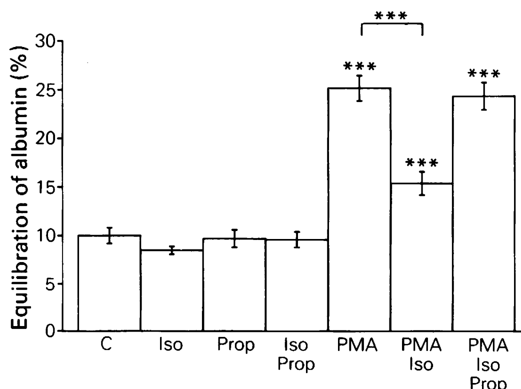

Figure 3 (±)-Isoprenaline (Iso, 20 μM) inhibits phorbol myristate acetate (PMA, 0.6 μM)-stimulated transfer of trypan blue-labelled albumin across monolayers of pig aortic endothelial cells and this is blocked by propranolol (Prop, 20 μM). Columns are the mean of 8–23 observations; vertical bars show s.e.mean. *** $P < 0.001$, denotes significant difference from control (C) monolayers, or a difference between groups joined by a bar.

Table 3 Effects of phorbol myristate acetate (PMA), forskolin, dibutyryl cyclic AMP (db cAMP), atriopeptin II (APII) and 8 bromo cyclic GMP (8 Br cGMP) on transfer of trypan blue-labelled albumin across monolayers of pig aortic endothelial cells

Stimulus	*Pretreatment*	*% equilibration of albumin*	n
None (control)	None	2.1 ± 0.3	6
None	Forskolin, 20 μM	1.8 ± 0.3	6
PMA, 0.6 μM	None	6.8 ± 0.4*** } **	6
PMA, 0.6 μM	Forskolin, 20 μM	4.5 ± 0.4*** }	6
None (control)	None	9.2 ± 1.3	6
None	db cAMP, 30 μM	8.5 ± 0.5	6
PMA, 0.6 μM	None	25.1 ± 0.6*** } **	6
PMA, 0.6 μM	db cAMP, 30 μM	19.6 ± 1.4*** }	6
None (control)	None	13.3 ± 1.7	6
None	APII, 20 nM	11.2 ± 2.0	6
PMA, 0.6 μM	None	49.1 ± 2.2***	6
PMA, 0.6 μM	APII, 20 nM	49.8 ± 1.8***	6
None (control)	None	8.0 ± 0.6	6
None	8 Br cGMP, 30 μM	7.2 ± 0.6	6
PMA, 0.6 μM	None	34.0 ± 1.3***	6
PMA, 0.6 μM	8Br cGMP, 30 μM	31.0 ± 2.9***	6

Following exposure of endothelial monolayers to PMA the % equilibration of trypan blue-labelled albumin in the lower chamber was measured spectrophotometrically. The effects of pretreatment with forskolin, dibutyryl cyclic AMP atriopeptin II or 8 bromo cyclic GMP, for 2 min before addition of PMA are also shown. Results are expressed as the mean ± s.e.mean. ** $P < 0.01$; ***$P < 0.001$, denotes significant difference from control or a difference between groups joined by a bar.

Modulation of A23187

Treatment with forskolin (20 μM) or 8-bromo cyclic GMP (30 μM) had no effect on resting or ionophore A23187 (20 μM)-induced transfer of labelled albumin across endothelial monolayers during a 90 min incubation period (Table 1).

Bradykinin, histamine, platelet activating factor and thrombin

Four endothelial cell stimulants, i.e. bradykinin (0.1 μM), histamine (100 μM), platelet activating factor (0.2 μM) and thrombin (1 u mi^{-1}), had no effect on transfer of labelled albumin across endothelial monolayers during a 90 min incubation period (Table 4).

Discussion

The use of endothelial cell monolayers grown on polycarbonate membranes as an *in vitro* model of the vascular permeability barrier is now well established (Shasby *et al*., 1985; Rotrosen & Gallin, 1986). An important characteristic of the permeability barrier i.e. the high permeability to small but not large molecular weight substances is conserved using this technique (Del-Vecchio *et al*., 1987). This model has been applied mainly to venous and pulmonary artery endothelial cells in attempts to elucidate the mechanisms underlying inflammation and pulmonary oedema (Shasby *et al*., 1982; Rotrosen & Gallin, 1986). The inflammatory mediator, histamine,

Table 4 Effects of histamine, bradykinin, thrombin and platelet activating factor (PAF) on transfer of trypan blue-labelled albumin across monolayers of pig aortic endothelial cells

Stimulus	*% equilibration of albumin*	n
None (control)	6.1 ± 0.7	5
Histamine, 100 μM	6.4 ± 0.6	6
Bradykinin, 0.1 μM	5.5 ± 1.0	6
Thrombin, 1 u ml^{-1}	6.6 ± 1.0	6
None (control)	11.7 ± 0.6	7
PAF, 0.2 μM	10.7 ± 0.4	7

Following exposure of endothelial monolayers to histamine, bradykinin, thrombin or platelet activating factor for 90 min the % equilibration of trypan blue-labelled albumin in the lower chamber was measured spectrophotometrically. Results are expressed as the mean ± s.e.mean.

has been observed to increase the transfer of high molecular weight markers across endothelial monolayers (Rotrosen & Gallin, 1986; Killackey *et al.*, 1986), consistent with its ability to increase vascular permeability *in vivo*. Inter-endothelial gap formation resulting from endothelial contraction mainly in post-capillary venules appears to be the basis of the increased vascular permeability *in vivo* (Majno & Palade, 1961). Recently, platelet activating factor, which promotes marked pulmonary oedema has been shown to induce a shape change, consistent with contraction, in cultures of pulmonary artery endothelial cells (Grigorian & Ryan, 1987). Changes in endothelial cell shape are triggered by calcium (Shasby *et al.*, 1985) or by activation of protein kinase C (Grigorian & Ryan, 1987).

In this study cultures of pig aortic endothelial cells restricted the passage of trypan blue-labelled albumin. In keeping with the *in vivo* finding that histamine and bradykinin promote plasma leakage only at venular endothelium (Svensjo *et al.*, 1979) we found that these agents did not increase the transfer of labelled albumin across aortic endothelial monolayers. Platelet activating factor and thrombin were also unable to promote increased albumin transfer despite having been shown to induce a shape change in and promote increased permeability of bovine pulmonary artery endothelial cells (Grigorian & Ryan, 1987; Minnear *et al.*, 1988; De-Michele *et al.*, 1988). Of these two agents only thrombin is known to have other effects on pig aortic endothelial cells e.g., stimulation of prostacyclin production (Pearson *et al.*, 1983).

Shasby *et al.* (1985) reported that calcium ionophore A23187 increased the transfer of albumin across monolayers of pig pulmonary artery endothelial cells and proposed that endothelial contraction was calcium-dependent. We found that ionophore A23187 induced a shape change, consistent with contraction, in pig aortic endothelial cells, and increased the permeability of monolayers to labelled albumin. This increased permeability was not solely due to inter-endothelial gap formation, but was also the result of detachment of cells. Elevation of endothelial cyclic AMP or cyclic GMP content using forskolin or 8-bromo cyclic GMP, respectively, had no effect on the ability of ionophore A23187 to induce increased transfer of labelled albumin. Bovine pulmonary artery endothelial cells have previously been reported to change from their normal polygonal morphology to an elongated spindle-like shape when exposed to the stimulant of protein kinase C (Grigorian & Ryan, 1987). We found that PMA induced a shape change in pig aortic endothelial cells. This occurred without detachment of cells and was associated with increased transfer of labelled albumin across endothelial monolayers. The ability to promote transfer of labelled albumin was concentration-dependent and was not shared by the inactive analogue, 4α-PDD and is likely to result from activation of protein kinase C. Phorbol esters stimulate production of superoxide radicals by endothelial cells (Matsubara & Ziff, 1986), and these can have adverse effects on endothelial cells (Shasby *et al.*, 1985) or their basement membrane (McCord, 1974; Greenwald & Moy, 1979), thus compromising the arterial endothelial barrier (Hennig & Chow, 1988). The lack of effect of superoxide dismutase and catalase on the PMA-induced increase in albumin transfer across aortic endothelial monolayers makes it unlikely that oxidant-induced injury was involved. It is more likely that activation of protein kinase C leads to phosphorylation of proteins involved in controlling cell shape. Two possible candidates are talin and vinculin, cytoskeletal proteins involved in regulation of cell shape and adhesion, since these are known substrates for protein kinase C (Litchfield & Ball, 1986; Werth *et al.*, 1983).

Elevation of cyclic AMP but not cyclic GMP content is known to relax epithelial cells and improve the integrity of tight junctions (Duffey *et al.*, 1981). Furthermore, *in vivo* studies have shown that stimulation of β_2-adrenoceptors inhibits the increase in vascular permeability induced by inflammatory agents, an effect caused by blockade of inter-endothelial gap formation at post-capillary venules (Svensjo & Grega, 1986; Grega, 1986; Marciniak *et al.*, 1978). *In vitro* studies with cultured pulmonary artery endothelial cells also suggest that elevation of cyclic AMP inhibits contraction and permeability (Stelzner *et al.*, 1988; Minnear *et al.*, 1988). In accord with these observations, we found that treatment with each of three agents designed to elevate cyclic AMP content i.e., dibutyryl cyclic AMP, forskolin and ($\pm$)-isoprenaline, caused a significant inhibition of PMA-induced transfer of labelled albumin across aortic endothelial monolayers without affecting resting transfer. The effect of ($\pm$)-isoprenaline was abolished in the presence of the β-adrenoceptor blocking agent, propranolol. Elevation of cyclic AMP content appears therefore to be a common mechanism whereby endothelial contractility and permeability is inhibited.

In contrast to the findings with cyclic AMP, elevation of cyclic GMP levels with either the membrane permeant analogue, 8 bromo cyclic GMP, or atriopeptin II (Martin *et al.*, 1988), failed to alter resting or PMA-induced transfer of labelled albumin across aortic endothelial monolayers. Cyclic GMP might, however, be involved in enhancing permeability at the post-capillary venule during inflammation (Chander *et al.*, 1988).

Vasodilator prostaglandins are known to potentiate vascular leakage during inflammatory states by

increasing blood flow (Williams, 1983). We found, however, that inhibition of cyclo-oxygenase using flurbiprofen had no effect on resting or PMA-induced transfer of labelled albumin across aortic endothelial monolayers.

In conclusion, our results using an *in vitro* model confirm well-established data obtained *in vivo* that inflammatory mediators have no effect on the permeability of aortic endothelium. Results with ionophore A23187 and PMA suggest that aortic endothelial contractility and permeability can be stimulated by elevation of calcium levels or activation of protein kinase C and that these two pathways are additive. Elevation of cyclic AMP but not cyclic GMP levels inhibits permeability increases induced by PMA. Pharmacological agents which elevate endothelial cyclic AMP levels and thereby inhibit transport of high molecular weight substances into the arterial wall might therefore warrant evaluation as anti-atherogenic drugs.

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