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Prostacyclin biosynthesis and reduced 5-HT uptake after complement-induced endothelial injury in the dog isolated lung

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1 Pulmonary prostacyclin (PGI_2) biosynthesis was evaluated in relation to endothelial integrity before and after complement activation in isolated plasma-perfused lung lobes of the dog.

2 The plasma was activated with zymosan (ZAP, $n = 4$), yeast cells (YAP, $n = 4$) or yeast with 3 μM indomethacin (Indo + YAP, $n = 3$). Immunoreactive 6-oxo-prostaglandin $F_{1\alpha}$ (i-6-oxo-$PGF_{1\alpha}$) and thromboxane B_2 (iTXB_2) were measured to monitor PGI_2 and TXA_2 biosynthesis.

3 The kinetic parameters K_m and V_{max} of 5-hydroxytryptamine (5-HT) uptake were calculated on the basis of multiple indicator diffusion data to evaluate endothelial integrity.

4 YAP and ZAP induced a biphasic increase of the arterial perfusion pressure. The immediate pressure peak was partly mediated by TXA_2 and the TXB_2 was subsequently cleared by the lung.

5 The apparent V_{max} of 5-HT uptake remained constant throughout the experiment. Thus, complement activation did not affect the number of endothelial 5-HT carrier sites available to the perfusate.

6 The apparent K_m of 5-HT uptake was enhanced in 9 lungs exposed to activated plasma complement for 20 min. This decreased affinity for 5-HT probably reflects endothelial injury. It was transient as the apparent K_m had returned to the baseline value after 60 min.

7 PGI_2 clearance and biosynthesis were virtually absent in the control period. PGI_2 formation increased drastically after infusion of ZAP or YAP and was proportional to the endothelial injury expressed as elevated K_m or pulmonary oedema. Thus, PGI_2 biosynthesis might be a marker of severe endothelial distress.

Introduction

Pulmonary endothelium can transform peptides, remove amines (Bakhle & Vane 1974; Gillis & Catravas, 1982) and release biologically active products, including prostacyclin (PGI_2, Moncada *et al.*, 1976) in addition to its function in gas exchange. The transient pulmonary distress during haemodialysis or after cardiopulmonary bypass is associated with reduced clearance capacity of pulmonary endothelium (Gillis & Catravas, 1982; Pitt *et al.*, 1982) and increased PGI_2 formation (Edlund *et al.*, 1981; Leithner *et al.*, 1980). Experimental endotoxin shock, a model that mimics the more severe adult respiratory distress syndrome (ARDS) to some extent (e.g. Brigham & Meyrick, 1986) also curtails the clearing capacity of pulmonary endothelium (Moalli *et al.*, 1984). Again PGI_2 is secreted in pulmonary lymph (Ikeda *et al.*, 1983; Brigham & Meyrick, 1986) or aortic blood, in which PGI_2 is normally undetectable (Bult *et al.*, 1980; 1985b; Bult & Herman, 1985; Flynn, 1985; Hüttemeier *et al.*, 1982). PGI_2 also appears in the circulation of patients suffering from sepsis associated with shock (Rie *et al.*, 1983; Slotman *et al.*, 1986).

Complement activation is a common denominator in these pulmonary disorders. It is assumed that the complement fragment C5adesArg, the plasma metabolite of C5a, induces pulmonary injury by promoting the attack of vascular endothelium by phagocytes (e.g. Craddock, 1982; Weinberg *et al.*, 1984; Slotman *et al.*, 1986; Stevens *et al.*, 1986). Selective complement activation with cobra venom factor (CVF)

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(Ulevitch & Cochrane, 1977; Larsen et al., 1985) or injection of zymosan activated plasma (ZAP, Cooper et al., 1980; Perkowski et al., 1983; Meyrick & Brigham 1984) as a source of C5adesArg do induce pulmonary hypertension, endothelial injury and PGI_2 biosynthesis (Cooper et al., 1980; Perkowski et al., 1983; Bult et al., 1985a). The injury is modest and reminiscent of the transient changes seen during haemodialysis. Additional factors are required for the full development of ARDS.

We investigated whether PGI_2 biosynthesis can be considered as an index of endothelial injury. To this end a plasma perfused dog lunglobe was exposed to ZAP or yeast activated plasma (YAP) as a source of C5adesArg. 5-Hydroxytryptamine (5-HT) uptake was studied with a multiple indicator-dilution technique, since impaired pulmonary clearance could serve as a sensitive index of endothelial injury before morphological alterations or oedema become apparent (Gillis & Catravas, 1982; Dawson et al., 1987). The 5-HT extraction was analysed with a mathematical model, which describes the saturable uptake in terms of the kinetic constants K_m and V_{max} of the Michaelis-Menten equation. K_m and V_{max} characterize the uptake independently of haemodynamic changes, in contrast to the conventional calculation of the fractional removal (Rickaby et al., 1984; Dawson et al., 1987).

Methods

Lung preparation

Mongrel dogs (23.5 ± 1.5 kg, $n = 14$) were anaesthetized with pentobarbitone sodium (30 mg kg^{-1}), heparinized (1000 iu kg^{-1}) and exsanguinated via a carotid artery. After centrifugation (10 min, 2000 g) autologous plasma was used to prime the perfusion system. The left lower lung lobe was removed from the chest, weighed and positioned in a perfusion chamber and perfusion with autologous plasma (500 ml) was started at a slow rate of 0.5 ml s^{-1} (Malcorps et al., 1984). The lobe was ventilated via the bronchus with 95% O_2 and 5% CO_2 with a Hoover ventilator (500 ml strokevolume, Palmer Ltd.). Bronchial pressures were adjusted to 0.5 mmHg at end-expiration and 5 mmHg at end-inspiration, leading to a plasma pH of approximately 7.4. The lobe was inflated several times to an end-inspiratory pressure of 10 mmHg to remove red blood cells trapped in the lobe. The first 200 ml effluent, containing most of the erythrocytes and PGI_2 formed during the short period of stasis, was discarded. Its reintroduction led to a persistent elevation of the 6-oxo-$PGF_{1\alpha}$ concentration. Donor plasma (1000 to 1200 ml), obtained from two heparinized anaesthetized dogs and stored at -20°C was brought to 37°C, filtered through cloth and slowly added to the circuit. The venous effluent was then allowed to drain into a reservoir from which it was pumped through a bubble trap into the lobar artery at an average rate of 5.00 ± 0.01 ml s^{-1} ($n = 14$), which was maintained throughout the experiment. This led to a lobar artery pressure of 6.3 ± 0.6 mmHg ($n = 14$) referenced to the top surface of the lobe. The venous pressure was kept at 1 mmHg (slightly above the end-expiratory pressure at the top of the lobe) by adjusting the height of the reservoir. Pressures in bronchus, artery and vein were continuously monitored (Malcorps et al., 1984).

For the 5-HT uptake studies, a bolus injector was situated in the tubing leading to the arterial cannula so that a 0.33 ml bolus could be injected rapidly (Malcorps et al., 1984). At the same moment the venous effluent was directed to a fraction collector, which operated at a rate of 2.25 samples per s for 20 s. At the end of the experiment (about 150 min after initiation of the perfusion), the lobe was removed, the plasma allowed to drain, and the lobe weight was again measured. The preparation is stable for at least 3 h and during this period there is no significant increase of lung weight (Malcorps et al., 1984).

Experimental protocol

The lungs were exposed to YAP ($n = 4$), YAP in the presence of indomethacin 3 μM (Indo + YAP, $n = 3$) or ZAP ($n = 4$). One lung was perfused with heat-inactivated plasma (15 min, 56°C), which was then treated with yeast and reintroduced in the pulmonary circuit. Another lobe was gradually exposed to complement activation by injection of purified CVF (0.1 unit ml^{-1} plasma). Finally, one lung was used to study the kinetics of TXB_2 uptake.

Before starting an experiment, the mixture of autologous and donor plasma was perfused through the lung lobe for at least 30 min in order to clear the 5-HT and TXB_2 (formed during plasma preparation) from the circuit. This did not influence the initial concentration of 6-oxo-$PGF_{1\alpha}$. Aliquots (5 ml) of plasma were removed from the venous effluent every 15 min for determination of PGI_2, TXB_2, haemolytic complement titre (CH50), and numbers of red blood cells and leukocytes. At 50 min, 700 to 1000 ml plasma was removed from the circuit and used for complement activation. In experiments with indomethacin, it was preceded by the first set of bolus injections and immediately thereafter indomethacin (3 μM) was added to the plasma. In all other experiments, the first pair of boluses was injected at 80 min, whereas in experiments with indomethacin the second set was then given; these data were used to

evaluate the basal kinetic parameters of 5-HT uptake. At 90 min the activated plasma was reintroduced in the circuit and additional plasma samples were removed 1, 2, 3, 4, 5 and 10 min later. Finally, bolus injections were given at 110 and 150 min (i.e. 20 and 60 min after exposure to activated complement).

In the experiment with heat-inactivated plasma the same protocol was used, but the plasma had been treated at 56°C for 15 min; in the experiment with CVF the plasma was not removed from the circuit, but 100 units purified CVF were injected at 90 min. The TXB_2 uptake was studied in one lung by injection of 1, 10 or 50 pmol TXB_2 together with indocyanine green and the TXB_2 concentration in the venous fractions was measured by radioimmunoassay.

Complement activation and complement titre

Plasma (700 to 1000 ml) was vigorously stirred with baker's yeast (20 mg ml^{-1}) or washed zymosan (3 mg ml^{-1}) for 5 min at 37°C. Yeast or zymosan particles were then removed by centrifugation (10 min, 2000 *g*). The clear supernatant was warmed to 37°C before re-introduction in the circuit.

The total haemolytic activity of the complement system in the recirculating plasma was monitored at 15 min intervals (Bult *et al*., 1985a). Results are given as haemolytic CH50 units ml^{-1} plasma. The CH50 provides an estimate of the overall activity of classical and lytic pathways of the complement system.

Radioimmunoassay

PGI_2 and TXA_2 were assessed by specific radioimmunoassays of 6-oxo-$PGF_{1\alpha}$ and TXB_2. In both assays 100 and 200 μl plasma and dilutions in 50 mM Tris pH 8 were measured directly as described (Bult *et al*., 1985a). During the experiment some ^3H gradually accumulated in the plasma. This background radioactivity was subtracted from the radioimmunoassay data.

Determination of 5-HT uptake

5-HT uptake was studied with a multiple indicator-dilution technique as described by Malcorps *et al.* (1984, method C). For each measurement two sequential boluses (330 μl) containing respectively 25 or 100 nmol unlabelled 5-HT along with 2.5 μCi [^{3}H]-5-HT (together forming the permeating indicator) and 0.5 mg indocyanine green dye (which binds to albumin and serves as non-permeating indicator confined to the vascular space) were injected as quickly as possible in the lobar artery within 5 min. Dispersal in the arteries and the combination of two boluses with a different 5-HT content resulted in a range of 5-HT and dye concentrations that were presented to the endothelium for a measurement of the unidirectional uptake. After passage through the pulmonary bed the venous effluent was fractionated. The venous concentration curves contain information about the convective transport (indocyanine green) as well as the endothelial 5-HT uptake (radiolabelled 5-HT). These data were analysed with a HP 1000 computer using a mathematical model which describes this saturable uptake process in a perfused lung in terms of the Michaelis-Menten equation. It enables calculation of the apparent kinetic constants K_m and V_{max}, independently of haemodynamic changes, as well as a factor alpha which compensates for the heterogeneity of capillary transit times (Rickaby *et al*., 1984; Dawson *et al*., 1987). V_{max} is the maximum rate of 5-HT uptake (nmol s^{-1} g^{-1}) and reflects the number of uptake sites available to the flowing plasma. K_m (μM) is the 5-HT concentration at $V_{max} . 2^{-1}$ and relates to the affinity of the carrier site for 5-HT.

Bioassay of C5a-like activity

C5a is spasmogenic for the guinea-pig isolated ileum (Vogt, 1974). The ileum was placed in a 10 ml organ bath for isotonic tension recording (Herman *et al*., 1979). After 40 min equilibration, a cumulative dose-response curve for histamine was made. After washout 1 ml plasma was added, followed by a second dose-response curve for histamine. Because of the rapid development of tachyphylaxis for C5a (Vogt, 1974), the strips were normally exposed only once to plasma.

Statistics

Results are given as mean $\pm$ s.e. mean. Student's *t* test was used to evaluate the effect of indomethacin, and the paired *t* test for time effects within each treatment (Diem & Lentner, 1975). The TXB_2 (pg ml^{-1}) and 6-oxo-$PGF_{1\alpha}$ (pg min^{-1} g^{-1}) data were first converted to logarithms in order to obtain normal distributions. The interdependence of two paired variables was assessed with a Spearman coefficient of rank correlation (R_S) (Diem & Lentner, 1975). Two-tailed tests and a 5 percent level of significance were chosen.

Materials

5-[1,2-^{3}H(N)]-hydroxytryptamine creatinine sulphate (15–30 Ci $mmol^{-1}$), 6-[5,8,9,11,12,14,15-^{3}H(N)]-oxo-$PGF_{1\alpha}$ (120–180 Ci $mmol^{-1}$), and [5,6,8,9,11,12,14,15-^{3}H(N)]-thromboxane B_2(TXB_2) (100–150 Ci $mmol^{-1}$) were obtained from New England Nuclear

(Brussels), indocyanine green (cardiogreen) from Becton Dickinson (Baltimore, U.S.A. 5-hydroxytryptamine (serotonin) creatinine sulphate from Aldrich-Europe, Janssen Pharmaceutica (Beerse, Belgium), zymosan and epsilon-aminocaproic acid (EACA) from Sigma (St. Louis, MO, U.S.A.), heparin from Leo (Copenhagen, Denmark), pentobarbitone from Abbott (Brussels, Belgium), cobra venom factor from Cordis Co. (Miami, FL, U.S.A.) and sheep red blood cells and rabbit anti-sheep red blood cell antiserum from Biomerieux Benelux (Brussels, Belgium). 6-Oxo-$PGF_{1\alpha}$ and TXB_2 were given by Dr J.E. Pike, Upjohn Co. (Kalamazoo, MI, U.S.A.), eicosatetraenoic acid by Roche (Welwyn Garden City, U.K.), BW-755C 3-amino-1-(m-trifluoromethyl)-phenyl-2-pyrazoline by Wellcome (Beckenham, U.K.) and indomethacin by Merck, Sharp and Dohme (Brussels, Belgium). FPL 55712 was from Fisons Ltd. (Loughborough, U.K.). Other chemicals were of analytical grade from U.C.B. (Brussels, Belgium) or Merck (Darmstadt, F.R.G.).

Results

Complement titres and C5a-like activity

In pilot experiments yeast and zymosan concentrations were selected which caused more than 75% reduction of the CH50 titre within 5 min. Heparinized dog plasma with 1 M epsilon-aminocaproic acid (EACA) (10% by volume) failed to contract the guinea-pig isolated ileum. Upon incubation with zymosan, yeast or CVF spasmogenic activity developed. It caused a biphasic contraction of the guinea-pig isolated ileum, which consistently diminished when the activated plasma, after wash-out, was applied again. The dose-response curve for histamine was not affected. The first, transient contraction was apparently mediated by histamine and dose-dependently suppressed by mepyramine (0.001 to 0.1 μM). The second component was suppressed by 1 and 10 μM indomethacin, 10 μM eicosatetraenoic acid and BW755C (above 10 μM). Atropine and FPL 55712 (0.1 to 10 μM) did not affect either phase. Maximum spasmogenic activity was observed after 30 min incubation with yeast (20 mg ml^{-1}). It should be noted that EACA suppressed the rate of overall complement activation (CH50 consumption). Incubation of plasma with yeast, zymosan or CVF in the absence of EACA did not lead to detectable spasmogenic activity at any time.

The initial CH50 titre (250 ± 11 units ml^{-1}, $n = 11$) of the perfusates was indeed absent (less than 25 units ml^{-1}) after treatment with either yeast or zymosan. Reintroduction of the activated and particle-free plasma led to a similar drop of the haemolytic complement activity for YAP, YAP with Indo, or ZAP (Table 1). The CH50 of heat-inactivated plasma was below the detection limit of the assay (less than 25 units ml^{-1}) throughout the experiment. The CH50 decreased gradually after CVF injection at 90 min (254, 252, 216 and 196 units ml^{-1} at 30, 80, 110 and 150 min, respectively).

Pressure and fluid accumulation

YAP caused an immediate rise of the perfusion pressure (Figure 1), which was most pronounced after about 1 min (6.7 ± 1.2 mmHg increase, $n = 4$, cf. Table 1). After a partial recovery and a second maximum between 5 and 15 min, the pressure gradually returned to the baseline in the subsequent 20 min. Indomethacin did not alter the baseline perfusion pressure (Table 1), but reduced the first (1.4 ± 0.7 mmHg, $n = 3$), but not the second pressure peak (Table 1, Figure 1). Yeast-treatment of heat-inactivated plasma (Figure 1) or CVF injection did not lead to pressure changes. ZAP caused a less pronounced acute rise of the perfusion pressure (increase 2.2 ± 0.9 mmHg), whereas the second vasoconstriction was comparable to that induced by YAP.

The lobe gained weight due to fluid accumulation during perfusion with YAP, but not in the presence of indomethacin. A variable oedema was observed after perfusion with ZAP (Table 1).

Thromboxane and PGI_2

Some TXB_2 was present in the plasma due to platelet activation during blood collection and centrifugation (1135 ± 212 pg ml^{-1}, $n = 11$). It was largely cleared by the lungs during the stabilization period (246 ± 44, 143 ± 25 and 88 ± 10 pg ml^{-1} after 15, 30 and 45 min perfusion). Injection of 1, 10 and 50 pmol TXB_2 in one lobe revealed that its removal was saturable. Apparent V_{max} (0.75 pmol g^{-1} s^{-1}) and K_m (4.7 nM) suggested the presence of a low number of carrier sites with a high affinity. Examples of the concentration of immunoreactive TXB_2 ($iTXB_2$) in the venous effluent are shown in Figure 1.

An $iTXB_2$ peak was measured 0.5 to 2 min after introduction of YAP, and the TXB_2 was cleared by the lung in the subsequent 15 min. The TXB_2 formation occurred mainly in the lung lobe, since $iTXB_2$ increased very little when plasma was incubated with yeast. This extra-pulmonary rise of $iTXB_2$ was not completely due to TXB_2, as it was also observed in plasma treated with 3 μM indomethacin (Figure 1). Indomethacin did not influence the baseline plasma level of $iTXB_2$, but strongly suppressed the rise due to YAP (Figure 1 and Table 2). The rise of $iTXB_2$

Table 1 Complement titre, perfusion pressure in lobar artery and increase in lobe weight after exposure of dog lung lobes to plasma with activated complement at 90 min

Parameter		Time (min)	*YAP* $n = 4$	*Indo + YAP* $n = 3$	*ZAP* $n = 4$
Complement titre					
CH50	units ml^{-1}	45	264 ± 23	236 ± 17	256 ± 15
CH50	units ml^{-1}	105	135 ± 5*	43 ± 10*	104 ± 33*
Decrease	units ml^{-1}	105	−129 ± 22	−193 ± 8	−152 ± 23
Perfusion pressure					
pPa	mmHg	45	6.3 ± 1.0	5.4 ± 0.9	5.5 ± 1.4
pPa	mmHg	90	6.5 ± 1.0	7.4 ± 1.2	6.0 ± 2.0
pPa	mmHg	peak 1	13.1 ± 1.8*	8.9 ± 1.1	8.3 ± 2.3
pPa	mmHg	peak 2	12.6 ± 0.9*	12.4 ± 0.6*	15.5 ± 4.5*
pPa	mmHg	110	7.6 ± 1.2	9.7 ± 0.6	10.9 ± 5.2
pPa	mmHg	150	6.5 ± 1.0	8.8 ± 0.8	6.8 ± 2.5
Lobe weight					
Increase	g	150	9.3 ± 2.3	−0.5 ± 1.5	20.0 ± 14.4
Increase	%	150	17.0 ± 5.0	−1.1 ± 3.3	64.0 ± 48.8

Peak 1, 0.5 to 1 min after plasma exchange; peak 2, 5 to 12 min after plasma exchange. YAP, yeast-activated plasma; Indo, 3 μM indomethacin; ZAP, zymosan-activated plasma; * significantly different from baseline measurement, paired Student's t test.

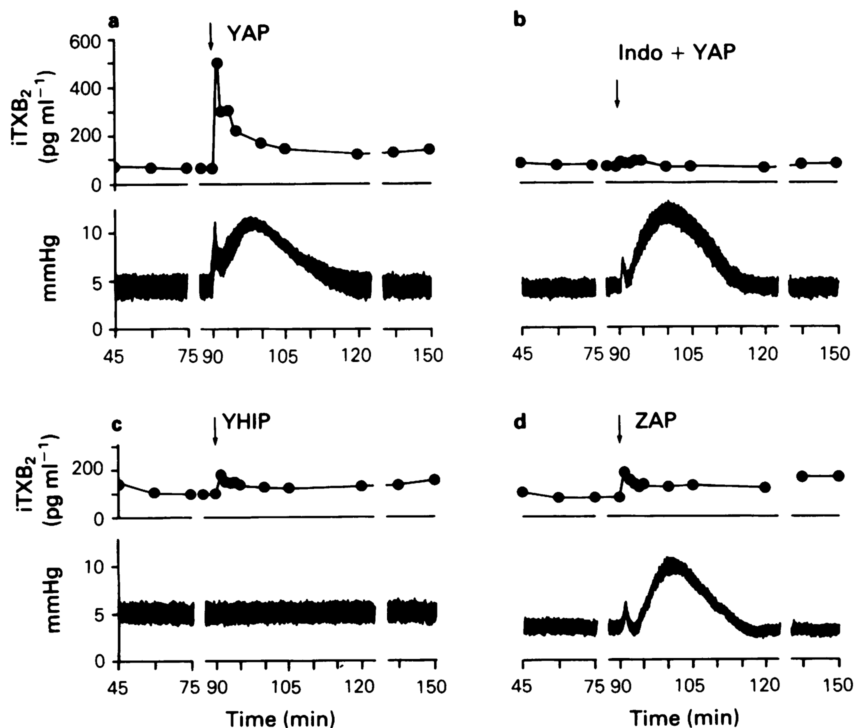

Figure 1 Pressure in the lobar artery and concentration of immunoreactive thromboxane B_2 ($iTXB_2$) in the venous effluent of four lung lobes before and after addition (arrows) of yeast-activated plasma (YAP, a), YAP in the presence of 3 μM indomethacin (Indo + YAP, b), yeast treated heat-inactivated plasma (YHIP, c) or zymosan-activated plasma (ZAP, d).

Table 2 Increase of immunoreactive thromboxane B_2 ($iTXB_2$) in the venous effluent after introduction of activated plasma at 90 min

Time (min)	*YAP* $n = 4$ ($pg\,ml^{-1}$)	*Indo + YAP* $n = 3$ ($pg\,ml^{-1}$)	*ZAP* $n = 4$ ($pg\,ml^{-1}$)
91	361 (214 – 817)	21 (9 – 52)*	77 (42 – 144)
110	72 (29 – 185)	2 (0 – 39)*	41 (19 – 86)
150	86 (27 – 269)	3 (0 – 33)*	50 (16 – 150)

Values represent geometric means and their 95% confidence limits. YAP, yeast-activated plasma; Indo, 3 μM indomethacin; ZAP, zymosan-activated plasma. *Significantly different from YAP, Student's t test of log ($pg\,ml^{-1}$ + 1).

was much less pronounced when heat-inactivated plasma, after a contact period of 5 min with yeast cells, was introduced into the pulmonary circuit. The peak in $iTXB_2$ also occurred after infusion of ZAP, but was smaller than with YAP.

Although the first effluent was discarded, some of the PGI_2 formed during the lobe removal and installation, was still trapped in the circuit. In contrast to $iTXB_2$, the initial concentration of PGI_2 plus 6-oxo-$PGF_{1\alpha}$ did not decrease ($662 \pm 138\,pg\,ml^{-1}$ at 15 min and $708 \pm 129\,pg\,ml^{-1}$ at 45 min, $n = 11$). Indomethacin had no effect on the i6-oxo-$PGF_{1\alpha}$ concentration ($-26 \pm 31\,pg\,ml^{-1}$ or $-4 \pm 5\%$ decrease between 60 and 90 min, $n = 3$). Since virtually all i6-oxo-$PGF_{1\alpha}$ accumulated in the circuit, the total amount of PGI_2 produced by the lobe was calculated from the increments in the concentration and the circulating volume. The PGI_2 production in the first 60 min was variable, but very low (between less than 1 and $101\,pg\,g^{-1}\,min^{-1}$). The mean production rate ($23 \pm 11\,pg\,g^{-1}\,min^{-1}$, $n = 11$) was statistically not different from zero.

Pulmonary PGI_2 biosynthesis increased dramatically after exposure to YAP or ZAP (Figure 2), and was maintained throughout the rest of the experiment. In the presence of indomethacin the rate of PGI_2 formation did change, but with some delay: no immediate increase was noted after the introduction of YAP but 60 min later the production of PGI_2 became pronounced. After CVF injection the PGI_2 formation increased from $1\,pg\,g^{-1}\,min^{-1}$ (between 15 and 90 min) to 23, 82, 420 and $505\,pg\,g^{-1}$ (15, 30, 45 and 60 min respectively after CVF).

Kinetic parameters of 5-HT uptake

Examples of the indicator dilution curves are shown in Figure 3. The non-diffusing dye was used as a reference for calculation of the arterial 5-HT concentration which entered the capillaries (Ca). The difference between Ca and Cv (the actual 5-HT concentration in the venous aliquot) is the amount taken up on passage through the lung. The ratio Ca × $(Ca - Cv)^{-1}$ × 100 gives the percentage extraction of 5-HT in each fraction; these percentages are shown for the bolus with a 'low' (25 nmol) and a 'high' (100 nmol) 5-HT content. Due to saturation of the facilitated uptake system, the extraction

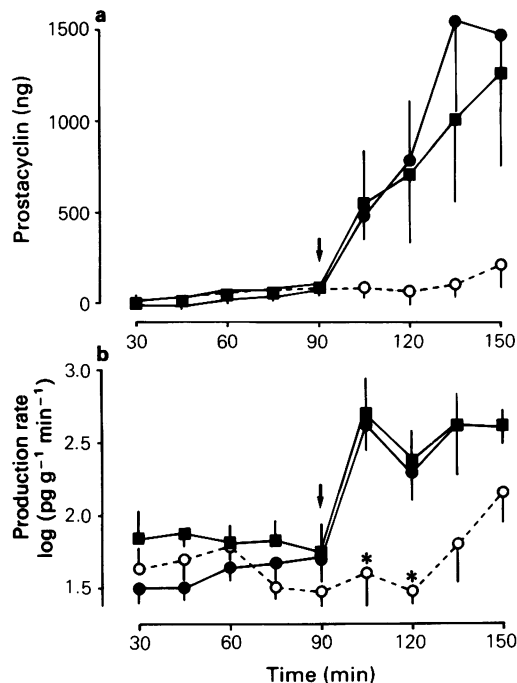

Figure 2 Biosynthesis of prostacyclin (PGI_2) in lung lobes before and after addition of yeast-activated plasma (YAP, ●, $n = 3$–4), 3 μM indomethacin (Indo) + YAP (○, $n = 3$) or zymosan-activated plasma (ZAP, ■, $n = 4$) at 90 min (arrow). The sum of immunoreactive PGI_2 and 6-oxo-$PGF_{1\alpha}$ was measured in the venous effluent at 15 min intervals. The total amount produced by the lung ($ng_{(t\,min)} - ng_{(15\,min)}$) is shown in (a), and the rate of PGI_2 formation (log ($[pg_{(t\,min)} - pg_{(t-15\,min)}]\,15^{-1}\,g^{-1}$)) in (b). The s.e. mean is only shown when it exceeded the height of the symbol. * Significantly different from YAP (Student's t test).

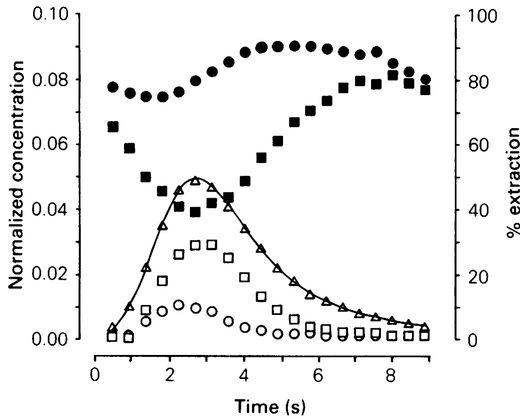

Figure 3 The normalized concentrations (C/m, ml^{-1}) of indocyanine green dye (△, solid line) and [^{3}H]-5-HT (○ = 25 nmol; □ = 100 nmol) in fractions of the venous outflow from an isolated lung lobe and the corresponding (● = 25 nmol; ■ = 100 nmol) percentage extraction (Ca × $(Ca - Cv)^{-1}$ × 100). C is the plasma concentration of the indicator and m the injected mass of indicator. Ca is the indocyanine green concentration times the mass of 5-HT injected divided by the mass of dye injected, and Cv is the 5-HT concentration in the venous effluent. The dye curves of both injections were virtually superimposable and, therefore only one is shown.

decreased when increasing 5-HT concentrations were presented to the capillaries. This can be noticed from reduced extraction with the higher 5-HT bolus at any given concentration of the reference indocyanine green (Ca), or from the decreased extraction within each bolus with increasing indocyanine green concentrations.

The indicator dilution data of each pair of bolus injections were analysed according to the lung model for 5-HT uptake to determine the apparent K_m and V_{max}, assuming that the facilitated carrier transport obeys Michaelis Menten kinetics. The baseline values for these parameters were 1.62 ± 0.20 μM (apparent K_m, $n = 11$) and 0.424 ± 0.040 nmol $g^{-1} s^{-1}$ (apparent V_{max}, $n = 11$). Neither K_m nor V_{max} were altered by indomethacin (Table 3). The apparent K_m was elevated 20 min after the exposure to activated complement (increase 1.02 ± 0.50 μM, $n = 11$). The increase was statistically significant for YAP (Table 3), but not for ZAP. In this group a very pronounced increase in one lung lobe caused more variation in the data. Thereafter, the apparent K_m returned to the baseline values (increase 0.12 ± 0.14 μM, $n = 11$). An enhanced apparent K_m was also noted after CVF injection (K_m 1.16, 1.61, and 1.03 μM, 10 min before and 20 and 60 min after CVF respectively). K_m did not change when heat-inactivated plasma was treated with yeast and then returned to the perfusion circuit (K_m 2.88, 2.85, and 2.92 μM, respectively 10 min before, as well as 20 and 60 min after addition of yeast-treated plasma).

The apparent V_{max} did not alter when assessed 20 min (increases 0.044 ± 0.029 nmol $g^{-1} s^{-1}$, $n = 11$) or 60 min (change −0.012 ± 0.025 nmol $g^{-1} s^{-1}$, $n = 11$) after exposure of pulmonary endothelium to activated complement (Table 3). Neither CVF (V_{max} 0.542, 0.581 and 0.464 nmol $g^{-1} s^{-1}$ at 80, 110 and 150 min respectively) nor yeast treatment of heat-inactivated plasma (V_{max} 0.541, 0.542, and 0.539 nmol $g^{-1} s^{-1}$ at 80, 110 and 150 min respectively) changed the apparent V_{max}.

Cell counts

The red blood cell count increased during the experiment, particularly after the introduction of activated plasma (724 ± 162, 1540 ± 463 and 1682 ± 972 erythrocytes μl^{-1}, at 90, 105 and 150 min

Table 3 Apparent K_m and V_{max} values of endothelial 5-HT uptake before and after exposure to yeast-activated plasma (YAP), 3 μM indomethacin (Indo) + YAP, or zymosan-activated plasma (ZAP) at 90 min

Parameter		*Time* (min)	*YAP* $n = 4$	*Indo + YAP* $n = 3$	*ZAP* $n = 4$
K_m	μM	50	—	1.05 ± 0.28	—
K_m	μM	80	1.65 ± 0.24	1.26 ± 0.25	1.87 ± 0.46
K_m	μM	110	2.49 ± 0.26*	1.47 ± 0.13	3.66 ± 1.68
K_m	μM	150	1.55 ± 0.26	1.50 ± 0.22	2.11 ± 0.55
V_{max}	$nmol.s^{-1}.g^{-1}$	50	—	0.41 ± 0.06	—
V_{max}	$nmol.s^{-1}.g^{-1}$	80	0.40 ± 0.08	0.42 ± 0.05	0.45 ± 0.07
V_{max}	$nmol.s^{-1}.g^{-1}$	110	0.41 ± 0.06	0.48 ± 0.04	0.52 ± 0.03
V_{max}	$nmol.s^{-1}.g^{-1}$	150	0.34 ± 0.08	0.45 ± 0.02	0.47 ± 0.06

* Significantly different from the baseline value at 80 min (paired Student's *t*-test, $p < 0.05$) and from Indo + YAP (Student's *t*-test).

respectively) and a gradual increase of the number of leukocytes was observed (32 ± 6, 41 ± 21 and 119 ± 55 cells μl^{-1} at 80, 105 and 150 min).

Correlations

Several of the variables were positively correlated, even when the lobe with extreme responses to ZAP was excluded from the analyses (i.e. $n = 10$). The apparent K_m of 5-HT uptake (measured after 20 min) and the final oedema showed a positive correlation ($R_S = 0.70$, $P = 0.038$, Figure 4a) and this was also seen for the acute increase of the perfusion pressure and the change in K_m ($R_S = 0.78$, $P = 0.030$). The increase of the venous concentration of TXB_2 at 1 min was positively correlated to the first ($R_S = 0.73$, $P = 0.028$), but not to the second ($P = 0.494$) rise of perfusion pressure. Positive correlations also existed between the $iTXB_2$ peak and the final oedema ($R_S = 0.876$, $P = 0.009$, Figure 4b) and between K_m and the rate of PGI_2 formation at 20 min ($R_S = 0.786$, $P = 0.020$) as well as the final oedema and the PGI_2 formation rate at 20 min ($R_S = 0.852$, $P = 0.011$).

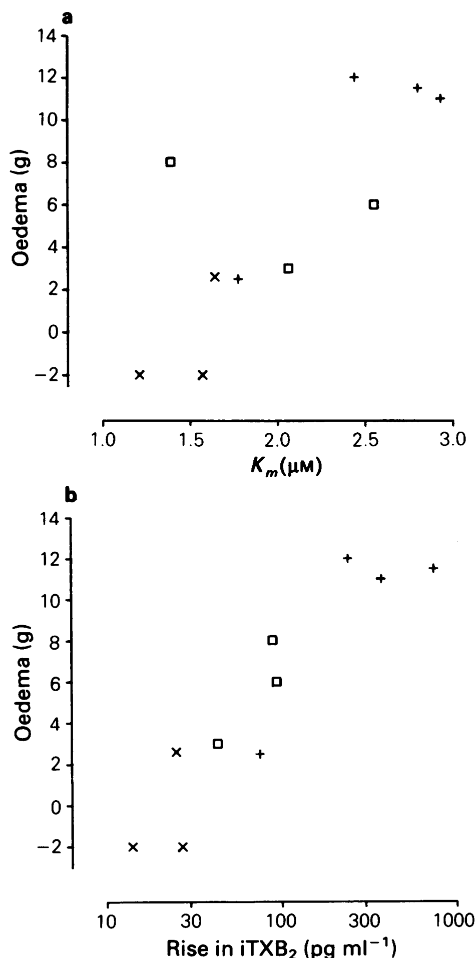

Figure 4 (a) Relationship between K_m, measured 20 min after plasma exchange, and oedema after 60 min. $R_S = 0.70$, $P = 0.038$. (b) Relationship between increase of immunoreactive thromboxane B_2 ($iTXB_2$) (logarithmic scale) and the increase of the lobe weight. $R_S = 0.85$, $P = 0.017$. (+) = yeast-activated plasma (YAP); (□) = zymosan-activated plasma, (×) = indomethacin (3 μM) + YAP.

Discussion

The dog lunglobes were perfused with plasma at a flow rate equivalent to the normal cardiac output and then exposed to ZAP or YAP, presumably containing C5adesArg. C5a-like activity was indeed detectable in plasma after treatment with yeast, zymosan or CVF. It contracted the guinea-pig ileum; the responses were liable to tachyphylaxis, part of the spasmogenic activity being due to histamine release and another part to cyclo-oxygenase products. It did not accumulate in plasma unless the carboxypeptidase inhibitor EACA was present. These are all characteristics of C5a of most species (Vogt, 1974; Stimler, 1986). Prevention of the rapid C5a degradation was not possible in the perfusion studies, as EACA caused vasoconstriction and severe endothelial injury with immediate oedema. Direct evidence for the presence of C5adesArg was not obtained, but the experiment with heat-inactivated plasma clearly indicated that the changes induced by YAP were complement-dependent. Moreover, selective and gradual complement activation with CVF caused similar changes in endothelial activity, i.e. a diminished affinity of the 5-HT carrier site for its substrate and induction of PGI_2 biosynthesis. In addition to C5adesArg, products liberated from yeast cells during complement induced lysis could have contributed further to the effects of YAP. This could explain the gradual differences between YAP and ZAP, in which soluble yeast products were absent.

The increased perfusion pressure after the plasma exchange is in accordance with the transient rise of the pulmonary artery pressure after ZAP infusion in sheep (Meyrick & Brigham, 1984; Egan *et al.*, 1985). It developed virtually without circulating leukocytes and cannot be due to embolization with aggregated granulocytes. The unchanged V_{max} of 5-HT uptake after 20 min also implied that large scale emboliza-

tion did not occur or that vascular recruitment was sufficient to compensate for occluded microvessels. The first vasoconstriction was partly mediated by TXA_2 because the venous peak of $iTXB_2$ in individual lobes was mirrored in the acute pressure response (Figure 1), the maximum of both parameters was positively correlated, and both were suppressed by indomethacin (Figure 1 and Table 2). The very acute pulmonary hypertension after *in vivo* ZAP or endotoxin injection is also partly mediated by TXA_2, in contrast to the secondary pulmonary vasoconstriction (Perkowski *et al.*, 1983; Winn *et al.*, 1983; Larsen *et al.*, 1985; Brigham & Meyrick, 1986). Our experiments eliminated platelets as a TXA_2 source, confirming *in vivo* results in sheep in which the thromboxane formation in response to ZAP was also independent of circulating formed elements (Egan *et al.*, 1985). The nature of the mediators responsible for the slowly developing second vasoconstriction is unclear. Indomethacin did not reduce the second pressure peak, thereby excluding involvement of vasoconstrictor cyclo-oxygenase products. In general, C5adesArg does not release histamine and as YAP and ZAP (without EACA) did not cause constriction of the guinea-pig isolated ileum, involvement of histamine in the vascular responses appears to be unlikely. Platelet activating factor (Paf) and SRS-A, a mixture of the leukotrienes C_4, D_4 and E_4 are possible candidates for the late stages. Indirect evidence suggests that C5adesArg may induce formation of Paf and lipoxygenase metabolites (Stimler-Gerard, 1986).

The increased lobe weight at the end of the experiment was positively correlated to the first pressure peak and the plasma $iTXB_2$ level. However, an increased perfusion pressure alone was not sufficient to induce plasma exudation, since the second pressure wave and its duration in the presence of indomethacin were as pronounced as the first peak in lobes treated with YAP alone, and yet extravascular fluid accumulation was absent. CVF-induced pulmonary plasma leakage in rabbits is also suppressed by cyclo-oxygenase inhibition (Larsen *et al.*, 1985). Both findings are at variance with the observation that the increased flow of protein-rich pulmonary lymph in ZAP-treated sheep is not mediated by TXA_2 or other cyclo-oxygenase products (Cooper *et al.*, 1980; Perkowski *et al.*, 1983). It is generally believed that acute pulmonary oedema is the result of increased pulmonary capillary pressure and/or increased exchange vessel permeability. Cyclo-oxygenase products may affect vascular permeability indirectly through alterations in hydrostatic gradient favouring net fluid infiltration (Perkowski *et al.*, 1983).

The mathematical model developed by Rickaby *et al.* (1984) allows evaluation of active processes on the endothelial surface in terms of the Michaelis-Menten kinetics, independently from the haemodynamic changes which affect the contact period. Intra-endothelial metabolism of 5-HT does not interfere with the assessment of 5-HT uptake, as intracellular 5-HT metabolism is slow relative to the uptake process and very little 5-hydroxyindoleacetic acid appears in the perfusate during first passage of the bolus curve (Dawson *et al.*, 1987). The validity of the model is suggested by the increased K_m in the presence of imipramine, an inhibitor of cellular 5-HT-uptake, and the reduced V_{max} after partial embolization of the lung (Rickaby *et al.*, 1984). Furthermore, the baseline apparent K_m found in the present experiments was in the order of magnitude of values obtained under steady state conditions (Malcorps *et al.*, 1984) or in cultured cells (Catravas & Watkins, 1985).

The number of carrier sites available to circulating 5-HT remained relatively constant, but their affinity for 5-HT decreased transiently after exposure to YAP, ZAP or selective complement activation with CVF. This can be concluded from the unchanged apparent V_{max} and the increased K_m after 20 min, but not after 60 min. It suggests that the luminal plasma membranes of pulmonary endothelial cells temporarily lose their optimal functions under the stress of complement activation. Indeed, the increase of K_m was positively correlated to the oedema measured at the end of the experiment. The decreased 5-HT uptake and angiotensin I converting enzyme (ACE) activities after endotoxin administration to rabbits are also due to an increased K_m rather than a decreased V_{max} (Moalli *et al.*, 1984). The transient nature of the injury is in accordance with the reversible pulmonary distress during haemodialysis, and the transient morphological injury of pulmonary endothelium after ZAP injection in sheep (Meyrick & Brigham, 1984). The factors responsible for the deleterious effect of YAP or ZAP on the endothelial membrane are unknown. The correlation between the TXA peak or the acute hydrostatic pressure peak and the subsequent increase of K_m (Figure 4b) suggest, but do not prove a causality. In cultured endothelial cells, the reduction of 5-HT uptake induced by reactive oxygen species is probably caused by membrane fluidity changes altering the 5-HT carrier or its movement in the lipid environment in which it functions (Block *et al.*, 1986).

Some i-6-oxo-$PGF_{1\alpha}$, formed during lobe removal and installation when circulation and ventilation had to be arrested, was trapped in the perfusate. Its concentration remained relatively stable, suggesting that PGI_2 and/or 6-oxo-$PGF_{1\alpha}$ were not cleared by pulmonary endothelium, or that elimination and formation rate were equal. The initial concentration did

not change after blockade of PGI_2 biosynthesis with indomethacin. This confirmed directly that PGI_2 and/or 6-oxo-$PGF_{1\alpha}$ are not actively cleared by pulmonary endothelium (Salmon *et al.*, 1979). Thus, arterial PGI_2 reflects its presence in mixed venous blood as well as its secretion by the lungs.

PGI_2 biosynthesis was often undetectable in the control period, in spite of the pulsations in the arterial bed produced by the roller pump and the rhythmic expansions of the alveoli. This mimicked the absence of detectable pulmonary PGI_2 formation in anaesthetized rabbits (Bult *et al.*, 1980; 1985a) and dogs, even after hyperventilation (Vercruysse & Bult, unpublished results). However, when dog pulmonary endothelium came into contact with ZAP or YAP, PGI_2 was immediately formed. Similar results have been obtained when humans are submitted to extracorporeal perfusion (Leithner *et al.*, 1980; Edlund *et al.*, 1981), after ZAP injection in sheep (Cooper *et al.*, 1980; Perkowski *et al.*, 1983) or after selective complement activation in the rabbit (Bult *et al.*, 1985a). Indeed, ZAP, C5a and C5adesArg are potent stimulators of PGI_2 release in rabbit aortic endothelium (Rampart *et al.*, 1983) and vascular strips (Hugli & Marceau, 1985). Thus, biologically relevant PGI_2 concentrations are not present in the arterial circulation, but are promptly generated in response to C5adesArg. It supports the hypothesis that local induction of endothelial PGI_2 release by activated complement is a mechanism able to initiate and maintain a substantial blood flow for recruitment of plasma factors and phagocytes to an acute inflammatory response (Rampart *et al.*, 1983; Bult & Herman, 1985; Hugli & Marceau, 1985).

The high rate of endothelial PGI_2 formation was maintained after ZAP or YAP, whereas PGI_2 formation in response to various stimuli by perfused rabbit arteries (Van Hove & Bult, unpublished results) or piglet lungs (Hellewell & Pearson, 1984) is normally short lasting and liable to rapid desensitization. It could mean that different areas of the lobar endothelium were sequentially activated to produce PGI_2 for a short period of time. The unchanged apparent V_{max} of pulmonary 5-HT uptake indicated that there was no overall alteration of the perfused endothelial surface, but this does not exclude that recruitment took place. The experiments with indomethacin confirmed that endothelial PGI_2 biosynthesis is readily blocked by non-steroidal anti-inflammatory drugs (e.g. Bult *et al.*, 1980; Cooper *et al.*, 1980; Ikeda *et al.*, 1983) but after 60 min perfusion PGI_2 biosynthesis began to rise. This may be due to instability of indomethacin in plasma, or its clearance and/or metabolism in the lung lobe. Furthermore, it pointed to the prolonged presence of stimuli for PGI_2, but not for TXA_2 biosynthesis, a finding consistent with *in vivo* administration of CVF (Bult *et al.*, 1985a) or endotoxin (Ikeda *et al.*, 1983; Bult *et al.*, 1985b; Bult & Herman, 1985; Flynn, 1985; Slotman *et al.*, 1985). In rabbits, PGI_2 formation in response to endotoxin depends to some extent on complement activation (Bult *et al.*, 1985b).

The present *in vitro* experiments indicate that healthy pulmonary endothelium releases virtually no PGI_2 unless it is injured. PGI_2 release was proportional to both markers of endothelial damage, increased K_m of the 5-HT carrier and oedema. Cells are fully equipped to release this signal, e.g. influx of extracellular calcium is not required (Van de Velde *et al.*, 1986). As PGI_2 indicates endothelial injury, downregulation of its biosynthesis is not required, as it will stop as the endothelial perturbation ends. Therefore, it is not surprising that adenylate cyclase stimulation by PGI_2 in the cells in which it is formed does not affect its rate of formation (Hong, 1983; Bult & Herman, 1985). It also implies that a deficiency in PGI_2 biosynthesis in itself is not sufficient to initiate endothelial damage or vascular injury such as atherosclerotic plaque formation. The PGI_2 released by injured endothelium may help to maintain sufficient blood flow through the affected blood vessel by reducing arterial resistance, by suppressing granulocyte activation and their attack of downstream uninjured endothelium (McGillen *et al.*, 1980; Rampart & Williams, 1986), and by opposing aggregation (Moncada *et al.*, 1976) but not adhesion of platelets. Local release of an autacoid with this spectrum of activities is beneficial, e.g. by reducing endotoxin-induced lung injury (Ikeda *et al.*, 1983), but systemic effects of PGI_2 appear to be detrimental (Bult *et al.*, 1980; Rie *et al.*, 1983; Bult & Herman, 1985; Slotman *et al.*, 1985; 1986).

In conclusion, the attractive hypothesis that healthy endothelium continuously releases PGI_2 to oppose platelet activation (Moncada *et al.*, 1976) seems to be no longer tenable. The present data and those of others (Brox & Nordoy, 1982; Eldor *et al.*, 1983) suggest rather that PGI_2 release is a marker of endothelial distress.

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