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Prostanoid synthesis by aortic rings in human blood: selective increase of prostacyclin mediated by a serum factor

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1 Synthesis of vascular epoprostenol (PGI_2) and platelet thromboxane (TX) A_2 is influenced by the coagulation cascade in incompletely understood ways. To elucidate this, prostanoids were determined in human blood anticoagulated by different drugs and incubated with and without rat aortic rings. Control incubations were performed in Hanks balanced salt solution. PGI_2 and TXA_2 synthesis were assessed by radioimmunoassay of their stable hydrolysis products 6-oxo-prostaglandin (PG) $F_{1\alpha}$ and TXB_2.

2 Fresh aortic rings incubated in Hanks solution with a thrombin inhibitor (TCK) synthesized similar quantities of 6-oxo-$PGF_{1\alpha}$ in the presence or absence of sodium citrate. In contrast, the intracellular calcium antagonist TMB-8 inhibited 6-oxo-$PGF_{1\alpha}$ synthesis.

3 In contrast to the finding in Hanks solution, sodium citrate inhibited 6-oxo-$PGF_{1\alpha}$ synthesis by fresh aortic rings incubated in blood anticoagulated with TCK. However, TXB_2 synthesis was not affected by citrate.

4 Blood incubated alone at 37°C in plain glass tubes generated a small amount of immunoreactive 6-oxo-$PGF_{1\alpha}$. A thromboxane synthase inhibitor, OKY1581, increased immunoreactive 6-oxo-$PGF_{1\alpha}$. However, blood anticoagulated with TCK and incubated similarly, generated no detectable 6-oxo-$PGF_{1\alpha}$ either in the presence or absence of OKY1581, showing that 6-oxo-$PGF_{1\alpha}$ synthesis in the previous experiments was dependent on the vascular rings.

5 OKY1581 had little or no effect on 6-oxo-$PGF_{1\alpha}$ synthesis in incubations of fresh aortic rings with blood anticoagulated with TCK, despite inhibition of TXB_2 synthesis. However, OKY1581 increased 6-oxo-$PGF_{1\alpha}$ synthesis by rings pretreated with acetylsalicylic acid (ASA) when incubated in blood, presumably by diversion of platelet endoperoxide to vascular PGI_2 synthase.

6 Sodium citrate did not influence the increase in 6-oxo-$PGF_{1\alpha}$ synthesis by ASA pretreated aortic rings caused by OKY1581 in whole blood. This implies that the PGI_2 stimulating activity of whole blood in the absence of citrate exerts its effect proximal to PGI_2 synthase.

7 It is concluded that a low molecular weight serum factor formed during activation of the intrinsic coagulation pathway in blood, modulates PGI_2/TXA_2 balance by an action on vascular cyclo-oxygenase, possibly by an effect on intracellular calcium.

Introduction

Haemostosis and thrombosis are determined by interdependent processes involving platelets, blood vessels, and the coagulation cascade. Metabolites of arachidonic acid may be important in these processes (Moncada & Vane, 1979). Such metabolites include the pro-aggregatory vasoconstrictor thromboxane A_2 (TXA_2), formed principally by platelets, and the antiaggregatory vasodilator epoprostenol (PGI_2), synthesized mainly in blood vessels. Both these prostanoids are unstable and although PGI_2 escapes uptake and metabolism in the lungs, its overall rate of synthesis is low (Fitzgerald *et al.*, 1981) and the plasma concentration of 6-oxo-$PGF_{1\alpha}$ in healthy humans is too low for PGI_2 to act as a circulating antiaggregatory hormone in this circumstance (Blair *et al.*, 1982). Thus in common with other prostaglan-

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dins, PGI_2 and TXA_2 probably exert their effects locally. It is hard, however, to envisage a locally acting mediator operating effectively at an interface between a damaged endothelial surface and flowing blood which will rapidly remove it from its site of synthesis. This focusses attention on the coagulation process: mediators secreted into an evolving fibrin gel may be free to diffuse to a local site of action without being rapidly diluted by bulk flow. Since prostanoids are not stored prior to release, their rate of synthesis is crucial in determining their concentration in the local milieu, raising the question of control of prostaglandin synthesis by the coagulation process.

There are several connections between the coagulation cascade and prostanoid synthesis. Thus, thrombin stimulates PGI_2 synthesis by cultured endothelial cells (Weksler *et al.*, 1978) and so does bradykinin (Hong, 1980). Thrombin is also important in the platelet release reaction and TXB_2 synthesis (Shuman & Levine, 1980; Remuzzi *et al.*, 1983). We previously obtained evidence of a low molecular weight substance formed during intrinsic coagulation that stimulates PGI_2 synthesis by fresh rat aortic rings (Ritter *et al.*, 1982a; 1983). This serum factor (SF) was not thrombin since it was dialysable and stable at 100°C for 5 min. Nor was it bradykinin, which is rapidly enzymically inactivated in plasma. The purpose of the present work was to extend these observations by measuring 6-oxo-$PGF_{1\alpha}$ and TXB_2 in incubations of aortic rings in whole blood using an anticoagulant that inhibits thrombin, thereby blocking the coagulation mechanism distally. The drug used was D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (TCK), a tripeptide which is an affinity label of thrombin (Kettner & Shaw, 1979). Incubations in blood anticoagulated with TCK were performed with and without added citrate, which blocks the coagulation mechanism at several stages, including proximal steps, by lowering ionised calcium (for review see Ratnoff, 1977). It was argued that in the absence of citrate, SF would be formed by activation of the intrisic pathway, but that the TCK would prevent coagulation. (Clot formation would have prevented equal agitation of the rings in experimental and control incubations). This approach was initiated because of a prior observation that 6-oxo-$PGF_{1\alpha}$ synthesis by aortic rings is little affected by external calcium concentration *per se* (Ritter *et al.*, 1982a). This was verified using control incubations in Hanks balanced salt solution, which contains divalent cations (calcium 1.26 mM; magnesium 0.9 mM), but not coagulation factors. Subsequent experiments were performed with inhibitors of prostaglandin and thromboxane synthesis to elucidate further the processes involved.

Methods

Aortic ring incubations

Aortic rings were prepared by methods similar to those described previously (Bunting *et al.*, 1976; Ritter *et al.*, 1982a, b). Male CD rats (Charles River, Portage, Michigan, U.S.A.), 150–250 g, were anaesthetized with ether. The aorta was removed rapidly and raised in Gey balanced salt solution (Gibco, Grand Island, N.Y., U.S.A.) with added $NaHCO_3$ (2 mM), at 4°C. It was cut into 0.7 mm rings with a McIlwain tissue chopper (Mickle Engineering Company, Guildford, Surrey). Rings were individually allocated to one of two, three or four groups, depending on the experiment, so as to minimize differences between groups. Usually four groups of 16 rings were prepared from each rat, and each group of rings was incubated in a volume of 1.5 ml. The rings were kept at 4°C in Gey solution for less than 30 min before the incubation. This was started by adding the rings to incubation fluid at 37°C in siliconized glass tubes (16 × 100 mm) from Becton-Dickinson Co (Rutherford, N.J., U.S.A.) and was performed with constant shaking. Incubation media were either Hanks balanced salt solution (Gibco, Grand Island, N.Y. U.S.A.) or freshly drawn human blood. Blood was obtained by venepuncture using a 21 gauge Butterfly needle (Abbott, North Chicago, Ill., U.S.A.) from healthy male volunteers aged 25–39 who had abstained from aspirin and other antiinflamatory drugs for at least 2 weeks. Blood was immediately added to tubes containing anticoagulant and/or other drugs, mixed and placed in a 37°C water bath. Aortic rings were added within 1–2 min. The anticoagulants used were the antithrombin drug D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone, 2HCl, $3H_2O$ (TCK) from Calbiochem-Behring Corp (La Jolla, Ca, U.S.A.) and trisodium citrate $2H_2O$ from Fisher Scientific Co (Fair Lawn, NJ, U.S.A.). The TCK was made up as a 10^{-2} M solution in distilled water and stored in siliconized tubes at −30°C. Once thawed, any unused residue was discarded. Trisodium citrate was made up as a 3.1% (w/v) stock solution (105 mM) and one volume added to nine volumes of blood. Since citrate distributes extracellularly, the expected plasma concentration is thus 16.5 mM with a packed cell volume of 40%. This was confirmed in two neutralized perchloric acid extracts of plasma using an enzymatic assay for citrate (Dagley, 1974), the values obtained being 16.3 mM and 16.7 mM. In comparisons in the presence or absence of citrate, an equal volume of physiological saline (sodium chloride 140 mM) was added to the control tube. Other drugs used were sodium (E)-3-[4-(3-pyridylmethyl) phenyl]-2-methacrylate

(OKY1581), a gift from Ono Pharmaceutical, Japan and 8-(N, N-diethylamino)-octyl-3, 4, 5 trimethoxybenzoate (TMB-8) which was purchased from the Aldrich Chemical Company (Milwaukee, Wis, U.S.A.). Both were dissolved in physiological saline and added in volumes of 5–15 μl. Equal volumes of saline were added to the control tubes. Samples were collected from the incubations at timed intervals. When sampling from blood it was difficult to avoid aspirating the rings. This was best achieved by gentle suction, with the tip of the pipette positioned near the bottom of the tube. Samples were immediately spun for 4 min at 8,800 *g* in an Eppendorf 5413 centrifuge, the plasma aspirated and stored at −20°C. Samples of Hanks solution were stored similarly but were not spun. In some experiments rings were pretreated with acetylsalicylic acid (ASA) from Sigma (St. Louis, Mo, U.S.A.). Rings were incubated at 37°C in 5 ml Gey solution containing ASA(10^{-3} M) for 30 min. The solution was then decanted and each group of rings washed 3 times in 5 ml changes of Gey solution before adding them to the blood in which they were subsequently incubated. At the end of each experiment the rings were blotted and weighed.

Incubations of blood alone

These control incubations were performed by a method similar to that of Patrono *et al.* (1980). The blood was drawn under the same conditions as described above and 1.5 ml incubated at 37°C in borosilicate tubes, 16 × 100 mm (Fisher Scientific Company, Pittsburgh, PA, U.S.A.). Serum or plasma were separated and stored as above.

Radioimmunoassay of 6-oxo-$PGF_{1\alpha}$ and TXB_2

Standards were from Upjohn (Kalamazoo, Mi, U.S.A.) and tritiated ligands from Amersham International (Evanson, Ill, U.S.A.). The antibody to 6-oxo-$PGF_{1\alpha}$ was a gift from Dr M.J. Dunn (Cleveland, Ohio, U.S.A.) and that to TXB_2 was a gift from Dr. W. Campbell (Dallas, Texas, U.S.A.). The specificity and sensitivity of these antibodies are published elsewhere (Beck *et al.*, 1980). The only cross reactions greater than 0.1% were between the antibody to 6-oxo-$PGF_{1\alpha}$ and $PGF_{2\alpha}$ (2%) and to other one-series prostaglandins. Assays were performed on unextracted samples. Where possible, three dilutions were selected that bracketed 50% displacement on the standard curve, using a concentration of anti-6-oxo-$PGF_{1\alpha}$ of 1:15,000 and of anti-TXB_2 of 1:225,000 in a final volume of 0.3 ml. In no case were values accepted outside 20–80% displacement of ^{3}H-ligand. Diluted samples from incubations with aortic rings were assayed for 6-oxo-$PGF_{1\alpha}$ in the presence of a final concentration of 1% human serum albumin (essentially fatty acid free) from Sigma (St. Louis, Mo, U.S.A.) to minimize interference with binding as described previously (Orchard *et al.*, 1982) Under these conditions, similarly diluted plasma from fresh unincubated blood gave values that were indistinguishable from controls where buffer replaced sample or standard, and there was agreement between the triplicate checks of different dilutions of unknowns. Serum from incubates of blood alone contained too little immunoreactive 6-oxo-$PGF_{1\alpha}$ to be substantially diluted and they were therefore assayed by the method of Patrono *et al.*, (1982) in which non-specific effects on binding are controlled for by the addition of charcoal treated (prostaglandin-free) plasma or serum (0.1 ml) to each standard and an equal volume of buffer to each unknown. Charcoal-treated sera/plasmas were prepared for each condition studied (in the presence and absence of TCK and of OKY1581). Charcoal 1 g (Norit A, Fisher Scientific Co, Fair Lawn, NJ, U.S.A.) was added to 10 ml serum. The mixture was allowed to stand on ice for 60 min before separation by centrifugation at 4°C. With this method the 6-oxo-$PGF_{1\alpha}$ in plasma from freshly drawn blood was always below the detection limit of the assay, defined as 20% tritiated ligand displacement ($77.3 \pm 4.9\,pg\,ml^{-1}$, mean ± s.e. mean, $n = 12$). Controls were performed that showed that in the absence of unlabelled 6-oxo-$PGF_{1\alpha}$ relevant concentrations of TMB-8, sodium citrate, TCK and OKY1581 had no effect on the binding of tritiated 6-oxo-$PGF_{1\alpha}$ to the antibody. In TXB_2 assays, it was unnecessary to use either albumin or charcoal-treated serum, since at the dilutions used, TXB_2 in plasma from freshly drawn blood was below the detection limit of the assay ($37.4 \pm 4.9\,pg\,ml^{-1}$, mean ± s.e. mean, $n = 5$), and there was agreement between differently diluted triplicate determinations. Controls showed that [^{3}H]-TXB_2 binding was unaffected by relevant concentrations of sodium citrate, TCK and OKY1581.

Statistics

Comparisons were by Student's *t* test, two tailed. Differences were considered significant when $P < 0.05$.

Results

Effect of citrate and TMB-8 on 6-oxo-$PGF_{1\alpha}$ synthesis by aortic rings in Hanks solution

Table 1 shows the results of 6 experiments in which

Table 1 6-oxo-$PGF_{1\alpha}$ synthesis in 60 min incubates of aortic rings in Hanks solution with D-phenylalanyl-L-prolyl-L-argine chloromethyl ketone (TCK) at 37°C in the presence and absence of sodium citrate (16.5 mM)

	6-oxo-$PGF_{1\alpha}$ (ng mg^{-1})		
Expt number	*Citrate*	*Control*	*Control/Citrate × 100%*
1	9.70	10.11	104.2
2	15.33	16.68	108.8
3	10.15	10.77	106.1
4	8.29	8.12	97.9
5	2.70	2.42	89.6
6	3.33	3.22	96.7
Mean ± s.e. mean	8.25 ± 1.92	8.55 ± 2.16	100.6 ± 2.9

rings were incubated in Hanks solution with TCK (7×10^{-5} M) at 37°C for 60 min. Paired experiments were performed comparing sodium citrate (16.5 mM) with controls where an equal volume of physiological saline had been added to the Hanks solution. 6-oxo-$PGF_{1\alpha}$ synthesis was similar ($P > 0.1$) in each condition. Figure 1 shows an experiment in which rings were incubated with or without citrate and with TMB-8 (5×10^{-4} M). It is apparent that the citrate had little if any effect on 6-oxo-$PGF_{1\alpha}$ synthesis, whereas TMB-8 inhibited it substantially. Further experiments demonstrated that the effect of TMB-8 was dose-related, with a steep concentration-effect curve plotted arithmetically in Figure 2. This curve was drawn by eye; the IC_{50} value was estimated to be 1.4×10^{-4} M.

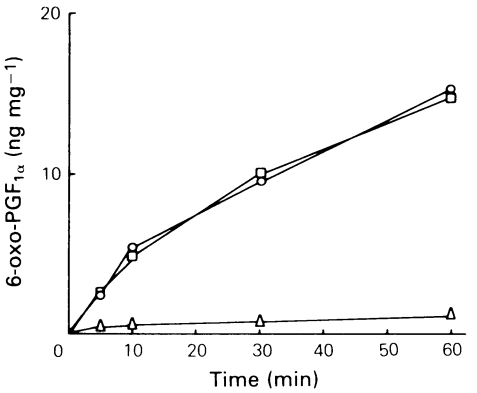

Figure 1 Effect of calcium modifying drugs on 6-oxo-$PGF_{1\alpha}$ synthesis by fresh aortic rings. Matched groups of rings were incubated in Hanks solution at 37°C. 6-oxo-$PGF_{1\alpha}$ synthesis was similar in the presence of 16.5 mM sodium citrate (○) and in the control (□). The intracellular calcium antagonist 8-(N, N-diethylaminal-octyl-3, 4, 5 trimethoxybenzoate (TMB_8) 5×10^{-4} M inhibited 6-oxo-$PGF_{1\alpha}$ synthesis substantially (△). The results of a representative experiment are shown.

Effect of citrate on 6-oxo-$PGF_{1\alpha}$ and TXB_2 synthesis in incubations of aortic rings with blood

Table 2 summarizes the results of 7 experiments in which rings were incubated in fresh human blood anticoagulated with TCK (7×10^{-5} M) at 37°C for 60 min. As with the incubations in Hanks solution, paired experiments were performed comparing sodium citrate (plasma concentration 16.5 mM) with controls where an equal volume of physiological saline had been added to the blood. 6-oxo-$PGF_{1\alpha}$

Table 2 Prostanoid synthesis in 60 min incubates of aortic rings in blood anticoagulated with D-phenylalanyl-L-prolyl-L-argine chloromethyl ketone (TCK) at 37°C in the presence and absence of sodium citrate (plasma concentration 16.5 mM)

	6-oxo-$PGF_{1\alpha}$ (ng mg^{-1})			*TXB_2* (ng ml^{-1})		
Expt number	*Citrate*	*Control*	*Control/Citrate × 100%*	*Citrate*	*Control*	*× 100%*
1	5.01	7.46	148.9	1.60	1.60	100.0
2	8.86	11.43	129.0	1.70	2.00	117.6
3	3.75	5.29	141.1	4.40	4.70	106.8
4	2.24	3.08	137.5	3.68	5.32	144.5
5	6.19	9.40	151.9	4.33	3.93	90.8
6	7.08	8.82	124.6	10.00	6.02	60.2
7	6.61	8.59	129.7	7.90	6.80	86.1
Mean ± s.e. mean	5.68 ± 0.83	7.72 ± 1.05	137.5 ± 3.9	4.80 ± 1.18	4.34 ± 0.74	100.9 ± 10.0

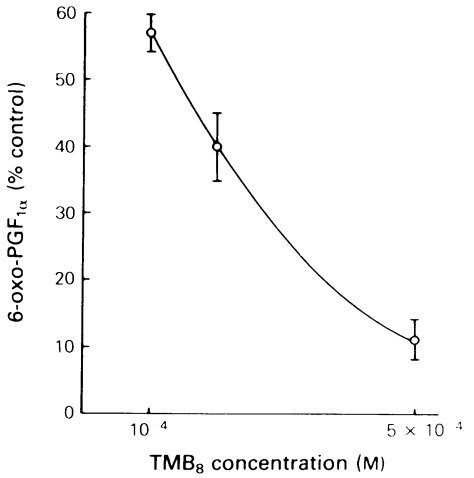

Figure 2 Effect of 8-(N, N-diethylamino)-octyl-3, 4, 5 trimethoxybenzoate (TMB-8) on 6-oxo-$PGF_{1\alpha}$ synthesis by fresh aortic rings. Matched groups of rings were incubated in Hanks solution for 60 min at 37°C with or without the addition of one of three concentrations of TMB-8. In each experiment the inhibition of 6-oxo-$PGF_{1\alpha}$ synthesis was determined as 6-oxo-$PGF_{1\alpha}$ in the presence of TMB-8/6-oxo-$PGF_{1\alpha}$ in control × 100%. The figure shows the mean percentage inhibition at each concentration of TMB-8; $n=5$ at 10^{-4} M, $n=6$ at 2×10^{-4} M and $n=4$ at 5×10^{-4} M. Vertical lines indicate s.e.mean.

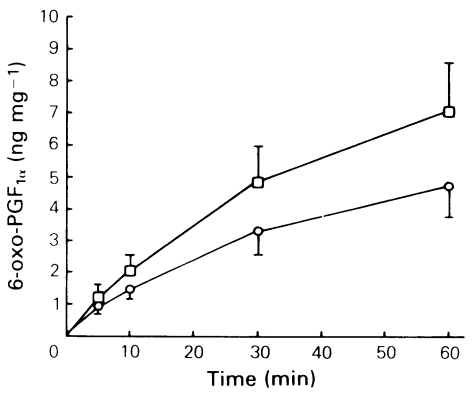

Figure 3 Time course of 6-oxo-$PGF_{1\alpha}$ synthesis in incubations of fresh aortic rings with human blood. Blood was anticoagulated with D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (TCK, 7×10^{-5} M). Paired incubations were performed in the presence of sodium citrate, plasma concentration 16.5 mM (○) and in control blood with an equal volume of physiological saline (□). Means are shown ($n=5$) but significance was determined by paired testing; differences at 10 min and greater were significant; vertical lines show s.e.mean.

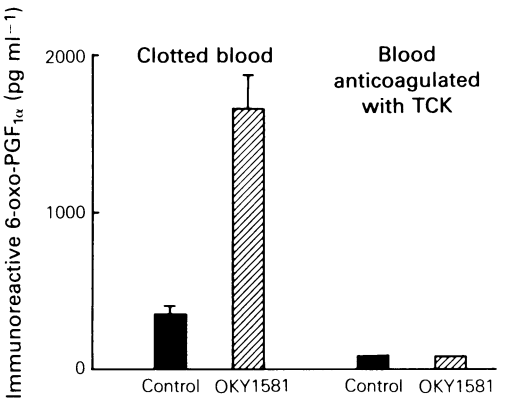

Figure 4 Immunoreactive 6-oxo-$PGF_{1\alpha}$ generated in blood alone. Fresh human blood from 8 subjects was incubated for 60 min in borosilicate tubes at 37°C, in the presence and absence of sodium (E)-3-[4-(3-pyridylmethyl) phenyl]-2 methacrylate (OKY1581) 10^{-5} M. With no other additions (left hand columns) the blood clotted, and immunoreactive 6-oxo-$PGF_{1\alpha}$ was detected in the serum as indicated (mean ± s.e.mean, $n=8$). With D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (TCK) 7×10^{-5} M, no clot formed by 60 min, and any 6-oxo-$PGF_{1\alpha}$ formed was less than the detection limit of the assay, as indicated by the columns at the right.

synthesis was consistently lower in the presence of citrate ($P<0.001$) while no difference in TXB_2 synthesis was evident ($P>0.5$). Figure 3 shows the time course of 6-oxo-$PGF_{1\alpha}$ synthesis in the presence and absence of sodium citrate in 5 experiments. 6-oxo-$PGF_{1\alpha}$ synthesis diverges in the two conditions with progressively more 6-oxo-$PGF_{1\alpha}$ synthesized in the absence of citrate (at 5 min $P>0.3$, from 10 min on $P<0.05$). In contrast, TXB_2 synthesis showed no consistent change between the two conditions as a function of time (data not shown).

Immunoreactive 6-oxo-$PGF_{1\alpha}$ synthesis in blood alone

Blood incubated at 37°C in borosilicate tubes clotted and the serum contained material that displaced [^{3}H]-6-oxo-$PGF_{1\alpha}$ from the antibody (Figure 4). In paired studies OKY1581, 10^{-5} M, increased the immunoreactive 6-oxo-$PGF_{1\alpha}$ from 350 ± 50 pg ml^{-1} serum (mean ± s.e.mean, $n=8$) to 1790 ± 240 pg ml^{-1} (mean ± s.e.mean, $P<0.001$). When blood was incubated similarly but in the presence of TCK, 7×10^{-5} M, it did not clot until after one hour. Plasma collected at 60 min contained no detectable immunoreactive 6-oxo-$PGF_{1\alpha}$ either in the absence or presence of OKY1581, 10^{-5} M. This is shown on the right of Figure 4 where the bars indicate the detection limit of the assay.

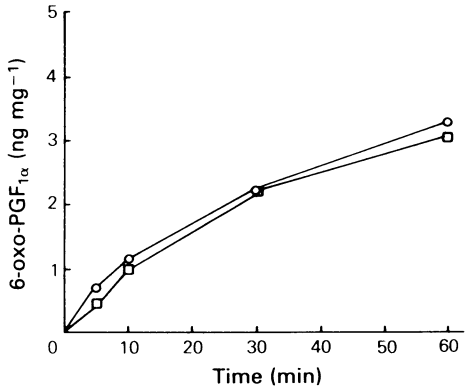

Figure 5 6-oxo-$PGF_{1\alpha}$ synthesis in incubation of aortic rings in blood in the presence and absence of sodium (E)-3-[4-(3-pyridylmethyl) phenyl]-2-methacrylate (OKY1581). Matched groups of rings were incubated in blood anticoagulated with D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (TCK) 7×10^{-5} M in the presence of OKY1581, 10^{-5} M (○) and in a control (□). One experiment is shown representative of two.

Effect of OKY1581 on prostanoid synthesis in incubations of fresh aortic rings with blood

Figure 5 shows an experiment in which matched groups of rings were incubated in blood anticoagulated with TCK, 7×10^{-5} M, in the presence and absence of OKY1581, 10^{-5} M. Little, if any, difference was apparent. TXB_2 in the 60 min samples was 3.93 ng ml^{-1} and undetectable (i.e. < 37 pg ml^{-1}) respectively. In a second experiment, the time course of 6-oxo-$PGF_{1\alpha}$ synthesis by control and OKY1581 treated rings was again very similar. In this experiment the 6-oxo-$PGF_{1\alpha}$ produced at 60 min was 6.2 and 6.6 ng mg^{-1} and the TXB_2 concentrations were 5.32 and 0.08 ng ml^{-1} in control and OKY1581 treated tubes, respectively.

Effect of OKY1581 on prostanoid synthesis in incubations of acetylsalicylic acid-treated aortic rings with fresh blood

Aortic rings pretreated with ASA, 10^{-3} M, at 37°C for 30 min synthesized only small amounts of 6-oxo-$PGF_{1\alpha}$ on subsequent incubation in blood. Under these conditions OKY1581 caused a substantial increase in 6-oxo-$PGF_{1\alpha}$ synthesis. Figure 6 shows an experiment in which the time course of 6-oxo-$PGF_{1\alpha}$ and of TXB_2 synthesis were determined in incubations of matched groups of such ASA-treated aortic rings with fresh whole blood (not exposed to ASA), in the presence and absence of OKY1581. OKY1581 inhibited TXB_2 synthesis (from 23.1 ng ml^{-1} to 71 pg ml^{-1} at 60 min), and there was a rise in 6-oxo-$PGF_{1\alpha}$ from 1.17 ng ml^{-1} to 5.67 ng ml^{-1}. Figure 7 shows the mean data from 7 experiments in which ASA pretreated aortic rings were subsequently incubated for 60 min with fresh blood at 37°C. Each experiment was performed using rings from a different rat, and blood from a different donor. OKY1581 (10^{-5} M) reduced TXB_2 from $10{,}900 \pm 1350$ pg ml^{-1} (mean ± s.e.mean) to 103 ± 17 pg ml^{-1} ($P < 0.001$) and increased 6-oxo-$PGF_{1\alpha}$ from 1440 ± 120 pg ml^{-1} to 3680 ± 320 pg ml ($P < 0.001$).

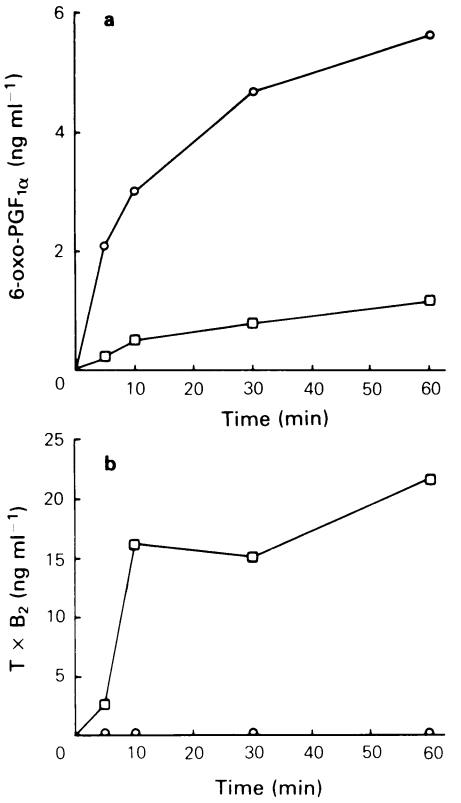

Figure 6 Time course of prostanoid synthesis in incubations of acetylsalicylic acid (ASA)-treated aortic rings in blood in the presence and absence of sodium (E)-3-[4-(3-pyridylmethyl) phenyl)-2-methacrylate (OKY1581) Matched groups of rings were preincubated with ASA, 10^{-3} M in Gey balanced salt solution at 37°C for 30 min. They were then incubated at 37°C with fresh blood anticoagulated with D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (TCK) 7×10^{-5} M in the presence of OKY1581, 10^{-5} M (○) or in control blood containing the same volume of vehicle (□). Inhibition of TXB_2 by OKY1581 (b) is accompanied by increased 6-oxo-$PGF_{1\alpha}$ synthesis (a). One experiment is shown, representative of three.

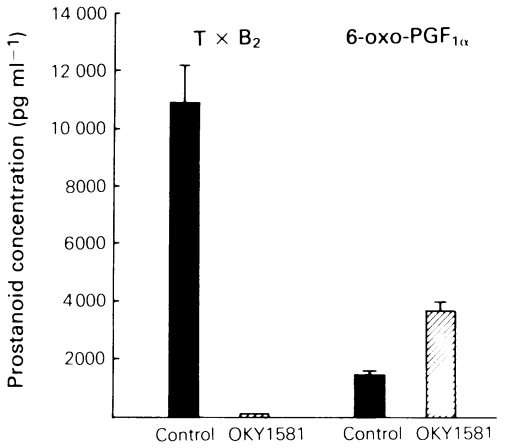

Figure 7 Prostanoid synthesis at 60 min in incubations of acetylsalicylic acid (ASA)-treated aortic rings in blood. Matched groups of rings were pretreated with ASA, 10^{-3} M in Gey balanced salt solution at 37°C for 30 min. They were then incubated at 37°C with fresh blood anticoagulated with D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (TCK) 7×10^{-5} M in the presence of sodium (E)-3-[4-(3-pyridylmethyl) phenyl] -2-methacrylate (OKY1581) 10^{-5} M (hatched columns) or in control blood containing vehicle only (solid columns). TXB_2 synthesis is shown on the left, 6-oxo-$PGF_{1\alpha}$ synthesis on the right. The columns show means with s.e.mean indicated by vertical lines ($n = 7$) except for TXB_2 with OKY1581, where the error is too small to indicate on this scale.

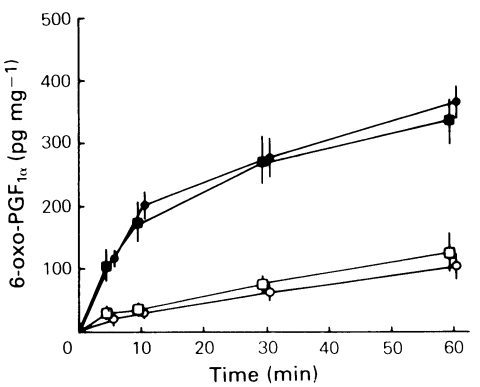

Figure 8 Lack of effect of citrate on sodium (E)-3-[4-(3-pyridylmethyl) phenyl]-2-methacrylate (OKY1581)-enhanced 6-oxo-$PGF_{1\alpha}$ synthesis. Acetylsalicylic acid-treated aortic rings were incubated in fresh blood at 37°C in the presence of OKY1581, 10^{-5} M (solid symbols) or in its absence (open symbols). Paired incubations were performed in the presence of citrate (○, ●) and in its absence (□, ■). Points are means with s.e. mean shown by vertical lines; $n = 4$.

Influence of citrate on 6-oxo-$PGF_{1\alpha}$ synthesis in incubations of acetylsalicylic acid-treated aortic rings with fresh blood, with and without OKY1581

In experiments similar to those described above, the effect of OKY1581 on 6-oxo-$PGF_{1\alpha}$ synthesis by ASA-treated rings, was determined in the presence or absence of sodium citrate, 16.5 mM. The results are shown in Figure 8. OKY1581, 10^{-5} M increased 6-oxo-$PGF_{1\alpha}$ synthesis both in the absence and in the presence of citrate ($P < 0.05$, $n = 4$). The magnitude of the increase was similar in each case ($P > 0.2$).

Discussion

Addition of sodium citrate to Hanks solution lowers the concentration of free divalent cations. This had no detectable effect on aortic PGI_2 synthesis (Table 1, Figure 1). This may appear surprising in view of the known calcium dependency of phospholipases (Brokerhoff & Jensen, 1974). However, we had previously noted only small effects of changing calcium concentration on 6-oxo-$PGF_{1\alpha}$ synthesis by aortic rings in EDTA buffered salt solutions (Ritter & Orchard, unpublished observations), and no effect on adding calcium chloride (20 mM) to dialysed serum (Ritter *et al.*, 1982a) . It is possible that the lack of effect of citrate on PGI_2 synthesis by the intact tissue is because intracellular rather than extracellular calcium is important for acylhydrolase activity. This is to be expected for platelet phospholipase C, which is a cytoplasmic calcium-dependent enzyme (Siess & Lapetina, 1983). It might also be true for phospholipase A_2 and for diglyceride lipase, which are also calcium-dependent (Brokerhoff & Jensen, 1974; Bell *et al.*, 1979) depending on their orientations in the membrane. The inhibition of PGI_2 synthesis by TMB-8 (Figures 1, 2) is consistent with this interpretation, since this drug is an inhibitor of intracellular calcium transport (Malagodie & Chiou, 1974; Chiou & Malagodie, 1975) and the ID_{50} for direct electrically elicited contractions of striated muscle determined by these workers was 1.1×10^{-4} M, similar to the value of 1.4×10^{-4} M found in the present study. TMB-8 (1 mM) has previously been found to inhibit PGI_2 synthesis by cultured vascular endothelium stimulated with thrombin or arachidonate (Brotherton & Hoak, 1982). The present results confirm these findings and extend them to PGI_2 synthesis from endogenous substrate released following the mechanical stimulus of chopping fresh aorta. They do not discount the possibility of some other mechanism of action of TMB-8 in its effect on PGI_2 synthesis. If intracellular calcium were the important determinant of this effect of TMB-8, the present findings do not indicate at what step or

steps in PGI_2 synthesis the calcium concentration is critical.

The lack of effect of sodium citrate on aortic PGI_2 synthesis in Hanks solution raised the possibility of using sodium citrate as an anticoagulant without the resulting change in free divalent cation concentrations directly influencing PGI_2 synthesis. Several steps in the coagulation cascade are calcium-dependent, including conversion of Factor IX to IXa, and PGI_2 stimulating activity is not generated in citrated plasma (Ritter *et al.*, 1982a). It was, therefore, predicted that if the early stages of coagulation did indeed cause the synthesis of a PGI_2 stimulating factor, then less PGI_2 synthesis should occur in incubations of aortic rings in blood with citrate and TCK than in blood anticoagulated with TCK alone. This prediction was borne out (Table 2, Figure 3). Further, the increase in PGI_2 was not paralleled by any change in TXA_2 synthesis (Table 2) showing that in these conditions (where there is a high concentration of PGI_2 in the blood because of the aortic tissue), the effect is selective. If similar situations occur *in vivo* (for instance, whithin a vessel wall at a site of endothelial rupture), such selectivity could have functional implications.

Although highly significant, the difference in 6-oxo-$PGF_{1\alpha}$ in incubations of rings in the presence and absence of sodium citrate is not as large as the difference observed in incubations of rings in serum versus plasma (Ritter *et al.*, 1982a). There are several possible reasons for this. In the earlier experiments serum was prepared by a 3 h incubation in polystyrene tubes, whereas in the present study siliconized glass tubes were used to minimize activation of the intrinsic coagulation pathway by contact with the container; instead, activation of the coagulation mechanism by both intrinsic and extrinsic pathways could occur by contact of the blood with the damaged vascular tissue (Hornstra, 1981). The stimulating factor can therefore be formed only during the 1 h for which the tissue is incubated, so less may be synthesized than during the preparation of serum. A second possible reason for the discrepancy is that it is likely that at the concentration used (7×10^{-5} M) TCK is not completely specific for thrombin but also has some effect on earlier steps in the pathway (Kettner & Shaw, 1979). This concentration was used because it permitted a 60 min incubation without clotting occurring. During the incubation the concentration of TCK is expected to fall as it irreversibly combines with thrombin as this is synthesized. If TCK completely inhibited earlier steps in the cascade it would be expected that, as in the presence of citrate, coagulation would not occur at all. However, this is not the case. Clotting did occur under these conditions if incubations were continued at 37°C beyond 60 min. It is likely, however, that the high concentraton of TCK present at the beginning of the incubation will have had some effect on the other proteases in the pathway besides thrombin and this may have retarded the synthesis of the PGI_2 stimulating factor.

The source of 6-oxo-$PGF_{1\alpha}$ in the incubations of rings in blood could have been either the vascular tissue or the blood, since whole blood is capable of synthesizing PGI_2 at least in the presence of thromboxane synthase inhibitors (Blackwell *et al.*, 1978; Defreyn *et al.*, 1982; Parry *et al.*, 1982). This was addressed by measuring 6-oxo-$PGF_{1\alpha}$ in incubations of blood alone (Figure 4). Immunoreactive material was detected in serum under these circumstances and, as expected from the studies cited, this was increased by the thromboxane synthase inhibitor OKY1581. Since concentrations of 6-oxo-$PGF_{1\alpha}$ detected in the absence of OKY1581 were low, it was not possible to determine whether there was concordance between triplicate determinations in differently diluted samples and it is possible that in this situation the immunoreactive 6-oxo-$PGF_{1\alpha}$ was really due to cross reactivity of the antibody with the very large concentrations of other prostanoids synthesized when blood clots. Indeed, using a highly specific mass spectrometric method, Orchard *et al.* (1983) reported that < 20 pg ml^{-1} of 6-oxo-$PGF_{1\alpha}$ was present in similarly prepared serum; However, in the presence of another thromboxane synthase inhibitor, UK38485, these workers confirmed the earlier reports of increased 6-oxo-$PGF_{1\alpha}$.

When blood alone was incubated with TCK, no 6-oxo-$PGF_{1\alpha}$ was detected (Figure 4, right hand side) either in the presence or absence of OKY1581. This finding is consistent with previous demonstrations of the importance of thrombin in the platelet release reaction and thromboxane synthesis during the clotting of blood *in vitro* (Shuman & Levine, 1980; Remuzzi *et al.*, 1983). More importantly in the context of the present study, it demonstrates that 6-oxo-$PGF_{1\alpha}$ synthesis in incubations of aortic rings with TCK-treated blood (Figure 3) was dependent on the presence of the rings. Thus, in TCK-treated blood alone, no 6-oxo-$PGF_{1\alpha}$ was detected, showing that the blood made no direct contribution to the PGI_2 synthesis in incubations of TCK-treated blood with vascular rings. However, a question remains whether some of the aortic PGI_2 synthesis is from endoperoxides provided by the platelets rather than from arachidonate liberated within the vascular tissue. Where this so, then OKY1581 might increase 6-oxo-$PGF_{1\alpha}$ synthesis by increasing available endoperoxide from platelets. This was not the case when fresh tissue was used (Figure 5) but the preponderence of PGI_2 synthesis in these experiments was such that, despite $> 98\%$ inhibition of TXB_2 by OKY1581, the

extra endoperoxide that could have been available for PGI_2 synthesis would have been too little to produce a detectable increase in 6-oxo-$PGF_{1\alpha}$.

Incubations were performed with aortic rings pretreated with ASA, so as to reduce the conversion of arachidonate by cylo-oxygenase, while leaving the opportunity for platelet derived endoperoxide to be converted to PGI_2 by vascular PGI_2 synthase. With this method, it was possible to demonstrate increased 6-oxo-$PGF_{1\alpha}$ synthesis coincident with inhibition of TXB_2 synthesis by OKY1581 (Figures 6, 7). This confirms the findings of Needleman *et al.* (1979) who detected 6-oxo-$PGF_{1\alpha}$ synthesis in incubations of strips of aorta from ASA-treated rabbits with human platelets prelabelled with [^{14}C]-arachidonate and treated with imidazole, using radiometric thin layer chromatography. It also relates to the findings of Marcus *et al.* (1980). These workers demonstrated synthesis of 6-oxo-$PGF_{1\alpha}$ from platelet-derived endoperoxides by ASA-treated cultured endothelial cells even in the absence of a thromboxane synthase inhibitor in stirred cell suspensions with a substantial endothelial cell/platelet ratio. The ability of vascular tissue in which the cyclo-oxygenase has been inhibited to synthesise PGI_2 when incubated in blood with a thromboxane synthase inhibitor, is presumably due to 'steal' of endoperoxide from platelets to vessel wall (Moncada & Vane, 1979). If vascular cylo-oxygenase becomes inactivated in pathological conditions, such as atheroma, but the PGI_2 synthase remains intact, this may have therapeutic implications.

The finding that the stimulation of prostaglandin synthesis by vascular rings in whole blood in the absence of citrate affected PGI_2 but not TXA_2 (Table 2, Figure 3) raised the possibility that the stimulating factor operates at the level of PGI_2 synthase, thereby accounting for product specificity. This would be compatible with an earlier finding that in serum, but not plasma, arachidonate continues to stimulate vascular PGI_2 synthesis even after 60 min (Ritter *et al.*, 1982a). This implies that the stimulation is due, at least in part, to an effect distal to the release of arachidonate, but does not distinguish between an effect on the cyclo-oxygenase or PGI_2 synthase. The finding that ASA-treated aortic rings synthesize PGI_2 when incubated with OKY1581-treated blood, offered an opportunity to study this by measuring 6-oxo-$PGF_{1\alpha}$ synthesis under these conditions in the presence and absence of citrate. Since the vascular cyclo-oxygenase is largely inhibited, the increased 6-oxo-$PGF_{1\alpha}$ synthesis caused by the OKY1581 depends on the vascular PGI_2 synthase and the supply of platelet-derived endoperoxide. If the stimulation seen in the absence of citrate were due to an effect on the PGI_2 synthase, it should, therfore, still be evident in this circumstance. If, on the other hand, the stimulation were due to a more proximal effect (e.g. on the vascular cyclo-oxygenase), then similar amounts of PGI_2 synthesis would be expected in the presence and absence of sodium citrate. In the event, no difference in 6-oxo-$PGF_{1\alpha}$ synthesis was detected (Figure 8), thus suggesting that the stimulating activity operates proximally to the PGI_2 synthase, most likely (in view of the earlier work discussed above) on the cyclo-oxygenase/hydroperoxidase enzyme. This action might entail either a direct protective effect in inhibiting the self-inactivation of the enzyme (Egan *et al.*, 1976) or might operate indirectly by some other cellular action. The striking effect of TMB-8 on PGI_2 synthesis (Figure 2) coupled with evidence that serum rapidly mobilizes intracellular calcium in quiescent fibroblasts (Lopez-Rivas & Rozengurt, 1983) raises the possibility that mobilization of intracellular calcium by the serum factor may indeed have some critical influence on vascular PGI_2 synthesis. In conclusion, a serum factor formed during blood clotting modulates PGI_2/TXA_2 balance by an action on vascular cyclo-oxygenase, possibly mediated by an effect on intracellular calcium.

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