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STIMULATION OF VASCULAR PROSTACYCLIN (PGI_2) PRODUCTION BY HUMAN SERUM

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Abstract—Prostacyclin (PGI_2) synthesis by chopped rings of rat aorta was measured by radioimmunoassay (RIA) of its stable hydrolysis product 6-oxo-prostaglandin $F_{1\alpha}$ (6-oxo-$PGF_{1\alpha}$). 6-oxo-$PGF_{1\alpha}$ production by matched groups of aortic rings incubated at 37° in human citrated plasma (PPP) or serum were compared. Serum was prepared by the addition of calcium chloride to citrated plasma and agitation for 3 hr at 37°; the serum was expressed from the coagulum so formed. At the start of an incubation, immediately after the addition of the aortic rings, no 6-oxo-$PGF_{1\alpha}$ was detectable either in plasma or in serum. As described previously, in plasma 6-oxo-$PGF_{1\alpha}$ rose asymptotically toward a plateau at about 30 min. In serum the rapid initial production was prolonged and the increase in 6-oxo-$PGF_{1\alpha}$ concentration was almost linear for 60 min ($r = 0.78$, $P < 0.001$). Production of 6-oxo-$PGF_{1\alpha}$ in serum at 4, 8, 30 and 60 min exceeded that in plasma by factors of 1.48, 1.67, 3.60 and 5.71 respectively ($P < 0.005$ at each time). Similar stimulatory activity was found in serum derived from platelet-rich plasma (PRP-S) and that derived from platelet-poor plasma (PPP-S). It was heat stable (100° for 5 min) but was lost following dialysis against an isotonic balanced salt solution. It was not restored by adding calcium chloride to such dialysed serum, and no stimulatory activity was generated if PPP was agitated at 37° for 3 hr without the addition of calcium chloride. The stimulatory activity was not inhibited by cycloheximide. It is concluded that a small heat-stable molecule is generated during coagulation of plasma that stimulates PGI_2 synthesis by rat aorta *in vitro*. Its mechanism of action does not depend on *de novo* protein synthesis.

PGI_2 is rapidly synthesised by chopped rat aortic rings incubated in human plasma [1]. However, in healthy humans the maximal secretion of PGI_2 into the circulation is less than 0.1 ng/kg/min [2] at which rate it is without systemic effect [3]. This suggests that the function of PGI_2 may be to act locally at sites of vascular injury, perhaps to limit the extent of thrombus formation. The effect on PGI_2 synthesis of factors formed during blood coagulation is pertinent in this context because the coagulation mechanism is activated at sites of vascular injury. Indeed, effects of thrombin [4] and of platelet derived growth factor [5] on PGI_2 synthesis by cultured endothelial cells have been described.

A method has recently been developed for studying 6-oxo-$PGF_{1\alpha}$ production by matched groups of rings of rat aorta that gives consistent results ($r = 0.96$) [1]. We describe the application of this method to a comparison of the effect of plasma with that of serum on 6-oxo-$PGF_{1\alpha}$ production. In the first group of experiments plasma was compared with serum derived from platelet rich plasma (PRP-S). This contains substances derived both from the coagulation cascade and from platelets [5]. The effects observed on 6-oxo-$PGF_{1\alpha}$ production in these incubation fluids were then elucidated by further studies with serum derived from platelet poor plasma (PPP-S). Some of the data have been communicated elsewhere in preliminary form [6].

MATERIALS AND METHODS

Preparation of plasma and serum. Blood was drawn by venepuncture from healthy humans and added to 3.8% trisodium citrate (10 vol. blood : 1 vol. citrate). Platelet-rich plasma (PRP) was prepared by centrifugation at 400 *g* for 7.5 min at room temperature. PPP was prepared by centrifugation at 1000 *g* for 20 min at 4°. Serum was prepared from PRP (PRP-S) or from PPP (PPP-S) by the method of Coughlin and co-workers [5]: 100 μl of calcium chloride (1 M) was added to 5 ml of plasma which was then incubated in a shaking water bath for 3 hr at 37°. In PRP-S, serum was expressed spontaneously from the resulting coagulum; in PPP-S serum was expressed by gentle pressure with a spatula. Serum was stored at 4° for up to 48 hr before use. Heat treated serum was prepared in a glass vial in a boiling water bath for 5 min. The heat treated serum was then expressed from the coagulum through a nylon mesh. Dialysed serum (5 ml) was prepared in a dialysis bag (Visking tubing 24/32, average pore radius 24 Å: The Scientific Instrument Centre, London) suspended in 2 litres of constantly stirred Gey's solution (Gibco, Uxbridge, U.K.) at 4° for 24 hr.

Preparation of aortic rings. These were prepared from male CD rats (Charles River, Margate, U.K.) by a similar method to that described previously [1]. A minor modification was that for experiments involving comparison of 4 incubation fluids; 1 mm rings from the aortas of two rats were combined to yield 4 groups of 24 rings, 12 rings in each group from each rat. As before, the rings were allocated

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individually to each group so as to minimize the difference between groups. Each group of rings was stored in 5 ml of Gey's solution on ice until the start of the incubation, when it was added to 2 ml of incubation fluid at 37° in a shaking water bath. Aliquots were removed at subsequent times for analysis by RIA of 6-oxo-$PGF_{1\alpha}$ after dilution in buffer and temporary acidification [1, 7]. The identity of the 6-oxo-$PGF_{1\alpha}$ was confirmed by gas chromatography negative ion chemical ion mass spectrometry [8].

Materials. Sodium arachidonate and cycloheximide were obtained from Sigma (London, U.K.) and calcium chloride (Analar grade) from BDH Chemicals Ltd (Poole, U.K.).

Statistics. All comparisons were by Student's paired *t*-test (two-tailed). Differences were considered significant when $P < 0.05$.

RESULTS

6-oxo-$PGF_{1\alpha}$ production by aortic rings in PPP and PRP-S

Eight paired incubations were performed with PPP and PRP-S prepared from blood drawn at the same venepuncture (Fig. 1). Measurements were made at 60 min in only five. PPP and PRP-S sampled immediately after addition of the aortic rings, and diluted in the same way as the 4 min samples, contained no detectable 6-oxo-$PGF_{1\alpha}$. At subsequent times there was always more 6-oxo-$PGF_{1\alpha}$ in PRP-S than in PPP ($P < 0.005$ for all times). This increase became progressively greater throughout the incubation. Thus the mean increase at 4, 8, 30 and 60 min was by factors of 1.48, 1.67, 3.60 and 5.71, respectively. While 6-oxo-$PGF_{1\alpha}$ production by aortic rings incubated in PPP rose asymptotically toward a plateau at around 30 min as described previously [1], in PRP-S the rapid initial production was maintained over 60 min with a production rate that was almost linear over this period ($r = 0.78$, $P < 0.001$).

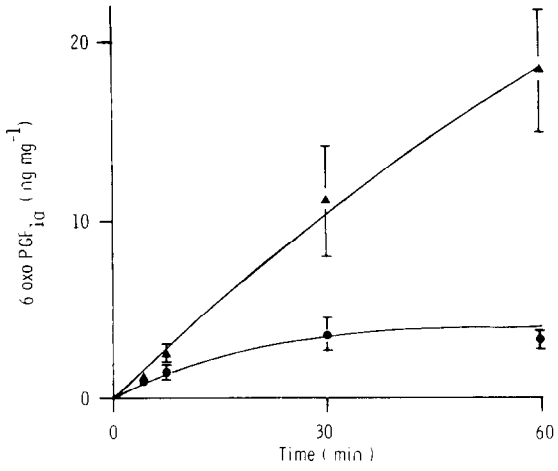

Fig. 1. Time course of production of 6-oxo-$PGF_{1\alpha}$ (ng/mg tissue) by rat aortic rings in PPP (●) and in PRP-S (▲) at 37°. Each point is the mean (± S.E.) of 8 measurements (4, 8 and 30 min) or 5 measurements (60 min).

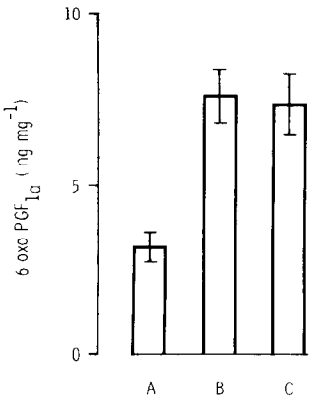

Fig. 2. Stimulation of 6-oxo-$PGE_{1\alpha}$ production by serum derived from platelet-rich and platelet-poor plasmas. The bars indicate mean (± S.E., $n = 12$) 6-oxo-$PGF_{1\alpha}$ (ng/mg tissue) produced by rat aortic rings incubated for 30 min at 37° in PPP (A), PPP-S (B), and PRP-S (C).

Comparison of PRP-S and PPP-S

A series of 12 incubations was performed. In each, 6-oxo-$PGF_{1\alpha}$ production was measured in PPP, PPP-S and PRP-S. As before, plasma and serum for each incubation were obtained from blood obtained at a single venepuncture and different subjects were used for each incubation. The results are shown in Fig. 2. More 6-oxo-$PGF_{1\alpha}$ was produced in PPP-S than in PPP ($P < 0.001$, $n = 12$) and in PRP-S than in PPP ($P < 0.001$, $n = 12$). However, there was no evidence that the production of 6-oxo-$PGF_{1\alpha}$ in PRP-S was different from that in PPP-S ($P > 0.6$, $n = 12$).

Effect of dialysis and heat treatment

A series of 4 incubations (Fig. 3) was performed to compare the production of 6-oxo-$PGF_{1\alpha}$ by aortic

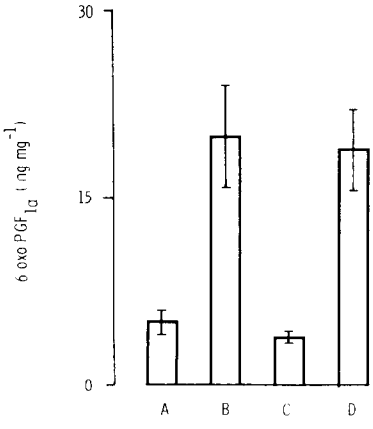

Fig. 3. Physical properties of serum stimulatory activity. The bars indicate mean (± S.E., $n = 4$) 6-oxo-$PGF_{1\alpha}$ (ng/mg tissue) produced by rat aortic rings incubated for 60 min at 37° in PPP (A), PPP-S (B), serum dialysed against Gey's solution (C), and serum heated in a boiling water bath for 5 min (D).

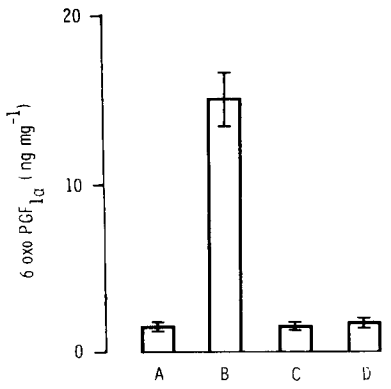

Fig. 4. Lack of effect of calcium on 6-oxo-$PGF_{1\alpha}$ production. The bars indicate mean (± S.E., $n = 4$) 6-oxo-$PGF_{1\alpha}$ (ng/mg tissue) produced by rat aortic rings incubated for 60 min at 37° in PPP (A), PPP-S (B), serum dialysed against Gey's solution (C) and serum dialysed against Gey's solution to which 20 mmoles $CaCl_2$ had been added per litre (D).

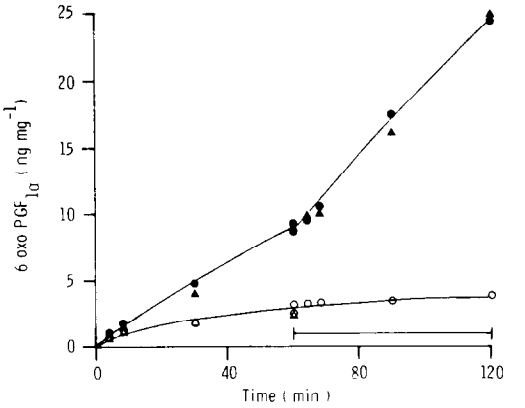

Fig. 6. Effects of cycloheximide and sodium arachidonate. Comparison of 6-oxo-$PGF_{1\alpha}$ production (ng/mg tissue) by rat aortic rings at 37° in PPP (○, △) and in PPP-S (●, ▲). At 60 min sodium arachidonate was added (final concentration 100 μM) as indicated by the horizontal bar. In two incubations cycloheximide (100 μg/ml) was present (△, ▲); in two it was not (○, ●).

rings incubated in PPP, PPP-S, PPP-S that had been dialysed against Gey's solution (PPP-SD), and PPP-S that had been heated in a boiling water bath for 5 min (PPP-SH). Dialysis removed the stimulatory factor from serum but heating did not. In a separate series of experiments (Fig. 4) addition of 100 μl of calcium chloride (1 M) to 5 ml dialysed serum, to restore the calcium concentration to that present in PPP-S, failed to restore the stimulatory activity. Incubation of PPP alone for 3 hr at 37° in a shaking water bath without the addition of calcium chloride did not generate stimulatory activity. The production of 6-oxo-$PGF_{1\alpha}$ on subsequent incubation of this plasma with aortic rings was similar to that observed in control PPP (Fig. 5 and compare Fig. 1).

Effect of cycloheximide on 6-oxo-$PGF_{1\alpha}$ production

Cycloheximide (100 μg/ml) had no detectable effect on the production of 6-oxo-$PGF_{1\alpha}$ by aortic rings incubated either in PPP or in PPP-S (Fig. 6, representative of three experiments). Figure 6 also shows the effect of sodium arachidonate (final concentration 100 μM) at 60 min. This had no effect on the subsequent production of 6-oxo-$PGF_{1\alpha}$ in PPP over a further 60 min. In contrast, addition of sodium arachidonate to aortic rings in PPP-S at 60 min caused a further rise in rate of 6-oxo-$PGF_{1\alpha}$ production.

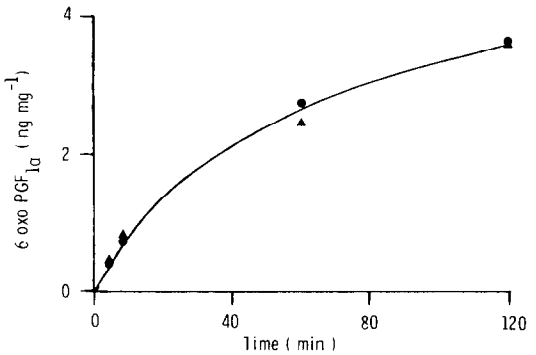

Fig. 5. Lack of effect of pre-incubation of PPP at 37°. Comparison of 6-oxo-$PGF_{1\alpha}$ production (ng/mg tissue) by rat aortic rings at 37° in PPP (▲) and in PPP that had been preincubated for 3 hr at 37° (●).

DISCUSSION

6-oxo-$PGF_{1\alpha}$ production by fresh rat aortic rings is inhibited by plasma [6] probably due to albumin [9]. In contrast 6-oxo-$PGF_{1\alpha}$ produced in serum is greater than in PPP (Figs. 1 and 2). This is best explained by a stimulatory activity in serum on 6-oxo-$PGF_{1\alpha}$ production by vascular tissue. An alternative explanation, namely that an inhibitory substance in plasma is destroyed during the coagulation process is ruled out by the experiments shown in Fig. 3 which demonstrate that following dialysis against a salt solution the ability of a sample of serum to support increased 6-oxo-$PGF_{1\alpha}$ production is lost. This is simply explained by the loss of dialysable stimulator but cannot be accounted for if the increased production in serum were due to loss of an inhibitor. Furthermore, we have shown elsewhere [6] that the production of 6-oxo-$PGF_{1\alpha}$ by aortic rings incubated in PRP-S for 30 min is significantly greater than that produced in Tris buffer (50 mM, pH 7.5); and this observation is explained by the presence of a stimulatory factor in serum.

The control experiments described demonstrate that the stimulatory activity depends on coagulation having occurred, and is not merely a consequence of the addition of calcium chloride *per se*. This is pertinent because phospholipase A_2 is a calcium dependent enzyme [10] and we have ourselves observed modest effects of changing external calcium concentration on 6-oxo-$PGF_{1\alpha}$ production by aortic rings (unpublished observations). However, the effect of calcium is qualitatively unlike the effect of serum on 6-oxo-$PGF_{1\alpha}$ production. The increased

production caused by calcium is proportionately similar throughout the incubation, whereas in serum 6-oxo-$PGF_{1\alpha}$ production becomes progressively greater as the incubation proceeds (Fig. 1). Furthermore, restoring the calcium concentration of serum deficient in stimulatory activity following dialysis, to that of control serum does not restore its stimulatory effect on 6-oxo-$PGF_{1\alpha}$ production (Fig. 4).

The stimulatory substance in our experiments is not thrombin [4] because the activity withstands 100° for 5 min and is dialysable. Neither is it platelet-derived growth factor which is released during platelet activation, and stimulates PGI_2 production by cultured cells [5]. Unlike thrombin it is stable at 100° [11], but its molecular weight is approximately 13,000–16,000 [11], and the activity we observed was similar in serum derived from PPP or from PRP (Fig. 2). It thus appears that the activity we observed on fresh aortic rings is due to some other and as yet unidentified factor, possibly a small peptide fragment cleaved from one of the clotting factors during activation.

The mechanism by which the serum factor increases 6-oxo-$PGF_{1\alpha}$ production may be to increase the activity of the rate limiting enzyme for the PGI_2 synthetic pathway. This is not due to enzyme induction as the effect is too rapid and cannot be inhibited by a large concentration of cycloheximide (Fig. 6). Initially, the rate limiting step is the release of arachidonic acid by phospholipase; thus addition of exogenous arachidonic acid causes increased 6-oxo-$PGF_{1\alpha}$ production [12]. As the incubation proceeds in PPP, a subsequent step in the prostaglandin synthetase pathway becomes rate limiting. The rate of 6-oxo-$PGF_{1\alpha}$ production slows and exogenous arachidonic acid has no effect on the production rate (Fig. 6). In serum, however, phospholipase activity remains rate limiting during the whole incubation, 6-oxo-$PGF_{1\alpha}$ production is linear with time and exogenous arachidonic acid increases the production rate even after 60 min (Fig. 6).

An explanation of these results is that clotting produces a substance that prevents either self destruction of the cyclooxygenase enzyme or the formation of alternative oxidation products [13]. The cyclooxygenase enzyme would then function at the same efficiency during the whole incubation and phospholipase cleavage of phospholipid would remain the rate limiting step. The alternative possibility that the substance produced in clotting causes increased prostaglandin synthetase activity by an allosteric effect cannot be completely discounted.

Remuzzi and co-workers [14] have described a factor in plasma that increases PGI_2 activity generated by aortic rings 'exhausted' by prior washing in Tris buffer. Plasma from patients with renal failure was more active in this respect than plasma from normal subjects [14]. Plasma also increases 6-oxo-$PGF_{1\alpha}$ production by cultured endothelial cells [15, 16]. This plasma factor is different from the factor in serum that we describe in this paper, which is only generated when the coagulation mechanism has been activated. Indeed in fresh aortic rings (as used in the present study) plasma from both healthy subjects and subjects with severe renal impairment caused approximately a 20% reduction of 6-oxo-$PGF_{1\alpha}$ production compared with Tris buffer [6, 9]. This difference between fresh tissue and exhausted rings or cultured cells makes interpretation of the physiological relevance of plasma factor [14–16] problematical. The serum stimulatory factor we describe here is active on fresh vascular tissue and may play a role in the control of thrombosis and haemostasis. Thus at sites of endothelial damage the coagulation pathway is activated: the extent of the thrombus formed may be limited to PGI_2 produced by neighbouring endothelial cells that have been damaged but not destroyed. Damaged vascular tissue in plasma synthesises a brief burst of PGI_2 [11]. The effect of local formation of the factor we describe, formed during the coagulation process, is to increase the rate of PGI_2 synthesis and perhaps more importantly to convert the pulse of PGI_2 production into a sustained response.

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