EPOPROSTENOL (PROSTACYCLIN, PGI₂) BINDING AND ACTIVATION OF ADENYLATE CYCLASE IN PLATELETS OF DIABETIC AND CONTROL SUBJECTS

G.L. SHEPHERD, P.J. LEWIS, I.A. BLAIR, C. de MEY & J. MacDERMOT

Department of Clinical Pharmacology,
Royal Postgraduate Medical School, London W12

- 1 The binding of epoprostenol (prostacyclin, PGI₂) to isolated fractured human platelets has been studied using tritiated PGI₂.
- 2 High and low affinity binding sites for PGI₂ have been identified (K_d values = 16 and 382 nM).
- 3 Analysis of the prostacyclin-dependent activation of adenylate cyclase suggests that enzyme activation is mediated by the high affinity binding site.
- 4 Platelet PGI₂ receptor binding and adenylate cyclase activation by PGI₂ are unchanged in diabetic and normal human platelets.
- 5 This work suggests that hyperaggregability of diabetic platelets is not due to any alteration of platelet prostacyclin receptor numbers or their activation.

Introduction

Epoprostenol (prostacyclin, PGI₂) an unstable metabolite of prostaglandin endoperoxides, is the most potent endogenous inhibitor of platelet aggregation. It binds to human platelets (Siegl *et al.*, 1979), activates adenylate cyclase (EC 4.6.11) (Gorman *et al.*, 1977) and increases cyclic AMP concentration (Tateson *et al.*, 1977). Vascular endothelium produces prostacyclin which could bind to platelets and increase cAMP levels. This increase in cAMP may inhibit cyclooxygenase (Best *et al.*, 1977) and membrane phospholipase (Lapetina *et al.*, 1977), and hence limit synthesis of platelet endoperoxide necessary for thromboxane synthesis. Thereby platelet aggregation would be inhibited (Best *et al.*, 1977).

Occlusive vascular disease is common in patients with diabetes mellitus. Diabetic platelets are hyperaggregable (Sagel *et al.*, 1975; Lagarde *et al.*, 1980; Silberbauer *et al.*, 1981) and have been shown to have increased thromboxane synthesis in humans (Halushka *et al.*, 1981) and animals (Harrison, 1978; Gerrard *et al.*, 1980). In human (Johnson *et al.*, 1979) and animal (Harrison *et al.*, 1978; Gerrard *et al.*, 1980) experiments, blood vessels from diabetics have been shown to produce less PGI₂ than normal vessels. We have studied the characteristics of the PGI₂ receptor and adenylate cyclase activity in fractured human platelets from diabetic and control subjects. The mechanisms of hyperaggregability in diabetics

could be caused by hyporesponsiveness to PGI₂ (Lagarde *et al.*, 1980) and a decreased sensitivity to PGI₂ antiaggregatory activity was shown in patients with diabetic retinopathy (Bonne *et al.*, 1978).

Methods

Patients and controls

The patients studied were young insulin dependent male diabetics with proliferative retinopathy. The retinopathy was assessed by ophthalmoscopy and fluorescein angiography. Control subjects were young male volunteers with normal blood glucose levels. In binding experiments the mean age of the diabetics studied was 35 years (range 31–35 years) and for the controls 31 years (range 25–37 years). In the adenylate cyclase experiments the mean age of the diabetics was 34 years (range 17–44 years) and controls, mean 26 years (range 23–31 years). In the binding experiments five diabetic subjects were compared with four controls; in the adenylate cyclase activation experiments, seven diabetic subjects were compared with six controls.

Preparation of platelet homogenate

Blood was drawn through a thin wall 21 gauge needle

from an antecubital vein and collected into EDTA giving a final concentration of 5 mM. Platelet-rich plasma (PRP) was prepared by centrifugation at 100 g for 15 min at 20°C, and the platelets were counted in a Coulter Counter (Coulter Electronics Ltd). The PRP was then centrifuged at 2400 g for 10 min at 20°C. The platelet pellet was suspended in 5 mM Tris-HCl buffer, pH 8.5 and disrupted at 4°C by 25 strokes in a tight fitting Dounce homogeniser. The platelet membrane homogenate was washed twice by re-suspension in 4 mM Tris HCl buffer at pH 8.5 and centrifuged at 30,000 g for 20 min at 4°C. The pellet was finally resuspended in 50 mM Tris-HCl buffer, pH 8.5, for the binding assay and 25 mM Tris HCl buffer, pH 8.5, for the adenylate cyclase assay. The suspensions were maintained at 4°C prior to the assay. A modification of the method of Lowry *et al.* (1951) was used to determine the protein concentration.

Binding of [³H]-PGI₂ to platelet membranes

11β-[³H]-PGI₂ (Blair *et al.*, 1981) with a specific activity of 8 Ci/mmol was used. Incubations of 100 μl containing 100–150 μg of platelet membrane protein together with 50 mM Tris-HCl buffer, pH 8.5, 10 mM MgSO₄ and [³H]-PGI₂ at a range of concentrations were incubated in triplicate at 20°C for 15 min. The reaction was terminated by the addition of 4 ml of 50 mM Tris-HCl buffer, pH 8.5, at 4°C. The membranes were filtered rapidly under reduced pressure through Whatman GF/B glass filter discs and washed 3 times rapidly with 4 ml of 50 mM Tris-HCl buffer, pH 8.5 at 4°C. The filtration procedure was completed within 20 s. Parallel incubations contained 10 μM synthetic PGI₂ and specific binding was determined by the binding that was displaced by this excess of unlabelled PGI₂. An experiment to measure the dissociation of the ligand-receptor complex was performed in a single incubation of 1100 μl containing the same molar concentrations as described above 15 nM [³H]-PGI₂. The reaction mixture was incubated for 20 min and at the zero time point an excess (100 μM final concentration) of unlabelled PGI₂ was added. At selected times therefore 100 μl was removed and the reaction terminated as before. Measurement of the rate of association of the ligand and receptor was made in incubations of 100 μl. The reaction was terminated at selected times as before.

Computer program analysis of binding data

The data were fitted by an iterative program for non-linear regression analysis (Koeppel & Hamann, 1980) to the following equation for a four parameter model identifying two independent binding sites. The value of bound ligand (b) is given by:

$$b = \left[\frac{(B_{\max 1} \times L)}{(K_{d1} + L)} \right] + \left[\frac{(B_{\max 2} \times L)}{(K_{d2} + L)} \right]$$

where $B_{\max 1}$ was the maximum binding for the high affinity site and K_{d1} the high affinity site dissociation constant. $B_{\max 2}$ was the maximum binding for the low affinity binding site and K_{d2} the low affinity site dissociation constant. L was the ligand concentration.

Adenylate cyclase assay

Enzyme activity was determined by a modification (Sharma *et al.*, 1975) of the method of Salomon *et al.* (1974). Incubations of 100 μl contained 50 mM Tris HCl buffer, pH 8.5; 5 mM magnesium sulphate; 20 mM creatinine phosphate (Sigma); 10 international Units creatine kinase (Sigma EC 2732); 1 mM cyclic adenosine 3',5'-monophosphate (Sigma); 0.25 mM Ro 20-1724 (a phosphodiesterase inhibitor) with a final concentration of 0.25% ethanol; 1.0 mM [α -³²P]-ATP (1.5 μCi/reaction mixture, Amersham International) and 100–200 μg of homogenate protein. Incubations were maintained at 30°C for 8 min pH 8.5. The reactions were terminated by the addition of 800 μl of 6.25% (w/v) trichloroacetic acid at 4°C. 100 μl of [³H]-cAMP (10,000 counts/min in 100 μl, Amersham International) was added to each tube. The mixtures were centrifuged at 100 g for 20 min at 20°C. The supernatant from each tube was loaded onto a Dowex AG 50 W-X4 column containing 1 ml of expanded resin. The columns had been regenerated previously with 6 ml of 1M HCl and then washed with 6 ml of water. The supernatant was allowed to run through the column and then 2.5 ml of water was run through. A further 3 ml of water was added and the eluate run onto a neutral alumina column containing 0.6 g dry neutral alumina. The alumina column had been regenerated previously with 10 ml of 100 mM imidazole-HCl buffer, pH 7.5, and then washed with 6 ml of water. Finally 4 ml of 100 mM imidazole HCl buffer, pH 7.5, was added to the alumina column and the eluate was collected in scintillation vials. Insta-Gel (10 ml) (Packard Instrument Co) was added to each vial. Counting was performed in a Packard liquid scintillation spectrometer.

The following generous gifts were received: PGI₂ from the Wellcome Research Laboratories; Ro 20-1724 from Roche Products Ltd.

Results

Normal subjects

The binding of [³H]-PGI₂ to normal platelet membranes was rapid (Figure 1) and the increase in binding with time has been presented as a pseudo first order rate plot (Figure 1, inset). The observed rate constant (k_{obs}) is given by the slope: $1.7 \times 10^{-3} \text{ s}^{-1}$ and from it the second order rate constant (k_{+1}) for the association of the ligand and the receptor was calculated ($k_{\text{obs}}/[PGI_2]$) to be $1.7 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$.

Figure 1 The association of [³H]-PGI₂ and platelet membrane receptors. Each data point represents a single measurement of specific binding. The inset shows the pseudo first order rate plot of the same data, where B_{eq} is the specific binding at equilibrium and B_t is the specific binding at any particular time t .

The dissociation of the ligand receptor complex (Figure 2) obeyed first order kinetics ($k_{-1} = 2.9 \times 10^{-3} \text{ s}^{-1}$). This was calculated from the half-time for the decrease in specific [³H]-PGI₂ binding to the receptor (240 s). The true dissociation constant (K_d) is given by the ratio of k_{-1}/k_{+1} and was 17 nM.

The binding of [³H]-PGI₂ to normal platelet membranes was measured at equilibrium with increasing concentrations of [³H]-PGI₂. The results shown (Figure 3) are from one experiment, and were typical of results from all four normal subjects. Scatchard analysis of the data was non-linear, consistent with two independent binding sites, one of high and one of low affinity. Computer analysis revealed that the mean K_d of the high affinity site was 16 nM (± 2.3) and of the low affinity site was 382 nM (± 94). The maximum binding capacity of the high affinity site was 1125 ± 67 fmol/mg protein. It follows that there are 3.3×10^3 high affinity receptor sites per platelet.

In comparative experiments of several prostaglandins as inhibitors of [³H]-PGI₂ binding, K_i values were calculated. The affinity of PGI₂ ($K_i = 2.3$ nM) was 40 times that of PGE₁ ($K_i = 83$ nM). 6-oxo-PGF_{1 α} and PGF_{2 α} did not inhibit [³H]-PGI₂ binding at concentrations up to 5 μM .

Diabetic patients

In the diabetic patients ($n = 5$) the dissociation constant for the high affinity site was 14 ± 1.3 nM compared with 16 ± 2.3 nM in the controls ($n = 4$). The K_d for the low affinity receptor was 248 ± 29 nM in the diabetics compared with 382 ± 94 nM in the controls. There was no difference in the number of

Figure 2 The dissociation of [³H]-PGI₂ and platelet membrane receptors. The plot shows the dissociation of the ligand-receptor complex with respect to time. Each data point represents a single measurement of specific binding. The inset shows a semi-logarithmic plot of the same data, where B_t is the [³H]-PGI₂ bound at any particular time t and B_0 is the [³H]-PGI₂ bound at time zero.

Figure 3 A Scatchard plot for the specific binding of [³H]-PGI₂ to platelet membrane receptors. The specific binding is estimated as fmol mg⁻¹ membrane protein and each data point represents a single measurement.

Figure 4 PGI₂ dependent activation of platelet adenylate cyclase. The data are presented as an Eadie-Hofstee plot. δV is the increase in adenylate cyclase activity above basal enzyme activity, and $[L]$ the PGI₂ concentration (nM).

high affinity sites in platelet membranes from diabetics (1122 ± 63 fmol/mg protein) or normal subjects (1125 ± 67 fmol/mg protein).

PGI₂ increased basal adenylate cyclase activity of platelet membranes from normal subjects (Figure 4). The concentration of PGI₂ required for half maximum enzyme activation (K_{act}) was 49 ± 7.3 nM compared with 38 ± 5.5 nM in the diabetics. The maximum increase in adenylate cyclase activity produced by PGI₂ was not significantly different in the fractured platelets of diabetics (194 ± 50 pmol cyclic AMP min⁻¹mg⁻¹ protein) or control subjects (295 ± 71 pmol cyclic AMP min⁻¹mg⁻¹ protein).

There were therefore no statistically significant differences in the binding of [³H]-PGI₂ or PGI₂-dependent activation of adenylate cyclase, in platelet membranes of diabetics or their age-matched controls.

Discussion

Prostacyclin binding to platelets in a variety of species has been shown (Schillinger *et al.*, 1980). Siegl *et al.* (1979), using whole human platelets, identified two

binding sites for PGI₂. Scatchard analysis of [³H]-PGI₂ binding to human platelet homogenates (Figure 3) in the present work supports this data. The Eadie Hofstee plot for the adenylate cyclase activation by PGI₂ (Figure 4) is linear. This suggests that enzyme activation is mediated by a simple non-cooperative bimolecular interaction between PGI₂ and a single receptor species. The similarity in the dissociation constant for the receptor binding (16 nM) and the concentration for half maximum adenylate cyclase activation (49 nM), suggests that adenylate cyclase activation is mediated by the high affinity receptors. No biological function for the low affinity PGI₂ receptor binding site has been identified.

A link between diabetic vascular disease and an abnormality of metabolism, or alternatively a defect in platelet or endothelial function has been sought by many. Isolated vascular tissue from diabetic patients produces less PGI₂ than control tissue (Johnson *et al.*, 1979) and similar results have been obtained in experimental diabetes (Harrison *et al.*, 1978). Diabetic platelets show increased thromboxane synthesis (Halushka *et al.*, 1981) and a decrease in sensitivity to PGI₂ antiaggregatory action (Bonne *et al.*, 1978). However, other workers have found similar sensitivity of platelets to inhibition of ADP induced aggregation by PGI₂ in diabetics and controls (Betteridge *et al.*, 1980). Heath *et al.* (1971) reported that platelet aggregation was more marked in patients with deteriorating diabetic retinopathy. There have been reports that circulating levels of PGI₂ are lower in diabetics (Dollery *et al.*, 1979) but the plasma levels of PGI₂ recorded at that time are now believed to be too high. Circulating 6-oxo-PGF_{1 α} is maintained below 3 pg/ml in normal subjects (Blair *et al.*, 1982) at which concentration (14 pM) there would be little or no binding or activation of platelet receptors. It seems unlikely that changes in PGI₂ concentrations in plasma of the order now being measured could result in significant alteration in platelet sensitivity to PGI₂.

The present report shows that [³H]-PGI₂ binding and adenylate cyclase activity of human platelets are similar in diabetics and controls. Hence the vascular complications of diabetics are probably not secondary to altered platelet sensitivity to PGI₂.

We wish to thank Dr Eva Kohner (RPMS) who allowed us to study her patients.

References

- BEST, L.C., MARTIN, T.J., RUSSELL, R.G.G. & PRESTON, F.E. (1977). Prostacyclin increases cyclic AMP levels and adenylate cyclase activity in platelets. *Nature*, **267**, 850–851.
- BETTERIDGE, D.J., EL TAHIR, K.E.H., RECKLESS, J.P.D. & WILLIAMS, K.I. (1980). A study of sensitivity of platelets from patients with diabetes mellitus to inhibition of ADP induced aggregation by prostacyclin. *Clin. Sci.*, **59**, 27P.
- BLAIR, I.A., BARROW, S.E., WADDELL, K.A., LEWIS, P.J. & DOLLERY, C.T. (1982). Prostacyclin is not a circulating hormone in man. *Prostaglandins*, (in press).
- BLAIR, I.A., HENSBY, C.N. & MACDERMOT, J. (1981). Synthesis of [$^{11}\beta$ - 3 H]-prostacyclin. *J. Label. Comp. Radiopharm.*, **18**, 361–370.
- BONNE, C., ROMQUIN, N. & REGNAULT, F. (1978). Modified sensitivity of diabetic platelets to prostacyclin. 10th Europ. Conf. Microcirculation. *Biblhca anat.*, **18**, 98–103.
- DOLLERY, C.T., FRIEDMAN, L.A., HENSBY, C.N., KOHNER, E., LEWIS, P.J., PORTA, M. & WEBSTER, J. (1979). Circulating prostacyclin may be reduced in diabetes. *Lancet*, **ii**, 1365.
- GERRARD, J.M., STUART, M.J., RAO, G.H.R., STEFFES, M.W., MAUER, S.M., BROWN, D.M. & WHITE, J.G. (1980). Alteration in the balance of prostaglandin and thromboxane synthesis in diabetic rats. *J. lab. clin. Med.*, **95**, 950–958.
- GORMAN, R.R., BUNTING, S. & MILLER, O.V. (1977). Modulation of human platelet adenylate cyclase by prostacyclin (PGX). *Prostaglandins*, **13**, 377–388.
- HALUSHKA, P.V., ROGERS, R.C., LOADHOLT, C.B. & COLWELL, J.A. (1981). Increased platelet thromboxane synthesis in diabetes mellitus. *J. lab. clin. Med.*, **97**, 87–96.
- HARRISON, H.E., REECE, A.H. & JOHNSON, M. (1978). Decreased vascular prostacyclin in experimental diabetes. *Life Sci.*, **23**, 351–356.
- HEATH, H., BRIGDEN, W.D., CANEVER, J.V., POLLOCK, J., HUNTER, P.R., KELSEY, J. & BLOOM, A. (1971). Platelet adhesiveness and aggregation in relation to diabetic retinopathy. *Diabetologia*, **7**, 308–315.
- JOHNSON, M., HARRISON, H.E., RAFTERY, A.T. & ELDER, J.B. (1979). Vascular prostacyclin may be reduced in diabetes in man. *Lancet*, **i**, 325–326.
- KOEPPE, P. & HAMANN, C. (1980). A program for non-linear regression analysis to be used on desk-top computers. *Computer Programs in Biomedicine*, **12**, 121–128.
- LAGARDE, M., BURTON, M., BERCIAUD, P., BLANC, M., VELARDO, B. & DECHAVANNE, M. (1980). Increase of platelet thromboxane A₂ formation and its plasmatic half-life in diabetes mellitus. *Thrombosis Res.*, **19**, 823–830.
- LAPETINA, E.G., SCHMITGES, C.J., CHANDRABOSE, K. & CUATRECASAS, P. (1977). Cyclic AMP and prostacyclin inhibit platelet membrane phospholipase. *Biochem. Biophys. res. Comm.*, **76**, 828–835.
- LOWRY, O.H., ROSENBOUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the folin phenol reagent. *J. biol. Chem.*, **193**, 265–275.
- SAGEL, J., COLWELL, J.A., CROOK, L. & LAIMINS, M. (1975). Increased platelet aggregation in early diabetes mellitus. *Ann. int. Med.*, **82**, 733–738.
- SALOMON, Y., LONDOS, C. & RODBELL, M. (1974). A highly sensitive adenylate cyclase assay. *Anal. Biochem.*, **58**, 541–548.
- SCHILLINGER, E. & PRIOR, G. (1980). Prostaglandin I₂ receptors in a particulate fraction of platelets of various species. *Biochem. Pharmacol.*, **29**, 2297–2299.
- SHARMA, S.K., NIRENBERG, M. & KLEE, W.A. (1975). Morphine receptors as regulators of adenylate cyclase activity. *Proc. Nat. Acad. Sci.*, **72**, 590–594.
- SIEGL, A.M., SMITH, J.B., SILVER, M.J., NICOLAOU, K.C. & AHERN, D. (1979). Selective binding site for [3 H]-prostacyclin on platelets. *J. clin. Invest.*, **63**, 215–220.
- SILBERBAUER, K., SCHERNTHANER, G., SINZINGER, H. & FREYLER, H. (1981). Platelet aggregation and reversible platelet aggregates in Type I diabetes staged by retinal fluorescein angiography. *Atherosclerosis*, **40**, 81–90.
- TATESON, J.E., MONCADA, S. & VANE, J.R. (1977). Effects of prostacyclin (PGX) on cyclic AMP concentrations in human platelets. *Prostaglandins*, **13**, 389–397.

(Received May 26, 1982,
accepted September 24, 1982)