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Human Platelet Secreted Proteins and Prostacyclin Production by Bovine Aortic Endothelial Cells[1,2] (41600)

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Abstract. The effects of specific human platelet-secreted proteins on prostacyclin (PGI_2) production by primary cultures of bovine aortic endothelial cells have been studied. Cells were incubated with various concentrations of highly purified preparations of platelet factor 4 (PF_4), low-affinity platelet factor 4 (LA-PF_4), β-thromboglobulin (βTG), platelet basic protein (PBP), and partially purified platelet-derived growth factor (PDGF) in the presence or absence of arachidonic acid (AA). The amount of 6-Keto-$PGF_{1\alpha}$, the stable degradation product of PGI_2, was determined in the cell incubation medium by means of a specific radioimmunoassay. Short-term (15 min) incubation of cell monolayers with either LA-PF_4 or βTG slightly reduced 6-keto-$PGF_{1\alpha}$ production. The effect was not dose-related and could not be observed after prolonged (24 hr) incubation of the cells with the same proteins. It was not seen in the cell suspensions. Moreover, 6-keto-$PGF_{1\alpha}$ production stimulated by AA was not affected by incubation with either of the proteins. PF_4 and PBP had no significant effect on 6-keto-$PGF_{1\alpha}$ production by endothelial cells. Human PDGF showed a slight tendency to stimulate 6-keto-$PGF_{1\alpha}$ release when cells were incubated for 24 hr with the protein; however, PDGF did not potentiate the stimulatory effect of AA on 6-keto-$PGF_{1\alpha}$ release by the cells. We suggest that platelet-derived proteins exert only a moderate and possibly nonspecific effect on PGI_2 production by endothelial cells.

Human platelets release several proteins upon stimulation with aggregating agents. A group of these proteins is characterized by the affinity for heparin and the ability to neutralize the anticoagulant properties of this glycosaminoglycan (1). These proteins are specific for platelets, are stored in the α granules and can be isolated from material released by human platelets by adsorption to insolubilized heparin and elution with a gradient of sodium chloride. PF_4 has the highest affinity for heparin and it is eluted with 1.5 *M* NaCl; the other proteins (LA-PF_4, βTG, PBP, and PDGF) have lower affinity and are eluted with 0.3–0.5 *M* NaCl. βTG has been shown to be a stable degradation product of LA-PF_4; it can be obtained from the latter by cleavage of four N-terminal amino acids (2). LA-PF_4 can in turn be derived from PBP by removal of a basic nonapeptide at the NH_2-terminal (3). βTG, LA-PF_4, and PBP are immunologically identical. PDGF is a highly cationic protein with a molecular weight of about 30,000 daltons (4). It is not related immunologically to βTG or to PF_4.

Both PF_4 (5) and βTG (6) have been reported to bind to endothelial cells. PGI_2, a major AA metabolite of endothelial cells in culture, is a potent inhibitor of platelet aggregation (7). Hope *et al.* (6) have found that human βTG at physiological concentrations reduced PGI_2 production by bovine aortic endothelial cells when measured by an assay based on the inhibition of platelet aggregation. Ager and Gordon (8) only partially confirmed this observation when they studied the effect of βTG on PGI_2 production by porcine

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endothelial cells, measured by a specific radioimmunoassay of PGI_2's stable hydrolysis product, 6-keto-$PGF_{1\alpha}$. They found an inhibitory effect with only one of four batches of βTG at very high concentrations of the protein. Coughlin *et al.* (9) have demonstrated that PDGF, another human platelet-secreted protein, stimulated 6-keto-$PGF_{1\alpha}$ production by both bovine aortic endothelial and smooth muscle cells. It has also been proposed that other, yet unidentified, plasmatic factors might influence PGI_2 production by endothelial cells (10–12). The purpose of our investigation was to study a possible effect of a number of specific human platelet-released proteins on PGI_2 production by primary cultures of bovine aortic endothelial cells.

Materials and Methods. *Materials.* Arachidonic acid (AA) (>99% pure, Nu-Chek Prep, Elysian, Minn.) was dissolved under nitrogen in 0.1 *M* sodium carbonate at a concentration of 10 m*M* and stored at −30°C under nitrogen. The 6-keto-$PGF_{1\alpha}$ used as a standard was a gift of Dr. J. Pike, Upjohn Company, Kalamazoo, Michigan. ^{3}H-6-keto-$PGF_{1\alpha}$ (100 Ci/mmole) was obtained from New England Nuclear, Boston, Massachusetts. Antiserum against 6-keto-$PGF_{1\alpha}$ was prepared as described previously (13). Purified human LA-PF_4 (14) and purified βTG (2) were kindly provided by Dr. B. Rucinski and Dr. J. C. Holt, Thrombosis Center, Temple University Medical School, Philadelphia, Pennsylvania. In addition, purified βTG and LA-PF_4, prepared according to Moore and Pepper (15), were kindly provided by Dr. C. Chesterman, Melbourne, Australia. All these preparations contained less than 5% contaminants. PF_4 (14) (purity >90%) was isolated from outdated platelet concentrates by chromatography on heparin–agarose and Sephacryl S 200. PBP (3, 16) was obtained from material released by thrombin-stimulated platelets. Three chromatographic steps, (CM Sephadex, heparin–agarose, and Sephadex G_{75}) yielded a preparation which was >80% homogeneous on dodecyl sulfate gel electrophoresis. Its mitogenic activity towards 3T3 cells was 200-fold less than that reported for PDGF (3). PF_4 and PBP were kindly provided by Dr. J. C. Holt. Partially purified PDGF, prepared according to Antoniades *et al.* (4), was purchased from Collaborative Research (Waltham, Mass.). As tested in our laboratory by methyl-^{3}H-thymidine incorporation assay (16), 1 unit of PDGF was equivalent to 1% calf serum. The preparation of PBP at the concentration of 1–2 μg had a mitogenic activity equivalent to 1% calf serum.

Cells. Bovine aortic endothelial cells were prepared according to a modification of the method of Gimbrone *et al.* (17). Thoracic aortas were collected from freshly slaughtered calves and placed in sterile containers in Dulbecco's modified Eagle's medium (DME, Flow Laboratories, Rockville, Md.) supplemented with Gentamicin (50 μg/ml, Gentamicin sulfate, Sigma Chemical, St. Louis, Mo.) and Fungizone (2.5 μg/ml, Gibco, Grand Island, N.Y.). All subsequent operations were carried under a laminar-flow hood. The aortas were freed of the surrounding tissues, opened through a longitudinal cut, and washed repeatedly with fresh medium. Aortas were then covered with a thin layer of a sterile solution of collagenase (Type II, Sigma Chemical, St. Louis, Mo., 0.1%) and trypsin (Type III, Sigma, 0.004%) in DME. After 10–15 min of incubation endothelial cells were harvested by gentle rolling strokes of a sterile cotton swab. The cells were washed off the swab into DME supplemented with 10% fetal bovine serum (Flow Lab Orstone), 25 m*M* L-glutamine (Microbiological Associates, Whittaker, Md.), 340 u/ml penicillin (Penicillin G, Sigma) and 80 μg/ml streptomycin (Streptomycin Sulfate, Sigma). Endothelial cells were then plated in tissue culture dishes (35 or 100 mm diam, Falcon, Becton Dickinson, Cockeysville, Md.) or in microwell plates (16 mm diam, Costar, Cambridge, Mass.) and incubated at 37°C in a moist incubator (10% CO_2/90% air). One 100-mm dish or eight 35-mm dishes or a 26-well plate were obtained from each aorta (15–20 cm long). Medium was changed the following day and every second day thereafter. Cultures were allowed to grow until confluency (5–7 days). The cell density was 2–3 $\times$ 10^5 cells per well or 5–10 $\times$ 10^5 cells per 35-mm dish at confluency. Only cells from primary cultures were used throughout the experiments.

The identity of endothelial cells was established by immunofluorescent demonstration of factor VIII antigen as described by Jaffe *et al.* (18), using rabbit anti-serum against bo-

vine factor VIII provided by Dr. E. Kirby, Department of Biochemistry, Temple University Medical School, Philadelphia, Pennsylvania. Contaminating nonfluorescent cells were less than 1%. Anti-bovine Factor VIII serum did not cross-react with bovine fibronectin as tested by immunodiffusion and by rocket immunoelectrophoresis.

Experimental system. Three different procedures were utilized.

Procedure A. Cells from confluent dishes were detached by incubation with a solution of trypsin (0.25%, Flow Laboratory) and EDTA (0.02%) in Hank's buffer (Flow Laboratory). After addition of 5% fetal bovine serum, cells were harvested from the dish, spun at 150*g* for 5 min and subsequently resuspended at the concentration of 1×10^6 cells/ml in a balanced salt solution (Tris 15 m*M*, NaCl 120 m*M*, KCl 4 m*M*, $MgSO_4$ 1.6 m*M*, NaH_2PO_4 2 m*M*, $CaCl_2$ 1.2 m*M*, glucose 10 m*M*, pH 7.4) containing 0.1% bovine albumin fatty acid-free, (Type V, Sigma). Cell viability was 90–95% as measured by erythrosin B dye exclusion test. Cells were allowed to rest for 15 min before the beginning of the experiment. Aliquots of 0.5 ml (0.5×10^5 cells) were then distributed in Eppendorf tubes and incubated for 15 min in a shaking water bath at 37°C in the presence of the proteins to be tested. Cell suspensions were centrifuged at 10,000*g* for 2 min; the supernatants were stored at −20°C for measurement of 6-keto-$PGF_{1\alpha}$. This procedure was similar to that used by Hope *et al.* (6) in his study describing inhibitory effect of βTG on the production of PGI_2-like activity in endothelial cells.

Procedure B. Confluent dishes (35 mm diameter) or wells (16 mm diameter) of bovine endothelial cells were washed twice with phosphate-buffered saline (PBS), pH 7.4, allowed to rest for 15 min, and subsequently incubated in 1 ml of PBS or Dulbecco Modified Eagle's Medium (DME) with the addition of different amounts of the proteins to be tested. Cells were incubated for 15 min in a shaking waterbath. The incubation fluid was harvested for measurement of 6-keto-$PGF_{1\alpha}$. In some experiments cells were further incubated in DME for 24 hr. The incubations were carried out in a tissue culture incubator at 37°C in 10% CO_2. After 24 hr the incubation fluid was collected and stored at −20°C for measurement of 6-keto-$PGF_{1\alpha}$. Triplicate dishes were used for each protein concentration. In each experiment cells incubated with medium or buffer alone were taken as controls; three dishes were incubated with a constant concentration of AA (10–20 μM) as a reference standard to evaluate the ability of cells to synthesize PGI_2. A total of 15 experiments were performed. Each protein concentration was tested in at least two experiments.

Procedure C. Bovine endothelial cells, prepared as in procedure B, were incubated with tested proteins and AA added simultaneously. Cells treated with AA alone were taken as controls. A total of six experiments was performed. At the end of the incubation period (15 min or 24 hr), the incubation fluid was collected from the dishes and stored at −20°C for measurement of 6-keto-$PGF_{1\alpha}$.

Harvesting of samples and cell count. At the end of each experiment (procedures B and C), the cultures were washed twice in PBS and incubated for 15–60 min in a solution of trypsin (0.25%) until cells detached. Number of cells was determined by counting the suspension in a hemocytometer by phase-contrast light microscopy. Cell viability was 90–95% in each experiment, as tested by erythrosin B dye exclusion test.

Radioimmunoassay. The endothelial cell supernates (procedure A) or supernates of incubation mixtures (procedures B and C) were assayed for the presence of 6-keto-$PGF_{1\alpha}$. The procedure was based on the one described elsewhere (19). Aliquots of 100 μl of 6-keto-$PGF_{1\alpha}$ standard (0.3–30 pmole/ml) or the unknown, 100 μl of [^{3}H]-6-keto $PGF_{1\alpha}$ (10,000–20,000 dpm/100 μl diluted in a suspension of 1% bovine γ-globulin) and 50 μl of antiserum at a dilution giving 50% binding of the labeled standard (usually 1:500–1:1000) were incubated for 1 hr at 37°C. Bound radioactivity was then separated from free by the addition of 250 μl of saturated ammonium sulfate (50% saturation level), followed by centrifugation at 10,000*g* for 2 min. Radioactivity in a 200-μl sample of the supernatant fluid was measured by liquid scintillation counting. The amounts of radioactivity precipitated in the presence of known amounts of unlabeled 6-keto-$PGF_{1\alpha}$ were plotted on a semilogarithmic paper and the unknown amounts of 6-

keto-PGF$_{1\alpha}$ in the samples were determined by interpolation from the standard curve. There was approximately 50% of binding of radiolabeled antigen in all assays and the limit of detection of unlabeled antigen was 0.3–0.5 pmole/ml at the dilution of antibody mentioned above. Using the above antiserum, PGF$_{2\alpha}$ and PGE$_2$ cross-reacted with 6-keto-PGF$_{1\alpha}$ at the levels of 1.5% and 1.2%, respectively. Thromboxane B$_2$, PGD$_2$, 6,15-diketo-PGF$_{1\alpha}$, and AA crossreacted less than 0.1% (20). Results were expressed as picomoles of 6-keto-PGF$_{1\alpha}$ per dish or well.

Statistics. The 6-keto-PGF$_{1\alpha}$ levels, first calculated in picomoles per dish, were converted to percentages of the average value of the respective controls. One-way analysis of variance and *t* tests were performed for each experiment separately and subsequently on groups of results with the same time of incubation and protein concentration. Tests for homogeneity of variance, and multiple range tests were also performed (21).

In a selected group of experiments, data obtained from the same cell dishes after two different incubation times were tested by two-way analysis of variance for repeated measures.

Results. In a first series of experiments, suspensions of endothelial cells prepared by partial trypsin digestion (procedure A) were incubated with different concentrations of LA-PF$_4$ (1–5 μg/ml) for 15 min. As shown in Table I, the levels of 6-keto-PGF$_{1\alpha}$ were not modified by treatment with LA-PF$_4$ at any of the concentrations tested. Three different preparations of LA-PF$_4$ were tested and found to be devoid of any activity (data not shown).

TABLE I. LEVELS OF 6-KETO-PGF$_{1\alpha}$ IN SUSPENSIONS OF BOVINE ENDOTHELIAL CELLS, AFTER INCUBATION WITH LA-PF$_4$ (PROCEDURE A)[a]

Protein concentration	6-Keto-PGF$_{1\alpha}$[b] production (pmole/0.5 ml)
Control	3.2 ± 0.8
LA-PF$_4$	
1 μg/ml	3.7 ± 1.3
2 μg/ml	3.8 ± 1.1
5 μg/ml	3.3 ± 0.6
10 μg/ml	3.9 ± 0.3

[a] Cells were suspended in a balanced salt solution containing 0.1% BSA at the concentration of 5 × 10^5 cells/0.5 ml and incubated for 15 min in a shaking incubator at 37°C. The supernatants were tested for the presence of 6-keto-PGF$_{1\alpha}$.

[b] Mean ± SEM of five determinations. No statistical differences were found using *t* test.

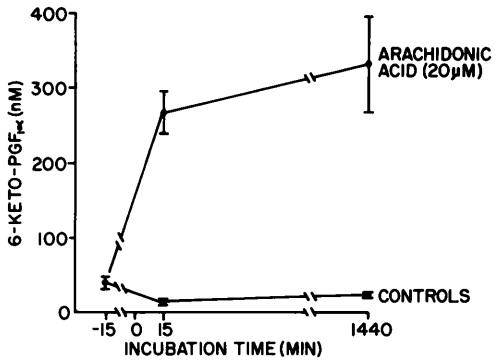

FIG. 1. Effect of various incubation times on the production of 6-keto-PGF$_{1\alpha}$ by bovine endothelial cells incubated either with serum-free medium (controls) or with medium containing AA (20 μ*M*). Levels of 6-keto-PGF$_{1\alpha}$ were measured 15 min before the washing procedure and after 15 min and 24 hr of incubation. Cells were grown in 35-mm dishes (5–8 × 10^5 cells/dish at confluency). Each point represents mean ± SEM of 6–25 determinations.

In all of the following experiments (procedures B and C), tested proteins or AA were added to the cell monolayers without detachment from the tissue culture dishes. The time-dependence of 6-keto-PGF$_{1\alpha}$ production was studied in cells incubated either with serum-free medium (controls) or with 20 μ*M* AA (Fig. 1). The basal level of 6-keto-PGF$_{1\alpha}$ before starting the experiment was 39.9 ± 7.0 pmole/dish (mean ± SEM of six determinations). After washing of the cells and incubating them for 15 min in the absence of AA, release of 6-keto-PGF$_{1\alpha}$ decreased slightly. Production increased and returned towards basal levels after 24 hr of incubation. Addition of 20 μM AA stimulated a 20-fold increase of 6-keto-PGF$_{1\alpha}$ production within 15 min; further incubation did not further increase 6-keto-PGF$_{1\alpha}$ production. The dose response of endothelial cells to the AA (1.5–100 μ*M*) was also studied (Fig. 2). Increased formation of 6-keto-PGF$_{1\alpha}$ was observed after 15 min of incubation with concentrations of 6–25 μ*M* AA, with a saturation level of 6-keto-PGF$_{1\alpha}$ above 25 μ*M* AA. The figure shows the results of a single experiment, which is representative of the three performed.

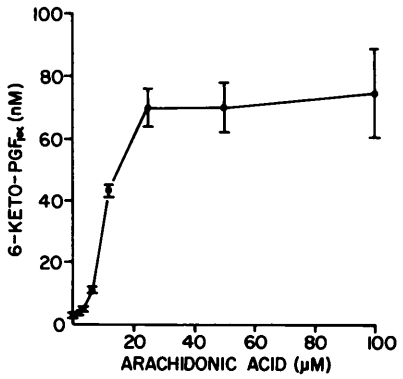

FIG. 2. Effect of increasing concentrations of AA (μM) production of 6-keto-$PGF_{1\alpha}$ (nM) by bovine endothelial cells. Cells grown in microwell clusters were incubated with AA for 15 min. Cell number was 2–2.5 × 10^5/ml. Each point represents mean ± SEM of three determinations.

The cell count did not affect the results of experiments even if the rate of growth of various batches of endothelial cells showed some variations. A statistically significant correlation between 6-keto-$PGF_{1\alpha}$ production and the number of cells was found either after 15 min or after 24 hr of incubation of endothelial cells in serum-free medium by means of linear regression (data not shown). In all experiments the control and tested samples containing approximately the same number of cells were processed in parallel.

Table II shows the results obtained after 15 min or 24 hr of incubation of endothelial cells with different concentrations of human platelet secreted proteins. None of the proteins tested had any effect on cell number after 24 hr of incubation. Cells were prepared according to procedure B; levels of 6-keto-$PGF_{1\alpha}$ were converted to percentages of the average value of the controls. Preparations of $LA\text{-}PF_4$ and βTG obtained in our laboratory (indicated as preparations "A" in the table) were compared with samples of the same proteins obtained from Dr. Chesterman ("B" preparations in the table). Each protein was tested at 1, 5, and 10 μg/ml. Both proteins had some inhibitory effect on the release of 6-keto-$PGF_{1\alpha}$ after 15 min, but not after 24 hr of incubation with the cells. PF_4, tested at the concentrations of 1, 5, and 10 μg/ml, did not have any effect on 6-keto-$PGF_{1\alpha}$ production at 15 min of incubation except for one experiment in

TABLE II. EFFECT OF HUMAN PLATELET-DERIVED PROTEINS ON 6-KETO-$PGF_{1\alpha}$ PRODUCTION BY BOVINE AORTIC ENDOTHELIAL CELLS (PROCEDURE B)[a]

Protein concentration	6-keto $PGF_{1\alpha}$ production (% of controls)[b] 15 min		24 hr	
βTG (A)[c]				
1 μg/ml	77.1 ± 15.0	(6)	121.7 ± 19.0	(6)
5 μg/ml	91.8 ± 6.3	(6)	—	—
10 μg/ml	45.7 ± 2.7[d]	(6)	222.7 ± 53.3	(6)
βTG (B)[c]				
1 μg/ml	53.0 ± 4.8[d]	(6)	135.4 ± 34.3	(3)
5 μg/ml	70.5 ± 14.0	(6)	173.8 ± 42.4	(3)
10 μg/ml	61.8 ± 11.9[d]	(6)	87.7 ± 20.3	(3)
$LA\text{-}PF_4$ (A)				
1 μg/ml	83.8 ± 8.4	(9)	130.6 ± 21.2	(6)
5 μg/ml	66.0 ± 8.0[d]	(15)	112.2 ± 23.4	(6)
10 μg/ml	93.1 ± 12.3	(10)	139.6 ± 29.6	(8)
$LA\text{-}PF_4$ (B)				
1 μg/ml	75.4 ± 9.2[e]	(6)	117.5 ± 20.1	(3)
5 μg/ml	61.3 ± 5.5[d]	(6)	162.2 ± 53.7	(3)
10 μg/ml	60.4 ± 6.2[d]	(6)	82.8 ± 15.8	(3)
PF_4				
1 μg/ml	100.1 ± 17.4	(5)	78.9 ± 20.4	(2)
5 μg/ml	115.0 ± 20.2	(9)	93.1 ± 8.2	(5)
10 μg/ml	124.6 ± 29.9	(6)	64.6 ± 6.0[a]	(3)
PBP				
0.01 μg/ml	119.3 ± 32.6	(5)	58.3 ± 12.8	(2)
0.10 μg/ml	106.4 ± 16.4	(9)	74.9 ± 14.5	(5)
1.00 μg/ml	110.6 ± 16.9	(6)	83.1 ± 26.7	(3)
PDGF				
0.01 u/ml	86.5 ± 23.3	(3)	140.7 ± 6.0[e]	(3)
0.10 u/ml	96.0 ± 13.8	(5)	178.7 ± 59.6	(2)
0.50 u/ml	90.6 ± 19.1	(6)	130.0 ± 31.1	(6)
1.00 u/ml	101.9 ± 17.7	(5)	147.3 ± 25.0	(3)

[a] Levels of 6-keto-$PGF_{1\alpha}$ were converted to percentages of the average level produced by the controls. The average levels of 6-keto-$PGF_{1\alpha}$ production by control bovine aortic endothelial cells were 15.5 ± 2.4 pmole/dish (mean ± SEM of 27 determinations) after 15 min of incubation and 25.0 ± 3.75 pmole/dish (mean ± SEM of 13 determinations) after 24 hr of incubation in serum-free medium. Average number of cells in the control group was 8.6 ± 2.8 × 10^5 cells/dish (mean ± SEM of 17 determinations) after 24 hr of incubation in serum-free medium.

[b] Mean ± SEM. Numbers in brackets indicate number of experiments.

[c] Preparations of $LA\text{-}PF_4$ and βTG obtained in our laboratory are indicated as "A," whereas preparations of the same proteins obtained from Dr. C. Chesterman are indicated as "B."

[d] Statistically different from the control sample at <0.05 level.

[e] Statistically different from the control sample at <0.1 level.

which the level of 6-keto-PGF$_{1\alpha}$ was significantly reduced after 24 hr of incubation with 10 μg/ml of PF$_4$. PBP and PDGF at the doses of 0.01–1 μg/ml and 0.01–1 u/ml, respectively, did not modify 6-keto-PGF$_{1\alpha}$ levels after 15 min of incubation. The production of 6-keto-PGF$_{1\alpha}$ was increased when the cells were incubated with PDGF for 24 hr, although statistically significant results were obtained only at one concentration of this agent. The reduction of 6-keto-PGF$_{1\alpha}$ production after 24 hr incubation was not statistically significant.

A two-way analysis of variance was applied in the experiments where 6-keto-PGF$_{1\alpha}$ production was measured in the same cell dishes after 15 min and 24 hr of incubation with the proteins. This analysis showed that βTG and LA-PF$_4$ significantly reduced 6-keto-PGF$_{1\alpha}$ after 15 min of incubation. No significant differences were found between the sample containing PBP, PF$_4$, and PDGF tested at two incubation times.

In further experiments, the proteins were evaluated for their effects on 6-keto-PGF$_{1\alpha}$ production induced by AA (procedure C). Table III shows the results of two separate sets of experiments. LA-PF$_4$ and βTG were tested at the concentration of 10 μg/ml in association with AA at the dose of 10 μ*M*. Addition of either LA-PF$_4$ or βTG did not modify AA-induced stimulation of 6-keto-PGF$_{1\alpha}$ production. In another set of experiments, endothelial cells were incubated with PF$_4$ (5 μg/ml), PBP (0.1 μg/ml), or PDGF (0.5 u/ml) in combination with 20 μ*M* AA. Neither PF$_4$ nor PBP modified AA-induced stimulation of 6-keto-PGF$_{1\alpha}$ production at any incubation time. An apparent potentiation found between PDGF and AA after 24 hr of incubation (135%) was not statistically significant.

TABLE III. EFFECT OF HUMAN PLATELET-DERIVED PROTEINS ADDED IN COMBINATION WITH AA ON 6-KETO-PGF$_{1\alpha}$ PRODUCTION BY BOVINE AORTIC ENDOTHELIAL CELLS (PROCEDURE C)[a]

Protein concentration	6-keto-PGF$_{1\alpha}$ production (% of controls)[b]	
	15 min	24 hr
AA + βTG (10 μg/ml)	112.4 ± 13.5 (7)	not determined
AA + LA-PF$_4$ (10 μg/ml)	93.6 ± 18.8 (9)	not determined
AA + PF$_4$ (5 μg/ml)	88.4 ± 15.1 (6)	87.2 ± 18.0 (5)
AA + PBP (0.1 μg/ml)	113.0 ± 12.4 (5)	96.0 ± 9.7 (5)
AA + PDGF (0.5 u/ml)	93.0 ± 9.8 (6)	134.8 ± 19.7 (5)

[a] Levels of 6-keto-PGF$_{1\alpha}$ were converted to percentages of the average level produced by cells treated with AA alone. Treatment of cells with 10 μ*M* AA (control samples done for experiments with LA-PF$_4$ and βTG) increased 6-keto-PGF$_{1\alpha}$ production from 8.6 ± 3.1 pmole/dish to 80.6 ± 15.3 pmole/dish in 15 min of incubation (mean ± SEM of six determinations). Incubation with 20 μ*M* AA (control samples done for experiments with PF$_4$, PBP, and PDGF) increased 6-keto-PGF$_{1\alpha}$ production from 34.5 ± 2.2 pmole/dish to 270.5 ± 28.3 pmole/dish after 15 min and 332.8 ± 64.1 pmole/dish after 24 hr of incubation (mean ± SEM of six determinations).

[b] Mean ± SEM. Numbers in brackets indicate numbers of experiments. No statistically significant differences were found using *t* test.

Discussion. Production of PGI$_2$ by bovine aortic endothelial cells in culture is influenced by several variables such as the incubation time, the cell number, and the number of serial passages in culture (22, 23). Our first effort was to define the experimental conditions to be used. All endothelial cells tested in our experiments were primary cultures of bovine aortas at Days 5–7 after explanation from the vessel wall. Primary cultures of endothelial cells are able to release higher amounts of PGI$_2$ and are supposed to be more representative of the situation *in vivo* than later subcultures. Two different procedures including preparations of cells in suspension and cells in monolayer were utilized. Release of 6-keto-PGF$_{1\alpha}$ from endothelial cells in suspension was of 2–10 ng/10^6 cells. Basal release of 6-keto-PGF$_{1\alpha}$ from cell monolayers was of 5–14 ng/ or 12–40 pmole/10^6 cells, similar to the levels reported by other investigators (9, 19, 22). Cells in suspension as well as cells in monolayers were able to increase 6-keto-PGF$_{1\alpha}$ production rapidly after stimulation with exogenous AA, thus indicating the integrity of their enzymatic system. An important variable seemed to be the incubation time. Higher levels of 6-keto-PGF$_{1\alpha}$ were found after 24 hr than after 15 min of incubation.

The effects of incubation of bovine aortic endothelial cells with human platelet-derived proteins differed according to the experimental system used and varied between one pro-

tein and another. Incubation of various concentrations of LA-PF_4 with endothelial cells in suspension (procedure A) did not have any effect on 6-keto-$PGF_{1\alpha}$ production, although the experimental conditions were similar to those described by Hope *et al.* (6). A significant reduction of 6-keto-$PGF_{1\alpha}$ production was found only when bovine endothelial cells in monolayers were incubated either with LA-PF_4 or with βTG for 15 min (procedure B). However, the inhibition was not dose-related, the effect of 1 μg/ml being often similar to the one at 10 μg/ml. Moreover, when the cells were incubated for 24 hr instead of 15 min no reduction was observed. The reasons for the differences between the two procedures are not clear. They may depend on a different ability of the cells in suspension or in monolayer to reequilibrate after the stress of manipulation and to react to the addition of stimuli in a short period of time (15 min). It is unlikely that the effects may be due to toxicity because the inhibition was more pronounced after 15 min then after 24 hr and because the effect was not related to the protein concentration tested. It is also unlikely that the effect might have been due to the same contaminant still present after the purification procedure. Ager and Gordon (8) suggested that different preparations of βTG may have different effects on 6-keto-$PGF_{1\alpha}$ independently of any physicochemical difference between the preparations. However, LA-PF_4 and βTG produced in our laboratory and preparations obtained from Dr. Chesterman had similar inhibitory effect on endothelial cells.

It is interesting to note that PF_4, a protein which binds to the endothelial cells (5), did not show any significant effect on 6-keto-$PGF_{1\alpha}$ production except for one experiment. PBP did not affect 6-keto-$PGF_{1\alpha}$ release after incubation for 15 min and slightly but not significantly reduced production after 24 hr of incubation with the cells.

A partially purified preparation of PDGF showed no significant effect after 15 min but moderately stimulated 6-keto-$PGF_{1\alpha}$ production after 24 hr of incubation. However, this stimulation was not significant except for one experiment (Table III). PDGF did not potentiate the effect obtained upon incubation with AA, at least at the protein concentration tested. It has been shown previously that PDGF does not stimulate growth of endothelial cells (24) and that endothelial cells do not possess PDGF receptors (25). Therefore absence of any effect of PDGF on the prostacyclin production by these cells could be anticipated. However, Coughlin *et al.* (9) observed a strong stimulation of 6-keto-$PGF_{1\alpha}$ production by endothelial cells incubated either with human serum or with purified preparations of human PDGF although DNA biosynthesis was not stimulated under these conditions. At present we are not able to explain the discrepancies obtained by Coughlin *et al.* (9) and in our experimental system.

In conclusion, our data indicate that, among the platelet-specific proteins studied, LA-PF_4 and βTG had a slight inhibitory effect on 6-keto-$PGF_{1\alpha}$ production by endothelial cells whereas PF_4, PBP, and PDGF did not significantly alter 6-keto-$PGF_{1\alpha}$ production by the cells. None of the effects found was consistent but varied according to the experimental conditions used. For this reason, any relevant role of the tested proteins in the prostacyclin production by bovine endothelial cells *in vitro* can be excluded. However, it is difficult to extrapolate our data to the *in vivo* situations.

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