Substrate for Endothelial Prostacyclin Production in the Presence of Platelets Exposed to Collagen Is Derived From the Platelets Rather Than the Endothelium

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Interactions between vascular endothelial cells and blood platelets have been investigated using a model microcirculation consisting of microcarrier beads colonized with human umbilical vein endothelial cells (HUVECs) and perfused with washed platelet suspensions. To simulate the effects of endothelial desquamation and exposure of subendothelium, fibrillar collagen in suspension was coinjected with the platelets. In this model, neither the passage of platelets alone nor collagen alone stimulated prostacyclin (PGI_2) production by the HUVECs. Platelets activated by coinjection with collagen released thromboxane A_2 (TXA_2), and this was associated with the simultaneous production of PGI_2 by the HUVECs. By means of double-isotope experiments with [^{3}H]arachidonic acid (AA) incorporated into platelets and [^{14}C]-AA into HUVECs, it was shown that all the PGI_2 generated was derived from platelet AA and/or endoperoxides. This interpretation was strengthened by the finding that PGI_2 production was not prevented by treatment of HUVECs with indomethacin followed by perfusion with collagen-stimulated platelets. AA metabolites in double-isotope label experiments were further characterized by reverse-phase chromatography, and it was shown that both cyclooxygenase and lipoxygenase products of the HUVECs were derived from platelet membrane lipid. Thrombin regularly produced transient PGI_2 release, but showed rapid tachyphylaxis. Platelet-derived compounds including ADP, ATP, and platelet-activating factor (PAF) did not produce PGI_2 release by HUVECs in this system. Thus, the transfer of AA and metabolites from collagen-stimulated platelets is likely to be the mechanism for PGI_2 production in the context of minor degrees of endothelial desquamation.

THE RELATIONSHIP between circulating blood platelets and the vessel wall is complex, but it is clear that endothelial cells maintain a nonadhesive luminal surface, this property relying on a number of unstimulated membrane and metabolic characteristics. The concept that endothelial-derived prostacyclin (PGI_2), continuously released, may result in circulating "anticoagulant" activity[1] has not proved tenable.[2] Measurable levels are not high enough. Nevertheless, PGI_2 release by endothelium is still regarded as a local and effective response to vascular "injury" or thrombotic stimulus, providing a defense against further local platelet deposition and initiating vasodilatation, which might in itself promote blood flow rather than stasis.[2,3] Defining the mechanisms and the agonists responsible for such a local PGI_2 release in a given situation is not as easy as might be expected, because potential stimuli differ and the vascular response to individual agonists varies with both the species and the anatomical origin of the endothelium.[4-6]

If the subendothelium becomes exposed to circulating blood or the endothelium loses its platelet-repelling properties, it is likely in many instances that the only stimulus to PGI_2 production by nearby endothelium is platelet derived. A number of platelet release products have been reported to stimulate endothelial PGI_2 release, including adenine nucleotides, ADP and ATP,[7] platelet-derived growth factor (PDGF),[8] platelet-activating factor (PAF)[6,9] and serotonin,[6] although responses are both species and site dependent. In other situations, further stimulus to PGI_2 production by endothelium may be contributed by other cells: for example, ADP from damaged red cells or PAF and leukotrienes released by granulocytes.

Experiments incubating cultured endothelial cells (EC) with human platelets[10,11] or slices of vascular tissue with platelets[12] have suggested the occurrence of the transfer of cyclic endoperoxides from stimulated platelets to EC, thus providing substrate for PGI_2 production. Some authors, however, have reported results showing that this is not a significant phenomenon.[13-15] For the most part, these experiments were carried out in static systems which suffered from an unfavorable ratio of culture medium to the surface area of the EC and which introduced potential artifacts by the physical or enzymatic manipulation of the cultured cells and from the accumulation of metabolites during the time course of the experiment.

To avoid these problems and to construct a physiological model to investigate interactions between EC and platelets in flowing blood, we used a perfusion system in which the luminal surface of the vessel wall was represented by microcarrier beads colonized with human umbilical vein endothelial cells (HUVECs).[5,16] In this way, we were able to document clearly that the transfer of arachidonate and metabolites from collagen-stimulated platelets to vascular EC is the major mechanism for the production of PGI_2 by EC in the immediate vicinity.

MATERIALS AND METHODS

HUVECs. Endothelial cells were harvested from umbilical cords <4 hours after delivery by collagenase digestion.[17] Primary cultures were initiated in 25-cm^2 culture flasks (Corning, NY). Two culture techniques were used. In the first method, the flasks were incubated overnight with human fibronectin 10 μg per square centimeter. The fibronectin was prepared according to the method of Engvall and Ruoslahti.[18] The culture medium was M199 (Flow Laboratories, North Ryde, Sydney), 0.1% penicillin/streptomycin (GIBCO, Grand Island, NY) 20% human umbilical cord blood serum (T.H. Forster, personal communication), and 5% CO_2 in air at 37 °C.

In the second method, the flasks were incubated overnight with

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1% gelatin (Haemaccel; Behringwerk AG, Marburg, FRG); the medium was M199, 0.1% penicillin/streptomycin, 20% fetal calf serum (FCS) from Flow Laboratories, 20 μg per milliliter endothelial cell growth factor (ECGF) obtained from BRL Laboratories (Gaithersburg, Maryland), heparin 90 μg per milliliter (Weddell, Wrexham, England) in 5% CO_2 in air at 37 °C.[19]

The cells were passaged with trypsin-EDTA (Flow Laboratories) and split in a ratio of 1:3. At passage 3, the contents of 3 × 75 cm^2 flasks of cells were seeded onto 3 g of microcarrier beads (Biosilon, Nunc, Denmark).[5,16] The microcarriers had been preincubated with the respective adhesive protein, and the culture was continued in microcarrier culture bottles (Techne, Cambridge, England), being stirred at 40 rpm for 20 seconds every 30 minutes. The cells on beads were visualized by staining a small aliquot in 0.5% methyl violet for 15 to 20 minutes, rinsing with tap water, and observing under a ×10 objective. HUVEC counts were performed by incubating a small aliquot of colonized beads in trypsin-EDTA at 37 °C for five minutes before the cells were resuspended and counted in a hemocytometer. Factor VIII antigen immunofluorescence was observed using rabbit anti-human factor VIII antigen (Dako, Denmark) and FITC-conjugated sheep anti-rabbit IgG (Miles Laboratories, Mulgrave, Australia).

[^{14}C]-Labeled AA Incorporation into HUVECs. Two and a half microcuries [^{14}C]AA (NEC-756, 390 mCi per millimole Lot No. 1931-109, New England Nuclear, Boston) was evaporated in a Speed Vac concentrator (Savant Instruments Inc, Farmingdale, NY) and then resuspended in 1 mL of serum-free M199. The medium containing the labeled AA was added to 19 mL of serum-free M199 containing 2.65 g of microcarrier beads with ~2.7 × 10^6 HUVECs. The beads bearing the HUVECs had previously been washed twice with phosphate-buffered saline (PBS). The cells were incubated at 37 °C for 75 minutes with intermittent stirring in the culture vessel. Aliquots of beads and medium were taken prior to and after incubation to ascertain cell association of [^{14}C] into the HUVECs. Under these conditions, cell association was ~37%.

Washed platelet suspension. Platelets were prepared by a modification of the method of Vargas and colleagues.[20] Prostacyclin (PGI_2) (Wellcome Laboratories, Kent, England) 300 ng per milliliter was added to citrated platelet-rich-plasma (PRP) and in each of two subsequent washes in Tyrode's buffer, which was supplemented with 0.01% fatty acid-free bovine serum albumin (BSA). Heparin, 25 U per milliliter was included in the first wash. In experiments in which platelets were prelabeled with tritiated AA, the platelets at the second wash were incubated with 50 μCi of tritiated AA (NEN, 83.8 Ci per millimole, NET 298 Lot No. 2145-160) for 45 minutes at 37 °C with intermittent agitation and then pelleted. The platelets were resuspended at 2 × 10^8 per milliliter in PGI_2-free Tyrode's buffer and stored in a double-walled siliconized container at 4 °C overnight before use. Prior to the experiment, platelets were resuspended with gentle pipetting and allowed to come to room temperature. Platelet aggregation was assessed using collagen at 5 or 10 μg/400 μL of platelet suspension.

Platelet aggregation. Platelet aggregation was carried out at 37 °C in a Payton single-channel aggregometer, Model 401 (Payton Associates, Scarborough, Ontario, Canada) with an Omniscribe flatbed recorder, Model EB5117-1 (Houston Inst., Austin, Texas).

Agonists. Agonists used to stimulate either platelet or HUVEC arachidonate metabolism were as follows: Thrombin was of bovine origin (Thrombostat, Parke-Davis, Sydney, Australia); adenosine 5'-diphosphate (ADP) disodium salt was from Calbiochem-Behring (Lot 102030); adenosine 5'-triphosphate (ATP) disodium salt was from Calbiochem-Behring (Lot 102197); PAF (L-α-phosphatidylcholine, β-acetyl-γ-*O*-alkyl) was from Sigma (St Louis), P9525 (Lot 53F8370) evaporated under N_2 and diluted in tissue culture medium (TCM) containing BSA 2.5 mg per milliliter; collagen suspension was equine in origin in isotonic glucose solution (Hormon-Chemie, Munich, FRG).

Platelet and white cell counts. Cell counts were performed in a Coulter-S plus Particle Counter using Isoton II as diluent (Coulter Electronics, Sydney, Australia).

Thromboxane B_2 (TXB_2) and 6-keto prostaglandin $gF_{1\alpha}$ (6-keto $PGF_{1\alpha}$). Arachidonate metabolites were assayed in unextracted perfusion fractions by radioimmunoassay (RIA) using Seragen antisera (Flow Laboratories). Cross-reactivity with 6-keto-$PGF_{1\alpha}$ antiserum at 50% B/B_0 was stated by the suppliers to be 2.2% for $PGF_{2\alpha}$, 0.6% for PGE_2, and <0.01% for both PGD_2 and TXB_2. Cross-reactivity with the TXB_2 antiserum was stated to be <0.1% for prostaglandins including 6-keto-$PGF_{1\alpha}$ and 5-, 12-, and 15-HETES. When [^{3}H] metabolites were present in column fractions, RIA was performed using [^{125}I]-labeled tracers (New England Nuclear RIA kits). Standard curves were constructed using as diluting buffer the serum-free culture medium and, where appropriate, including a dilution of the anticoagulant mixture used to collect the platelet suspensions from perfusion (see below). When platelets were perfused through the HUVEC column, the eluate 6-keto-$PGF_{1\alpha}$ concentrations were corrected for the small residual contamination in the platelet suspension (see Washed platelet suspension section above).

^{125}I-Assays were counted in an LKB Wallac Gamma Sample Counter Model GTL 300-500 equipped with AS-12 Pulse Height analyser and SC-20/SC-23 scalers.

Analysis of radiolabeled AA metabolites from column fractions. Perfusion fractions were prepared for analysis by extraction in 80% ethanol (vol/vol)[21] and stored at −70 °C under argon before centrifugation at 3,000 g for five minutes, evaporation in the Speed Vac concentrator at 37 °C and reconstituted in 250 μl of starting solvent. The sample was subjected to reverse-phase chromatography on a Pharmacia PepRPC HR 5/5 column, C_2/C_{18}, operating with a Pharmacia FPLC (fast protein liquid chromatography) System. The column was developed with a stepwise methanol gradient as follows: flow rate 0.5 mL/min; methanol 47%, 0 to 30 minutes; methanol 60%, 31 to 65 minutes; methanol 68%, 66 to 85 minutes; methanol 100%, 86 to 95 minutes. The remainder of the solvent was buffered water, acetic acid (1:0.0008) brought to pH 6.2 with a 50% vol/vol solution of 33% ammonia [modified from Henke et al[21]]. Fractions were mixed with 8 vol of Amersham ACSII scintillant and counted in a Packard Minaxi, Model D4430 scintillation counter using a dual-label program in which [^{3}H] and [^{14}C] labeled metabolites were present. Tritiated standards, AA, prostaglandins ($PGF_{2\alpha}$, PGD_2, PGE_2), 5-,12-,15-HETE, and leukotrienes (LTB_4, LTC_4, LTD_4, and LTE_4) were obtained from NEN. 6-Keto $PGF_{1\alpha}$ and TXB_2 were from Amersham (Sydney, Australia). Standard mixtures were subjected to extraction procedures identical to perfusion fractions. High-performance liquid chromatography (HPLC) grade methanol was obtained from Mallinckrodt (South Oakleigh, Victoria), HPLC grade acetic acid from Ajax Chemicals (Sydney), and ethanol (AR grade) from May & Baker (Melbourne). Water was triple-distilled, deionized using Elgacan C114 cartridge, and filtered through a Millipore 0.22-μm filter.

The recoveries of total DPM of radiolabeled standards 6-keto-$PGF_{1\alpha}$, TXB_2, 12-HETE, and 15-HETE following extraction from M199 and reverse-phase chromatography were 87.9%, 93.3%, 98%, and 96%, respectively. Recoveries of the standards from Tyrode's buffer, 0.01% BSA, were 88.8%, 90.2%, 99.2% and 93.2%, respectively.

Experimental perfusion system. The system is illustrated diagramatically in Fig 1. A perspex column with an internal diameter of 4 mm and a length of 90 mm accommodated ~1 g of microcarrier beads colonized with HUVECs or, in control situations, beads alone coated with fibronectin or gelatin. The column could be extended

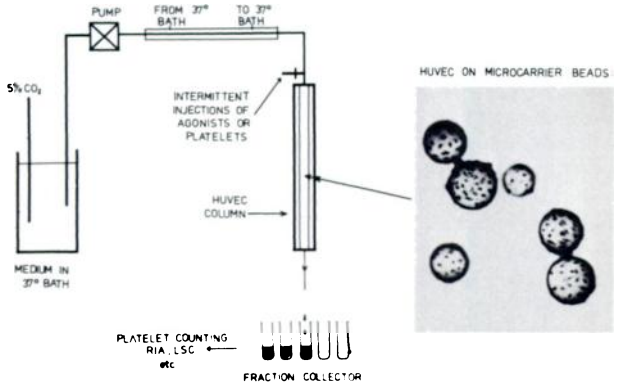

Fig 1. Diagram of the system used to perfuse human umbilical vein endothelial cells colonized on microcarrier beads packed in a perspex column. Two columns of beads were perfused simultaneously.

and doubled in length if required. A Holter pump (model 912) was used to perfuse two columns simultaneously with serum-free M199 at 0.4 to 0.6 ml/min. The medium was gassed with 5% CO_2 in a 37 °C water bath and was delivered to the column at 37 °C by passage through a heated water jacket. An injection of prewarmed agonists or platelet suspensions was made into a side arm at the top of the column using a 1-mL tuberculin syringe, the flow rate being maintained. Column perfusate was collected in 1-mL fractions. In experiments in which nucleotides were used as agonists, columns were perfused with Dulbecco's modified Eagle's medium (DMEM) because this medium contains no added nucleotides. If a platelet suspension was perfused through the column, an aliquot of each fraction was taken into Isoton for platelet counting. To the remainder of the fraction was added EDTA/theophylline/prostaglandin E_1 (ETP)[22] anticoagulant brought to 4 °C and centrifuged at 11,000 g for five minutes before the supernatant was taken off for assay. The perfusion system was cleaned with 0.1 mol/L of HCl between experiments, and sterility was maintained during perfusion by aseptic technique.

RESULTS

HUVECs were regularly used at the third passage to provide adequate numbers of cells on microcarriers. The mean number of cells per column was 3.07 ± 1.58 × 10^6 (N = 17). Both culture methods resulted in similar proliferation rates, 5.3 ± 2.8 days (N = 22) between passages in media supplemented with CBS and 4.6 ± 2.1 (N = 28) days with fetal calf serum/endothelial cell growth factor (FCS/ECGF) heparin. The morphology of the HUVECs, however, was noticeably different. The cells in CBS maintained typical cobblestone appearance, whereas those grown in FCS/ECGF/heparin became elongated. In both, however, there was expected factor VIII antigen staining by immunofluorescence.

Platelet preparations. The method for platelet preparation was suitable for these studies because the platelets remained as a single cell suspension and retained their reactivity to collagen (and other agonists, although these were not regularly tested). The platelet recovery was 58.2% ± 8.6% (N = 16) of the initial PRP, and there was contamination with 3 ± 1.3 white cells per 10^3 platelets (N = 9). The uptake of [^{3}H] AA, when included, was between 50% and 60%.

Experiments with unlabeled platelet suspensions passed through the HUVEC column. A 1-mL suspension of 2 × 10^8 platelets was fully recovered after passage through the HUVEC column over six fractions (6 mL) (Fig 2A). There was the concurrent release of 0.63 ng TXB_2. When 10 μg of collagen suspension was coinjected with 2 × 10^8 platelets (from the same platelet preparation), 55% of the platelets were retained in the column. This was accompanied by 7.71 ng TXB_2 and a simultaneous PGI_2 release measured as 3.7 ng 6-keto-$PGF_{1\alpha}$. A control column perfused with collagen (10 μg) suspension produced no 6-keto-$PGF_{1\alpha}$.

When 6 × 10^8 platelets coinjected with 30 μg collagen suspension were passed over a HUVEC column that had been pretreated with 10 μmol/L of indomethacin for 60 minutes, there was 6-keto-$PGF_{1\alpha}$ production (12.3 ng) and retention of 30% of the platelets (Fig 2B). In experiments in which platelet suspensions were coinjected with collagen (examples Figs 2 and 3), although there was a net retention of platelets during the perfusion, there was a tendency for platelets to continue to appear from the column over a period of 10 to 20 minutes in some instances (Fig 3) with a second peak following the release of PGI_2, suggesting the possibility of disaggregation or the release of adherent platelets.

Isotopically [^{3}H]-AA-labeled platelets and [^{14}C]-AA-labeled HUVECs interaction. A 10-minute perfusion of a HUVEC column was carried out with a total of 10^9 platelets (2 × 10^8/mL) coinjected with 50 μg of collagen suspension. The platelets had been incubated with [^{3}H]-AA for 45

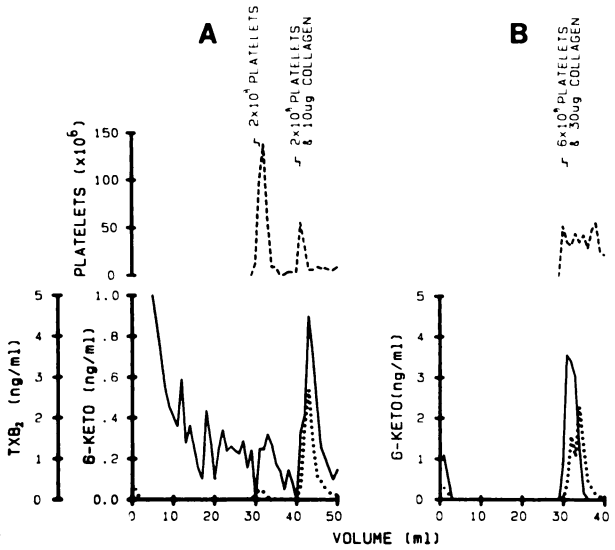

Fig 2. (A) One milliliter of Tyrode's buffer containing 2 × 10^8 platelets in suspension was perfused through a column containing human umbilical vein endothelial cells on microcarrier beads over 2 minutes, the column otherwise being continuously perfused with medium 199 at the same flow rate. A second perfusion of platelets, this time with coinjection of 10 μg collagen, followed 20 minutes later. The platelet recovery from the column is shown in the upper panel (------). Lower panel: production of thromboxane [radioimmunoassay (RIA) of thromboxane B_2] ..... and prostacyclin (RIA of 6-keto-$PGF_{1\alpha}$) during the experiment. (B) A similar column of human umbilical vein endothelial cells was pretreated with 10 μmol/L of indomethacin for 60 minutes before perfusion with 6 × 10^8 platelets and 30 μg of collagen over 6 minutes. The symbols are as in A.

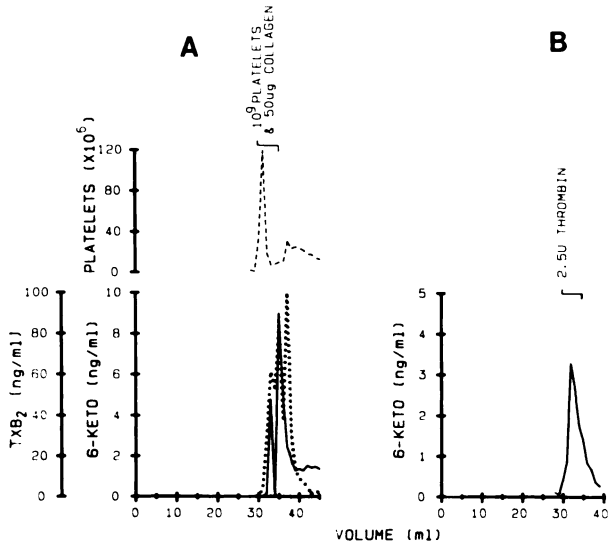

Fig 3. (A) A 10-minute perfusion of a human umbilical cord endothelial cells (HUVECs) column with a total of 10^9 platelets (coinjected with 50 μg collagen) was carried out. The platelets were prelabeled with [^{3}H]-arachidonic acid (AA), and the HUVECs were prelabeled with [^{14}C]-AA. The platelet recovery is shown in the upper panel and the production of thromboxane [radioimmunoassay (RIA) of thromboxane B_2] and prostacyclin (RIA of 6-keto-$PGF_{1\alpha}$) shown in lower panel. Symbols as in Fig 2. (B) A similar column of [^{14}C]-AA prelabeled HUVECs was perfused with thrombin, 2.5 U. Results of RIA of 6-keto-$PGF_{1\alpha}$ performed on column fractions are shown.

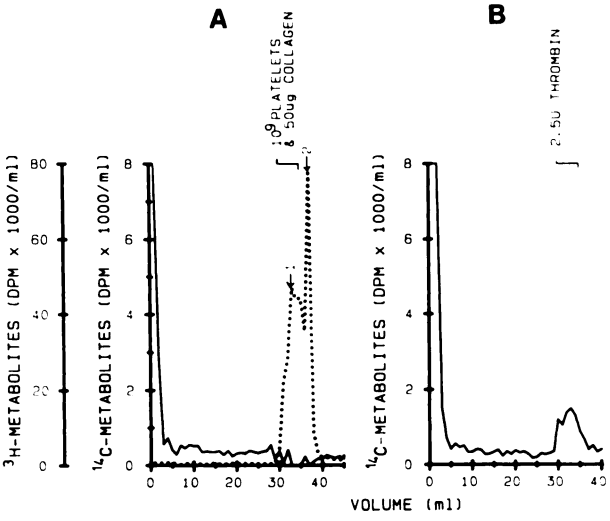

Fig 4. Aliquots of the human umbilical vein endothelial cell (HUVEC) column fractions from the experiments shown in Fig 3 were counted for the release of [^{3}H] [platelet-derived arachidonic acid (AA) or metabolites] and for [^{14}C] (HUVEC-derived AA or metabolites) as a response to injections of collagen and platelets (A) or thrombin (B). Peaks labeled 1 and 2 were subjected to reverse phase chromatography (Fig 5).

minutes and the HUVECs with [^{14}C]-AA for 75 minutes prior to the experiment. Sixty percent of the platelets were retained in the column, and there was simultaneous TXA_2 (518.9 ng) and PGI_2 (33.2 ng) production; the latter persisted for more than 15 minutes after the injection of platelets had finished (Fig 3A). As a control for the ability of the HUVECs to respond, an identical column was perfused with 0.5 U/mL of thrombin for ten minutes (Fig 3B). There was a transient release (12.6 ng) of PGI_2 that was not sustained throughout the injection.

Following centrifugation (as described in Methods section) of the fractions collected during these experiments, aliquots were counted for the release of [^{3}H]-AA metabolites (platelet-derived) and [^{14}C]-AA metabolites (HUVEC-derived) (Fig 4). The passage of collagen-stimulated platelets resulted in only [^{3}H]-labeled metabolites (Fig 4A), whereas the injection of thrombin resulted in the release of [^{14}C]-labeled metabolites (Fig 4B).

When an early fraction from the passage of collagen-stimulated platelets (fractions 32 and 33 pooled, marked 1, Fig 4A) was extracted and subjected to reverse-phase (RP) chromatography, the profile demonstrated the following features (Fig 5A): 39.3% of the [^{3}H]-AA was converted to metabolites. Of the total, 7.4% eluted in the buffer front, 2.9% eluted with a retention time identical to [^{3}H]-TXB_2, a small peak [unidentified, but presumably 12-hydroxyheptadecatrienoic acid (HHT)] comprised of 3.2% of DPM and the major peak of 10.3% eluted with a retention time similar to that of standard 12-HETE. An identical profile was obtained from similarly prelabeled platelets stimulated by collagen in a column with fibronectin-coated beads without HUVECs.

A later fraction (fraction 37, marked 2, Fig 4A) from the passage of [^{3}H]-AA platelets (collagen stimulated) and [^{14}C]-AA HUVECs was subjected to RP chromatography. In addition to the TXB_2, probable HHT, and 12-HETE, three new [^{3}H] peaks were identified (Fig 5B). These corresponded to 6-keto $PGF_{1\alpha}$ (4.4%), PGD_2/PGE_2 (2.6%), and 15-HETE (2.9%), respectively.

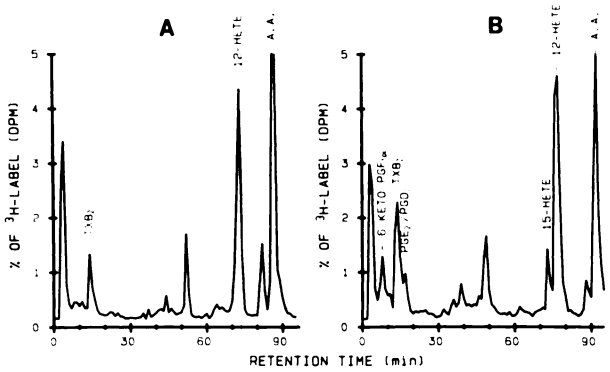

Fig 5. Two human umbilical vein endothelial cells (HUVEC)-column fractions (32 through 33 and 37, labeled 1 and 2, respectively) from the experiment shown in Fig 4a, were extracted and subjected to reverse-phase (RP)-chromatography in an FPLC (fast protein liquid chromatography) liquid chromatography system. The profiles shown represent the metabolism of the [^{3}H]-arachidonic acid (AA) (platelet-derived) during the passage of collagen-stimulated platelets through the HUVEC column. (A) represents the RP-chromatography profile derived from the pooled fractions 32 and 33 and (B) from fraction 37. The retention times of radiolabeled standards of prostaglandins, leukotrienes, and HETES are marked. The major unidentified peak at 52 minutes (A) and 50 minutes (B) is probably 12-hydroxyheptadecatrienoic acid.

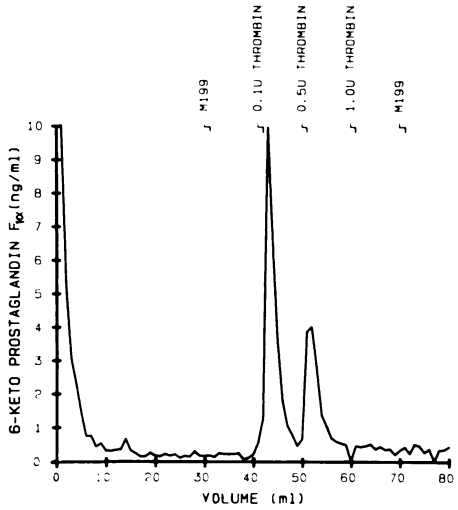

Fig 6. A column of human umbilical vein endothelial cells was stimulated with three successive doses of thrombin (0.1 U, 0.5 U, 1.0 U) at 20-minute intervals. The resulting prostacyclin production (radioimmunoassay of 6-keto-$PGF_{1\alpha}$) is shown.

Prostacyclin released by HUVECs to perfusion with agonists. Following institution of flow through the perfusion system, a substantial but short-lived release of PGI_2 as measured by 6-keto $PGF_{1\alpha}$ was invariable [20.2 ± 6.4 ng, (N = 6)]. After 20-minute perfusion, little or no 6-keto $PGF_{1\alpha}$ was detectable in the perfusate. On 10 of 18 occasions, the minimal change in the flow due to the control injection of culture medium alone resulted in a transient release of PGI_2 [1.6 ± 2.5 ng (N = 18)]. Thrombin, 0.1 to 0.5 U always resulted in substantial but transient release of PGI_2 [5.73 ± 7.4 ng (N = 14)]. A dose–response using the same column of cells was not constructed because there was rapid desensitization of the HUVECs so that a second injection of thrombin released little or no PGI_2 (Fig 6). Other putative agonists assessed, ADP (10^{-3} to 10^{-6} mol/L), ATP (10^{-3} to 10^{-6} mol/L) and PAF (10^{-5} to 10^{-8} mol/L) produced occasional but no reproducible responses in excess of control injections (N = 44).

DISCUSSION

We have shown that intact, washed platelets pass through a HUVEC column without significant activation and without adherence to components in the column. There was no release of PGI_2 by the EC as a consequence of the proximity of unactivated platelets, nor did fibrillar collagen in suspension result in PGI_2 release by the ECs. When the collagen, representing local subendothelium in this model, was coinjected with platelet suspension, there were physical and metabolic consequences. About 50% of the platelets were retained in the column, and there was massive production of both TXA_2 and PGI_2. Both these products were derived from arachidonic acid of platelet membrane origin. The evidence to support this conclusion is threefold.

First, when the HUVECs were preincubated with indomethacin, the PGI_2 response was not significantly different from that seen when untreated HUVECs were perfused with collagen-activated platelets. This suggests that HUVEC cyclooxygenase activity is not essential and that the substrates for the HUVEC PGI_2 synthetase in this experiment are lipid endoperoxides generated by and transferred from the platelets. These experiments were not strictly quantitative and furthermore do not reflect a physiological situation; rather, they are the result of pharmacological manipulation. Therefore, a second line of evidence was sought using isotopically labeled AA incorporated into both platelets and endothelium as an index of AA release and metabolism.

The passage of collagen-stimulated platelets labeled with [^{3}H]-AA did not cause the release of [^{14}C]-AA or its metabolites from the HUVEC column, suggesting that neither the proximity of aggregating platelets nor products released from them resulted in stimulation of endothelial membrane phospholipase. There was, however, the expected release of substantial [^{3}H]-AA metabolites from the activated platelets. During the early phase of the perfusion, these metabolites were shown to be identical to those produced by stimulated platelets alone on the basis of RP chromatography of the isotopically labeled products. There were three prominent metabolites: TXB_2, 12-HETE, and a third, assumed to be HHT.

After some minutes of perfusion there were additional peaks representing 6-keto-$PGF_{1\alpha}$, PGE_2 or PGD_2 and 15-HETE. These products have been reported to be produced by pulmonary ECs,[23] and represent the metabolism of either AA or cyclic endoperoxides transferred from the activated platelets. The production of 15-HETE by ECs could be important in the regulation of the inflammatory process locally in the endothelium. 15-HETE has been shown to regulate leukotriene biosynthesis in leukocytes, mast cells, and 12-lipoxygenase pathway in platelets.[24-26] In addition, 15-HETE enhances human neutrophil degranulation to PAF.[27] It would seem that generation of this product by ECs could be protective by several mechanisms. RIA of the fractions showed again the presence of TXB_2 and 6-keto-$PGF_{1\alpha}$, suggesting that the metabolism of recently incorporated isotopically labeled AA is at least representative of the metabolism of the nonlabeled pool.

Finally, the injection of putative agonists which are platelet-derived did not result in reproducible PGI_2 release. Pearson et al[7] showed that piglet aortic ECs responded to both ADP and ATP with PGI_2 release, the pattern of response being consistent with purinergic P_2 receptors. The lack of response to ADP and ATP seen in our experiments is in agreement with Acharya and MacIntyre[6] and Wecksler and colleagues[28] using HUVECs suggesting species or site specificity. Acharya and MacIntyre[6] and Test and Bang[9] recorded PGI_2 release induced by PAF. We were unable to confirm that this occurs as an acute response using concentrations up to 10^{-5} mol/L. Other platelet products with possible effects on EC PGI_2 release are α-granule proteins β-thromboglobulin,[29] and PDGF.[8] Any influence of these proteins may be exerted over a longer time course and there is no evidence for or against an important role for these proteins from our present experiments. However, basal PGI_2 release by piglet aortic endothelial cells in culture was identical whether the medium was supplemented with homologous serum or plas-

ma-derived serum, suggesting that platelet release products do not exert a significant chronic effect.[30]

Thrombin regularly caused a striking but transient (despite continued exposure) release of PGI_2 with the development of tachyphylaxis. This phenomenon may be related to receptor occupancy and internalization or to inhibition of the peroxidase component of cyclooxygenase by its products[31] or by the accumulation of lipid peroxides due to lipoxygenase.[32] Our results confirm the phenomenon of transfer of AA metabolites from activated platelets to local ECs as shown by others[10-12] in less physiological models. They suggest that in the context of minor endothelial damage or desquamation, this is likely to be the only mechanism for EC PGI_2 release. In vivo experiments reported by Deckmyn and colleagues[33] support this conclusion. One explanation for the efficiency of the transfer of cyclic endoperoxides not being demonstrated by some workers[13-15] is the possible requirement of direct cell–cell contact.[11] This requirement would be readily met by the model we have used, one which mimics closely the dynamics of the microcirculation.[16] Clearly, the elaboration of thrombin in the event of more extensive tissue injury will contribute to PGI_2 release. Repeated stimulation, however, is likely to exhaust this contribution, and once again the system may rely on platelet arachidonate metabolites for continued PGI_2 production.

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