Transcellular Activation of Platelets and Endothelial Cells by Bioactive Lipids in Platelet Microparticles

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Abstract

Microparticles are released during platelet activation in vitro and have been detected in vivo in syndromes of platelet activation. They have been reported to express both pro- and anticoagulant activities. Nevertheless, their functional significance has remained unresolved.

To address the mechanism(s) of cellular activation by platelet microparticles, we examined their effects on platelets and endothelial cells. Activation of human platelets by diverse stimuli (thrombin, 0.1 U/ml; collagen, 4 μg/ml; and the calcium ionophore A23187, 1 μM) results in shedding of microparticles. Pretreatment of these particles, but not membrane fractions from resting platelets, with (s)PLA_2 evokes a dose-dependent increase in platelet aggregation, intracellular [Ca^{2+}] movement, and inositol phosphate formation.

These effects localize to the arachidonic acid fraction of the microparticles and are mimicked by arachidonic acid isolated from them. However, platelet activation requires prior metabolism of microparticle arachidonic acid to thromboxane A_2. Thus, pretreatment of platelets with the cyclooxygenase (COX) inhibitor, indomethacin (20 μM), the thromboxane antagonist SQ29,548 (1 μM), or the protein kinase C inhibitor GF109203X (5 μM) prevents platelet activation by microparticles. However, platelet microparticles fail to evoke an inositol phosphate response directly, via either of the cloned thromboxane receptor isoforms stably expressed in human embryonic kidney (HEK) 293 cells. Prelabeling platelets with [$^2H_8$] arachidonate was used to demonstrate platelet metabolism of the microparticle-derived substrate to thromboxane. Platelet microparticles can also induce expression of COX-2 and prostacyclin (PGI_2) production, but not expression of COX-1, in human endothelial cells. These effects are prevented by pretreatment with actinomycin D (12 μM) or cycloheximide (5 μg/ml). Expression of COX-2 is again induced by the microparticle arachidonate fraction, which it may then use to synthesize PGI_2. Both PGE_2 and iloprost, a stable PGI_2 analog, evoke human umbilical vein endothelial cell COX-2 expression, albeit with kinetics that differ from the response to platelet microparticles. These studies indicate a novel mechanism of transcellular lipid metabolism whereby platelet activation may be amplified or modulated by concentrated delivery of arachidonic acid to adjacent platelets and endothelial cells. (*J. Clin. Invest.* 1997. 99:2118–2127.)

Key words: platelet microparticles • platelets • human umbilical vein endothelial cells • cyclooxygenase-2 • arachidonic acid

Introduction

A curious feature of eukaryotic cells is that, after activation, they may shed components of their plasma membranes into the extracellular space (1, 2). Such fragments may include cytoplasmic elements and are colloquially known as microparticles. Although the formation of such cellular components may involve the translocation of proteins to the cell membrane and the assembly of multimeric complexes (3), the release of microparticles is not associated with cell death, although it may coincide with altered cellular viability (4). Microparticles appear to result from an exocytotic budding process. Thus, microparticles shed by human platelets, which have an average diameter of ~ 0.1 μm, contain the cytoplasmic marker factor (F) XIII A_2, but not the plasma fibrin–stabilizing F XIII A_2 B_2 (5). Recently, the possibility that microparticles might themselves evoke cellular responses in the immediate microenvironment of their formation has been suggested. For example, activation of endothelial cells with thrombin results in vesicle shedding, which, in turn, activates neutrophils and enhances their propensity to adhere to the endothelial cells (6). Similarly, microparticles shed from platelets activated with *Staphylococcus aureus* α-toxin may induce platelet aggregation. Pretreatment of the microparticles with secretory (s) phospholipase A_2 (PLA_2)[1] was necessary for them to evoke this response (7). Both pro- and anticoagulant proteins have been detected in platelet microparticles (8), and circulating microparticles have been reported in syndromes of platelet activation (9–12).

The present study was designed to explore the mechanism(s) of cellular activation by platelet microparticles. We report that they induce platelet activation by the concentrated transcellular delivery of arachidonic acid and its subsequent metabolism to thromboxane A_2. Platelet microparticles may also activate endothelial cells. For example, microvesicular arachidonic acid induces the expression of cyclooxygenase (COX)-2, which, in turn, may process the arachidonate to prostacyclin. Microvesicular shedding may represent a novel mechanism whereby activated platelets and, perhaps, other cells may influence their local environment via transcellular lipid metabolism.

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1. *Abbreviations used in this paper:* AP-1, activator protein-1; COX, cyclooxygenase; HEK, human embryonic kidney; HUVEC, human umbilical vein endothelial cells; IP, inositol phosphate; MP, microparticles; PAF, platelet-activating factor; PGI_2, prostacyclin; PKC (or PKA), protein kinase C (or A); PLA_2, phospholipase A_2; PRP, platelet rich plasma; TP, thromboxane receptor; WP, washed platelets.

Methods

Reagents

Calcium ionophore A23187, (s)PLA_2, indomethacin, *N N* diisopropyl-ethylamine, pentafluorylbenzyl bromide, EDTA, yohimbine, cycloheximide, actinomycin-D, leupeptin, Nonidet P-40, soybean trypsin inhibitor, and aprotinin were purchased from Sigma Chemical Co. (St. Louis, MO). CV-6209, a platelet activating factor (PAF) receptor antagonist, was obtained from BIOMOL Research Labs. Inc. (Plymouth Meeting, PA). Collagen was purchased from Biodata Corp. (Hatboro, PA). The thromboxane receptor (TP) antagonist SQ29,548, the prostacyclin (PGI_2) analog Iloprost, [$^2H_8$]arachidonic acid, [$^2H_4$]TxB_2, [$^2H_4$]6-keto $PGF_{1\alpha}$, AA, and PGE_2 were all purchased from Cayman Chemical Co., Inc. (Ann Arbor, MI). Fura-2/AM, GF109203X (bisindolylmaleimide 1) and H-89 (*N*-[2-(*p*-bromocinnamylamino) ethyl]-5-isoquinoline-sulfinamide) were purchased from Calbiochem Corp. (La Jolla, CA). The Dowex 1-X8 AG anion exchange resin was purchased from Bio-Rad Laboratories (Hercules, CA). HBSS and Hepes were obtained from Life Technologies, Inc. (Grand Island, NY). The activator protein-1 (AP-1) antibody was a kind gift from Dr. T. Kunicki (Scripps Research Institute, La Jolla, CA). Mouse monoclonal antibodies against COX-1 and COX-2 were kindly provided by Dr. J. Maclouf (INSERM, Unit 348, Paris, France). Rabbit monoclonal antibody against (s)PLA_2 was kindly provided by Dr. R. Kramer of Lilly Research Laboratories (Indianapolis, IN). The TPβ cDNA was a generous gift of Dr. A. Ware of the Beth Israel Hospital (Boston, MA). Human embryonic kidney (HEK) cells were from the American Type Culture Collection (Rockville, MD). Densitometric analysis was performed with Scanner Control Version 1.00 (Molecular Dynamics, Sunnyvale, CA). All solvents were of HPLC grade and obtained from T.J. Baker (Danvers, MA). The reverse phase HPLC column was from Beckman Instruments, Inc. (Fullerton, CA), and the LK60 silica gel 60 A plates (0.25-mm thick) were from Whatman Inc. (Clifton, NJ).

Platelet functional studies

Isolation of platelets and platelet aggregation. Platelets were harvested as described previously (13). Briefly, blood was collected from healthy volunteers into a plastic syringe containing 3.8% buffered sodium citrate as anticoagulant (ratio 1:9). The volunteers had not taken any drugs for at least 14 d. Platelet rich plasma (PRP) was prepared by centrifugation at 130 *g* for 15 min, and platelet-poor plasma by centrifugation of PRP at 900 *g*. Washed platelets (WP) were isolated from PRP after centrifugation and resuspended in calcium- and magnesium-free Hepes buffer at pH 7.4. The platelet number was always adjusted to 3×10^8 platelets/ml. Platelet aggregation was studied at 37°C using WP in a PAP-4 model aggregometer (Biodata Corp.) in siliconized cuvettes with continuous stirring. A number of inhibitors were employed to elucidate the mechanism of platelet aggregation induced by microparticles. These included SQ29,548 (1 μM), a specific TxA_2/PGH_2 receptor antagonist (14), indomethacin (20 μM), a nonspecific COX inhibitor (15), yohimbine (140 nM), an α_2 adrenergic receptor antagonist (16), CV-6209 (120 nM), a PAF receptor antagonist (17), GF109203X (5 μM), a protein kinase C (PKC) inhibitor (18), and H-89 dihydrochloride (10 μM), a PKA inhibitor (18). They were all incubated for 1 min at 37°C before addition of platelet agonists: platelet aggregation was followed for 5 min. The supernatant was collected for measurement of TxB_2, the stable hydrolysis product of TxA_2.

Preparation of platelet microparticles and their identification. Platelet microparticles were isolated after platelet aggregation, as previously described (7). Washed platelets were or were not treated with indomethacin (20 μM). The agonists used for platelet activation were thrombin (0.1 U/ml) and/or collagen (4 μg/ml) or the calcium ionophore A23187 (1 μM). Aggregation was followed for 10 min, after which the platelets were sedimented at 1,500 *g* for 15 min and, from the resulting supernatant, microparticles were isolated at 13,000 *g* for 45 min. The pellet was resuspended in endotoxin free Hepes, pH 7.4, and then treated with (s)PLA_2 (2 μg/ml) in the presence of 1 mM $CaCl_2$ at 37°C for 1 h. The microparticles were washed twice to remove (s)PLA_2 and resuspended in Hepes buffer at the end of the incubation. The protein content was measured using a microbicinochoninic assay (Pierce Chemical Co., Rockford, IL) with BSA as standard. Samples were analyzed on a flow cytometer (FACScan®; Becton Dickinson & Co., Mountain View, CA) as previously described to characterize the microparticles (19). Briefly, platelet microparticles were incubated with AP-1 (antibody to glycoprotein GP-Ib), for 45 min at room temperature, and then fixed with 1% paraformaldehyde. Microparticles were identified by gating on GP1b (FITC-AP-1)-positive events and were distinguished by forward scatter size analysis. Each platelet microparticle preparation was assayed for endotoxin contamination by the *Limulus* amebocyte lysate assay. Final endotoxin contamination was always < 0.02 U/mg protein. Experiments were also performed with platelet microparticles that were preincubated with polymixin B (50 μg/ml) for 1 h, before their addition to platelets or to human umbilical vein endothelial cells (HUVEC).

Platelet intracellular calcium and inositol phosphate formation. PRP was incubated with 2 μM Fura-2/AM at 37°C for 45 min, washed to remove the extracellular Fura-2, and resuspended in HBSS, pH 7.4. Fluorescence measurement was carried out at 37°C in a spectrofluorimeter equipped with a magnetic stirrer (LS 50-B, Perkin-Elmer Corp., Beaconsfield, UK). The fluorescence signal was monitored at 510 nm with excitation wavelengths of 340 and 380 nm. The ratios of maximum and minimum fluorescence were determined by the addition of 250 μM digitonin in the presence of 1 mM $CaCl_2$ and in the presence of 10 mM EDTA ($pH > 8.5$), respectively, as described previously (13).

PRP was incubated with *myo*-[2-^{3}H]inositol (50 μCi/ml) at 37°C for 3 h, and platelets were harvested as described above. They were then washed in Hepes buffer, pH 7.4, and resuspended in Hepes containing 20 mM LiCl for 15 min. These conditions inhibit conversion of inositol phosphates to free inositol. Platelets were incubated with microparticles for 1 min at 37°C. Total inositol phosphates were separated on a Dowex 1-X8 AG anion exchange resin (anion form) as previously described (20). Total inositol phosphates were eluted with 4 ml of 2 M formic acid after washing of the column with 40 mM ammonium formate to remove both [^{3}H]inositol and [^{3}H]glycerophosphorylinositol. 1 ml of the eluant was added to 9 ml of scintillant and the radioactivity was determined using a scintillation counter (Beckman Instruments, Inc., Fullerton, CA).

Lipid analysis

Lipids were extracted according to a modified version of the method of Bligh and Dyer (21). Lipid separations were performed as previously described (22). Briefly, the lipid extract dissolved in 300 μl chloroform was applied to a 100 mg NH_2 solid phase extraction cartridge (International Sorbent Technology, Glamorgan, UK) that had been pretreated with *n*-hexane (2 ml). Different mobile phases were used to elute the following fractions: 4 ml of chloroform/isopropanol (2:1) (neutral lipids), 4 ml of 2% acetic acid in ethyl ether (free fatty acids), and 4 ml of methanol (polar lipids). The neutral lipid fraction was dried and reconstituted in 200 μl of hexane and applied to a second pretreated NH_2 column. The following fractions were eluted: 4 ml of hexane (cholesteryl esters), 6 ml of 1% ethyl ether and 10% dichloromethane in hexane (triglycerides), 6 ml of 5% ethyl acetate in hexane (cholesterol), 4 ml of 15% ethyl acetate in hexane (diglycerides), and 4 ml of chloroform/methanol (2:1) (monoglycerides). The free fatty acid fraction recovered from the NH_2 column was dried under a stream of nitrogen and redissolved in 20 μl of chloroform/methanol (2:1, vol/vol) and applied to a silica thin-layer chromatography plate. Plates were developed with the mobile phase 80% ethyl acetate, 20% heptane, and 0.1% acetic acid and the standards made visible with a solution of $CuSO_4$/85% H_3PO_4. The silica plate was divided into 4-cm sections, ethyl acetate extractions with 0.1% acetic acid were made of each and tested for biological activity as assessed by platelet aggrega-

tion. The extract with biological activity was dried and resuspended in 50 μl of the HPLC mobile phase. A 1050 HPLC (Hewlett-Packard Co., Palo Alto, CA) on line with an HP1050 ultraviolet (UV) detector was used for all HPLC experiments. Reverse phase chromatography used an Ultrasphere ODS 5 μm column (Beckman Instruments, Inc.), 4.6 mm × 25 cm. The flow rate was 1 ml/min. A linear gradient mobile phase 18% acetonitrile/H_2O to 100% acetonitrile in 40 min was employed (23). Fractions from 5 to 50 min were collected and dried under a stream of nitrogen. Each fraction was resuspended in 5 μl of ethanol and added to a suspension of washed platelets to assess platelet aggregation.

Gas chromatography/mass spectrometry

All gas chromatography/mass spectrometry studies were performed on a Fisons MD-800 (VG Organic, Manchester, UK) equipped with a split/splitless injector operated in the splitless mode at 260°C. The interface was maintained at 300°C, the ion source at 250°C. The mass spectrometer was operated in the negative ion chemical ionization mode, using ammonia as the reagent gas. Analysis of prostanoids and arachidonic acid was performed as previously described (24). Briefly, a 30-m DB-5 capillary column of 0.25-mm inner diameter with 0.25 μm of coating was used. The temperature program ran from 190 to 320°C at 20°C/min. The ions monitored were *m/z* 614 for TxB_2 and 6-keto $PGF_{1\alpha}$, *m/z* 618 for [$^2H_4$]TxB_2 and [$^2H_4$]6-keto $PGF_{1\alpha}$, *m/z* 621 for [$^2H_7$]6-keto $PGF_{1\alpha}$, *m/z* 622 for [$^2H_8$]TxB_2, *m/z* 303 for AA, and *m/z* 311 for [$^2H_8$]AA. TxB_2 and 6-keto $PGF_{1\alpha}$ were both analyzed as the pentafluorylbenzyl ester (PFB)/trimethylsilyl ether derivatives (25), while AA was analyzed as the PFB derivative, as described previously (26).

Cell culture and stimulation

HUVEC were prepared according to the method of Jaffe et al. (27). Briefly, cells were harvested from human umbilical veins with 1 mg/ml collagenase A at 450 U/mg (Boehringer Mannheim, Indianapolis, IN). They were grown to subconfluence in T-25 flasks (Costar Corp., Cambridge, MA) coated with 2 mg/ml gelatin and maintained in medium 199 (Boehringer Mannheim) containing 10% heat inactivated human serum, 2 mM glutamine, 100 μg/ml streptomycin, and 100 U penicillin. The medium contained 10 μg/ml heparin and 50 μg/ml endothelial growth supplement. Cells were grown in a humidified atmosphere at 37°C in 5% CO_2. HUVEC were characterized by their cobblestone appearance. Passage cells were subcultured into 6-well plates (3–4 × 10^5 cells/well) and were allowed to grow under the conditions described above. Heparin and endothelial growth supplement were removed 48 h before stimulation and replaced with 5% heat inactivated human serum. Cells were harvested at the second passage at subconfluence and incubated in culture medium containing 0.75% bovine serum albumin. Platelet microparticles, resuspended in Hepes buffer, were incubated with HUVEC for 2, 6, 12, and 24 h. Supernatants were collected to determine PGI_2 production, as reflected by release of its hydrolysis product, 6-keto $PGF_{1\alpha}$. SQ29,548 (2 μM) and cycloheximide (5 μg/ml) were added to the HUVEC 45 min before stimulation by microparticles. Actinomycin D (12 μM), yohimbine (140 nM), and CV-6209, the PAF receptor antagonist (30 nM), were coincubated with platelet microparticles as described.

Western blot analysis

Western blot analysis of COX proteins was carried out as previously described (28). Monolayers of HUVEC in 35-mm 6-well plates were lysed in ice-cold buffer (20 mM Tris, pH 8.0, 1 mM EDTA, 1% Nonidet P-40, 0.25% SDS, 0.5 mM DTT, 10 μg/ml soybean trypsin inhibitor, 1 μg/ml leupeptin, and 10 μg/ml aprotinin) after incubation with platelet microparticles. The protein content was determined using a microbicinchoninic acid assay (Pierce Chemical Co.) with bovine serum albumin as the standard. 40 μg of protein lysate was used for analysis of COX-2 and 20 μg for COX-1 analysis by SDS-PAGE. 7% and 3% acrylamide was used, respectively, for the separating and stacking gels. Proteins were transferred onto a nitrocellulose membrane with a semidry transfer unit (Hoefer Scientific Instruments, San Francisco, CA). Blots were then saturated for 2 h with 5% fat free milk in Tris buffered saline (Tris-HCl, pH 7.5, 250 mM NaCl, 0.1% Tween 20) and incubated with a mouse monoclonal antibody directed against either COX-1 or COX-2 at a concentration of 1/10,000 for 1 h at room temperature. Both mAbs are specific for their respective enzymes, no cross-reactivity between the two mAbs has been described (28, 29). The mAb we used for COX-1 does not recognize human COX-2 (29). The mAb against COX-2 recognizes the sequence of the carboxyl-terminal portion of the human COX-2 (C)-NASSSRSGLDDINPTVLLK (amino acid sequence 580–598), which is absent from COX-1. Blots were then incubated with sheep anti–mouse IgG_1 conjugated with horseradish peroxidase at 1/2,000 (0.1 ml/cm^2) for 1 h at room temperature. Chemiluminescent substrates were used to reveal positive bands that were visualized after the exposure to Hyperfilm ECL (Amersham Corp., Arlington Heights, IL).

Western blot analysis of (s)PLA_2 in platelet microparticles was also performed. 10, 20, 40, and 60 μg of microparticle protein was used for analysis of (s)PLA_2. The separating and stacking gels employed 15 and 3% acrylamide, respectively. Proteins were transferred onto a nitrocellulose membrane with a semidry transfer unit. Blots were then saturated for 2 h with 5% fat free milk in Tris buffered saline (Tris-HCl, pH 7.5, 250 mM NaCl, 0.1% Tween 20) and incubated with a rabbit monoclonal antibody directed against (s)PLA_2 at a concentration of 1/10,000 for 1 h at room temperature. Blots were then incubated with rabbit anti–mouse IgG_1 conjugated with horseradish peroxidase at 1/5,000 (0.1 ml/cm^2) for 1 h at room temperature. Chemiluminescent substrates were used to reveal positive bands that were visualized after exposure to Hyperfilm ECL.

Cell culture and transfections

HEK 293 cells were routinely grown in Dulbecco's modified Eagle's medium. Transfections were carried out as previously described (30). The cDNAs encoding the placental (TPα; reference 31) or endothelial (TPβ; reference 32) human thromboxane receptor isoforms were subcloned into the EcoRI-EcoRI or the EcoRI-XbaI sites, respectively, of the G418-resistant mammalian expression vector pcDNA111 (Invitrogen Corp., San Diego, CA). Receptor expression was verified in several cell lines by Northern blot analysis, using the respective cDNAs as probes.

Inositol phosphate formation

The HEK-transfected cells in the presence of DMEM inositol free medium were labeled with *myo*-[2-^{3}H]inositol (1 μCi/ml) at 37°C for 24 h. The medium was then removed and replaced with inositol free DMEM containing 20 mM LiCl for 15 min at 37°C. These conditions inhibit conversion of inositol phosphates to free inositol. The HEK cells were then incubated with platelet microparticles for 10 min at 37°C. Total inositol phosphates were separated on a Dowex 1-X8 AG anion exchange resin (anion form) as previously described (20). The measurement of total inositol phosphates was carried out as outlined above for platelets.

Statistical analysis

Results are expressed as mean±SEM. Statistical comparisons were made by using analysis of variance with subsequent application of Student's *t* test as appropriate.

Results

Effects of microparticles on human platelets. Washed human platelets stimulated with either A23187 or thrombin in the presence or absence of indomethacin (20 μM) resulted in microparticle formation. We confirmed microparticle formation by FACS® analysis of AP-1 binding as previously described (19). Thus, platelet microparticle formation is not cyclooxygenase dependent. Microparticles may evoke biological responses irrespective of the cyclooxygenase activity of their

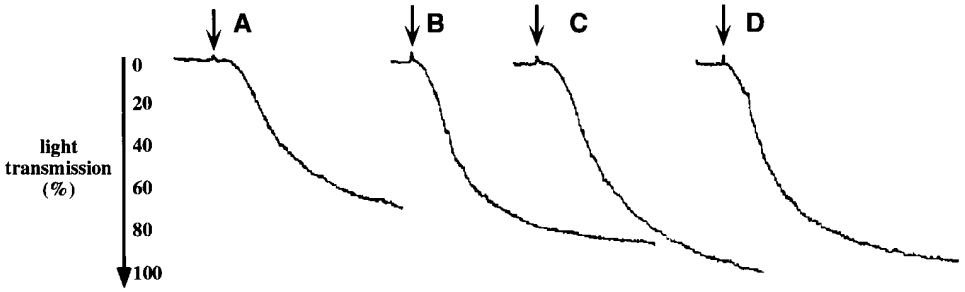

Figure 1. Platelet aggregation induced by microparticles. (*A*) A23187-generated platelet microparticles from aspirinated platelets induce platelet aggregation. (*B*) A23187-generated platelet microparticles from nonaspirinated platelets induce platelet aggregation. (*C*) A23187 (1 μM) induced aggregation of nonaspirinated platelets. (*D*) Thrombin (0.1 U/ml) induced aggregation of nonaspirinated platelets. The traces are representative of four experiments. The arrows indicate the addition of microparticles or the platelet agonist to washed platelets.

platelet source (Fig. 1). Thus, incubation of washed human platelets with microparticles (5–40 μg protein/ml) results in a dose-dependent increase in platelet aggregation and TxB_2 formation (Fig. 2). The magnitude of the response evoked by concentrations of microparticles typically shed by activated platelets in vitro (260 μg microparticles protein/3 × 10^8 platelets) corresponds to that evoked by chemical (A23187) and biological (thrombin) agonists. Irreversible aggregation is only observed when the microparticles are first treated with (s)PLA_2 (2 μg/ml). (s)PLA_2 alone does not cause platelet aggregation. We excluded the presence of endogenous (s)PLA_2 in the microparticle preparation by Western blot analysis (Fig. 3). Addition of the thromboxane receptor antagonist SQ29,548 (1 μM) results in a 79.8±9.8% (n = 6) reduction in microparticle-induced platelet aggregation (Fig. 4). Similarly, the COX inhibitor, indomethacin (20 μM), results in an 81±6.8% reduction and the PKC inhibitor, GF109203X (5 μM), results in a 66.6±7.4% (n = 6) reduction in platelet aggregation. A corresponding decrease in TxB_2 production is also observed (data not shown). The α_2-adrenergic receptor antagonist, yohimbine (140 nM), the PAF antagonist CV-6209 (120 μM), and the PKA antagonist, H-89 dihydrochloride (10 μM), all fail to prevent microparticle-induced platelet aggregation (data not shown). Microparticles cause a dose-dependent increase in intracellular calcium when added to human platelets loaded with Fura-2/AM (Fig. 5). To investigate the origin of this calcium movement, Fura-2–loaded platelets were pretreated with 1 mM $CaCl_2$ to increase a potential influx of extracellular calcium. Similarly, they were pretreated with 2 mM EGTA to chelate extracellular calcium. No change in the microparticle-evoked calcium signal is observed upon treatment with either reagent. This implies that platelet microparticles induce mobilization of calcium from intracellular stores. When washed human platelets were incubated with microparticles, we observed a dose-dependent increase in platelet inositol phosphates (IPs) (Table I). Similarly, U46619 caused a marked increase in platelet IP formation.

Platelet microparticles and thromboxane receptor activation. Since SQ29,548 causes a significant reduction in platelet aggregation induced by microparticles, we addressed the possibility that they might activate directly either of the two cloned thromboxane receptor isoforms. Formation of IPs was measured in HEK 293 cells, which stably expressed the recombinant TPα and TPβ isoforms. Platelet microparticles, in concentrations up to 40 μg/ml, fail to increase total IPs in either case. The PGH_2/TxA_2 analog, U46619, by contrast, markedly increases IP formation in cells expressing either isoform (Fig. 6).

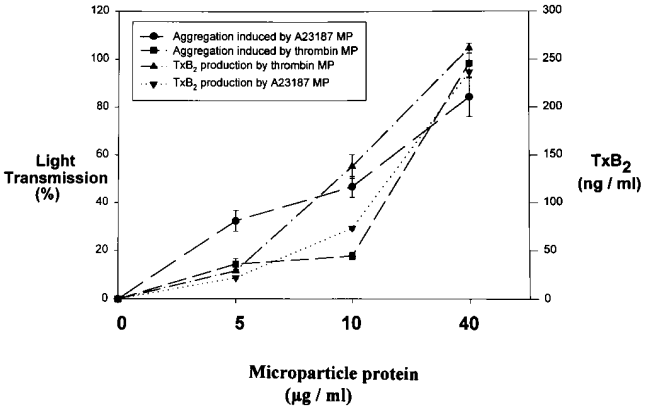

Figure 2. Dose-dependent increase in platelet aggregation and platelet TxB_2 production. The aggregation trace was followed for 5 min and expressed as the percentage increase in light transmission. The platelet supernatant was collected after 5 min for TxB_2 measurement. Each point represents the mean±SEM of six experiments.

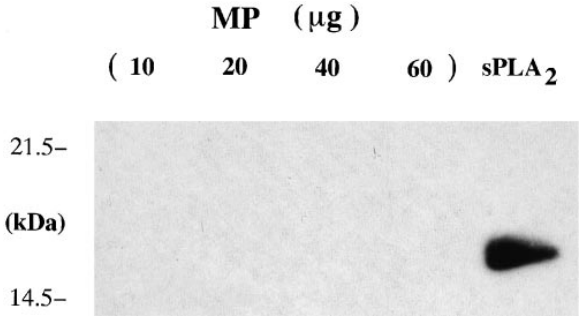

Figure 3. Western blot analysis of (s)PLA_2 in platelet microparticles. 10, 20, 40, and 60 μg of microparticle protein were loaded on a 15% SDS-PAGE. 10 μg of human (s)PLA_2 is used as a positive control. The figure is representative of two experiments.

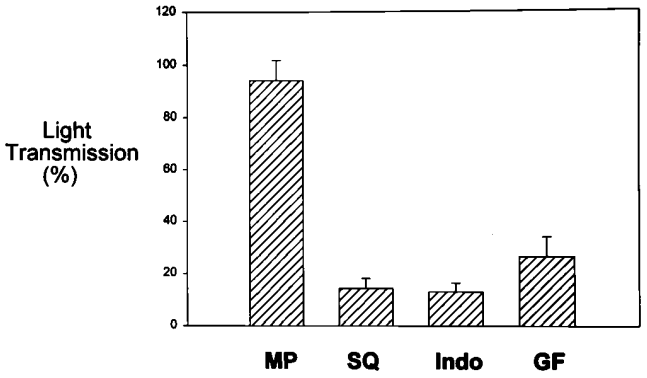

Figure 4. Inhibition of platelet aggregation. WP were incubated with the thromboxane antagonist SQ29,548 (*SQ*) (1 μM), the COX inhibitor indomethacin (*Indo*) (20 μM), or the PKC inhibitor GF109203X (*GF*) (5 μM), and then stimulated with microparticles (MP) (20 μg). Platelet aggregation was followed for 5 min and expressed as the percentage increase in light transmission. Each point represents the mean±SEM of eight experiments.

Identification of the biological mediator present in platelet microparticles. Neither the neutral nor the polar lipid fractions extracted from microparticles and recovered from the NH_2 column express biological activity when added to washed platelets. However, the free fatty acid fraction dose-dependently increases platelet aggregation (data not shown). To identify the component of this fraction that was responsible for the activity, we subjected it first to TLC. The band that evokes platelet aggregation has the same R_f value as an arachidonic acid standard. Next, the fraction was subjected to purification by reverse phase HPLC. Only one fraction results in a dose-dependent increase in platelet aggregation. This had a retention time of 39.9 min, which coincides with the retention time for the arachidonic acid standard (Fig. 7). Finally, the HPLC active fraction was analyzed as the pentafluorobenzyl ester. Mass spectral analysis employed electron capture, negative ion chemical ionization with selected ion monitoring at *m/z* 303 and *m/z* 311, for the deuterated internal standard (Fig. 8). The intense fragment ions found at *m/z* 311 and *m/z* 303 arise from the loss of the pentafluorobenzyl radical, leaving the carboxylate anion in high abundance (Fig. 9, *A* and *B*, respectively).

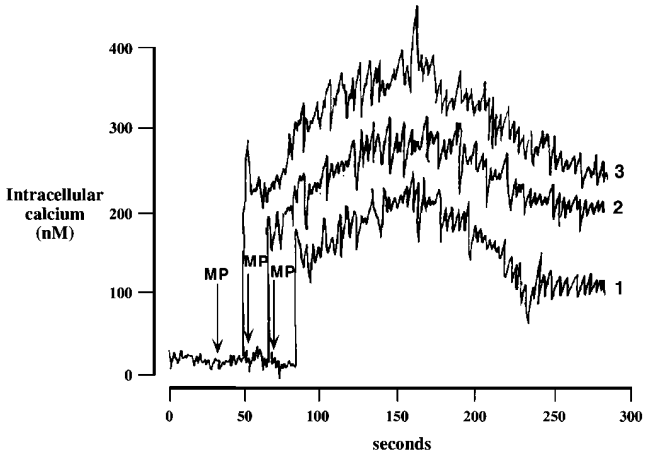

Figure 5. Platelet intracellular calcium response by MP. Human platelets were loaded with Fura-2/AM and stimulated with MP 5 μg (trace *1*), 10 μg (trace *2*), and 20 μg (trace *3*). The data shown are representative of three experiments.

Table I. Inositol Phosphate Formation by Human Platelets Stimulated with Microparticles and U46619

Microparticles (μg/ml)	Percent increase in IPs
5	45.8±10.2
10	65.3±6.1
20	92.2±1.6
40	111.6±6.1
U46619 (1 μM)	121.3±4.8

Dose-dependent stimulation of [^{3}H]inositol phosphate formation by platelet microparticles. Platelets were prelabeled with *myo*-[2-^{3}H]inositol (50 μ Ci/ml), and experiments were carried out in the presence of LiCl (20 mM). The level of total inositol phosphate formation was measured by Dowex-1 anion exchange chromatography as described in Methods. Each point represents the mean±SEM of three separate experiments.

Arachidonic acid distribution and its transcellular metabolism. To investigate the distribution of arachidonic acid between platelets and microparticles, PRP was incubated with 1 μCi/ml [^{3}H]AA for 1 h at 37°C. Platelets were harvested and washed to remove the free arachidonate. Microparticles were formed by aggregating platelets with A23187 (1 μM) in the presence of 20 μM indomethacin. The percentage of radioactivity associated with the platelet pellet was 40±1.5%, while the amount released in microparticles was 32±2.1% (Table II). The amount found in the supernatant was 29.7±1.8%. In two experiments, treatment of the platelet microparticles with (s)PLA_2 increased microparticle-associated radioactivity by 23 and 31%. Similar results were obtained when washed platelets were stimulated with thrombin (data not shown). Microparticles generated from WP loaded with 2 μM [$^2H_8$]AA were

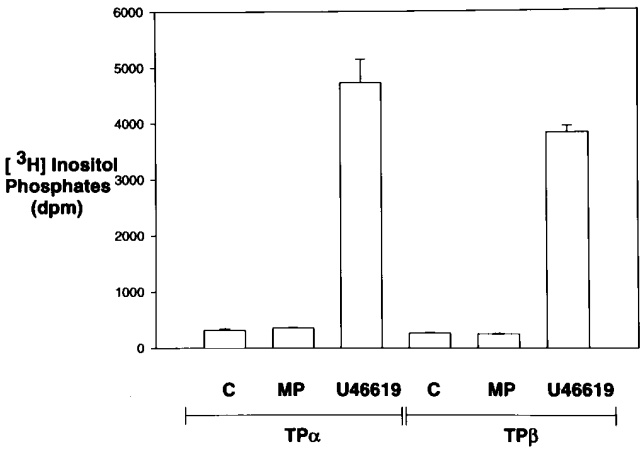

Figure 6. Total inositol phosphate formation by HEK 293 cells stimulated with platelet microparticles or the PGH_2/TxA_2 analog, U46619. HEK 293 cells transfected with the placental (*TPα*) or the endothelial (*TPβ*) receptors were prelabeled with *myo*-[2-^{3}H]inositol (1 μCi/ml) and stimulated with MP (40 μg/ml) or U46619 (1 μM). Controls (*C*) are unstimulated HEK 293 cells. Each point represents the mean±SEM of four experiments.

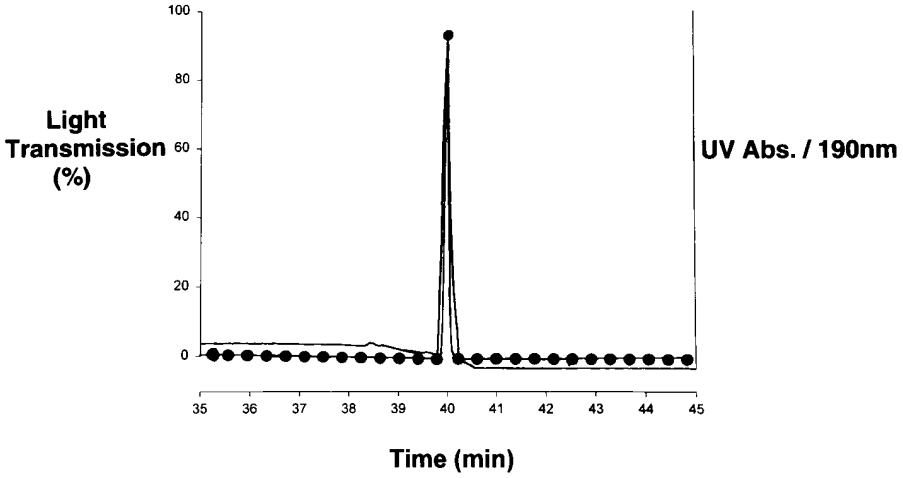

Figure 7. Coelution of the biological mediator isolated from microparticles (●) with the UV absorption profile of authentic arachidonic acid (−). Reverse phase HPLC of the underivatized compound was run with a gradient mobile phase: 18% acetonitrile/82% water to 100% acetonitrile in 40 min. Fractions were collected each minute and monitored at 190 nm.

added to freshly prepared platelets to address the possibility that they might donate arachidonate for transcellular metabolism. [$^2H_8$] TxB_2 is undetectable before platelet activation, but rises to 423.3±43.3 pg/ml in the supernatant after platelet aggregation for 5 min with 10 μg/ml protein. This result implies that intact platelets possess the capacity to use arachidonate from platelet microparticles to generate TxA_2, a platelet agonist and vasoconstrictor. We also investigated whether HUVEC might use the AA present in platelet microparticles. Similarly, HUVEC enzymatically metabolizes [$^2H_8$]AA, since the stable hydrolysis product of prostacyclin (PGI_2), [$^2H_7$]6-keto $PGF_{1\alpha}$, was detected in the supernatant after exposure to platelet microparticles. The concentration of [$^2H_7$]6-keto $PGF_{1\alpha}$ rises time dependently from an undetectable level at time 0 h to 62.7±2.5 pg/ml at 2 h, and 266.7±20.8, 226.7±15.3, and 69.7±9.4 pg/ml at 6, 12, and 24 h, respectively.

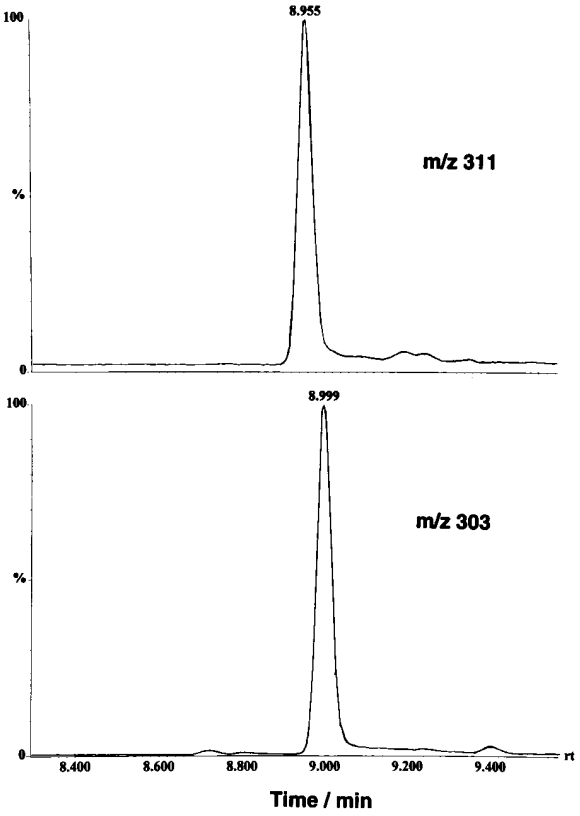

Figure 8. Selected ion monitoring of the active fraction isolated from platelet microparticles. [$^2H_8$]AA (5 ng) was added to the HPLC active fraction and analyzed as the pentafluorobenzyl ester derivative. The upper trace shows a peak at *m/z* 311 corresponding to [$^2H_8$]AA-labeled internal standard. The lower trace shows a peak at *m/z* 303 corresponding to unlabeled arachidonic acid.

Induction of cyclooxygenase activity in HUVEC by platelet microparticles. HUVEC were incubated with platelet microparticles (6 μg/ml) for various time periods (2, 6, 12, and 24 h) to address the mechanism by which PGI_2 formation might be induced. Western blot analysis indicates that platelet microparticles do not induce the expression of COX-1 (data not shown). COX-2 expression, however, increases in response to platelet microparticles. Thus, the protein is apparent at very low levels in controls, perhaps reflecting some degree of cellular activation as previously described (28). However, expression rose significantly from control levels (136.67±26.03 densitometric units), reaching a maximum at 6 h (1,293.67±84.48 densitometric units, $P = 0.0001$) (Fig. 10 *A*). No difference in COX-2 expression was observed when microparticles were first treated with polymixin B (50 μg/ml) for 1 h before addition to HUVEC to rule out any contamination by LPS (data not shown). Expression of COX-2 protein is associated with PGI_2 synthesis, as measured by 6-keto $PGF_{1\alpha}$ in the supernatant (see Fig. 12). Indomethacin (20 μM) was always added to the medium before the addition of platelet microparticles to

Table II. Arachidonic Acid Released from Human Platelets Activated with A23187

Fractions	Percent [^{3}H]arachidonic acid
Microparticles	32.0±2.1
Supernatant	29.7±1.8
Platelets	40.0±1.5

Platelets (3×10^8) were stimulated with A23187 (1 μM) for 10 min at 37°C. Microparticles, a pellet obtained after 100,000 *g* centrifugation; supernatant, obtained at 100,000 *g* centrifugation. Each point represents the mean±SEM of four separate experiments.

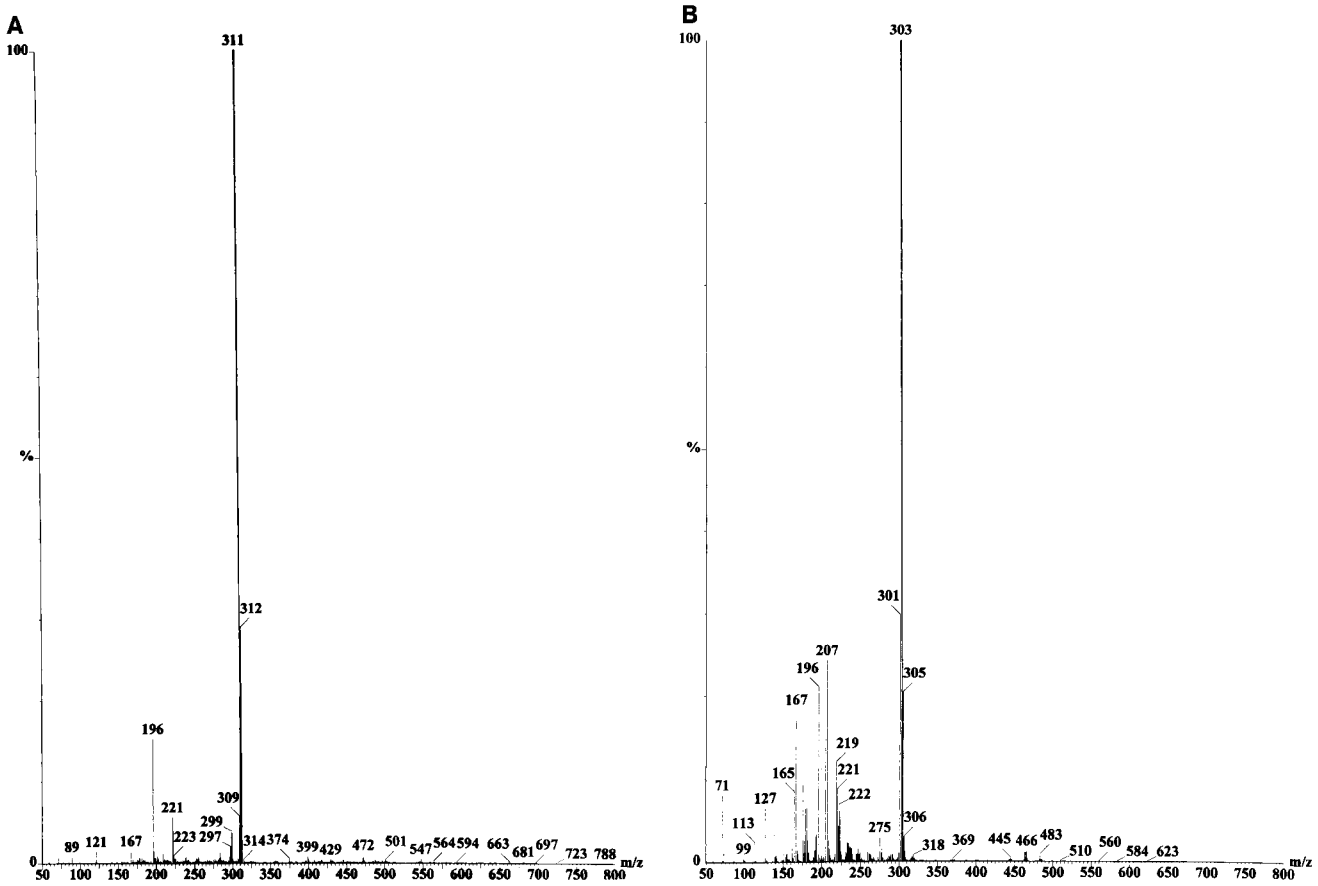

Figure 9. Electron capture negative ion chemical ionization mass spectrum. *A* shows a spectrum of [$^2H_8$]AA derivatized as the pentaflourylbenzyl ester derivative, while *B* shows a spectrum of the active principle isolated from the platelet microparticles.

inhibit constitutive activity of COX(s). Actinomycin D (12 μM) and cycloheximide (5 μg/ml) inhibited COX-2 expression and 6-keto $PGF_{1\alpha}$ production induced by platelet microparticles (173.97±5.96 and 161.09±11.06 densitometric units, respectively, 6 h after stimulation) (Fig. 10; Table III). By contrast, SQ29,548 (2 μM), yohimbine (140 nM), and CV-6209 (30 nM) all failed ($P > 0.05$) to reduce either COX-2 expression (1,058.53±104.87, 1,097.62±108.97, and 917±116.93 densitometric units, respectively, 6 h after stimulation), or 6-keto $PGF_{1\alpha}$ production from the maximal values stimulated by platelet microparticles (Fig. 10; Table III). To investigate further the contribution of COX-2 to PGI_2 production, we used L-745,337, a specific inhibitor of COX-2 (33). L-745,337 (10 μM) markedly reduced the ability of A23187-generated platelet microparticles to augment HUVEC 6-keto $PGF_{1\alpha}$ production. This was reduced by 81.2%±5.5 at 2 h, by 74.9%±4.4 at 6 h, by 75.6%±3.5 at 12 h, and by 70.3%±3.2 at 24 h. Similar results were obtained when L-745,337 was added to HUVEC in the presence of thrombin-generated microparticles (data not shown).

Arachidonic acid induction of COX-2 expression. Since we observed that the AA fraction was the mediator of microparticle-induced activation of washed platelets, we decided to investigate its role in the endothelial response. Arachidonic acid was isolated from both A23187 and thrombin-generated platelet microparticles, as previously described, and added to HUVEC at various time points. Indomethacin (20 μM) was present in the medium before the addition of microparticle arachidonate to prevent AA metabolism. The same time course of COX-2 expression was observed with arachidonic acid as for intact platelet microparticles. Again, the protein is

Table III. Effect of SQ29,548, Yohimbine, CV-6209, Actinomycin D, and Cycloheximide on 6-keto $PGF_{1\alpha}$ Production by HUVEC When Stimulated with Platelet Microparticles (6 μg/ml)

	6-Keto $PGF_{1\alpha}$ (ng/ml)	
Preincubation conditions	A23187	Thrombin
Microparticles alone	16.87±1.35	13.95±1.29
MP + SQ29,548	14.35±3.15	10.56±2.51
MP + yohimbine	16.21±3.24	13.25±2.39
MP + CV-6209	15.95±2.42	12.89±1.43
MP + actinomycin D	1.37±0.10	1.84±0.50
MP + cycloheximide	1.97±0.21	1.73±0.27

SQ29,548 (2 μM), yohimbine (140 nM), and CV-6209 (30 μM) were incubated with HUVEC for 45 min before addition of platelet microparticles. Actinomycin D (12 μM) and cycloheximide (5 μg/ml) were coincubated with platelet microparticles. 6-Keto $PGF_{1\alpha}$ production was measured in the supernatant by gas chromatography/mass spectrometry after a 6-h incubation period. Each point represents the mean±SEM of six separate experiments.

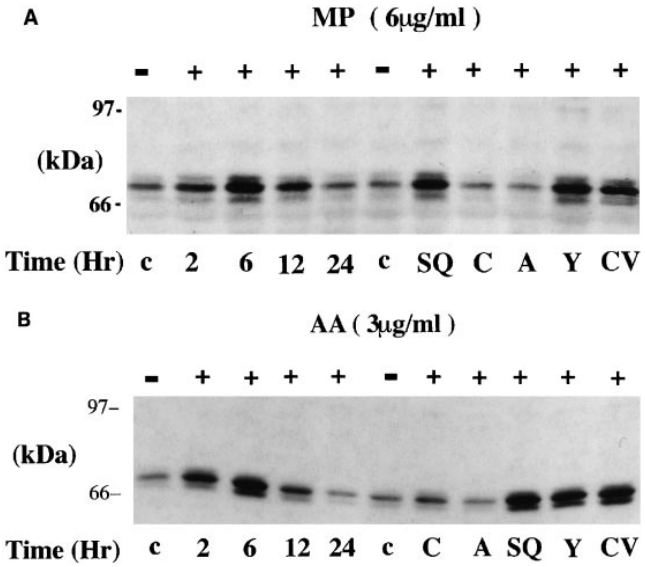

Figure 10. Western blot analysis of COX-2 protein expression in HUVEC treated with platelet MP (6 μg/ml) (*A*) and AA isolated from MP (3 μg/ml) (*B*). HUVEC (3–4 × 10^5 cells/well) were incubated with medium only (control, *c*); with MP alone for 2, 6, 12, or 24 h; with MP + either SQ29,548 (*SQ*), cycloheximide (*C*), actinomycin D (*A*), yohimbine (*Y*), or CV-6209 (*CV*) for 6 h. The lower blot shows HUVEC incubated with medium only (control, *c*), with arachidonate isolated from platelet microparticles for 2, 6, 12, or 24 h, with AA + either SQ29,528(*SQ*), cycloheximide (*C*), actinomycin D (*A*), yohimbine (*Y*), or CV-6209 (*CV*) for 6 h. The figure is representative of five experiments.

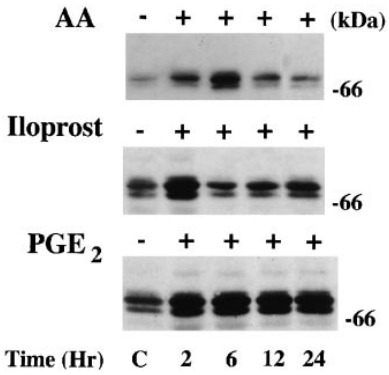

Figure 11. Western blot analysis of COX-2 protein expression in HUVEC treated with microparticle AA (3 μg/ml), Iloprost (100 nM), and PGE_2 (10 μM). HUVEC (3–4 × 10^5 cells/well) were incubated with medium only (control, *C*), AA, Iloprost, or PGE_2 for 2, 6, 12, and 24 h. The figure is representative of two experiments.

apparent at very low levels in controls (139±7.21 densitometric units), while maximum expression of COX-2 induced by AA is observed at 6 h (1,142.33±41.01 densitometric units, $P = 0.00001$) (Fig. 10, *B*). Consistent with the data obtained with microparticles, SQ29,548, yohimbine, and CV-6209 all fail ($P > 0.05$) to suppress the expression of COX-2 induced by arachidonic acid (1,077.62±109.33, 1,033.79±62.91, and 1,033±39.80 densitometric units, respectively, at 6 h). Actinomycin D and cycloheximide, by contrast, both reduce expression of the COX-2 protein (116.50±9.28 and 244.09±16.85 densitometric units, respectively, at the same time point) (Fig. 10, *B*). To investigate further the likelihood that microparticle-dependent induction of COX-2 was indeed mediated by AA itself and not one of its metabolites, we coincubated HUVEC with a PGI_2 analogue, Iloprost (100 nM), and PGE_2 (10 μM) for 2, 6, 12, and 24 h as for AA (Fig. 11). Both Iloprost and PGE_2, when added to HUVEC, induce COX-2 expression. However, the kinetics of protein expression are different from those evoked by microparticle arachidonate. Maximum induction of COX-2 expression by Iloprost occurs at 2 h, while PGE_2 induces COX-2 over a 24-h period. This contrasts with the results for AA, or intact microparticles where maximum COX-2 expression and 6-keto $PGF_{1\alpha}$ production (Fig. 12) occurs at 6 h and decreases to near basal levels at 24 h.

Discussion

Microparticles shed by aggregating platelets may, in turn, cause platelet activation. The active principle in the microparticles is identified as arachidonic acid. Rather than stimulate platelets directly, we demonstrate that the arachidonic acid must first be subjected to metabolism to thromboxane A_2. Thus, pretreatment of platelets with the cyclooxygenase inhibitor, indomethacin, or the thromboxane antagonist SQ29,548 inhibits the ability of the microparticles to induce platelet aggregation. These data are consistent with the observation that the microparticles fail to evoke cellular activation directly via either of the cloned thromboxane receptor isoforms. They are also consistent with the dose-dependent increase in platelet calcium concentration, inositol phosphates, and thromboxane formation evoked by the microparticles and by inhibition of microparticle-induced platelet aggregation by an inhibitor of protein kinase C. Thromboxane receptor activation by ligands results in an increased phospholipase C activity with a consequent increase in intracellular calcium and activation of protein kinase C (34). Prelabeling of esterified platelet arachidonic acid with stable isotopes indicates that the arachidonate in the microparticles may serve as a direct source of thromboxane formation in microparticle-activated platelets.

Our results in platelets are consistent with a previous report that microparticles may evoke biological responses only

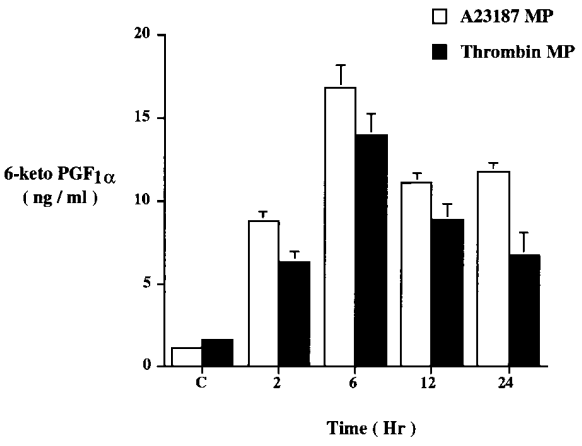

Figure 12. Time course of 6-keto $PGF_{1\alpha}$ production in HUVEC stimulated with platelet MP (6 μg/ml). The supernatant was assayed for 6-keto $PGF_{1\alpha}$ by gas chromatography/mass spectrometry at the indicated times after exposure to MP formed by platelets activated either by the calcium ionophore A23187 (□) or thrombin (■). Values are reported as mean±SEM from five experiments.

after treatment with (s)PLA_2 and sphingomyelinase (7). Experiments with radiolabeled arachidonic acid suggest that addition of (s)PLA_2 to the microparticles enhanced the availability of the lipid for transcellular exchange. Microparticles alone, without such pretreatment, or (s)PLA_2 alone failed to induce platelet aggregation. We used 2 μg/ml of (s)PLA_2 in our system. This is comparable to the levels of (s)PLA_2 that circulate in the plasma of patients with systemic inflammation (35, 36). Cellular activation may result in translocation of (s)PLA_2 to the membrane. (s)PLA_2 is shed by activated platelets, but tends to remain cell associated in endothelial cells (37). Either source could facilitate release of microparticle arachidonate in vivo.

Platelet-derived microparticles may also influence endothelial function. We have demonstrated that microparticles induce de novo expression of COX-2, but not COX-1, in indomethacin-pretreated endothelial cells. Again, arachidonic acid appears to be a sufficient active principle. Results with pharmacological inhibitors suggest that this is not mediated via activation of thromboxane, platelet activating factor, or alpha adrenergic receptors. Although the mechanism by which arachidonic acid induces COX expression remains to be elucidated, experiments with stable isotope–labeled substrate indicate that the arachidonate in the microparticles may serve as a substrate for the induced enzyme, contributing to augmented PGI_2 formation. Interestingly, both PGE_2 and PGI_2 (iloprost), products of COX metabolism of arachidonate in endothelial cells, may upregulate COX-2 in HUVEC. However, the time course of induction of protein expression suggests that they do not account for the predominant response to microparticles in this experiment. However, it is possible that such metabolites might augment the response to microparticle arachidonate in vivo. Direct effects of arachidonic acid on cellular function have been described previously. These include modulation of ion channel function, lipidation of enzymes and signaling proteins, and growth factors (38, 39). The role of arachidonic acid and related lipids in gene induction has received particular attention (40, 41).

Arachidonic metabolites have previously been shown to exhibit the potential for transcellular metabolism. Thus, Marcus and colleagues have previously demonstrated that platelet-derived endoperoxides may be used by endothelial cells as a substrate for PGI_2 synthesis (42); such a concept underlay the development of thromboxane synthase inhibitors. Similarly, platelet lipoxygenase products may undergo further processing by neutrophils or erythrocytes to give rise to novel hydroxy eicosatetraenoic acids of altered biological function (43). Neutrophil-derived lipoxygenase products may be further metabolized by erythrocytes and platelets or endothelial cells (44). Our studies extend these observations. Firstly, they demonstrate that microparticles may serve as a delivery system for bioactive lipids between cells. Although arachidonic acid accounts for the biological activities that we have studied, this concept may extend to other lipids. Thus, Lorant et al. have identified PAF-like lipids in the microparticles shed by activated endothelial cells that facilitate cellular adhesive interactions (45). Secondly, they demonstrate that arachidonic acid itself, rather than one of its downstream metabolites, may be subject to transcellular transfer. Finally, they illustrate a mechanism by which the consequences of platelet activation may be amplified or modulated. It is likely that such a process may extend to other cells in the milieu of a forming thrombus. These experiments were conducted in vitro, in the absence of such forces as shear and flow. However, it is likely that shear will enhance the release of microparticles (46). The existence of flow vortices in proximity to the evolving thrombus may enhance the likelihood of interaction with adjacent vascular endothelium (47). Furthermore, circulating platelet microparticles have been detected in a variety of clinical syndromes of platelet activation. This raises the possibility that flow-dependent dispersal of platelet microparticles may represent a mechanism for transcellular effects of biologically active lipids, remote from the immediate microenvironment of their formation.

Acknowledgments

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