Transcellular activation of platelets and endothelial cells by bioactive lipids in platelet microparticles.

O P Barry, …, J A Lawson, G A FitzGerald


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 microg/ml; and the calcium ionophore A23187, 1 microM) results in shedding of microparticles. Pretreatment of these particles, but not membrane fractions from resting platelets, with (s)PLA2 evokes a dose-dependent increase in platelet aggregation, intracellular [Ca2+] 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 A2. Thus, pretreatment of platelets with the cyclooxygenase (COX) inhibitor, indomethacin (20 microM), the thromboxane antagonist SQ29,548 (1 microM), or the protein kinase C inhibitor GF109203X (5 microM) 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. Prelabelling platelets with [2H(8)] arachidonate was used to demonstrate platelet metabolism of the microparticle-derived substrate to thromboxane. 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)PLA₂ evokes a dose-dependent increase in platelet aggregation, intracellular [Ca²⁺]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 A₂. 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 [³H]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 (PGI₂) 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 PGI₂. Both PGE₂ and iloprost, a stable PGI₂ 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). 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 A₂, but not the plasma fibrin-stabilizing F XIII A₂B₂ (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 A₂ (PLA₂)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 A₂. 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; PGI₂, prostacyclin; PKC (or PKA), protein kinase C (or A); PLÅ₂, phospholipase A₂; PRP, platelet-rich plasma; TP, thromboxane receptor; WP, washed platelets.
Methods

Reagents
Calcium ionophore A23187, (s)PLA₂, indomethacin, N N diospropyl-ethyline, pentfluorobenzyl bromide, EDTA, yohimbin, cycloheximide, actinomycin-D, leupetin, Nonidet P-40, soybean trypsin inhibitor, and aprotonin 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. (Hartboro, PA). The thromboxane receptor (TP) antagonist SQ29,548, the prostacyclin (PGI₂) analog iloprost, [H]arachidonic acid, [H]-TxB₂, [H]-6-keto-PGF₁α, AA, and PGE₂ were all purchased from Cayman Chemical Co., Inc. (Ann Arbor, MI). Fura-2/AM, GF109203X (bisindolylmaleimide 1) and H-89 (a specific TxA₄ receptor antagonist, was obtained from BIOMOL Research Labs. Inc. (Rockville, MD). Densitometric analysis was performed with Scan- nerg Control Version 1.00 (Molecular Dynamics, Sunnyvale, CA). All solvents were of HPLC grade and obtained from T.J. Baker (Daven- vers, MA). The reverse phase HPLC column was from Beckman In- struments, 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 sodi- um 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 × 10⁷ platelets/ml. Platelet aggregation was stud- ied 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 ag- gregation induced by microparticles. These included SQ29,548 (1 µM), a specific TxA₄/PGH₁ receptor antagonist (14), indomethacin (20 µM), a nonspecific COX inhibitor (15), yohimbin (140 nM), or an α₂ adren- ergic 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 superna- tant was collected for measurement of TxB₂, the stable hydrolysis product of TxA₂.

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, af- ter 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)PLA₂ (2 µg/ml) in the presence of 1 mM CaCl₂ at 37°C for 1 h. The microparticles were washed twice to re- move (s)PLA₂ and resuspended in Hepes buffer at the end of the incu- bation. The protein content was measured using a microbicinoc- ninic 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 de- scribed to characterize the microparticles (19). Briefly, platelet micro- particles were incubated with AP-1 (antibody to glycoprotein GP-Ib), for 45 min at room temperature, and then fixed with 1% paraformal- dehyde. Microparticles were identified by gating on GFP (FITC- AP-1)-positive events and were distinguished by forward scatter size analysis. Each platelet microparticle preparation was assayed for en- dotoxin contamination by the Limulus amebocyte lysate assay. Final endotoxin contamination was always < 0.02 U/ml protein. Experi- ments were also performed with platelet microparticles that were preincubated with polymyxin B (50 µg/ml) for 1 h, before their ad- dition 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 spectro- fluorometer 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 addi- tion of 250 µM digitonin in the presence of 1 mM CaCl₂ and in the presence of 10 mM EDTA (pH > 8.5), respectively, as described pre- viously (13).

PRP was incubated with myo-[2-3H]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 con- taining 20 mM LiCl for 15 min. These conditions inhibit conversion of inositol phosphates to free inositol. Platelets were incubated with mi- croparticles for 1 min at 37°C. Total inositol phosphates were sepa- rated 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 am- monium formate to remove both [H]inositol and [H]glycerophos- phorylinositol. 1 ml of the eluant was added to 9 ml of scintillant and the radioactivity was determined using a scintillation counter (Beck- man 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 previ- ously described (22). Briefly, the lipid extract dissolved in 300 µl chlo- roform was applied to a second silica plate. Lipid separations were per- formed with a mobile phase 80% ethyl acetate, 20% hexane, and 0.1% acetic acid and the standards made visible with a stream of nitrogen and redissolved in 20 l of hexane. 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-
tants were collected to determine PGI
buffer, were incubated with HUVEC for 2, 6, 12, and 24 h. Supernatants were removed 48 h before stimulation and replaced with 5% heat inactivated bovine serum albumin. Platelet microparticles, resuspended in Hepes subconfluence and incubated in culture medium containing 0.75% arachidonic acid was performed as previously described (24). Briefly, cells were harvested from human umbilical veins with 1 mg/ml collagenase A at 450 U/mg (Boehringer Mannheim, Indianapolis, IN). They 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 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)-NASSRRSGLDINPTVLLK (amino acid sequence 580–598), which is absent from COX-1. Blots were then incubated with sheep anti-mouse IgG, conjugated with horseradish peroxidase at 1/2,000 (0.1 ml/cm²) 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)PLA₂ in platelet microparticles was also performed. 10, 20, 40, and 60 µg of microparticle protein was used for analysis of (s)PLA₂. 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)PLA₂ at a concentration of 1/10,000 for 1 h at room temperature. Blots were then incubated with rabbit anti-mouse IgG, conjugated with horseradish peroxidase at 1/5,000 (0.1 ml/cm²) 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 (TPR; reference 31) or endothelial (TPB; 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-[2-³H]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
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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 TxB₂ formation (Fig. 2). The magnitude of the response evoked by concentrations of microparticles typically shed by activated platelets in vitro (260 μg microparticle protein/3 × 10⁹ 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)PLA₂ (2 μg/ml). (s)PLA₂ alone does not cause platelet aggregation. We excluded the presence of endogenous (s)PLA₂ 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 TxB₂ production is also observed (data not shown). The α₂-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 CaCl₂ 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 PGH₂/TxA₂ analog, U46619, by contrast, markedly increases IP formation in cells expressing either isoform (Fig. 6).
Identification of the biological mediator present in platelet microparticles. Neither the neutral nor the polar lipid fractions extracted from microparticles and recovered from the NH₂ 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 Rf 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 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).

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 [3H]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)PLA₂ 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 [3H]AA were

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.

Figure 6. Total inositol phosphate formation by HEK 293 cells stimulated with platelet microparticles or the PGH₂/TxA₂ analog, U46619. HEK 293 cells transfected with the placental (TPα) or the endothelial (TPβ) receptors were prelabeled with myo-[2-3H]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 three separate experiments.

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

<table>
<thead>
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<th>Microparticles (μg/ml)</th>
<th>Percent increase in IPs</th>
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<td>5</td>
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<td>40</td>
<td>111.6±6.1</td>
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<tr>
<td>U46619 (1 μM)</td>
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Dose-dependent stimulation of [3H]inositol phosphate formation by platelet microparticles. Platelets were prelabeled with myo-[2-3H]inositol (50 μCi/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.
added to freshly prepared platelets to address the possibility that they might donate arachidonate for transcellular metabolism. [3H] TxB2 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 TxA2, a platelet agonist and vasoconstrictor. We also investigated whether HUVEC might use the AA present in platelet microparticles. Similarly, HUVEC enzymatically metabolizes [3H]AA, since the stable hydrolysis product of prostacyclin (PGI2), [3H]-6-keto PGF1α, was detected in the supernatant after exposure to platelet microparticles. The concentration of [3H]-6-keto PGF1α 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.

**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 PGI2 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 PGI2 synthesis, as measured by 6-keto PGF1α in the supernatant (see Fig. 12). Indomethacin (20 μM) was always added to the medium before the addition of platelet microparticles to

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Percent [H]arachidonic acid</th>
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<tr>
<td>Microparticles</td>
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<tr>
<td>Supernatant</td>
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<tr>
<td>Platelets</td>
<td>40.0±1.5</td>
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Platelets (3 × 10^9) 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.
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)

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

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.
Discussion

Microparticles shed by aggregating platelets may, in turn, cause platelet activation. The active principle in the microparticle is identified as arachidonic acid. Rather than stimulate platelets directly, we demonstrate that the arachidonic acid must first be subjected to metabolism to thromboxane A₂. 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 isofoms. 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). Prelabelling 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 11. Western blot analysis of COX-2 protein expression in HUVEC treated with microparticle AA (3 μg/ml), Iloprost (100 nM), and PGE₂ (10 μM). HUVEC (3–4 × 10⁵ cells/well) were incubated with medium only (control, C), AA, Iloprost, or PGE₂ for 2, 6, 12, and 24 h. The figure is representative of two experiments.](image)

![Figure 12. Time course of 6-keto PGF₁α production in HUVEC stimulated with platelet MP (6 μg/ml). The supernatant was assayed for 6-keto PGF₁α 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.](image)
after treatment with (s)PLA₂ and sPLA₂ and sphingomyelinase (7). Experiments with radiolabeled arachidonic acid suggest that addition of (s)PLA₂ to the microparticles enhanced the availability of the lipid for transcellular exchange. Microparticles alone, without such pretreatment, or (s)PLA₂ alone failed to induce platelet aggregation. We used 2 μg/ml of (s)PLA₂ in our system. This is comparable to the levels of (s)PLA₂ that circulate in the plasma of patients with systemic inflammation (35, 36). Cellular activation may result in translocation of (s)PLA₂ to the membrane. (s)PLA₂ 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 PGI₂ formation. Interestingly, both PGE₁ and PGI₂ (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 PGI₂ synthesis (42); such a concept underlay the development of thromboxane synthase inhibitors. Similarly, platelet lipoygenase products may undergo further processing by neutrophils or erythrocytes to give rise to novel hydroxy eicosatetraenoic acids of altered biological function (43). Neutrophil-derived lipoygenase 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.

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References

15. Walters, M.N., and D.A. Willoughby. 1965. Indomethacin, a new anti-