Role of Platelet Microparticles in the Production of Thromboxane by Rabbit Pulmonary Artery

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Abstract—This study examined the role of platelet microparticles in thromboxane A2 (TXA2) production. Incubation of microparticles with [14C]arachidonic acid and A23187 produced 14C-labeled TXB2, the stable metabolite of TXA2. To investigate the possibility that endothelial cells (ECs) transfer arachidonic acid to platelet microparticles and promote TXB2 synthesis, ECs with their cellular lipids prelabeled with tritiated arachidonic acid were incubated with microparticles. In the absence of microparticles, there was no production of tritiated TXB2 by the ECs. However, when microparticles were coincubated with prelabeled ECs, tritiated arachidonic acid was metabolized to tritiated TXB2. Aspirin was then used to inhibit cyclooxygenase. ECs coincubated with aspirin-treated platelet microparticles did not produce TXB2, as measured by radioimmunoassay. In contrast, aspirin-treated ECs coincubated with microparticles produced TXB2, and its production was enhanced by methacholine (10^-4 mol/L), indicating that endothelially derived arachidonic acid, and not endothelially derived prostaglandin endoperoxide, was transferred to the microparticle and further metabolized to TXA2. Additional studies with rabbit aorta and pulmonary artery investigated whether microparticles contributed to vascular contractions. Preincubation with microparticles enhanced arachidonic acid–induced contractions in the aorta and methacholine-induced contractions in the pulmonary artery. The thromboxane receptor antagonist SQ29548 and the thromboxane synthase inhibitor dazoxiben blocked these effects. Because TXA2 is an important mediator in various pathophysiologic states, including hypertension, the ability of platelet microparticles to act as a cellular source of TXA2 might provide new insight into the role of platelets and platelet microparticles in the control of vascular tone. (Hypertension. 2004;43[part 2]:428-433.)

Key Words: thromboxanes ■ platelets ■ microparticles ■ vasoconstriction ■ arachidonic acid

The control of pulmonary vascular resistance involves the interaction of various vasoconstrictors, such as thromboxane (TX) A2, and vasodilators, such as prostacyclin. Because both prostacyclin and TXA2 are cyclooxygenase metabolites of arachidonic acid (AA), it has been postulated that a balance of these 2 compounds contributes to the regulation of vascular tone. Abnormalities in the balance of these factors might have a role in certain pathologic states. There are a number of instances wherein increased synthesis of TXA2 is associated with pulmonary disease. For example, clinical studies have shown that in primary pulmonary hypertension, there is increased synthesis of TXA2 and decreased synthesis of prostacyclin. Administration of endotoxin to the isolated, perfused rabbit lung can result in the activation of inflammatory cells and increased production of various mediators, including TXA2. One consequence of the inflammatory response in the lung is damage to the endothelium of pulmonary vessels and pulmonary arterial hypertension. Pretreatment with a cyclooxygenase inhibitor prevents the vasoconstrictor response to endotoxin in isolated, perfused lungs.

Despite the clear role for TXA2 in various pathophysiologic states, the cellular source of TXA2 has never been clearly defined. We studied the generation of TXA2 by intact segments of rabbit pulmonary arteries and found that in functional experiments, AA- and methacholine-induced contractions were mediated by TXA2. Intact pulmonary arteries synthesized TXA2 in response to AA and methacholine, whereas removal of the endothelium blocked both the vasoconstrictor response and the synthesis of TXA2. However, there was no evidence that endothelial cells (ECs) produced TXA2, either by direct measurement by high-pressure liquid chromatography (HPLC) or radioimmunoassay (RIA) or by immunodetection of TX synthase. Although several other blood cell types could be involved, our studies in isolated segments as well as studies by others in buffer- (nonblood) perfused lungs suggest that some resident cell must be involved. Platelets are likely candidates, because TXA2 is their major AA metabolite. Related to this is the concept that when platelets are activated, microparticles are produced from the plasma membrane and released into the extracellular space. The level of platelet-derived microparticles is increased in a number of different disease states, including acute myocardial infarction, stroke, and severe hypertension. Platelet microparticles can influence vascular function by...
participating in thrombus formation and leukocyte adhesion, as well as interacting with the vascular endothelium. In the current study, we hypothesized that platelet microparticles might also be a source of TXA2 in the pulmonary artery and contribute to the vasoconstrictor responses to AA and methacholine. This study provides evidence that platelet microparticles act as a cellular source of TXA2 in both the pulmonary artery and aorta and might provide new insight into the role of platelets and platelet microparticles in the control of vascular tone.

Methods

Animals

Two- to 3-month-old, male New Zealand White rabbits were obtained from New Franken Rabbitry (New Franken, Wis). The animals were housed at the Medical College of Wisconsin Animal Care Facilities, maintained on a standard rabbit chow diet, and given tap water ad libitum. Rabbits were anesthetized with sodium pentobarbital (120 mg/kg IV), and the heart and lungs were removed as a unit and placed immediately in Krebs bicarbonate buffer of the following composition (in mmol/L): NaCl 118, KCl 4, CaCl2 3.3, NaHCO3 24, KH2PO4 1.4, MgSO4 1.2, and glucose 5; pH 7.4. The main pulmonary artery was identified at its origin from the right ventricle, and both left and right pulmonary arteries were dissected to their most distal ends. The pulmonary artery distal to the first branching of the left or right pulmonary artery was used, and this is referred to as the intrapulmonary artery. In some cases, the thoracic aorta was removed and placed immediately in Krebs bicarbonate buffer. The pulmonary artery and aorta were carefully cleaned of adhering fat and connective tissue.

Platelet Microparticle Isolation

Platelet microparticles were prepared by a previously described method, with some modifications. Blood was collected from New Zealand White rabbits in 3.2% citrate and centrifuged at 150g for 10 minutes. The supernatant contains platelet-rich plasma. Platelets were sedimented by centrifugation of platelet-rich plasma at 1500g for 10 minutes, washed, and resuspended in buffer of the following composition (in mmol/L): NaCl 118, KCI 4, CaCl2 3.3, NaHCO3 24, KH2PO4 1.4, MgSO4 1.2, and glucose 5; pH 7.4. The main pulmonary artery was identified at its origin from the right ventricle, and both left and right pulmonary arteries were dissected to their most distal ends. The pulmonary artery distal to the first branching of the left or right pulmonary artery was used, and this is referred to as the intrapulmonary artery. In some cases, the thoracic aorta was removed and placed immediately in Krebs bicarbonate buffer. The pulmonary artery and aorta were carefully cleaned of adhering fat and connective tissue.

Metabolism of [14C]AA

Platelet microparticles (1 mg/mL) were resuspended in HEPES buffer and incubated at 37°C for 15 minutes with [14C]AA (0.05 μCi, 10^7 mol/L) and calcium ionophore A23187 (20 μmol/L). After incubation, HEPES buffer was removed, acidified to pH 2.0 with glacial acetic acid, and extracted over ODS extraction columns (BondElut), as previously described. The ODS columns were washed sequentially with 15 mL water and ethanol. The acidified sample (made to 15% vol/vol with ethanol) was then added to the column and washed sequentially with 5 mL each of 15% ethanol and water. The AA metabolites were eluted with 6 mL ethyl acetate, evaporated to dryness under a stream of N2, and stored at −40°C until analyzed by reverse-phase HPLC (Beckman Instruments). The prostaglandin (PG) metabolites of AA were separated on a reverse-phase HPLC system with a Nucleosil-C18 column (5 μm, 4.6×250 mm; Phenomenex). Solvent A was water containing 0.025 mol/L phosphoric acid and solvent B was acetonitrile. The program consisted of a 40-minute isocratic phase with 31% B in A, followed by a 20-minute linear gradient to 100% B and a 10-minute isocratic phase with 100% B. The flow rate was 1 mL/min. Column eluate was collected in 0.5-mL fractions, and radioactivity was determined by liquid scintillation spectrometry.

EC Experiments

ECS were isolated and cultured from rabbit pulmonary arteries by a modification of methods previously described. ECs were plated on 25-cm² culture flasks and left undisturbed for 48 hours at 37°C in an atmosphere of 5% CO2 in air. Once confluent, EC monolayers were obtained (5 to 10 days), the cells were detached with Pucks-EDTA solution and trypsin and transferred to 75-cm² culture flasks to incubate them for identification of AA metabolites. Monolayers were incubated overnight with 0.5 μCi tritiated AA to label their phospholipids. Under these labeling conditions, only 10% of the added radioactivity remains in the labeling buffer, and the remainder is incorporated into EC lipids. After the prelabeling period, ECs were washed 4 times with HEPES buffer containing 1% fatty acid–free bovine serum albumin. The washed cells were incubated in fresh, protein-free HEPES buffer in the presence or absence of microparticles (500 μg per incubation) and treated with methacholine (10⁻⁴ mol/L). Radioactivity released into the incubation medium was extracted and analyzed by reverse-phase HPLC, as described earlier. Elution times of radioactive peaks were compared with the retention times of known PG standards.

In an additional series of experiments, pulmonary artery ECs were grown in 24-multiwell plates. Microparticles (50 μg per well) or HEPES buffer was layered over the ECs or added to empty wells. Cells were immediately treated with buffer or methacholine (10⁻⁴ mol/L) and incubated for 30 minutes at 37°C. The buffer was removed, and the synthesis of TXB2 was measured by a specific RIA according to the method of Campbell and Ojeda. In an additional experiment, platelet microparticles were treated with 100 μmol/L aspirin for 30 minutes at 37°C. The microparticles were washed twice to remove unreacted aspirin, and the aforementioned experimental protocol was repeated. Finally, ECs were treated with 100 μmol/L aspirin for 30 minutes at 37°C. The monolayer was repeatedly washed to remove unreacted aspirin, and the aforementioned experimental protocol was repeated.

Vascular Reactivity Protocol

Rings of rabbit aorta were obtained (3 to 4 mm) and incubated in HEPES buffer containing platelet microparticles (1 mg/mL) for 30 minutes at 37°C. An equal number of rings were incubated under similar conditions but without platelet microparticles. The vessels were then suspended in 6-mL organ baths containing Krebs bicarbonate buffer warmed to 37°C and aerated with a 95% O2–5% CO2 mixture. Isometric tension was measured with force-displacement transducers (Grass Instruments) and amplifiers (AD Instruments ETH-400) and recorded on a Macintosh computer with MacLab 8e software. Resting tension was adjusted to its length tension maximum of 2 g. The vessels were allowed to equilibrate for 1 hour. Contractions were produced by increasing the KCl concentration of the bath to 40 mmol/L. KCl-induced contractions were repeated until maximal, reproducible responses were obtained. AA (1 and 10 μmol/L) was added to test for vascular contractions. Some vessels were pretreated with the TX receptor antagonist SQ29548 (10⁻⁴ mol/L) for 10 minutes before addition of AA. Because KCl contractile responses remained stable throughout the experiment, results were expressed as a percentage of the KCl contraction. In a separate experiment, rings of aorta preincubated with or without platelet microparticles (1 mg/mL) were placed in HEPES buffer, pH 7.4, and incubated at 37°C for 15 minutes with [14C]AA (0.05 μCi, 10⁻⁷ mol/L) and calcium ionophore A23187 (20 μmol/L). Radioactivity released into the incubation medium was extracted and analyzed by reverse-phase HPLC, as described before.

An additional series of experiments investigated methacholine-induced contractions in rings of rabbit pulmonary artery incubated with or without platelet microparticles, as described before for rabbit aorta. The vascular reactivity methods were similar to those described for the rabbit aorta, except that resting tension was adjusted.
to the pulmonary artery's length tension maximum of 1 g. Increasing concentrations of methacholine (0.1 to 100 μmol/L) were used to produce contractions. Some vessels were pretreated with the TX synthase inhibitor dazoxiben (10 μmol/L) for 10 minutes before addition of methacholine.

**Materials**

[14C]AA was obtained from DuPont NEN; tritiated AA and tritiated TXB2 were from Amersham; AA (free acid) was from Nu-Check Prep, Inc; β-methacholine, A23187, AA (sodium salt), and aspirin were from Sigma; and SQ29548 and TXB2 were from Cayman Chemical Co. AA (free acid) was prepared in ethanol previously sparged with N2. AA (sodium salt) was prepared in distilled water previously sparged with N2. The stock solution and dilutions of AA were made fresh for each experiment and kept on ice under an N2 atmosphere. All cell-culture reagents were purchased from GIBCO. Flasks used in cell culture were from Corning. All other chemicals were of reagent grade.

**Statistical Analysis**

Data are expressed as mean±SEM. Statistical analysis of the data was performed by ANOVA to determine differences within groups, followed by Student t test to determine differences between groups. A value of P<0.05 was considered statistically significant.

**Results**

Results showed that when platelet microparticles were incubated with [14C]AA and A23187, a major radioactive peak identified by reverse-phase HPLC comigrated with TXB2 (Figure 1). To determine the role of microparticles and methacholine in the production of TXB2 from cultured rabbit pulmonary artery ECs, cell lipids were prelabeled with tritiated AA. After a 15-minute incubation at 37°C in the presence of methacholine, EC monolayers (Figure 2A) released a number of radioactive products. The major products comigrated with 6-keto-PGF1α and PGE2. However, there was no radioactive peak that comigrated with TXB2.

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Additional studies by RIA confirmed that production of TXB2 by intact pulmonary artery required an interaction between ECs and adherent platelet microparticles. ECs and platelet microparticles were incubated as described in Methods, and TXB2 production was measured by RIA. As observed with the HPLC studies, ECs alone did not produce TXB2 (Figure 3A). In the presence of ECs and platelet microparticles, TXB2 was enhanced 25-fold (0.4±0.2 versus 25±2 pg/mL, ECs alone versus ECs + microparticles, n=5; Figure 3A). Methacholine, when added with the platelet microparticles, caused a slight enhancement of TXB2 production, which was not statistically significant (25±2 versus 30±2 pg/mL, microparticles versus microparticles + methacholine; NS, n=5; Figure 3A). Because aspirin irre-

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**Figure 1.** Metabolism of [14C]AA by rabbit platelet microparticles. Platelet microparticles were prepared as described in Methods. The resulting microparticle pellet was resuspended in HEPES buffer to give a final concentration of 1 mg/mL. Microparticles were incubated at 37°C for 15 minutes with [14C]AA and A23187. The PG metabolites of AA were separated by reverse-phase HPLC. Half-minute fractions were collected and analyzed by liquid scintillation spectrometry. Migration times of known standard eicosanoids are shown above the chromatograms. TXB2=21.2 minutes; AA=61 minutes. This experiment was repeated 3 times with similar results.

**Figure 2.** Effect of methacholine (10^-4 mol/L) on tritiated AA metabolism in prelabeled, rabbit pulmonary artery endothelial cells (RPAECs) incubated alone (A) or with platelet microparticles (B). PG metabolites of tritiated AA were separated by reverse-phase HPLC. Half-minute fractions were collected and analyzed by liquid scintillation spectrometry. Migration times of known standard eicosanoids are shown above the chromatograms. 6-keto-PGF1α=10.5 minutes; TXB2=21.5 minutes; PGE2=30.5 minutes; AA=61 minutes. This experiment was repeated 3 times with similar results.
versibly inactivates cyclooxygenase, it was used as a tool to investigate the interaction between these cells. Results showed that when ECs were coincubated with aspirin-treated platelet MPs, methacholine failed to induce TXB2 synthesis (Figure 3C). In contrast, when aspirin-treated ECs were coincubated with platelet microparticles, methacholine-induced TXB2 production was greater than that observed with just the aspirin-treated ECs and platelet microparticles (12.6±0.4 versus 31.4±7.2 pg/mL, microparticles versus microparticles + methacholine; P<0.05, n=5; Figure 3B).

The next series of experiments evaluated the effect of platelet microparticles in rabbit aorta. Unlike the rabbit pulmonary artery, rabbit aorta does not produce TXA2.12 However, when the aorta was preincubated with platelet microparticles before the [14C]AA metabolism studies, TXB2 production was observed (Figure 4), indicating that a sufficient number of platelet microparticles adhered to the aorta. A number of other radioactive products were also produced but not identified. When vascular responses to AA in aortas incubated with platelet microparticles were determined, AA caused a concentration-dependent contraction that was not observed in control aortas (maximal contraction, 44.4±3.2% versus 9.8±7.9%, aorta + microparticles versus aorta alone; P<0.05, n=3 to 10; Figure 5). Pretreatment with the TX receptor antagonist SQ29548 (10 μmol/L) blocked the responses to AA (Figure 5). A separate study used rabbit pulmonary artery rings and found that in the vessels preincubated

![Figure 3](image-url)  
**Figure 3.** TXB2 production in rabbit pulmonary artery ECs (RPAECs) coincubated with rabbit platelet microparticles (MPs). ECs were grown in multiwell plates and incubated with rabbit platelet MPs. In some experiments, methacholine (MECH, 10^-4 mol/L) was included. Production of TXB2 was measured in the incubation medium by RIA. Data points are mean±SEM for n=5. *P<0.05, MECH-treated versus MPs. B, ECs or MPs were pretreated with 100 μmol/L aspirin (ASA) before the coincubation studies.

![Figure 4](image-url)  
**Figure 4.** Metabolism of [14C]AA in rabbit aorta preincubated with platelet microparticles. Rings of rabbit aorta were preincubated in either HEPES buffer alone or HEPES buffer containing platelet microparticles (1 mg/mL) for 30 minutes at 37°C. After incubation, aortas were then incubated in fresh buffer at 37°C for 15 minutes with [14C]AA and A23187. PG metabolites of AA were separated by reverse-phase HPLC. Half-minute fractions were collected and analyzed by liquid scintillation spectrometry. Migration times of known standard eicosanoids are shown above the chromatograms. 6-keto-PGF1α=10.5 minutes; TXB2=22.5 minutes; AA=70 minutes. This experiment was repeated twice with similar results.
cubated with platelet microparticles, there was an enhanced contractile response to methacholine (maximal contraction, 71.8±8.5% versus 46.3±8.7%, pulmonary artery + microparticles versus pulmonary artery alone; *P<0.05, n=6 to 10; Figure 6). Pretreatment with the TX synthase inhibitor dazoxiben (10 μmol/L) blocked the response to methacholine in the vessels preincubated with microparticles (Figure 6).

Discussion
Previous studies investigating the cellular source of TXA2 in rabbit pulmonary arteries indicated that ECs and vascular smooth muscle cells are not the source, but that TXA2 generation is dependent on the interaction of the vascular endothelium with adherent platelets. These results were extended by the current study, which has shown that platelet microparticles released after platelet activation might also contribute to TXA2 synthesis by blood vessels. By using a variety of techniques, the major findings of this study are as follows: (1) platelet microparticles synthesize TXA2; (2) pulmonary ECs transfer AA to platelet microparticles, which is subsequently metabolized by the microparticles to TXB2; (3) AA-induced contractions are enhanced in rabbit aorta preincubated with platelet microparticles; (4) methacholine-induced contractions are enhanced in rabbit pulmonary artery preincubated with platelet microparticles; and (5) inhibition of TXA2 blocked the enhanced contractions observed in the presence of microparticles in both the aorta and pulmonary artery.

Platelet activation in response to certain agonists such as collagen, thrombin, and calcium ionophore A23187 results in a well-regulated process of shedding of membrane vesicles called microparticles. Circulating platelet microparticles have been detected in a number of pathophysiologic conditions, thus fueling the need to understand the function of these particles under both normal and pathologic states. In relation to a role in modulating vascular function, Boulanger and coworkers showed a direct effect of microparticles derived from patients with acute myocardial infarction to attenuate endothelium-dependent relaxations in isolated arteries. A similar effect was noted when microparticles were obtained from women with preeclampsia. The mechanism responsible for diminished endothelium-dependent relaxations was not identified. However, there is evidence that platelet microparticles and ECs interact in the biosynthesis of AA. Previous studies showed that platelet microparticles transferred AA to ECs, resulting in increased production of the vasodilator eicosanoid prostacyclin. This interaction would not explain the ability of these microparticles to impair relaxations. However, the current study, which shows that AA transfer also occurs from ECs to platelet microparticles, resulting in increased production of the vasoconstrictor TXA2, might provide a mechanism for how these particles attenuate endothelium-dependent relaxations.

The studies in isolated aortas added further support to the concept that microparticles, through generation of TXA2, are responsible for AA-induced contractions. Under normal conditions, rabbit aorta does not synthesize TXA2 and does not show an endothelium-dependent contractile response to either AA or methacholine. This contrasts with the behavior of other blood vessels, in which contractions to methacholine or AA have been observed. Therefore, we used the rabbit aorta to determine whether the presence of microparticles could contribute to alterations in vascular tone. Other studies showed that when isolated platelets were incubated with rabbit aorta, AA produced a contraction that was blocked by inhibitors of TX. Our studies provide the first evidence that platelet microparticles produce a similar effect. As further confirmation, these studies were repeated in rabbit pulmonary arteries, but the response to methacholine was examined. Our previous studies identified TXA2 as the mediator of methacholine-induced contractions in the rabbit pulmonary artery. The ability of platelet microparticles to enhance vascular contractions to both AA and methacholine in blood vessels obtained from different parts of the vasculature provides additional support of a role for platelet microparticles in the modulation of vascular tone.

Perspectives
Patients with cardiovascular disease are at higher risk of thrombosis, resulting in platelet activation and microparticle
formation. Studies have reported increased numbers of circulating platelet microparticles in several disease states, including diabetes and hypertension. The experimental approach of the current study describes an important role for platelet microparticles in modulating vascular tone by demonstrating the ability of platelet microparticles and ECs to participate in the transcellular metabolism of AA to the potent vasoconstrictor TXA2. In hypertension, a critical balance of vasodilators and vasoconstrictors exists, and any upset in this balance contributes to additional complications of the disease. Our results provide further understanding of how platelet microparticles interact with blood vessels, as well as providing some insight into how these microparticles might participate in the pathogenesis of diseases like hypertension.

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