Methacholine-Induced Contraction of Rabbit Pulmonary Artery: Role of Platelet-Endothelial Transcellular Thromboxane Synthesis

Sandra L. Pfister, David D. Deinhart, William B. Campbell

Abstract—Arachidonic acid- and methacholine-induced contractions of rabbit pulmonary arteries are mediated by thromboxane (TX) A2. Although removal of the endothelium abolishes the contractions, endothelial cells isolated from pulmonary arteries do not synthesize TXA2. Further studies described here showed that the expression of TX synthase was evident in platelets and intact pulmonary artery but not in endothelial cells. These studies examined the role of platelet TXA2 production in the vasoconstrictor response to methacholine. Endothelial cells were incubated with platelets in the presence or absence of methacholine. Methacholine caused an increase in TXB2 production. Pretreatment of endothelial cells with aspirin (100 μmol/L) before the addition of platelets did not impair the ability of methacholine to increase TXB2 synthesis. Conversely, if platelets were pretreated with aspirin, methacholine failed to stimulate TXB2. Using endothelial cells with their cellular lipids labeled with [3H]arachidonic acid, methacholine did not stimulate the production of [3H]TXB2. When the endothelial cells were incubated with methacholine and control platelets, [3H]TXB2 was detected. If aspirin-treated platelets were incubated with endothelial cells, methacholine did not increase the production of [3H]TXB2. However, pretreatment of the endothelial cells with aspirin did not affect the ability of methacholine to induce [3H]TXB2 release. This suggests that methacholine stimulated the endothelial cell to release arachidonic acid, which was transferred to the platelets and metabolized to TXA2. To test whether this cell-cell interaction is necessary for methacholine-induced contractions, rabbits were administered aspirin (20 mg/kg) for 2 days. On day 4, methacholine-induced contractions of pulmonary arteries were depressed in aspirin-treated compared with control subjects. Control arteries synthesized 6-keto-prostaglandin F1α and TXB2. Aspirin treatment inhibited both pulmonary artery and platelet TXB2 production but had no effect on vessel 6-keto-prostaglandin F1α. These studies implicate platelets as a vascular source of TXA2 and indicate that both endothelial cells and platelets may be required for methacholine-induced TXA2 synthesis and vasoconstriction (Hypertension. 1998;31[part2]:206-212.)

Key Words: thromboxane A2, cyclooxygenase, platelets, arachidonic acid, endothelial cells, endothelium-derived contracting factor

In recent years, the importance of various factors synthesized and released from the blood vessel endothelium that contribute to the regulation of vascular tone has become apparent. In pulmonary vessels, we have identified an endothelium-derived contracting factor as thromboxane (TX) A2. The control of pulmonary vascular resistance involves the interaction of various vasoconstrictors, such as TXA2, and vasodilators, such as prostacyclin. Because both prostacyclin and TXA2 are cyclooxygenase metabolites of arachidonic acid, it is postulated that a balance in these two compounds contributes to the regulation of vascular tone. Abnormalities in the balance of these factors may then have a role in certain pathological states. For example, an increased synthesis of TXA2 is associated with pulmonary disease, and TXA2 has been shown to be involved in pulmonary vasoconstriction observed in a number of animal models of pulmonary hypertension.

The major source of TXA2 is the platelet, polymorphonuclear leukocyte (PMN), and monocyte. Although there are numerous reports of TXA2 production by blood vessels, there is still controversy as to whether endothelial cells synthesize TXA2. Several researchers have reported that cultured endothelial cells produce not only prostacyclin but also TXA2. In contrast, we and others have failed to detect TXB2 synthesis by endothelial cells. Cultured rabbit pulmonary arterial endothelial cells synthesized 6-keto-prostaglandin (PG) F1α, the stable metabolite of prostacyclin, but not TXB2. Immunohistochemical studies indicated the presence of cyclooxygenase, but not TX synthase, in pulmonary artery endothelial cells. Campbell and coworkers reported that primary cultures of umbilical endothelial cells produced TXB2. However, when the cells were passaged, TXB2 production was lost. The synthesis of TXB2 in the primary cultures was...
Associated with adherent platelets. With passage, adherent platelets were lost, as was TXB₂ synthesis. Because it is important to identify the cellular source of TXA₂ in pulmonary vessels to assess its role in pulmonary function, the present study was designed to test the hypothesis that TXA₂ synthesis by intact pulmonary arteries requires an interaction between the endothelial cells and adherent platelets. Additional experiments are described that use aspirin-treated rabbits to study the role of the platelet in the vasoconstrictor response to arachidonic acid and methacholine in the intact pulmonary artery.

### Vascular Reactivity

Two-month-old New Zealand White rabbits were treated with aspirin (5, 10, or 20 mg/kg PO) or its vehicle on days 1 and 2. On day 4, the pulmonary vessels and platelets were isolated as described above. Strips of artery (30 mg wet weight) or isolated platelets (500 × 10⁶ cells) obtained from the control and aspirin-treated rabbits were placed in HEPES buffer and incubated at 37°C for 15 minutes with [³H]arachidonic acid (0.05 μCi, 10⁻¹⁵ mol/L) and the calcium ionophore A23187 (20 μmol/L). The incubation buffer was collected and analyzed using RP-HPLC. To quantitate 6-keto-PGF₁α and TXB₂ production, vessels (3 mg wet weight) from control and aspirin-treated rabbits were incubated at 37°C in HEPES buffer containing vehicle or arachidonic acid (10⁻¹⁵ mol/L) for 15 minutes. The synthesis of 6-keto-PGF₁α and TXB₂ was measured by RIA. Additionally, rings (2 to 3 mm) of pulmonary arteries were obtained and suspended in 15 mL organ baths containing Krebs-bicarbonate buffer at 37°C and continuously aerated with 95% O₂/5% CO₂. Isometric tension was measured with Grass force-displacement transducers and recorded with a Grass polygraph (model 7D). Resting tension was adjusted to its length tension maximum of 1 g. The vessels were allowed to equilibrate for 1 hour. Contraction responses 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. Concentration-response curves were obtained by the cumulative addition of either arachidonic acid (10⁻¹⁰ to 10⁻⁷ mol/L), methacholine (10⁻⁶ to 10⁻⁴ mol/L), or U46619 (10⁻⁶ to 10⁻⁵ mol/L). Because KCl concentration-response curves were stable throughout the experiment, results were expressed as a percentage of the KCl contraction. Methacholine was dissolved in distilled water, and a volume of 0.05 mL was added to the tissue bath. Arachidonic acid and U46619 were dissolved in ethanol and given in a volume that yielded a final ethanol concentration of the bath of <0.07%.

### Platelet-Endothelial Cell Experiments

Blood was collected from New Zealand White rabbits by heart puncture at 150 g for 10 minutes. The supernatant contains platelet-rich plasma. Platelets were sedimented by centrifugation of platelet-rich plasma at 200 x g for 10 minutes, washed, and resuspended in buffer of the following composition (mmol/L): HEPES 10, NaCl 150, KCl 5, CaCl₂ 2, MgCl₂ 1, and glucose 11, pH 7.4. Endothelial cells were isolated and cultured from the rabbit pulmonary arteries by modification of methods described previously. Endothelial cells were grown in 24-well plates. Washed platelets (6 x 10⁸ platelets/well) or HEPES buffer were layered over the endothelial cells 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 TXB₂ and 6-keto-PGF₁α production was measured by specific radioimmunoassays (RIA) using the method of Campbell and Ojeda. In an additional experiment, platelet-rich plasma was treated with 100 μmol/L aspirin for 30 minutes at 37°C. The platelets were washed twice to remove the unreacted aspirin, and the above experimental protocol was repeated. Finally, endothelial cells were treated with 100 μmol/L aspirin for 30 minutes at 37°C. The cells were repeatedly washed to remove the unreacted aspirin, and the above experimental protocol was repeated.

These studies were confirmed with [³H]arachidonic acid. Endothelial cells were incubated overnight with 0.5 μCi [³H]arachidonic acid 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 endothelial cell lipids. After the prelabeling period, cells were washed four times with HEPES buffer containing 2% fatty acid-free BSA. The washed cells were incubated in fresh protein-free HEPES buffer in the presence or absence of platelets (1 x 10⁶ platelets/
peptide was conjugated covalently to keyhole limpet hemocyanin and injected into rabbits. Serum from the rabbits was screened for antibody production using an enzyme-linked immunosorbent assay. Preliminary results indicated that rabbits produced an antibody that selectively recognized rabbit platelet TX synthase. The primary antibody was used at a dilution of 1:1000 for 1 hour at 4°C. After washing, the blot was incubated for 30 minutes with the secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG antibody) at a dilution of 1:3000. After again washing with TTBS, the blot was incubated for 1 minute with DuPont Renaissance Chemiluminescent reagents. The membrane was subsequently exposed to Kodak Biomax MR imaging film and developed. Prestained protein markers were used for molecular mass determination.

Statistics
Data are expressed as the mean ± SEM. Statistical analysis of the data was performed with an analysis of variance to determine differences within the groups followed by Dunnett's modification of the t test to determine differences between groups. A value of P < .05 was considered statistically significant.

Materials
[14C]Arachidonic acid was obtained from DuPont NEN (Boston, MA); [3H]6-keto-PGF1α and [3H]TXB2 were from Amersham (Arlington Heights, IL); arachidonic acid was from Nu-Chek Prep, Inc. (Elysian, MN); 6-keto-PGF1α, A23187, and aspirin were from Sigma; U46619, 6-keto-PGF1α, and TXB2 were from Cayman Chemical Company (Ann Arbor, MI). Thromboxane synthase was from BioRad Research Laboratories, Inc. (Plymouth Meeting, PA). All cell culture reagents were purchased from Gibco (Grand Island, NY). Flasks used in cell culture were from Corning (Corning, NY). All other chemicals were of reagent grade.

Results
Using a specific polyconal anti-TX synthase antibody, Western blot analysis showed the presence of immunoreactive bands in lysates prepared from rabbit platelets and rabbit pulmonary arteries (Fig 1). These bands corresponded to the 60-kDa TX synthase protein. Human platelet microsomes enriched in TX synthase were included as a positive control. In pulmonary endothelial cells, the presence of TX synthase was not detected. This experiment was repeated 3 times, and similar results were observed.

We determined whether platelets synthesized TXB2 in response to methacholine. When platelets were incubated with varying concentrations of methacholine, we failed to detect any stimulation of TXB2 synthesis by RIA (data not shown). In contrast, arachidonic acid caused a concentration-related increase in TXB2 synthesis by platelets (data not shown). To determine whether the production of TXB2 by intact pulmonary artery required an interaction between endothelial cells and adherent platelets, endothelial cells and platelets were incubated as described under Methods. As shown previously, endothelial cells alone did not produce TXB2, under either basal (Table 1) or methacholine-stimulated conditions. In the presence of endothelial cells and platelets, TXB2 was enhanced approximately 2-fold by the addition of methacholine (Table 1). Because aspirin irreversibly inactivates cyclooxygenase, it was used as a tool to investigate the interaction between these cells. Results showed that when endothelial cells were coincubated with aspirin-treated platelets, methacholine failed to induce TXB2 synthesis (Table 1). In contrast, when aspirin-treated endothelial cells were coincubated with platelets, methacholine-induced TXB2 production was not impaired (Table 1). The production of 6-keto-PGF1α was measured in the coincubation studies (Table 1). The addition of platelets to endothelial cells did not alter the production of 6-keto-PGF1α. The endothelial cells were pretreated with aspirin, 6-keto-PGF1α production decreased (data not shown). In the presence of normal platelets, aspirin-treated endothelial cell production of 6-keto-PGF1α was still attenuated (Table 1).

These studies were confirmed with [14C]arachidonic acid and the results shown in Fig 2. In radiolabeled endothelial cells incubated with methacholine (10 μmol/L), there was no production of [14C]TXB2 (Fig 2A). When the endothelial cells were incubated with methacholine in the presence of control platelets, [14C]TXB2 was a major synthetic product (Fig 2B). If aspirin-treated platelets were added to normal endothelial cells, methacholine failed to stimulate the production of [14C]TXB2 (Fig 2C). In contrast, aspirin pretreatment of endothelial cells blocked the production of the cyclooxygenase products 6-keto-

<table>
<thead>
<tr>
<th>Treatment</th>
<th>6-Keto-PGF1α (pg/mL)</th>
<th>TXB2 (pg/mL)</th>
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</thead>
<tbody>
<tr>
<td>Endothelial cells + platelets</td>
<td>184 ± 10</td>
<td>172 ± 10</td>
</tr>
<tr>
<td>Endothelial cells + platelets + MECH</td>
<td>254 ± 21</td>
<td>340 ± 40*</td>
</tr>
<tr>
<td>ASA-endothelial cells + platelets + MECH</td>
<td>64 ± 4</td>
<td>672 ± 33*</td>
</tr>
<tr>
<td>Endothelial cells + ASA-platelets + MECH</td>
<td>158 ± 11</td>
<td>32 ± 4*</td>
</tr>
<tr>
<td>Endothelial cells alone</td>
<td>141 ± 49</td>
<td>ND</td>
</tr>
</tbody>
</table>

ASA before the coincubation studies, ND not detected.*P < .05, MECH-stimulated vs control.
Figure 2. Effect of methacholine (10^{-4} \text{ mol/L}) on [3H]arachidonic acid metabolism in prelabeled rabbit endothelial cells incubated alone (A) or in the presence of platelets (B). In C, radiolabeled endothelial cells were pretreated with aspirin (100 \mu \text{mol/L}) for 30 minutes before incubation with control platelets. In D, radiolabeled endothelial cells were incubated with platelets that were previously pretreated with aspirin. The PG metabolites of [3H]arachidonic acid were separated by RP-HPLC. Migration time of known standard eicosanoids are shown above the chromatograms.

Figure 3. Arachidonic acid- (top) and methacholine- (bottom) induced contractions in pulmonary arteries obtained from control and aspirin-treated rabbits. Results are expressed as contractile response (% KCl), and data points are the mean±SEM for n=12.
pared with the control rabbits (Table 2). Likewise, in the platelets from the high-dose aspirin-treated rabbits, [14C]TXB2 production was depressed compared with control rabbits (Fig 4C and 4D).

Because 6-keto-PGF1α production was lower in the high-dose aspirin-treated rabbits compared with control rabbits, additional experiments were performed in which rabbits were treated with lower doses of aspirin. The vascular reactivity responses to methacholine in rabbits administered the low-dose regimen (5 mg/kg, PO) or the medium-dose regimen (10 mg/kg, PO) are shown in Fig 3 (bottom). The lower dose of aspirin did not reduce the contractile response to methacholine compared with the control rabbits, whereas the medium dose produced an approximate 50% reduction in methacholine-induced contractions. The low-dose aspirin treatment did not alter either the pulmonary artery or platelet production of [14C]TXB2 compared with the control rabbits (data not shown). In pulmonary arteries obtained from rabbits treated with the 10 mg/kg dose of aspirin, [14C]6-keto-PGF1α production was similar when compared with the control rabbits (Fig 5), however, the platelet [14C]TXB2 production was decreased in the treated rabbits compared with the control rabbits.

**Table 2. 6-Keto-PGF1α and TXB2 Production in Control and Aspirin-Treated Rabbit Pulmonary Artery**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>6-Keto PGF1α (pg/mg)</th>
<th>TXB2 (pg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal</td>
<td>AA</td>
</tr>
<tr>
<td>Control</td>
<td>2332 ± 426</td>
<td>3374 ± 474</td>
</tr>
<tr>
<td>Aspirin-treated</td>
<td>1034 ± 231</td>
<td>1427 ± 262</td>
</tr>
</tbody>
</table>

Segments of pulmonary arteries from control or aspirin-treated rabbits were incubated with vehicle or arachidonic acid (AA) (10^-3 mol/L) for 15 minutes at 37°C. Production of 6-keto-PGF1α and TXB2 were measured in the incubation media by radioimmunoassay. Values are the mean±SEM for n=6 vessel segments.

**Discussion**

Our original study reported that endothelium-dependent contractions are produced by arachidonic acid and methacholine in rabbit intrapulmonary artery. Using a variety of techniques, including vascular reactivity studies with inhibitors, bioassay,
endothelial cells release arachidonic acid on cholinergic stimulation and that the platelets convert this arachidonic acid to TXB_2.

To further support this conclusion, aspirin-treated endothelial cells and platelets produced TXB_2 in response to methacholine, indicating that endothelial cell cyclooxygenase is not required and suggesting that endothelial cell-derived arachidonic acid is the source of TXB_2. This conclusion is represented schematically in Fig 6.

The last series of experiments used aspirin-treated rabbits to study the role of the platelet in the vasoconstrictor response to arachidonic acid and methacholine in the intact pulmonary artery. It has been documented that aspirin can selectively inhibit platelet cyclooxygenase because of the inability of platelets to regenerate their cyclooxygenase. Thus, aspirin has a longer duration of action in platelets than in endothelial cells. A treatment regimen was designed to selectively inhibit platelet, but not endothelial, cyclooxygenase. In rabbits treated with high and medium doses of aspirin, platelet and pulmonary artery [³³C]TXB_2 synthesis was inhibited. Pulmonary artery production of [³³C]6-keto-PGF_1α was reduced in the high-dose aspirin-treated rabbits compared with the control rabbits. However, at the lower doses of aspirin, pulmonary artery 6-keto-PGF_1α production was similar between the treated and control rabbits. The vasoconstrictor responses to methacholine and arachidonic acid were reduced in vessels from the medium- and high-dose aspirin-treated rabbits but not in vessels from control rabbits or in rabbits treated with the low-dose aspirin regimen. The response to U46619 was the same in the vessels from control and aspirin-treated animals. Because platelet TXA_2 production is decreased in the aspirin-treated rabbits that exhibited decreased contractile response to methacholine, these results would implicate platelets as the cellular source of TXA_2 production. It is important to note that a recent report by Barry et

Figure 6. Schematic representation of the proposed mechanism of how methacholine stimulates its receptor on the endothelial cell to cause the release of arachidonic acid (AA) that is either further metabolized to prostacyclin (PGH_2) in the endothelial cell or transferred to the platelet to be metabolized to TXA_2. TXA_2 acts on its receptor in smooth muscle cells to cause constriction. PGH_2, PG endoperoxide, PL, phospholipids, COX, cyclooxygenase, TXS, TX synthase, PCS, PGH_2 synthase.
Platelet-Derived Thromboxane and Pulmonary Artery Contractions

al11 has shown that microparticles released from platelets also play a role in the transcellular metabolism of arachidonic acid. Specifically, platelet microparticles were shown to transfer arachidonic acid to the platelets to be used for TXA2 synthesis. Although this mechanism was not explored in the present study, it gives further support for how platelets can influence vascular function.

Although the major source of TXA2 is the platelet, PMN and monocyte also produce TXA2. Therefore, PMNs or monocytes may also be possible sources of TX synthase. These studies support the concept of transcellular metabolism of arachidonic acid in the pulmonary vasculature. Because TXA2 is an important mediator of pulmonary hypertension caused by adherent platelets, which subsequently synthesize the contracting arachidonic acid, it is possible that platelets may also provide further insight into the role of platelets in the regulation of pulmonary vascular tone.

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References

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