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 TXB₁. 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 TXB\textsubscript{2} synthesis. Because it is important to identify the cellular source of TXA\textsubscript{2} in pulmonary vessels to assess its role in pulmonary function, the present study was designed to test the hypothesis that TXA\textsubscript{2} 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.

**Methods**

**Animals**

Two-month-old male New Zealand White rabbits were obtained from New Franken Rabbity (New Franken, WI). The animals were housed in the Medical College of Wisconsin Animal Care Facilities and maintained on a standard rabbit chow diet and given tap water ad libitum. Rabbits were anesthetized with intravenous sodium pentobarbital (120 mg/kg), and the heart and lungs were removed as a unit and placed immediately in Krebs-bicarbonate buffer of the following composition (mmol/L): NaCl 118, KCl 4, CaCl\textsubscript{2} 3.3, NaHCO\textsubscript{3} 24, KH\textsubscript{2}PO\textsubscript{4} 1.4, MgSO\textsubscript{4} 1.2, glucose 11, 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 end. The pulmonary artery distal to the first branching of the left or right pulmonary artery was used, and this referred to as the intrapulmonary artery.

**Platelet-Endothelial Cell Experiments**

Blood was collected from New Zealand White rabbits in 3% citrate and centrifuged at 150g for 10 minutes. The supernatant contains platelet-rich plasma. Platelets were sedimented by centrifugation of platelet-rich plasma to 2000g for 10 minutes, washed, and resuspended in buffer of the following composition (mmol/L): HEPES 10, NaCl 150, KCl 5, CaCl\textsubscript{2} 2, MgCl\textsubscript{2} 1, and glucose 11, pH 7.4. Endothelial cells were isolated and cultured from the rabbit pulmonary arteries by a modification of methods described previously. Endothelial cells were grown in 24-well plates. Washed platelets (6X10\textsuperscript{8} 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\textsuperscript{-5} mol/L) and incubated for 30 minutes at 37°C. The buffer was removed, and TXB\textsubscript{2} and 6-keto-PGF\textsubscript{1α} production was measured by specific radioimmunoassays (RIA) using the method of Campbell and Ogeda. 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 [H]arachidonic acid. Endothelial cells were incubated overnight with 0.5 µCi [H]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 1% fatty acid-free BSA. The washed cells were incubated in fresh protein-free HEPES buffer in the presence or absence of platelets (1 X 10\textsuperscript{5} platelets/
Platelet-Derived Thromboxane and Pulmonary Artery Contractions

Figure 1. Representative Western blot analysis using a specific polyclonal anti-rabbit TX synthase antibody. Lysate fractions were prepared from rabbit platelets, rabbit pulmonary arteries, and rabbit pulmonary endothelial cells. Human platelet microsomes containing TX synthase were included as a positive control. The immunoreactive proteins were visualized using the chemiluminescence detection system described under Methods. Lane 1, platelets (10 μg); lane 2, pulmonary artery (10 μg); lane 3, TX synthase (4 μg); lane 4, pulmonary artery endothelial cells (10 μg); lane 5, platelets (25 μg).

peptide was conjugated covalently to keyhole limpet hemocyanin and injected with adjuvant into rabbits. Sera 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 20 minutes with the secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG antibody) at a dilution of 1:2000. After again washing with TBS, 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 among the groups followed by Dunnett's modification of the t test to determine differences between groups. A value of P<0.05 was considered statistically significant.

Materials
[14C]Arachidonic acid was obtained from DuPont NEN (Boston, MA); [1H]6-keto-PGF1α and [1H]TXB2 were from Amersham (Arlington Heights, IL); arachidonic acid was from Nu-Chek Prep, Inc. (Elysian, MN); β-methacholine, A23187, and aspirin were from Sigma; U46619, 6-keto-PGF1α and TXB2 were from Cayman Chemical Company (Ann Arbor, MI). Thromboxane synthase was from Biomol 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 polyclonal 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 of 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 (Table 1). The addition of platelets to endothelial cells did not alter the production of 6-keto-PGF1α. If 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 [3H]arachidonic acid and the results shown in Fig 2. In radiolabeled endothelial cells incubated with methacholine (10 μmol/L), there was no production of [3H]TXB2 (Fig 2A). When the endothelial cells were incubated with methacholine in the presence of control platelets, [3H]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 [3H]TXB2 (Fig 2C). In contrast, aspirin pretreatment of endothelial cells blocked the production of the cyclooxygenase products 6-ke-
The next series of experiments was designed to use aspirin-treated rabbits to further study the role of platelets in the vasoconstrictor response to arachidonic acid and methacholine in the intact pulmonary artery. Arachidonic acid and methacholine produced concentration-related contractions in the control rabbits (Fig 3). However, in the rabbits treated with the highest dose of aspirin (20 mg/kg), there was a significant attenuation of both arachidonic acid- (maximal contraction 66 ± 5.4% versus 15 ± 2.4%, control versus aspirin-treated, P < 0.05) and methacholine- (maximal contraction 55.4 ± 5.8% versus 16.3 ± 4.0%, control versus aspirin-treated, P < 0.05) induced contractions (Fig 3). The vasoconstrictor response to the TX-mimetic U46619 was the same in the control and high-dose aspirin-treated animals (data not shown). To assess cyclooxygenase inhibition, pulmonary arteries and platelets from the control and aspirin-treated rabbits were incubated with [14C]arachidonic acid and extracted metabolites analyzed by RP-HPLC. Both control (Fig 4A) and high-dose aspirin-treated (Fig 4B) pulmonary arteries synthesized [14C]6-keto-PGF1α, however, its synthesis was less in the vessels from the aspirin-treated rabbits. When measured by RIA, it also seemed that 6-keto-PGF1α production was reduced in the aspirin-treated rabbit pulmonary arteries (Table 2). High-dose aspirin treatment inhibited the production of [14C]TXB2 (Fig 4B). By HPLC analysis, TXB2 production was 45 ± 18 cpm/mg for the control pulmonary arteries and 23 ± 8 cpm/mg tissue for the aspirin-treated pulmonary arteries. When measured by RIA, there was an approximate 60% reduction in TXB2 production in the pulmonary arteries obtained from aspirin-treated rabbits compared to the control pulmonary arteries.
pared with the control rabbits (Table 2). Likewise, in the platelets from the high-dose aspirin-treated rabbits, \([^{14}C]\)TXB\(_2\) production was depressed compared with control rabbits (Fig 4C and 4D).

Because 6-keto-PGF\(_{1\alpha}\) 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) is 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 \([^{14}C]\)TXB\(_2\) compared with the control rabbits (data not shown). In pulmonary arteries obtained from rabbits treated with the 10 mg/kg dose of aspirin, \([^{14}C]\)6-keto-PGF\(_{1\alpha}\) production was similar when compared with the control rabbits (Fig 5), however, the platelet \([^{14}C]\)TXB\(_2\) production was decreased in the treated rabbits compared with the control rabbits.

**Table 2. 6-Keto-PGF\(_{1\alpha}\) and TXB\(_2\) Production in Control and Aspirin-Treated Rabbit Pulmonary Artery**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>6-Keto PGF(_{1\alpha}) (pg/mg)</th>
<th>TXB(_2) (pg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2332±426</td>
<td>330±53</td>
</tr>
<tr>
<td>Aspirin-treated</td>
<td>1034±231</td>
<td>139±14</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-PGF\(_{1\alpha}\) and TXB\(_2\) 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,
HPLC. RIA, and gas chromatography/mass spectrometry analysis, our previous work conclusively showed that TXA₂ is the mediator of these contractions. However, the question remained as to which cell type(s) in the intact pulmonary artery produce TXA₂. Although there are numerous reports of TXA₂ production by blood vessels, controversy exists as to whether endothelial cells synthesize TXA₂. Some researchers have reported that cultured endothelial cells produce not only prostacyclin but also TXA₂. In contrast, other studies did not detect TXA₂ synthesis by endothelial cells. In our previous study, immunofluorescence of rabbit pulmonary endothelial cells failed to detect the presence of TX synthase, whereas the presence of cyclooxygenase was clearly shown. The present study further supported the absence of TX synthase in cultured rabbit pulmonary artery endothelial cells. Using a specific polyclonal anti-TX synthase antibody, Western blot analysis showed the presence of immunoreactive bands in lysates prepared from rabbit platelets and intact rabbit pulmonary artery. These bands corresponded to the 60-kD TX synthase protein purified from human platelets and co-migrated with a human platelet microsome enriched in TX synthase. However, in lysates prepared from the rabbit pulmonary artery endothelial cells, no immunoreactive band was observed. Our previous experiments also showed that endothelial cells incubated with [³²P]arachidonic acid did not produce [³²P]TXB₂. When a more sensitive RIA method was used to quantitate TXB₂ production under both basal and stimulated conditions, no detectable TXB₂ was measured. Taken collectively, these studies would be consistent with some cell type other than endothelial cells releasing TXA₂. Because TXA₂ is the major arachidonic acid metabolite in platelets, we tested the hypothesis that TXA₂ synthesis by intact pulmonary arteries requires an interaction between endothelial cells and platelets.

We showed that methacholine stimulated TXB₂ synthesis in intact pulmonary artery. However, incubation of platelets with methacholine does not stimulate TXB₂ production, and cholinergic receptors have not been reported to exist on platelets. Therefore, a question remains as to how methacholine enhances the release of TXA₂ from the platelet in the intact pulmonary artery. PGH₂ from stimulated platelets may be taken up by endothelial cells and used to produce PGI₂, however, this transfer of PGH₂ does not operate in the reverse direction, i.e., platelets do not take up PGH₂ from endothelial cells for TXA₂ synthesis. It is possible that endothelial cells will, however, transfer arachidonic acid to platelets and promote TX synthase synthesis. We investigated this interaction in cultured endothelial cells and platelets during methacholine stimulation. Methacholine stimulated TXB₂ synthesis in platelets and endothelial cells but not in platelets alone or in endothelial cells alone, supporting our hypothesis that an interaction between these two cell types is required for methacholine-induced TXA₂ synthesis. Additional experiments used aspirin as a tool to confirm that arachidonic acid and not the cyclooxygenase metabolite PGH₂ was transferred to the platelet and further metabolized to TXA₂. Aspirin irreversibly inactivates cyclooxygenase by acetylation the enzyme. Pretreatment of platelets with aspirin blocks methacholine-induced TXB₂ synthesis when platelets and endothelial cells are cocultured, indicating that platelet cyclooxygenase is required. These data suggest that endothelial cells release arachidonic acid on cholinergic stimulation and that the platelets convert this arachidonic acid to TXB₂.

To further support this conclusion, aspirin-treated endothelial cells and platelets produced TXB₂ in response to methacholine, indicating that endothelial cell cyclooxygenase is not required and suggesting that endothelial cell-derived arachidonic acid is the source of TXB₂. 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. This 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 [⁴¹C]TXB₂ synthesis was inhibited. Pulmonary artery production of [⁴¹C]TXB₂, however, 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-PGF₁α 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 the 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 TXA₂ 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 TXA₂ production. It is important to note that a recent report by Barry et
al31 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 offers 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 synthesis in the rabbit pulmonary artery. Because this potential mechanism was not examined in the present study, the role of PMNs and monocytes cannot be eliminated.

Therefore, we propose that methacholine-induced contractions of rabbit pulmonary artery are mediated by the release of arachidonic acid from endothelial cells and its transfer to adherent platelets that subsequently synthesize the contracting factor TXA2. TXA2 is released by the platelet and has a direct effect on vascular smooth muscle TXA2 receptors. 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 a variety of pathophysiological conditions,48 the identity of the platelet and not the endothelium, as a cellular source of TXA2, may provide further insight into the role of platelets in the regulation of pulmonary vascular tone.

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References


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