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

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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(part 2):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.

**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, CaCl2 3.3, NaHCO3 24. KH2PO4 1.4, MgSO4 1.2, glucose 11, pH 7.4. The main pulmonary artery was identified at its orifice 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 is 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.28 The supernatant contains platelet-rich plasma. Platelets were sedimented by centrifugation of platelet-rich plasma (2000g for 10 minutes, washed, and resuspended in buffer of the following composition (mM): 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 a modification of methods described previously.1 Endothelial cells were grown in 24-well plates. Washed platelets (5 × 10⁶ 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.12 13 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. Finaly, 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 continued 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 radioactive remains in the labeling buffer and the remainder is incorporated into endothelial cell lipids.24 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 × 10⁶ platelets/
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
[3H]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); 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.[25,26] 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 alone did not produce TXB2 under either basal (Table 1) or methacholine-stimulated conditions.1 Although 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 production of 6-keto-PGF1α was still attenuated (Table 1). 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-
to-PGF$_{1\alpha}$ and PGE$_2$, however, pretreatment of the endothelial cells with aspirin did not affect the ability of methacholine to induce TXB$_2$ release in the presence of normal platelets (Fig 2D). The production of $[^3$H]6-keto-PGF$_{1\alpha}$ by endothelial cells was not affected by the addition of platelets (Fig 2B). When the endothelial cells were pretreated with aspirin and incubated with normal platelets, no production of $[^3$H]6-keto-PGF$_{1\alpha}$ was observed (Fig 2D).

The next series of experiments was designed to use aspirin-treated rabbits to further study the role of platelets in the vasoconstictor 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 66.1±5.4% versus 15.2±3.0%, 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 $[^3$C]arachidonic acid and extracted metabolites analyzed by RP-HPLC. Both control (Fig 4A) and high-dose aspirin-treated (Fig 4D) pulmonary arteries synthesized $[^3$C]6-keto-PGF$_{1\alpha}$, however, its synthesis was less in the vessels from the aspirin-treated rabbits. When measured by RIA, it also seemed that 6-keto-PGF$_{1\alpha}$ production was reduced in the aspirin-treated rabbit pulmonary arteries (Table 2). High-dose aspirin treatment inhibited the production of $[^3$H]TXB$_2$ (Fig 4B). By HPLC analysis, TXB$_2$ production was 45.3±18 cps/mg for the control pulmonary arteries and 23.0±8 cps/mg tissue for the aspirin-treated pulmonary arteries. When measured by RIA, there was an approximate 60% reduction in TXB$_2$ production in the pulmonary arteries obtained from aspirin-treated rabbits compared to controls.

![Figure 2](http://hyper.ahajournals.org/)

**Figure 2.** Effect of methacholine ($10^{-4}$ mol/L) on $[^3$H]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 ($100 \mu$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 $[^3$H]arachidonic acid were separated by RP-HPLC. Migration time of known standard eicosanoids are shown above the chromatograms.

![Figure 3](http://hyper.ahajournals.org/)

**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, $[^{14}C]$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></td>
<td>Ral</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-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,
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 [3H]TXB2 synthesis was inhibited. Pulmonary artery production of [3H]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 TXA2 production is decreased in the aspirin-treated rabbits that exhibited decreased contractile response to methacholine, these results would implicates platelets as the cellular source of TXA2 production. It is important to note that a recent report by Barry et
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alK 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 TXA2-3,11 Therefore, PMNs or monocytes may also be possible sources of TX synthase 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 adherent platelets that subsequently synthesize the contracting arachidonic acid from endothelial cells and its transfer to the platelets to be used for TXA2 synthesis, although this potential mechanism was not explored in the present study, it gives further insight into the role of platelets in the regulation of pulmonary vascular tone.

Acknowledgments

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