Endothelium-Dependent Contractions in Rabbit Pulmonary Artery Are Mediated by Thromboxane A₂

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This study was designed to characterize the endothelium-dependent contracting factor (EDCF) released by arachidonic acid (AA) and methacholine (MeCH) in the rabbit pulmonary artery. AA and MeCH contract the rabbit pulmonary artery; however, the effects of both are blocked by denuding the vessels and by administration of indomethacin (a cyclooxygenase inhibitor), dazoxiben (a thromboxane [TX] synthase inhibitor), and SQ29548 (a TXA₂/prostaglandin [PG] H₂ receptor antagonist). When segments of rabbit pulmonary artery were incubated with [¹⁴C]AA and the [¹⁴C] metabolites were resolved by reverse-phase high-performance liquid chromatography (HPLC), radioactive products were observed that comigrated with 6-keto-PGF₁α and TXB₂, the stable metabolites of prostacyclin and TXA₂. The TXB₂ radioactive peak was rechromatographed on normal-phase HPLC and again migrated with TXB₂. Finally, the structures of derivatized [¹⁴C]6-keto-PGF₁α and [¹⁴C]TXB₂ peaks were confirmed by gas chromatography/mass spectrometry. The synthesis of [¹⁴C]6-keto-PGF₁α and [¹⁴C]TXB₂ was inhibited by removal of the endothelium and by indomethacin. Dazoxiben inhibited the synthesis of [¹⁴C]TXB₂ but not [¹⁴C]6-keto-PGF₁α. Using specific radioimmuneassays, AA and MeCH stimulated 6-keto-PGF₁α and TXB₂ release. Indomethacin blocked the production of both 6-keto-PGF₁α and TXB₂, whereas dazoxiben only blocked TXB₂. In a superfusion/bioassay system, AA stimulated an endothelium-intact donor vessel to release a labile substance that contracted an indomethacin-treated endothelium-denuded recipient vessel. The EDCF released by AA had an approximate half-life of 30 seconds. Cultured rabbit pulmonary arterial endothelial cells synthesized 6-keto-PGF₁α, but not TXB₂. Immunohistochemical studies indicated the presence of cyclooxygenase, but not TX synthase, in pulmonary artery endothelial cells. TXA₂ appears to be the EDCF released by AA and MeCH in rabbit pulmonary artery; however, TXA₂ is not produced by endothelial cells but may arise from cells that adhere to the luminal surfaces, such as platelets or macrophages. (Circulation Research 1993;72:1023–1034)

Key Words • methacholine • arachidonic acid • prostacyclin • endothelial cells • cyclooxygenase • thromboxane synthase

Over the past several years, the importance of the vascular endothelium in the regulation of vascular tone has been clearly demonstrated. The discovery of endothelial vasodilators, such as prostacyclin and endothelin-derived relaxing factor, as well as endothelial vasoconstrictors, such as endothelin, has greatly enhanced our understanding of the role of the endothelium in modulating vascular tone. Both arachidonic acid (AA) and methacholine (MeCH) act in an endothelium-dependent manner to affect responses in vascular smooth muscle. Early studies showed that AA caused endothelium-dependent contractions in canine femoral and pulmonary veins, ¹ canine basilar arteries,² and rabbit aortas.³ These contractions were blocked by the cyclooxygenase inhibitor indomethacin, but there were conflicting reports on their ability to be blocked by inhibitors of thromboxane (TX) synthase or lipooxygenase.²,⁴

Acetylcholine also caused endothelium-dependent contractions in the rabbit pulmonary artery,⁴ the canine basilar artery,² and the aorta from spontaneously hypertensive rats.⁵ Acetylcholine-induced contractions of spontaneously hypertensive rat aortas were blocked by indomethacin and the TXA₂/prostaglandin (PG) H₂ receptor antagonist SQ29548, whereas the TX synthase inhibitor dazoxiben failed to block contraction. As a result, it was suggested that PGH₂ might be the endothelium-dependent contracting factor (EDCF) in aortic tissue of the spontaneously hypertensive rat. Other studies have shown that dazoxiben inhibits the endothelium-dependent contractions to MeCH in rabbit pulmonary artery,⁶ suggesting that the EDCF may be TXA₂. The possibility exists that different EDCFs may mediate the response in the spontaneously hypertensive rat aorta and rabbit pulmonary artery. Taken collectively, these studies suggest that both AA and MeCH release an EDCF that is a cyclooxygenase metabolite and acts via the TXA₂/PGH₂ receptor. Although there are pharmacological insights into the nature of EDCF, there is

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conflicting chemical information on its structure. As a result, the identity of EDCF is still controversial.

The purpose of the present study was to more completely characterize the EDCF released by AA and MeCH in the rabbit pulmonary artery. Using a variety of chemical and pharmacological means, we determined the properties of the EDCF released by MeCH and AA.

**Materials and Methods**

**Vascular Reactivity**

Three-month-old male New Zealand White rabbits were anesthetized with intravenous sodium pentobarbital (120 mg/kg). The heart and lungs were removed as a unit and placed immediately in Krebs’ bicarbonate buffer of the following composition (mM): NaCl 118, KCl 4, CaCl2 3.3, NaHCO3 24, KH2PO4 1.4, MgSO4 1.2, and 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 is referred to as the intrapulmonary artery. After dissection, the tissue was cleaned of adherent lung parenchyma and connective tissue; care was taken not to disturb the endothelial layer. Rings of intrapulmonary artery (2–3 mm) were obtained and suspended in 15-ml organ baths containing Krebs’ bicarbonate buffer, which was warmed to 37°C and continuously aerated with a 95% O2–5% CO2 mixture. Isometric tension was measured with force-displacement transducers (Grass Instrument Co., Quincy, Mass.) and recorded with a polygraph (model 7D, Grass Instrument) interfaced with an analog-to-digital converter (Coulbourne Instruments, Inc., Lehigh Valley, Pa.) and CODAS software to an IBM computer. In some experiments, the endothelium was removed by gently rubbing the intimal surface with the tip of small forceps. Resting tension was adjusted to its length tension maximum of 1 g. This value was determined by increasing the length of the rings in a stepwise fashion and measuring the active tension generated by exposing the rings to 20 mM KCl. The vessels were allowed to equilibrate for 1 hour. Contractions were produced by increasing the KCl concentration of the bath to 40 mM. KCl-induced contractions were repeated until maximal reproducible responses were obtained. This response averaged 1.33 ± 0.07 g tension. Concentration–response curves were obtained by the cumulative addition of either AA (10−6–10−5 M) or MeCH (10−5–10−3 M). Some vessels were pretreated with inhibitors for 10 minutes before the administration of AA or MeCH. These inhibitors included the following: indomethacin (10−5 M), a cyclooxygenase inhibitor; dazoxiben (10−3 M), a TX synthase inhibitor; and SQ29548 (10−7 M), the TXA2/PGJ2 receptor antagonist. Because the KCl contractile response remains stable throughout the experiment, results were expressed as a percentage of the KCl contraction. MeCH was dissolved in distilled water, and a volume of 0.05 ml was added to the tissue baths. AA, indomethacin, dazoxiben, and SQ29548 were dissolved in ethanol and given in a volume that gave a final ethanol concentration of the bath of less than 0.07%.

**Bioassay of EDCF**

A segment (2 cm) of rabbit pulmonary artery complete with its branch vessels was obtained, cleaned of adherent fat and connective tissue, and cannulated at the proximal end with polyethylene tubing. The segment was perfused in vitro in the direction of blood flow in vivo at a constant rate of 2.2 ml/min by a roller pump (Harvard Apparatus, South Natick, Mass.). Three detector rings in which the endothelium had been carefully removed were suspended in three sequential organ chambers with enough tubing spaced between each chamber to give a 15–20-second delay between each detector ring.6 Resting tension of the detector rings was adjusted to 1 g. Isometric tension was measured in the detector rings with Grass force-displacement transducers and recorded with a Grass polygraph. The detector rings were positioned to receive effluent from the perfused pulmonary artery donor segment. The drugs were added either to the perfusate directly through the donor segment or distal to the donor segment directly onto the detector rings. Indomethacin (10−4 M) was added distal to the donor segment on the detector rings to block smooth muscle metabolism of AA. As a result, AA failed to alter the tone of the detector rings when added directly to those tissues. After reproducible contractions to KCl were obtained, AA (10−5 M) was added to the perfusate of the donor segment, and changes in tension of the detector rings were measured over various transit times. Comparisons were made between the TX mimetic U46619, synthetic PGH2, and the vasoconstrictor factor released by AA. Results were expressed as the percent contraction of the KCl response to correct for differences in the sensitivity of the vessels.

**Metabolism of [14C]AA**

Strips of intrapulmonary artery (30 mg wet weight) were placed in buffer of the following composition (mM): HEPES 10, NaCl 150, KCl 5, CaCl2 2, MgCl2 1, and glucose 6, pH 7.4, and incubated at 37°C for 15 minutes with [14C]AA (0.05 μCi, 10−7 M) and the calcium ionophore A23187 (20 μM). Separate experiments evaluated the effect of removal of the endothelium and of the various inhibitors of AA metabolism, including indomethacin, dazoxiben, and SQ29548. In these experiments, the inhibitors were added 10 minutes before the addition of [14C]AA and A23187. After incubation, the HEPES buffer was removed, acidified to pH 2.0 with glacial acetic acid, and extracted over BondElut octadecylsila (ODS) extraction columns as previously described.7 The ODS columns were washed sequentially with 5 ml water and ethanol. The acidified sample (made 15% [vol/vol] with ethanol) was then added to the column and washed sequentially with 5 ml each of 15% ethanol, water, and petroleum ether. The AA metabolites were eluted with 6 ml ethyl acetate, evaporated to dryness under a stream of nitrogen, and stored at −40°C until analyzed by reverse-phase high-performance liquid chromatography (HPLC) (Beckman Instruments, Fullerton, Calif.). The PG metabolites of AA were separated using a reverse-phase HPLC system with a Nucleosil-C18 column (5 μm, 4.6×250 mm, Phenomenex, Inc., Torrance, Calif.). Solvent A was water containing 0.025 M phosphoric acid, and solvent B was acetonitrile. The
program consisted of a 40-minute isocratic phase with 31% solvent B in solvent A, followed by a 20-minute linear gradient to 100% solvent B and a 10-minute isocratic phase with 100% solvent 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, or the radioactivity in the eluate was detected with a Ramona-D radioactivity detector (Raytest U.S.A., Inc., Pittsburgh, Pa.). Elution times of radioactive peaks were compared with retention times of known PG standards. Any metabolites that comigrated with known standards were then collected and extracted with cyclohexane/ethyl acetate (50:50). The compounds were then rechromatographed using a normal-phase HPLC system with a silica gel column (Ultrasphere-Si, 5 μm, 4.6×250 mm, Beckman Instruments). The program consisted of solvent C (hexane/toluene/acetic acid, 50:50:0.5) and solvent D (toluene/ethyl acetate/acetonitrile/methanol/acetic acid, 30:40:30:2:0.5) with a gradient from 25% solvent D in solvent C to 80% solvent D in solvent C over 40 minutes. The flow rate was 2 ml/min. Fractions (0.5 ml) were collected, radioactivity was determined by liquid scintillation spectrometry, and radioactive peaks were compared with known standard elution times.

Gas Chromatography/Mass Spectrometry

For analysis by gas chromatography/mass spectrometry (GC/MS), pulmonary arteries were obtained from eight rabbits and incubated for 15 minutes at 37°C in HEPES buffer containing [14C]AA (0.05 μCi, 5×10⁶ M) and A23187 (20 μM). The extracted metabolites were chromatographed on reverse-phase HPLC as described above. The fractions corresponding to 6-keto-PGF1α and TXB2 were pooled, extracted with cyclohexane/ethyl acetate (50:50), and derivatized for GC/MS. First, the samples were converted to the methoxamine derivative by treating the compounds with 2% methoxamine in pyridine for 12 hours at room temperature. After extraction into methylene chloride, the samples were dissolved in 100 μl acetonitrile, 5 μl disopropylethylamine, 5 μl N,N-dimethylformamide, and 3 μl pentfluorobenzyl bromide and allowed to react for 40 minutes at 40°C. The compounds were hexane and converted to the trimethylsilyl ethers by incubating for 60 minutes at 60°C with bis(trimethylsilyl)trimfluoroacetamide. GC/MS analysis was performed with a Finnigan SSQ-700 quadrupole mass spectrometer. The samples were analyzed by negative ion chemical ionization mass spectroscopy using a 14-m capillary DB-5 column with a linear gradient of 100–290°C over 10 minutes. The reagent gas was methane.

Radioimmunoassay of TXB2 and 6-Keto-PGF1α

Strips of rabbit intrapulmonary artery (3 mg wet weight) were incubated in HEPES buffer containing increasing concentrations of AA or MeCH for 15 minutes. In some experiments, inhibitors of AA metabolism were added to the buffer for 10 minutes before the addition of AA or MeCH. The synthesis of TXB2 and 6-keto-PGF1α was measured by specific radioimmunoassay (RIA) using the method of Campbell and Ojeda. The antibodies for TXB2 and 6-keto-PGF1α were produced in rabbits in our laboratory. The sensitivity of the assay is 1 pg/0.3 ml for TXB2 and 5 pg/0.3 ml for 6-keto-PGF1α. The cross-reactivity of the antisera with known AA metabolites is less than 0.1%.

Cell Culture/Immunohistochemistry

Endothelial cells were isolated and cultured from rabbit pulmonary arteries by a modification of methods previously described. The arteries were dissected to their most distal ends within the lung, and all branch vessels were ligated at their origin from the main left or right pulmonary artery. The arteries were then removed and placed in RPMI 1640 media containing l-glutamine and HEPES (25 mM) and antibiotics (penicillin G, streptomycin, amphotericin, nystatin, and gentamicin) for approximately 15 minutes. The rinsed arteries were then cleaned of connective tissue and placed on damp sterile gauze in Petri dishes in a laminar flow hood. Collagenase (0.1%) in RPMI media was introduced into the vessel lumen with a polyethylene-tipped syringe, and both ends of the vessel were ligated with nylon sutures. The arteries were incubated at 37°C in an atmosphere of 5% CO2 in air for a minimum of 30 minutes, the lumen was then flushed twice with RPMI culture medium containing antibiotics, and the detached cells were collected into centrifuge tubes. The cells were sedimented by centrifugation at 200g for 10 minutes, washed once with RPMI medium, and resuspended in RPMI medium containing 20% fetal calf serum. The endothelial cells were plated on 25-cm² culture flask coated with 1% gelatin. The cultures were left undisturbed for 48 hours at 37°C in an atmosphere of 5% CO2 in air. The media were then changed every other day. Endothelial cells started growing from small clumps of cells and spread to confluence within 5–10 days. Monolayer cultures exhibited a typical cobblestone morphology of endothelial cells. Once confluence was obtained, the cells were detached with Puck-EDTA solution and trypsin and transferred to multiwell containers and culture flasks in order to proceed with additional experiments. The presence of endothelial cell markers was used to confirm purity of the cell cultures. Incorporation of 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate acetylated low density lipoprotein (dil-Ac-LDL) by the endothelial cells was determined by immunofluorescence.

Rabbit pulmonary artery smooth muscle cells were cultured by treating vessels as described for pulmonary artery endothelial cells. After collection of endothelial cells, strips of denuded vessels were placed into gelatin-coated flasks with medium 199 containing 10% fetal calf serum with l-glutamine (1%), tyrosine (0.1%), and antibiotics (0.15% nystatin and 0.15% gentamycine). Smooth muscle cells migrated to the flasks within 3–5 days. Once growth was established on the coated flasks, the vessels were removed, and the cells were fed as described for endothelial cells. Purity of smooth muscle cells was confirmed by positive staining for smooth muscle cell α-actin.

Immunohistochemical studies were performed on endothelial and smooth muscle cells in multiwell containers. The cells were washed with phosphate-buffered saline (PBS) consisting of 20 mM NaCl, 2.7 mM KCl, 10 mM phosphate buffer, and 0.2% bovine serum albumin (BSA), pH 7.4, and then fixed with ice-cold ethanol/
methanol (1:1) solution for 5 minutes. After fixation, the vessels were again washed with PBS containing 0.2% BSA, and the wells were treated with rabbit anti–TX synthase antibody (1:100, TX synthase polyclonal antibody, Cayman Chemical Co. Inc., Ann Arbor, Mich.), rabbit anticyclooxygenase antibody (1:150, cyclooxygenase polyclonal antibody, Cayman Chemical), or preimmune rabbit serum diluted in PBS for 30 minutes at 37°C. After the cells were washed with PBS containing 0.2% BSA for 20 minutes, they were treated with normal goat serum (1:20, Jackson Immunological) in PBS for 20 minutes and then incubated with Texas Red goat anti-rabbit antibody (1:100, Jackson Immunological) for 30 minutes at 37°C. The addition of normal goat serum helped decrease nonspecific binding of the secondary fluorescent antibody. After this step, the cells were washed with PBS containing 0.2% BSA for 30–45 minutes and viewed by phase-contrast and fluorescent microscopy (×20) with a Nikon Diaphot microscope. The epifluorescence of the cells was viewed with a rhodamine filter.

As a positive control for TX synthase, platelet rich plasma was obtained from rabbits by collecting blood in 3.2% citrate (9:1, pH 7.4). The blood was centrifuged at 150g for 10 minutes, and aliquots of platelet-rich plasma (1 ml) were added to multiwells containing glass cover-slips previously coated with albumin (2% BSA in Krebs’ bicarbonate buffer for 18 hours at 4°C). After a 2-hour incubation period, platelet-rich plasma was removed, and the multiwell containers with adherent platelets were washed with PBS/0.2% BSA and then fixed with ice-cold ethanol/methanol (1:1) for 5 minutes. Immunohistochemistry was performed using a similar procedure as described for the endothelial and smooth muscle cells.

In additional series of experiments, pulmonary artery endothelial cells or smooth muscle cells grown in monolayers were incubated with [14C]AA as described above for pulmonary artery tissue. The extracted media was resolved into its [14C] metabolites by reverse-phase HPLC as discussed above. Pulmonary artery endothelial cells also were grown in 24-cm2 multiwell flasks, incubated in HEPES buffer at 37°C, and stimulated with either vehicle, AA (1 μM), or MeCH (10 μM) for 30 minutes. The buffer was removed, and the production of 6-keto-PGF1α and TXB2 was measured by RIA.

**Statistics**

Data are expressed as mean±SEM. Statistical analysis of the data was performed with an analysis of

![Figure 1](http://circres.ahajournals.org/lookup/doi/10.1161/01.RES.72.5.1026)
variance to determine differences within the groups, and a Student's t test was used to determine differences between groups.

Materials

[^14]C]AA was obtained from New England Nuclear, Boston; [^3]H]6-keto-PGF_1α and [^3]H]TXB_2 were obtained from Amersham Corp., Arlington Heights, Ill.; AA was obtained from Nu-Check; β-MeCH, A23187, and indomethacin were obtained from Sigma Chemical Co., St. Louis, Mo.; SQ29548 was provided by S.J. Lucania of Bristol-Myers-Squibb; dazoxiben was obtained from Pfizer Laboratories, New York; U46619, PGH_2, 6-keto-PGF_1α, and TXB_2 were obtained from Cayman.

Results

Vascular Response to AA, MeCH, and U46619

AA caused endothelium-dependent concentration-related contractions of rabbit pulmonary artery (Figure 1B). The maximum contraction was 97.1±4.6% (p<0.05) of the response to KCl (40 mM) and occurred at 10^-3 M. The ED_50 for AA-induced contractions was 4.0x10^-7 M. These contractions were significantly inhibited by removal of the endothelium as well as pretreatment with indomethacin, dazoxiben, or SQ29548 (p<0.05, Figure 1B). Little or no contractions to AA were observed with the TXA_2/PGH_2 receptor antagonist SQ29548 (maximal response, 3.1±1.1%; p<0.05; Figure 1B). At 10^-3 M AA, the contractions were reduced 89.9% by removal of the endothelium, 85.6% by indomethacin, 79.5% by dazoxiben, and 96.8% by SQ29548. No significant difference was seen between the various treatment groups.

Similar contractions were seen when cumulative concentrations of MeCH were added to rabbit intrapulmonary rings. The maximal response was 95.5±4.6% of the KCl contraction and occurred with 3x10^-3 M (p<0.01, Figure 1A). The ED_50 for MeCH was 3.0x10^-5 M. As with AA, the contractions to MeCH were inhibited by removal of the endothelium and pretreatment with indomethacin, dazoxiben, and SQ29548 (p<0.01). At 10^-4 M MeCH, removal of the endothelium and pretreatment with indomethacin, dazoxiben, and SQ29548 reduced MeCH-induced contractions by 67.6%, 95.2%, 73.9%, and 86.8%, respectively. Because removal of the endothelium did not completely inhibit MeCH-induced contractions, the effect of MeCH in denuded vessels pretreated with the muscarinic antagonist atropine (10^-3 M) was investigated. Atropine completely attenuated the contractile response to MeCH (data not
A23187 resulted when added to the perfusate of an endothelium-intact donor vessel. In contrast, it had no effect when added directly to the detector vessel superfusate (Figure 2, top panel). PGH₂, U46619, and KCl, however, contracted the detector vessels when added directly. When perfused through the donor vessel, indomethacin inhibited the AA-induced contractions (data not shown). By comparing the constrictor responses in sequential detector vessels that were separated by a known time delay, the approximate half-life (t½) could be determined (Figure 2, bottom panel). The soluble, transferable contracting factor that was released from the perfused donor vessel by AA had a t½ of approximately 30 seconds. In contrast, the t½ of synthetic PGH₂, when added to the detector vessel superfusate, was greater than 60 seconds. Comparatively, at 60 seconds, PGH₂ had an overall decrease in activity of 56%, whereas the EDCF released from AA had a decrease in activity of 100%.

Metabolism of AA by Pulmonary Arteries

Incubation of rabbit pulmonary artery with [14C]AA and A23187 resulted in the formation of a number of PG metabolites. The PG metabolites of AA were separated by reverse-phase high-performance liquid chromatography as explained in “Materials and Methods.” Migration times of known standard eicosanoids are shown above the chromatograms.

**Figure 3.** Chromatograms showing the metabolism of [14C]arachidonic acid ([14C]AA) by rabbit pulmonary arteries. PG, prostaglandin; TX, thromboxane. Pulmonary artery with endothelium (control) and without endothelium (denuded) was incubated with HEPES buffer at 37°C for 15 minutes with [14C]AA and A23187. In some experiments, tissue was also preincubated with various inhibitors including indomethacin (10⁻⁵ M) and dazoxiben (10⁻⁶ M). The PG metabolites of AA were separated by reverse-phase high-performance liquid chromatography as explained in “Materials and Methods.” Migration times of known standard eicosanoids are shown above the chromatograms.

**Figure 4.** Chromatograms showing the metabolism of [14C]arachidonic acid ([14C]AA) by rabbit pulmonary arteries. PG, prostaglandin; TX, thromboxane. Strips of rabbit pulmonary artery were incubated with [14C]AA and A23187 for 15 minutes, and the PG metabolites were separated using reverse-phase high-performance liquid chromatography (panel A). The metabolite that corresponded to TXB₂ was then collected, extracted using cyclohexane/ethyl acetate, and rechromatographed using normal-phase high-performance liquid chromatography as explained in “Materials and Methods” (panel B).
cyclooxygenase metabolites, as identified by reverse-phase HPLC (Figure 3A). The major radioactive metabolite comigrated with 6-keto-PGF\(_{1\alpha}\) (the stable metabolite of prostacyclin). Other radioactive metabolites comigrated with TXB\(_2\) (the stable metabolite of TXA\(_2\)) and PGE\(_2\). Pretreatment of rabbit pulmonary artery with indomethacin eliminated the synthesis of these cyclooxygenase products (Figure 3B). Dazoxiben pretreatment eliminated the synthesis of [\(^{14}\)C]TXB\(_2\) without altering [\(^{14}\)C]6-keto-PGF\(_{1\alpha}\) synthesis (Figure 3C). When the endothelium was mechanically removed from the vessels, the metabolism of [\(^{14}\)C]AA was reduced, and only a small radioactive peak that comigrated with 6-keto-PGF\(_{1\alpha}\) was observed (Figure 3D). Pretreatment with SQ29528 had no effect on the metabolism of [\(^{14}\)C]AA (data not shown).

In a separate experiment, the radioactive peak that comigrated with TXB\(_2\) was collected, extracted with cyclohexane/ethyl acetate (50:50), and rechromatographed using a normal-phase HPLC system (Figure 4). The radioactive product eluting at 24 minutes in this system comigrated with the authentic TXB\(_2\) standard. Additionally, a radioactive peak was observed at 5 minutes. This peak was not seen if authentic [\(^{3}\)H]TXB\(_2\) standard was chromatographed using the normal-phase HPLC system. However, if authentic [\(^{3}\)H]TXB\(_2\) standard was first chromatographed on reverse-phase HPLC, collected, extracted with cyclohexane/ethyl acetate, and

![Graph](http://circres.ahajournals.org/)

**Figure 5.** Negative ion chemical ionization mass spectra of the metabolite comigrating with thromboxane B\(_2\) from rabbit pulmonary artery. M/Z, mass/charge ratio. The metabolite was converted to its pentafluorobenzyl bromide ester-methoxyamine-trimethylsilyl ether before analysis by gas chromatography/mass spectrometry.

![Graph](http://circres.ahajournals.org/)

**Figure 6.** Bar graphs showing release of 6-keto-prostaglandin F\(_{1\alpha}\) (solid bar) and thromboxane B\(_2\) (hatched bar) from rabbit pulmonary arteries by methacholine and arachidonic acid. Segments of rabbit pulmonary artery were incubated with increasing concentrations of methacholine (panel A) and arachidonic acid (panel B) for 15 minutes at 37°C. Production of 6-ketoprostaglandin F\(_{1\alpha}\) and thromboxane B\(_2\) was measured in the incubation media by radioimmunoassay. Data points are mean±SEM for 21 segments (*p<0.05).
rechromatographed on normal-phase HPLC, then the early radioactive peak was observed (data not shown).

The structures of 6-keto-PGF\(_{1\alpha}\) (data not shown) and TXB\(_2\) (Figure 5) were confirmed by GC/MS analysis of the corresponding pentfluorobenzyl bromide ester-methoxamine-trimethylsilyl ethers. The derivatized biological samples comigrated on capillary GC with authentic derivatized 6-keto-PGF\(_{1\alpha}\) and TXB\(_2\). Negative ion chemical ionization MS produced a major characteristic ion of mass/charge ratio of 614 (M-1, loss of pentafluorobenzyl bromide) for both 6-keto-PGF\(_{1\alpha}\) and TXB\(_2\), as well as for their standards.

**Radioimmunoassays**

To quantitate the production of prostacyclin and TXA\(_2\) by the rabbit pulmonary artery, strips of vessel were incubated with increasing concentrations of AA and MeCH, and the amounts of 6-keto-PGF\(_{1\alpha}\) and TXB\(_2\) released into the buffer were measured by RIA. Both AA and MeCH caused concentration-related increases in 6-keto-PGF\(_{1\alpha}\) and TXB\(_2\) production (Figure 6). The basal release of 6-keto-PGF\(_{1\alpha}\) exceeded TXB\(_2\). This relation was maintained after stimulation by AA and MeCH. At the maximal concentration used (10^{-5} M), AA elicited an approximate sixfold increase in 6-keto-PGF\(_{1\alpha}\) production and a 14-fold increase in TXB\(_2\) production as compared with control values (Figure 6, p<0.05). Similarly, MeCH (10^{-3} M) increased 6-keto-PGF\(_{1\alpha}\) and TXB\(_2\) by twofold and eightfold, respectively (Figure 6, p<0.05).

Indomethacin pretreatment significantly decreased the production of both 6-keto-PGF\(_{1\alpha}\) (percent inhibition, 80% versus 86% for AA versus MeCH; p<0.05) (Figure 7) and TXB\(_2\) (percent inhibition, 92% versus 99% for AA versus MeCH; p<0.05) in AA- and MeCH-stimulated vessels. In contrast, in vessels incubated with dazoxiben, only the production of TXB\(_2\) was inhibited (percent inhibition, 96% versus 97% for AA versus MeCH; p<0.05).

**Rabbit Pulmonary Arterial Endothelial Cells**

Cells isolated from the lumen of rabbit pulmonary arteries were maintained in culture and demonstrated a similar morphological appearance to endothelial cells cultured from other blood vessels. When viewed under phase-contrast microscopy, they had a polygonal epithelioidal shape and formed a monolayer with a typical cobblestone appearance. Furthermore, they accumulated diI-Ac-LDL, which is a marker for endothelial cells, and appeared as dense fluorescent cytoplasmic granules. This accumulation was blocked by the Ac-LDL uptake inhibitor dextran sulfate. Smooth muscle cells were characterized by their slower growth rate relative to endothelial cells. By phase-contrast microscopy, smooth muscle cells were several times larger than endothelial cells. Their filamentous cytoplasm was con-

**Figure 7.** Bar graphs showing the effect of inhibitors on the release of 6-keto-prostaglandin F\(_{1\alpha}\) (solid bar) and thromboxane B\(_2\) (hatched bar) from rabbit pulmonary arteries. Segments of pulmonary artery were incubated with increasing concentrations of methacholine (MeCH) (panel A) and arachidonic acid (AA) (panel B). Some vessels were also pre-treated for 10 minutes with 10^{-5} M indomethacin (Indo) or 10^{-5} M dazoxiben (Daz). Production of 6-keto-prostaglandin F\(_{1\alpha}\) and thromboxane B\(_2\) in the incubation media was measured by radioimmunoassay. Results are mean±SEM for 21 segments (*p<0.05 vs. control, **p<0.05 vs. MeCH or AA for six segments).
firmed by positive staining for smooth muscle cell α-actin.

Pulmonary artery endothelial cells grown in culture were treated with anticyclooxygenase and anti–TX synthase antibodies to evaluate the presence of these enzymes in the cells (Figure 8). The cells showed a strong fluorescent response with the anticyclooxygenase antibody; however, no fluorescence was observed with the anti–TX synthase antibody or the preimmune serum-negative control cells. Thus, this method failed to indicate the presence of TX synthase in pulmonary arterial endothelial cells. In contrast, platelets treated with the anti–TX synthase antibody exhibited a fluorescent response, whereas no fluorescence was seen with preimmune serum-negative control cells (Figure 9). When smooth muscle cells grown in culture were treated with anti–TX synthase or preimmune serum, no fluorescent response was observed (Figure 9).

Isolated rabbit pulmonary artery endothelial cells grown in culture were incubated with [14C]AA, the media were extracted, and the [14C]metabolites were resolved by HPLC (Figure 10). The major radioactive peaks comigrated with 6-keto-PGF$_{1α}$ and PGE$_2$. The radioactive peak that comigrated with PGE$_2$ was larger than that seen in rabbit pulmonary artery tissue incubated in a similar fashion. This finding is consistent with our previous results with human pulmonary endothelial cells. Smaller peaks comigrated with PGF$_{2α}$ and PGD$_2$. No radioactive peak comigrating with TXB$_2$ was detected. In smooth muscle cells incubated with [14C]AA, there were two major radioactive peaks that migrated with 6-keto-PGF$_{1α}$ and PGE$_2$. No radioactive peaks comigrated with TXB$_2$ (data not shown).

Because RIA is a more sensitive measurement of 6-keto-PGF$_{1α}$ and TXB$_2$ production, cultured rabbit pulmonary artery endothelial cells were incubated with either AA (1 μM) or MeCH (10 μM). The basal production of 6-keto-PGF$_{1α}$ was 148.1±24.5 pg per well. Both AA and MeCH caused an approximate twofold
increase in 6-keto-PGF₁α production (315.0±20.1 versus 391.5±49.3 pg per well for AA versus MeCH). In contrast, the values measured for TXB₂ production by either vehicle, AA or MeCH, were at or below the limits of detectability of our assay. Therefore, the RIA results support the HPLC data showing that cultured pulmonary endothelial cells produce 6-keto-PGF₁α but do not produce TXB₂ under either basal or stimulated conditions.

Discussion

The present study confirms the observation that EDCF is produced by AA and MeCH in the rabbit intrapulmonary artery. Prior reports have presented conflicting evidence about the nature of this contracting factor. For example, DeMey and Vanhoutte¹ observed endothelium-dependent contractions evoked by AA in canine femoral and pulmonary veins. The responses were blocked by indomethacin but unaffected by inhibitors of TX synthase or lipoxygenase, suggesting that endothelial cells metabolized AA to a vasoconstrictor cyclooxygenase product that was not TXA₂. In contrast, Katusic et al² reported AA-dependent contractions in canine basilar arteries that were blocked by inhibitors of both cyclooxygenase and TX synthase. Although numerous studies have shown that MeCH elicits endothelium-dependent relaxations,¹⁴ under certain conditions MeCH will also mediate endothelium-dependent contractions.²⁴ Altiere et al⁴ found that the EDCF released by MeCH was blocked by both indomethacin and the TX synthase inhibitor dazoxiben, leading them to conclude that TXA₂ was the contracting factor. Other investigators have found that indomethacin but not dazoxiben inhibited MeCH-induced contractions, suggesting that the effect was mediated by a cyclooxygenase product that was not TXA₂.² Auch-Schwelk et al⁵ examined endothelium-dependent contractions to MeCH in spontaneously hypertensive rats and reported that dazoxiben had no effect.

Figure 9. Immunohistochemistry of isolated platelets and pulmonary artery smooth muscle cells. Comparison between phase (panels A, C, E, and G) and epifluorescence (panels B, D, F, and H) microscopy. Panel A is the phase microscopy of platelets incubated with anti-thromboxane synthase (1:100), and panel B is the fluorescence microscopy. Panels C and D are platelets incubated with preimmune rabbit serum. Smooth muscle cells treated with anticyclooxygenase (1:150) and anti-thromboxane synthase are shown in panels E and F and panels G and H, respectively.
on contractions, whereas the TXA$_2$/PGH$_2$ receptor antagonist SQ29548 inhibited the response. When measured by RIA, MeCH stimulated the release of 6-keto-PGF$_{1\alpha}$ but not PGE$_2$, PGF$_{2\alpha}$, or TXB$_2$ from the rat aorta. The lack of effect of dazoxiben as well as the inability of MeCH to release TXB$_2$ led the authors to conclude that PGH$_2$, not TXA$_2$, was the EDCF.

In our study, the endothelium-dependent contractions elicited by both AA and MeCH were blocked by indomethacin, dazoxiben, or SQ29548. This would appear to indicate that TXA$_2$ was a contracting factor of pulmonary arteries. Further evidence indicated that this factor was a labile transferable factor released by the endothelium with a t$_1/2$ that was shorter than the t$_1/2$ of PGH$_2$ but comparable to the t$_1/2$ of TXA$_2$. To confirm the production of TXA$_2$ by rabbit pulmonary artery, strips of pulmonary artery equivalent in size to the rings were incubated with AA and MeCH, and the production of 6-keto-PGF$_{1\alpha}$ and TXB$_2$ was measured by RIA. Results showed concentration-dependent production of 6-keto-PGF$_{1\alpha}$ and TXB$_2$. TXB$_2$ production was inhibited by dazoxiben and indomethacin in a manner similar to that seen in the vascular reactivity studies. Additional evidence that TXA$_2$ was produced by rabbit pulmonary artery was provided by chromatographic analysis using two different HPLC systems. In both, a metabolite of AA comigrated with a known TXB$_2$ standard. When pulmonary artery was incubated with dazoxiben, the metabolite that comigrated with TXB$_2$ was inhibited. Finally, analysis of this metabolite by GC/MS confirmed that the rabbit pulmonary artery produced TXB$_2$.

Thus, the evidence from these experiments provide strong support that TXA$_2$ is the EDCF released by AA and MeCH in rabbit pulmonary artery. In all the previous reports, the identity of EDCF was based solely on experiments with TX syntheses inhibitors and TXA$_2$/PGH$_2$ receptor antagonists. Using a variety of techniques, including vascular reactivity studies with inhibitors, bioassay, and HPLC, RIA, and GC/MS analysis, we conclusively show that TXA$_2$ is EDCF in rabbit pulmonary artery. More important, it is the only study to provide chemical evidence for the identity of EDCF. It should be noted that there could be an intrinsic difference in the nature of EDCF between the various types of vascular beds. Thus, it is possible that the EDCF released by the aorta of the SHR is PGH$_2$ and that released from the rabbit pulmonary artery is TXA$_2$.

The question remains as to which cell type(s) in the pulmonary artery produces TXA$_2$. Possible candidates include endothelial cells or some type of cell adhering to or associated with the vessel intima. Concerning the latter possibility, platelets or macrophages are likely candidates, since TXA$_2$ is their major AA metabolite. Conflicting evidence exists over the ability of endothelial cells to produce TXA$_2$. In our studies, immunofluorescence of rabbit pulmonary endothelial cells failed to show the presence of TX syntheses, whereas the presence of cyclooxygenase was clearly shown. In addition, endothelial cells grown in culture and incubated with [14C]AA did not produce [14C]TXB$_2$. When a more sensitive RIA method was used to quantitate TXB$_2$, production under both basal and stimulated conditions, no detectable TXB$_2$ was measured. These studies would be consistent with some cell type other than endothelial cells releasing TXA$_2$. Alternatively, it is possible that TX syntheses is lost with culture of the endothelial cells. This possibility appears unlikely since cyclooxygenase, prostacyclin synthase, and PGE$_2$ isomerase are maintained under the same culture conditions.

In summary, we conclude that the EDCF released from rabbit pulmonary artery in response to AA and MeCH is TXA$_2$. Our data suggest that endothelial cells may not be the source of this vasoconstrictor factor, since neither TX syntheses nor TXB$_2$ production is detected in rabbit pulmonary endothelial cells grown in culture. Further experiments examining the presence of adherent or resident cell types in the intima of the pulmonary artery should reveal the cellular source of EDCF.

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References


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