Thromboxane mediates pulmonary hypertension and lung inflammation during hyperacute lung rejection

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Collins, Brendan J., Matthew G. Blum, Richard E. Parker, Andrew C. Chang, Kelly S. A. Blair, George L. Zorn III, Brian W. Christman, and Richard N. Pierson III. Thromboxane mediates pulmonary hypertension and lung inflammation during hyperacute lung rejection. J Appl Physiol 90: 2257–2268, 2001.—The role of thromboxane (Tx) in hyperacute rejection of pig lung by human blood was studied in an ex vivo model, wherein lungs from juvenile piglets were perfused with fresh heparinized human blood. In this model, hyperacute lung rejection was characterized by an abrupt rise in pulmonary vascular resistance (PVR; >1 cmH2O·ml−1·min) and prolific Tx elaboration (>15 ng/ml) within 5 min and loss of function within 10 min. Although papaverine significantly blunted the rise in PVR (<0.2 cmH2O·ml−1·min), Tx production was not inhibited (>20 ng/ml), and florid tracheal edema was usually evident within 20 min. In contrast, both inhibition of Tx synthesis (Tx < 3 ng/ml) with OKY-046 and blockade of the Tx receptor with SQ-30741 (Tx > 20 ng/ml) were not only associated with significantly lower peak PVRs (<0.2 cmH2O·ml−1·min) but also with attenuated increase in lung wet-to-dry ratio and airway edema. In concert, elaboration of histamine and tumor necrosis factor was blunted, and median survival in pigs perfused with human blood alone was significantly lower (20 ng/ml), whereas hearts and kidneys function well in analogous systems after similar manipulations of the complement activation pathway (30, 32, 33). Lung HAR (HALR) is characterized by rapid, marked elevation in pulmonary vascular resistance (PVR) and profuse capillary leak (30). The elevation in PVR observed with acute lung injury by cobra venom factor or endotoxin and after intravenous administration of xenogeneic blood in sheep is associated with thromboxane A2 (TxA2) elaboration; TxA2 has also been implicated as a direct cause of increased capillary permeability (2, 10, 15, 22, 26, 39, 43). This consideration led us to investigate thromboxane’s contribution to elevated PVR, increased vascular permeability, and elaboration of inflammatory mediators during HAR of pig lung by human blood. Furthermore, we investigated the primary cellular source of thromboxane in this model.

METHODS

Ex vivo lung perfusion model. We used an ex vivo perfusion system to model HAR of pig lung by human blood (3) (Fig. 1). Median sternotomy was performed in juvenile piglets (3–5 kg) under general endotracheal anesthesia with isoflurane. After heparinization (1,000 U/kg) and adequate circulation to indicate this fact.

xenotransplantation; microvascular permeability; macrophage; platelet; neutrophil; eicosanoid

HYPERTROPHIC REJECTION (HAR) of pig organs by humans is generally believed to be caused primarily by activation of human complement by a “natural” antibody bound to porcine endothelium (37). Supporting this view is the fact that pig hearts and kidneys can sustain the life of primates when complement activation is prevented with soluble complement receptor type 1, preempted using depletion with cobra venom factor, or downregulated as a consequence of expression of human complement regulatory proteins (HCRPs) in organs from transgenic pigs (7, 20, 25, 33, 44).

Among various organs from HCRP-transgenic pigs studied to date, porcine lung is poorly protected from acute injury during ex vivo perfusion with human blood and when transplanted into baboons (31, 43). Furthermore, neither absorption of antispecies antibody nor treatment with soluble complement receptor type 1 (R. Pierson, unpublished observations) fully protects the lung from injury, whereas hearts and kidneys function well in analogous systems after similar manipulations of the complement activation pathway (30, 32, 33). Lung HAR (HALR) is characterized by rapid, marked elevation in pulmonary vascular resistance (PVR) and profuse capillary leak (30). The elevation in PVR observed with acute lung injury by cobra venom factor or endotoxin and after intravenous administration of xenogeneic blood in sheep is associated with thromboxane A2 (TxA2) elaboration; TxA2 has also been implicated as a direct cause of increased capillary permeability (2, 10, 15, 22, 26, 39, 43). This consideration led us to investigate thromboxane’s contribution to elevated PVR, increased vascular permeability, and elaboration of inflammatory mediators during HAR of pig lung by human blood. Furthermore, we investigated the primary cellular source of thromboxane in this model.

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the lungs were continuously ventilated (Harvard cannula introduced through the left ventricular apex into the hilum; a pressure-limited circuit was chosen to simulate the arterial side of the circuit was set 50 cm above the lung with 95% O2-5% CO2 for 2–3 min before sample collection. Maximum mean pulmonary arterial pressure generated by perfusion pressure is limited to 50 cmH₂O. During conditions of increased pulmonary vascular resistance (PVR), pulmonary arterial perfusion pressure was typically 200 Torr during and after the first hour and was typically corrected by occasional addition of 5–10 meq of sodium bicarbonate. O₂)

For experiments using autologous blood perfusion, ~200 ml of blood were collected into heparin from the carotid artery of 5–10 meq of sodium bicarbonate. The perfusion apparatus consisted of a venous water jacket reservoir maintained at 37°C, with blood circulated via a roller pump (Renal Systems, Minneapolis, MN) at 100 ml·min⁻¹·kg⁻¹ piglet wt. An overflow in the pulmonary arterial side of the circuit was set 50 cm above the lung hilum; a pressure-limited circuit was chosen to simulate the maximum mean pulmonary arterial pressure generated by an unconditioned right ventricle in the lung transplant setting. Pulmonary vein effluent returned to the reservoir via a cannula introduced through the left ventricular apex into the left atrium. The lungs were continuously ventilated (Harvard Apparatus, South Natick, MA) 20 times/min at a tidal volume of 10 ml/kg piglet weight with 21% O₂-5% CO₂. Gas exchange by the lung was quantified by transcutaneous ventilation with 95% O₂-5% CO₂ for 2–3 min before sample collection. Arterial Po2, arterial Pco2, and pH were determined on an ABL30 blood-gas analyzer (Radiometer, Copenhagen, Denmark). Left ventricular and pulmonary arterial pressures were continuously measured via pressure transducers, and transpulmonary blood flow was assessed by ultrasonic flow probe measurements (Carolina Medical Electronics, King, NC) on the pulmonary artery inflow and pulmonary vein outflow conduits. PVR was calculated as

\[
PVR(\text{cmH}_2\text{O} \cdot \text{ml}^{-1} \cdot \text{min}^{-1}) = \frac{(\text{Ppa} - \text{Ppv})}{Q}
\]

where Ppa is pulmonary arterial pressure, Ppv is pulmonary venous pressure, and Q is flow.

Loss of lung survival was defined as 1) loss of oxygenation across the lungs (<10 Torr Po2 pulmonary vein-pulmonary artery gradient; normal ≥ 200 Torr), 2) loss of transpulmonary flow (<5 ml·kg⁻¹·min⁻¹ for two consecutive time points, which corresponds to a PVR > 2 cmH₂O·ml⁻¹·min⁻¹), or 3) alveolar flooding with appearance of edema fluid in the trachea (“tracheal edema”). Use of carbon dioxide at 5% in the inspired gasses was necessary to maintain pH balance (7.3–7.45) in the absence of metabolically active tissues other than blood and lung in the circuit. The Pco2 in pulmonary vein effluent was typically 32–45 Torr throughout the experiment; the Po2 was 85–120 Torr on 21% inspired O₂ fraction and typically rose to >250 Torr after 1 min of ventilation with 95% O₂ balanced with 5% CO₂, with normally functioning lungs. A mild metabolic acidosis was often seen after the first hour and was typically corrected by occasional addition of 5–10 meq of sodium bicarbonate.

To more precisely quantify oxygen transport function, in 24 additional experiments a pediatric oxygenator incorporated in the circuit was suffused with 95% N₂-5% CO₂ to deoxygenate pulmonary vein effluent, and the lungs were ventilated with 21% O₂-5% CO₂.

**Perfusate preparation.** Human blood was collected by trained phlebotomists from volunteer donors in the Vanderbilt Clinical Research Center, in accordance with protocols approved by the Vanderbilt Committee for the Protection of Human Subjects. Approximately 400 ml of fresh human blood from one donor were collected into 10,000 USP IU heparin (Elkins-Sinn, Cherry Hill, NJ) and expanded with ~180 ml of blood-type matched fresh-frozen plasma, which was heparinized (5,000 IU) before being recalculated with 450 mg calcium chloride [to neutralize CPDA-1 (citrate, phosphate, dextrose, adenine) anticoagulant and restore ionized calcium levels to the normal range]. For all experiments, the perfusate was circulated for ~15 min before baseline sample collection and introduction of the lung into the circuit.

**Experimental groups.** Positive control (heterologous) experiments were performed with unmodified fresh human blood; negative controls consisted of isolated piglet lungs perfused with autologous pig blood. OKY-046 [10 mg (2–3.3 mg/kg); Kissei Pharmaceutical, Matsumoto, Japan], a TXa2 synthase inhibitor, was administered intravenously to the piglet 5 min before surgical isolation of the lungs; 10 mg (16 μg/ml) were added as a bolus to the human blood perfusate 5 min before initiation of lung perfusion (n = 5). Interaction between thromboxane and its receptor was prevented by SQ-30741, a competitive TXa2-receptor antagonist [5 μg (1.6 μg/kg) to the piglet and 6.5 μg to the blood perfusate; n = 5; Squibb and Sons, Princeton, NJ], Papaverine hydrochloride [15 mg (3–5 mg/kg) to the piglet and 30 mg (50 μg/ml) to the blood; YorPharm, Buffalo Grove, IL], a smooth muscle relaxant, was employed to prevent vasconstriction by a mechanism that does not affect prostaglandin endoperoxide metabolism (n = 6) (27). Doses of OKY-046, SQ-30741, and papaverine added to blood were chosen based on literature review, extensive experience in endotoxic shock models (R. E. Parker), and limited pilot experiments. Doses administered to piglets were chosen based on similar considerations.

![Fig. 1. Ex vivo lung perfusion apparatus. The roller pump is set at 100 ml·min⁻¹·kg⁻¹ piglet wt. Peak pulmonary arterial perfusion pressure is limited by the height of the overflow tubing (O); under conditions of increased pulmonary vascular resistance (PVR), pulmonary arterial perfusion pressure is limited to 50 cmH₂O. During periods of low pulmonary blood flow, reservoir blood is continually warmed and recirculated through the "top off" (P). Transpulmonary blood flow and pulmonary arterial, left atrial, and airway pressures are monitored continuously (not shown).](image-url)
To effect platelet depletion from the human blood perfusate, heparinized human blood was Pall filtered with RC-400 filters (n = 5; Pall). Pall filtration removed ~95% of platelets and leukocytes, as confirmed by automated hemocytometry. In an additional set of seven experiments using Pall-filtered human blood to perfuse unmodified pig lungs, pulmonary vein effluent was collected in a second reservoir. The effluent was immediately returned to the main reservoir after a single passage through a second circuit consisting of a Pall filter and a second roller pump. The pulmonary vein filtration was discontinued after 10 min, and venous effluent then returned directly to the reservoir for the remainder of the experiment. Treatment of the piglet with acetylsalicylic acid (ASA; 10 mg/kg iv in 0.9% saline) 2 h before perfusion was employed to examine the effect of platelet depletion on the pace and character of HAR (41). Piglets pretreated with liposomes containing only PBS functioned as an additional control to rule nonspecific deactivation of PIMs. Blood circulated through the experimental apparatus, but without the lung (n = 3), served as a further control for biochemical assays.

An additional series of experiments was performed to quantify the effect of thromboxane inhibition on the rate of intraparenchymal fluid accumulation by using the change in wet-to-dry weight ratio in individual experiments as a proxy for relative vascular permeability. Biopsies were obtained from dependent portions of the lung 10 and 30 min after initiation of perfusion with autologous blood (n = 2) and human blood treated with papaverine (n = 4) or 1-benzylimidazole (1-BIA; a selective thromboxane synthase inhibitor; n = 3; no. 70510, Cayman Chemical, Ann Arbor, MI). The biopsy weight before and after 24 h of desiccation in a vacuum chamber was measured for each sample, and the wet weight was divided by the dry weight to derive the wet-to-dry weight ratio. The change in wet-to-dry ratio between the two biopsies from each experiment was compared among groups using a nonparametric one-way t-test for unpaired samples. When fluid was present in the airway, this was collected; if no fluid was present after the second biopsy was performed, the biopsy weight before and after 24 h of desiccation in a vacuum chamber was measured for each sample, and the wet weight was divided by the dry weight to derive the wet-to-dry weight ratio.

Eicosanoid quantitation. Samples for eicosanoid determination were collected with meclofenamate and EDTA from the precirculated reservoir (time 0) and pulmonary venous effluent at 1, 5, 10, 20, 30, and 60 min and hourly thereafter until a survival endpoint was met. Blood samples (1 mL/sample) were promptly placed on ice and centrifuged at 4°C, and plasma was stored at −70°C. Eicosanoid measurements in all experiments were conducted by stable isotope dilution gas chromatography-mass spectrometry. Plasma TxA2 and prostacyclin production were assayed by measurement of their stable metabolites, TxB2 and 6-keto-PGF1α, respectively. TxB2 and 6-keto-PGF1α samples were further purified via thin-layer chromatography (Whatman LK6DF silica obtained from VWR, Oak Ridge, TN) in the organic portion of H2O-ethyl acetate-hexane-acetic acid (7.5:6.75:3.12:1.5 vol/vol/vol/vol) and extracted in methanol. Both TxB2 and 6-keto-PGF1α samples were concentrated under vacuum and treated with acetonitrile, pentfluoroxy benzyl bromide (Sigma Chemical), and diisopropylphosphorylamine (Aldrich Chemical, Milwaukee, WI) (4:3:2 vol/vol/vol) for 30 min at 37°C. The methoxy-pentfluoroxybenzyl esters were then concentrated and purified by thin-layer chromatography with a mobile phase of ethyl acetate-methanol (98:2 vol/vol). Derivatization of each sample was completed by trimethylsililation with bis (trimethylsilyl)trifluoroacetamide (Supelco, Bellefonte, PA) in pyridine. Gas chromatography-mass spectrometry analyses were conducted on a 15-m fused silica capillary column (“QB 1.1” in a Varian Vista 6000 gas chromatograph coupled to a Nermag R10-10 mass spectrometer operated in the negative ion chemical ionization mode. Selected ions monitored for mass-to-charge ratio of 614/618. Eicosanoid levels were normalized to internal standard and expressed as nanograms per milliliter human plasma.

Histology. Lung biopsies for histology were gently inflated with diluted Tissue-Tek optimal cutting temperature compound (Sakura, Tokyo, Japan) instilled into a visible airway, snap frozen in liquid nitrogen, and stored at −80°C until study. Twelve-micrometer cryostat sections were employed for staining with either a Cy3-conjugated anti-COX-2 monoclonal antibody (no. 160112, Cayman Chemical) or hematoxylin and eosin after in situ terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay (Boehringer Mannheim, following manufacturer’s suggested methods) for detection of apoptotic PIMs after Clodronate pretreatment. PIMs were identified in hematoxylin-and-eosin-stained sections of lung of a piglet killed minutes after intravenous injection of a 1% solution of Monastral blue pigment in 0.9% saline; this lung was not exposed to heterologous or other perfusion.

In additional experiments using interventions described here, and with various complement inhibitory strategies, routine lung histology was performed on Formalin-fixed biopsies obtained at intervals after initiation of perfusion.

Histamine, tumor necrosis factor, and C3a assay. Sera stored in EDTA were assayed by commercial ELISA [histamine: Immunootech, Marseille, France; tumor necrosis factor (TNF)-α: R&D Systems, Minneapolis, MN; and C3a: Quidel, San Diego, CA] according to the manufacturer’s instructions.

Animal care and use. All experiments were conducted under protocols approved by the Vanderbilt Animal Care and Use Committee, and in accordance with guidelines from the “Guide for the Care and Use of Laboratory Animals” (DHENV Publication No. (NIH) 85–23, Revised 1985, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20205).

Statistical analyses. All values are expressed as means ± SE. Comparisons within groups were conducted by Wilcoxon signed rank test, and those among groups were conducted by a Mann-Whitney rank sum test, except where otherwise noted. Statistical significance was defined as P < 0.05.

RESULTS

Thromboxane’s role in HALR. Porcine lungs perfused under physiological conditions with fresh human blood (heterologous blood, positive controls) exhibited a rapid...
fall in transpulmonary blood flow within the first minutes of perfusion, associated with a rise in PVR from 0.07 ± 0.01 cmH2O·ml−1·min at baseline to 5.6 ± 1.3 cmH2O·ml−1·min at 10 min (Fig. 2A). Lungs in this group met the loss of flow survival endpoint at 10.0 ± 2.7 min (Fig. 2B) and often exhibited tracheal edema as well when ventilation was subsequently discontinued. In contrast, lungs perfused with autologous blood universally survived with preserved gas exchange and low airway pressures to the 240-min endpoint, demonstrating that the physiological perturbations observed with human blood are not an artifact of the experimental preparation (Fig. 2B). In lungs perfused with unmodified human blood, TxB2 levels in pulmonary vein effluent rose to 22.8 ± 3.9 ng/ml at 1 min (not shown) and plateaued at 44.4 ± 7.9 ng/ml at 10 min (P < 0.05 vs. autologous controls, <1.5 ng/ml at 10 min) (Fig. 2C). Administration of a competitive inhibitor of thromboxane synthase (OKY-046) or blockade of the thromboxane receptor (SQ-30741) markedly blunted the rise in PVR otherwise seen with heterologous blood (Fig. 2A). SQ-30741-pretreated lungs maintained PVRs between 0.08 ± 0.02 and 0.09 ± 0.02 cmH2O·ml−1·min for the duration of perfusion; OKY-046-pretreated lungs exhibited PVRs between 0.05 ± 0.01 and 0.06 ± 0.01 cmH2O·ml−1·min (P = 0.027 for SQ-30741 and OKY-046 experiments vs. heterologous controls at 10 min). OKY-046 prevented thromboxane production (<2 ng/ml) through the first hour (Fig. 2C) and beyond (not shown), whereas receptor blockade (SQ-30741) led to levels (~40 ng/ml) that tended to be higher than those of human blood controls (P = 0.07 at 10 min). Survival was significantly improved with either approach to thromboxane blockade (P < 0.01 for both vs. control; P = 0.1 for OKY-046 vs. SQ-30741). Although all OKY-046- and SQ-30741-treated lungs exhibited gradual fluid sequestration, some survived beyond 4 h in each group (Fig. 2C), and tracheal edema was generally not seen. Throughout the first 10 min of perfusion, prostacyclin production in the OKY-046 group was significantly elevated with respect to all other groups (P < 0.02) (Fig. 2D).

If the prolonged survival associated with thromboxane blockade was due primarily to prevention of the rise in PVR, rather than to modulation of thromboxane-dependent inflammatory or thrombotic mechanisms, a vasodilator should yield survival similar to thromboxane inhibition. We employed papaverine, an eicosanoid-independent smooth muscle relaxant, to examine this possibility. Papaverine treatment substantially blocked the rise in PVR (Fig. 3A), yet survival was not significantly prolonged (20.8 ± 5.7 vs. 10.0 ± 2.7 min for positive controls; P = 0.15) (Fig. 3B). TxB2 levels were similar to human blood controls, increasing markedly from 1.33 ± 0.51 ng/ml at baseline to 36.4 ± 11.9 ng/ml at 10 min (P = 0.1 vs. heterologous control) (Fig. 3C). Papaverine-treated lungs failed when prolific serous tracheal edema developed, demonstrating loss of vascular-epithelial barrier function. The possibility that papaverine was toxic to the lung at the dose used was excluded in autologous perfusion experiments (n = 3, data not shown).

The increase in wet-to-dry ratio associated with papaverine treatment (mean 0.85, range 0.50–1.61) was significantly greater than that seen in lungs perfused with autologous blood (mean 0.06, range −0.17–0.28; P < 0.02), demonstrating that HAR is associated with increased fluid and solute exchange. (Wet-to-dry ratios from lungs perfused with untreated human blood are not informative, due to absence of transpulmonary...
blood flow.) Thromboxane inhibition with 1-BIA blunted the increase in wet-to-dry ratio to 0.31 (range 0.13–0.42), a difference that approached significance ($P = 0.06$) relative to papaverine. The ratio between tracheal fluid and serum protein averaged 0.55 (range 0.3–0.67) in unmodified human blood control experiments, whereas with papaverine the ratio was 1.53 (range 0.9–2.3). Despite elevation of capillary hydrostatic pressure caused by transient venous outflow obstruction (see METHODS), tracheal edema fluid was not obtainable within the first hour of perfusion with 1-BIA-treated blood. On histology, lungs perfused with unmodified or papaverine-treated human blood exhibited profound dilatation of intraparenchymal and subpleural lymphatics at 10 min (Fig. 4). Lymphatic dilatation and intraparenchymal hemorrhage were consistently less prominent with thromboxane synthase inhibition (OKY, SQ, or 1-BIA) relative to papaverine treatment (not shown).

These findings were confirmed by additional experiments in which the capacity of the lung to transport oxygen was rigorously assessed by incorporating a pediatric oxygenator in the perfusion circuit and using this to deoxygenate the blood perfusate. Preserved oxygen transport function (Table 1) was observed with either method of thromboxane inhibition, demonstrating that oxygen transport function and, by implication, pulmonary microvascular barrier integrity were protected by selective thromboxane blockade. As we and others have previously found that complement activation mediates hyperacute lung rejection (3, 7, 30, 43), and no direct intervention to prevent complement-mediated injury was used in these experiments, protection from lung injury by thromboxane blockade was unexpected.

To determine whether a thromboxane agonist is sufficient to increase PVR or impair pulmonary vascular-epithelial barrier function in this model, a stable analog of thromboxane, carbocyclic TxA$_2$ (Cayman Chemicals), was added to autologous blood-perfused experimental preparations ($n = 3$) as graduated boluses (1–50 µg). PVR increased incrementally with escalating carbocyclic TxA$_2$ dose, and parenchymal and...
tracheal edema were grossly evident at the conclusion of these experiments.

The primary source of thromboxane in HALR. The primary source of thromboxane could reside either in the formed elements of the human blood perfusate or within the pig lung, where macrophages are the most likely source. Depletion of platelets and leukocytes (95% reduction) from the human blood by Pall filtration was associated with a rapid rise in PVR to 1.24 ± 0.52 cmH₂O·ml⁻¹·min by 10 min (Fig. 3A), a level intermediate between heterologous controls (P < 0.02 vs. heterologous controls) and groups with thromboxane blockade (P = 0.12 vs. OKY-046; P = 0.09 vs. SQ-30741) (Fig. 2A). The early rise in PVR partially resolved between 30 and 60 min, with subsequent transpulmonary blood flows averaging about one-third of those observed with autologous blood. Lung survival with Pall filtration was prolonged relative to unmodified human blood, with a mean survival of 222 ± 6 min (Fig. 3B). Pall filtration reduced thromboxane production by 75% to 11.11 ± 3.45 ng/ml at 10 min (Fig. 3C) (P = 0.07 vs. heterologous controls).

To evaluate the lung’s contribution to thromboxane elaboration, eicosanoid production in the lung was inhibited with ASA (10 mg/kg), a noncompetitive inhibitor of COX, which was administered intravenously to piglets 2 h before study. An ASA effect on the human blood perfusate is unlikely given the short plasma half-life (~20 min) of ASA coupled with flushing of pig blood from the lungs (35). Furthermore, salicylate levels in human blood perfusate were below assay detection limits (<0.02 mg/ml, data not shown). When unmodified human blood was used to perfuse ASA-treated lungs, lung injury was attenuated, with graft survival to 162 ± 43 min (Fig. 3B). Thromboxane production was reduced to 1.47 ± 0.44 ng/ml throughout the first hour of perfusion (Fig. 3C). Interestingly, PVR elevation was observed during the first hour, which spontaneously reversed (Fig. 3A).

That PVR rises with ASA treatment but not with selective inhibition of thromboxane (OKY-046, SQ-30741) demonstrates that thromboxane inhibition is not sufficient to prevent loss of transpulmonary blood flow. We hypothesized that ASA may also inhibit production of other COX-dependent vasoregulatory eicosanoids. Specifically, arachidonic acid metabolites required for prostacyclin synthesis (the prostaglandin endoperoxides PGG₂ and PGH₂) are unavailable when COX is inhibited by ASA. During perfusion of ASA-treated lungs, prostacyclin levels (as 6-keto-PGF₁α) remained <50 pg/ml throughout the first 40 min of perfusion (P < 0.03 relative to autologous and heterologous controls and all other experimental groups; Fig. 3D). After 1 h, prostacyclin production was detected, increasing to levels similar to those seen in other groups. This was temporally associated with induction of COX-2 expression in the lung (Fig. 5) and correlated

Table 1. Mean step up in Po₂ across the lung over time after initiation of perfusion

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>n</th>
<th>At 1 min</th>
<th>At 5 min</th>
<th>At 10 min</th>
<th>At 15 min</th>
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<tr>
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<td>7</td>
<td>45 ± 22</td>
<td>24 ± 19</td>
<td>8 ± 9</td>
<td>3 ± 11</td>
<td>2 ± 5</td>
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<td>Papaverine</td>
<td>5</td>
<td>53 ± 24</td>
<td>27 ± 23</td>
<td>17 ± 24</td>
<td>13 ± 26</td>
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<tr>
<td>SQ-30741</td>
<td>5</td>
<td>50 ± 10</td>
<td>48 ± 13*</td>
<td>54 ± 14†</td>
<td>59 ± 10*†</td>
<td>49 ± 16†</td>
</tr>
<tr>
<td>OKY-046</td>
<td>7</td>
<td>81 ± 31</td>
<td>65 ± 38*</td>
<td>48 ± 36*</td>
<td>43 ± 31*</td>
<td>40 ± 34*</td>
</tr>
</tbody>
</table>

Values are means ± SE, calculated as Po₂ (pulmonary vein) – Po₂ (pulmonary artery) measured in samples collected simultaneously. n = No. of animals. Δ, mean step up. *P < 0.05 vs. heterologous. †P < 0.05 vs. papaverine. P values are by 2-tailed Student’s t-test.

Fig. 4. Lymphatic dilatation during hyperacute lung rejection. Biopsies of pig lung 10 min after initiation of perfusion with autologous (A) or unmodified heterologous (B) blood. Marked dilatation of a lymphatic channel (*) is evident. Patchy engorgement of interalveolar septae with erythrocytes and focal hemorrhage into alveoli and perivascular sheaths (B, top left) is also demonstrated.
with the fall in PVR in these lungs. Together these data suggest that not only inhibition of thromboxane elaboration but also preserved prostacyclin production are critical to the maintenance of transpulmonary blood flow during HALR.

**Effect of PIM depletion on thromboxane production.** To selectively deplete PIMs from the lung, dichloromethylenebisphophonate (Clodronate) in large multilamellar liposomes was given intravenously 2 days before study. Clodronate is an inorganic bisphosphonate that, when encapsulated in liposomes, selectively depletes macrophages in vivo (41). Six hours after intravenous infusion of Clodronate liposomes, positive terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling staining localized to large mononuclear cells in alveolar septae (Fig. 6, B and C); these apoptotic cells corresponded in quantity and location with PIMs previously identified in untreated lungs by their phagocytosis of Monastral blue pigment (Fig. 6D), suggesting that the apoptotic cells are PIMs. Lungs treated with liposomes containing only PBS demonstrated HAR similar in pace and in physiological and biochemical characteristics to that observed for lungs perfused with unmodified human blood, as described above (Fig. 7). Lungs pretreated with Clodronate liposomes exhibited a modest, transient rise in PVR to $0.11 \pm 0.02 \text{cmH}_2\text{O} \cdot \text{ml}^{-1} \cdot \text{min}$ at 10 min, similar to that observed with autologous pig blood perfusion or thromboxane blockade (Fig. 7A). Median survival was 240 min (3 of 5 experiments), with gradual sequestration of reservoir volume in the lung, low airway pressures, and little edema fluid in the airways (Fig. 7B). Thromboxane production in the Clodronate-treated lungs was similar to autologous blood perfusion ($P > 0.32$ for all time points) and was markedly reduced relative to levels observed in PBS-liposome-treated or untreated control groups ($P < 0.01$ at 10 min) (Fig. 7C). In contrast to ASA treatment, prostacyclin elaboration (Fig. 7D) was not inhibited by Clodronate. Thus Clodronate pretreatment inhibits production of thromboxane but not prostacyclin, and PVR elevation and loss of microvascular barrier function were attenuated in the absence of any direct inhibition of antibody- or complement-mediated lung injury. Interestingly, only 40–50% of the human platelets in the perfusate were sequestered in the Clodronate-treated lungs, compared with >95% with OKY-046 or SQ-30741 treatment.

In previous studies (unpublished observations), we had observed the rapid appearance of a population of leukocytes in pulmonary vein effluent in experiments using Pall-filtered human blood. We hypothesized that porcine cells might be activated and released from the lung after interaction with Pall-filtered human blood and could be contributing to activation of PIMs and thus to thromboxane release and increased vascular resistance on return to the lung. When pulmonary vein effluent was Pall filtered for the first 10 min of lung perfusion, thromboxane levels were reduced by 90% relative to preperfusion Pall filtration alone ($1.03 \pm 1.03 \text{ ng/ml}$ at 10 min; $P < 0.05$). PVR elevation was blunted ($P = 0.15 \pm 0.08 \text{ cmH}_2\text{O} \cdot \text{ml}^{-1} \cdot \text{min}$; $P < 0.01$ vs. preperfusion Pall filtration alone; median survival (90 min) was similar to preperfusion Pall filtration alone. This result shows that porcine cells mobilized from the lung during the initial interaction with human blood, in addition to human formed blood elements, participate in elaboration of thromboxane in this model.

**Thromboxane and mediators of inflammation.** To investigate why thromboxane inhibition is associated with prolonged survival in the setting of endothelial interaction with heterologous antibody and complement, elaboration of several inflammatory mediators associated with pulmonary parenchymal injury were evaluated (Table 2). Histamine production, an index of mast cell activation, was significantly inhibited (50–90%) in each group in which thromboxane production was inhibited (OKY-046, Clodronate, ASA), or its receptor was blocked (SQ-30741). Produced primarily by activated neutrophils, lymphocytes, and macrophages, TNF-α elaboration was significantly inhibited by Clo-

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**Fig. 5.** Cy3-labeled anti-COX-2 monoclonal antibody staining of ASA-treated pig lung. **A:** preperfusion biopsy of ASA-pretreated lung ($\times 20$ magnification) demonstrating minimal COX-2 positivity. **B:** ASA-pretreated lung after 4 h of perfusion with unmodified human blood demonstrates marked COX-2 expression ($\times 40$ magnification).
Fig. 6. Localization of pulmonary intravascular macrophages (PIMs) and demonstration of apoptotic cells in lungs after Clodronate-liposome treatment. A: apoptotic cells were not seen in lungs of piglets pretreated with PBS-loaded liposomes. B and C: apoptotic cells localized to alveolar septae were identified in biopsies of Clodronate liposome-pretreated lungs (B: ×40; C: ×63 magnification). Apoptotic cells are identified by in situ terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay. D: PIMs in normal pig lung, visualized by phagocytosis of monastral blue pigment given intravenously 15 min before biopsy.

dronate and OKY-046 relative to other human blood groups and tended to be lower with SQ-30741 (one outlier prevented this difference from reaching statistical significance). Pall filtration effectively eliminated TNF-α production, suggesting that, in contrast to findings in an ex vivo liver perfusion model, the primary source of TNF-α during hyperacute lung rejection is leukocytes in the human blood (29). We observed a consistent trend toward reduced C3a production with each thromboxane blocking strategy but not with papaverine or Pall filtration; a statistically significant reduction in C3a was demonstrated only for PIM depletion with Clodronate. Thus thromboxane inhibition is associated with diminished activation of the TNF-α-, histamine-, and complement-mediated proinflammatory cascades, all of which likely contribute to progression of lung injury. Papaverine effectively blocked both TNF-α and histamine elaboration.

**DISCUSSION**

These results demonstrate that thromboxane mediates the rise in PVR observed during HAR of the lung and influences biochemical indexes of inflammation and the pace of intraparenchymal fluid sequestration. Both increased PVR and inflammation are mediated at least in part through the thromboxane receptor, because results with SQ-30741 approximate those with OKY-046. The influence of thromboxane on inflammation and rate of increase in lung water can be dissociated from pure rheological effects, as shown by improved oxygen transport and protection from tracheal edema with thromboxane blockade relative to papaverine. We find that Clodronate-sensitive cells in the lung are critical to the production of thromboxane during the interaction of pig lung with human blood. We infer that these cells are PIMs activated in the pig lung during the initial interaction with human blood, as ASA therapy targeted selectively at the lung reduces thromboxane by 96%. However, Pall-filterable blood constituents, both human and porcine, contribute significantly to overall thromboxane production, because Pall filtration of the human blood before lung perfusion inhibits thromboxane production by 70% and additional transient pulmonary vein filtration effectively prevents thromboxane production. Together these data suggest that eicosanoids elaborated by the lung are required to trigger thromboxane production by blood elements, presumably by platelets and perhaps by activated monocytes and macrophages. Whether neutrophils, which are also effectively depleted by this intervention, contribute to this interaction remains to be defined. In aggregate, our experiments suggest that PIMs in the lung elicit TxA2 synthesis in platelets or other human and porcine formed blood elements through COX- and TxA2-dependent mechanisms. Prolific thromboxane production, accelerated by PIMs in the pig lung, may account for this organ’s particular susceptibility to injury in this model of HAR.

Reduced thromboxane effect generally correlates with a blunted inflammatory response, as reflected in reduced elaboration of TNF-α, histamine, and C3a in every experimental group in which thromboxane pro-
duction was inhibited or its receptor blocked. Smooth muscle relaxation with papaverine also inhibits TNF-α and histamine, suggesting that altered rheology associated with elevated PVR contributes significantly to these facets of the proinflammatory HALR milieu. In contrast, papaverine does not blunt C3a production, whereas thromboxane inhibition does have a modest effect that reaches significance with PIM depletion. Because C3a, C5a, and other membrane-bound components of the complement cascade are mechanistically linked to increased vascular permeability, we postulate that thromboxane’s proinflammatory effect is mediated in part through activation of the alternative complement pathway. This hypothesis is supported by the association between thromboxane and alternative pathway complement activation in other models (2, 15, 26, 39) and by our demonstration of properdin deposition in pig lungs perfused with human blood (3).

When pulmonary COX activity is inhibited using ASA, the importance of endothelial prostacyclin elaboration to preservation of transpulmonary blood flow is apparent. In contrast to any selective antithromboxane strategy (OKY-046, SQ-30741, or Clodronate), each of which results in preserved transpulmonary blood flow, inhibition of both prostacyclin and thromboxane is associated with significantly elevated PVRs. Recovery of perfusion was associated with de novo expression of COX-2 in the lung, delayed elaboration of prostacyclin, and a subsequent rise in thromboxane. Thus, in the setting of thromboxane blockade, pulmonary prostacyclin production appears to be required for physiological transpulmonary flow of human blood through the pig.

Table 2. Elaboration of inflammatory mediators (C3a, histamine, TNF-α) during hyperacute lung rejection

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>n</th>
<th>ΔC3a, ng/ml 0–10 min</th>
<th>P Value</th>
<th>ΔHistamine, ng/ml 0–10 min</th>
<th>P Value</th>
<th>ΔTNF-α, pg/ml 0–10 min</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heterologous</td>
<td>5</td>
<td>2,236 ± 533</td>
<td></td>
<td>255 ± 27</td>
<td>0.005</td>
<td>416 ± 163</td>
<td>0.05</td>
</tr>
<tr>
<td>Autologous</td>
<td>5</td>
<td>NA</td>
<td></td>
<td>88 ± 21</td>
<td>0.005</td>
<td>&lt;15.6*</td>
<td>0.05</td>
</tr>
<tr>
<td>Papaverine</td>
<td>6</td>
<td>3,467 ± 1,127</td>
<td>0.41</td>
<td>8 ± 3</td>
<td>&lt;0.001</td>
<td>4 ± 16</td>
<td>0.031</td>
</tr>
<tr>
<td>OKY-046</td>
<td>5</td>
<td>970 ± 726</td>
<td>0.21</td>
<td>22 ± 13</td>
<td>&lt;0.001</td>
<td>60 ± 32</td>
<td>0.07</td>
</tr>
<tr>
<td>SQ-30741</td>
<td>5</td>
<td>1,120 ± 686</td>
<td>0.23</td>
<td>34 ± 31</td>
<td>&lt;0.001</td>
<td>140 ± 134</td>
<td>0.23</td>
</tr>
<tr>
<td>ASA</td>
<td>5</td>
<td>1,099 ± 266</td>
<td>0.12</td>
<td>126 ± 34</td>
<td>0.019</td>
<td>208 ± 101</td>
<td>0.29</td>
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<tr>
<td>Pall filtration</td>
<td>5</td>
<td>2,669 ± 781</td>
<td>0.65</td>
<td>154 ± 38</td>
<td>0.06</td>
<td>&lt;15.6*</td>
<td>0.05</td>
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<tr>
<td>Clodronate</td>
<td>5</td>
<td>197 ± 176</td>
<td>0.006</td>
<td>51 ± 13</td>
<td>&lt;0.001</td>
<td>89 ± 68</td>
<td>0.11</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = no. of animals. ASA, acetylsalicylic acid; Δ, change; TNF-α, tumor necrosis factor-α. P values are by 2-tailed Student’s t-test vs. heterologous. NA, not done, as porcine C3a is not accurately assayed in human C3a ELISA. *Analyzed by porcine-specific TNF-α ELISA.
Thromboxane is closely associated with experimental and clinical pulmonary dysfunction (4, 5, 9, 19, 39). Whether thromboxane directly affects pulmonary microvascular integrity is controversial (2, 17, 22, 34, 38); our data support the view that it does, at least in the context of antispecies antibody binding and complement activation, and that its effect on permeability is mediated through the thromboxane receptor. As a consequence of its postvenular vasoconstrictive effects, TxA2 likely also increases microvascular fluid transudation by causing increased hydrostatic pressure in pulmonary capillary beds, and this may account in part for the trend toward improved survival with OKY-046 relative to SQ-30741.

Thromboxane is closely associated with the observation in a canine model of oleic acid-induced acute lung injury, where inhibition of COX by ASA is associated with augmented PVR elevation (23). We infer that preventing synthesis of PGIL substrate by COX inhibition results in increased vascular smooth muscle tone or permits constriction in response to the endothelial activation associated with HALR (36, 40). Whether manipulation of the balance between eicosanoids can be used to protect the lung from PVR elevation or other manifestations of HALR will require further investigation. If thromboxane causes increased vascular permeability, we would predict that PGIL supplementation, even to supraphysiological levels, will probably fail to prevent lung injury in the absence of selective thromboxane blockade.

Our results support the idea that endoperoxide shunts can have important physiological consequences. Lungs pretreated with the thromboxane synthase inhibitor OKY-046 demonstrated the lowest PVRs and highest prostacyclin levels observed in this series of experiments. Substrate diversion from platelets to endothelium has been well documented in vitro and in vivo (28). We speculate that, in this model of pulmonary HAR, thromboxane synthase inhibition affords diversion of prostaglandin endoperoxide to prostacyclin pathway precursors at the interface among PIMs, formed blood elements, and endothelium. As prostacyclin is a vasodilator and potent antagonist of platelet aggregation, increased production may preserve perfusion of microvascular beds, and this may account in part for the trend toward improved survival with OKY-046 relative to SQ-30741.

How thromboxane might directly influence permeability has not yet been defined at the molecular level, but plausible cellular mechanisms are suggested by several observations. There is compelling evidence for the presence of functional TxA2 receptors on vascular endothelium, that TxA2 can directly activate endothelium via its receptor, and that TxA2 can cause alterations in endothelial physiology and structure likely to contribute to pulmonary microvascular leak (14, 18, 42). Blocking thromboxane in the context of endothelial activation by antibody and complement may thus attenuate structural and biochemical endothelial alterations crucial to the loss of microvascular barrier function. With regard to thromboxane’s putative proinflammatory effects, myeloid lineage cells in blood and lung (monocytes, platelets, macrophages, mast cells, and neutrophils) bear thromboxane receptors. Our results with Pall filtration of human blood and of pulmonary vein effluent suggest that myeloid constituents of blood and lung contribute to intersecting positive feedback loops, which together determine the pace of microvascular injury and lung failure.

Based on these considerations and the observations reported here, our working hypothesis is that TxA2-receptor-mediated endothelial and PIM activation occurs as an initial event during HAR of the pig lung by human blood, causing alterations in endothelial cytoskeletal structure that result in plasma and cellular extravasation between endothelial cells (6, 24). By inhibiting amplification of the complement cascade and prothrombotic pathways and blunting release of other inflammatory mediators (TNF-α, histamine) at a proximal stage in the HAR response, selectively blocking thromboxane allows recruitment of anti-inflammatory cellular defenses to a degree sufficient to facilitate prolonged lung function in a “discordant” xenogeneic environment (1).

The trigger for thromboxane production remains to be elucidated. The thromboxane cascade might be driven by events specific to the immunological interaction between pig and humans (antibody binding, complement activation), physiological incompatibility in coagulation pathway regulation, or interaction of human formed blood elements with molecules constitutively expressed on pig lung (30, 31, 39, 43). The deposition of the complement membrane attack complex on macrophages is known to stimulate phospholipase A2 activity, providing arachidonic acid for eicosanoid synthesis, which may in turn be the initiating event for thromboxane production in this model (13). Experiments wherein endothelial and systemic complement activation are optimally inhibited (e.g., perfusion of lungs with high-HCRP transgene expression with soluble complement receptor type 1-treated blood) will address this important issue.

The eicosanoid pathway, like the complement system, is a primitive yet potent component of the innate immune system. There exists circumstantial evidence that thromboxane may contribute to the increased vascular resistance and subsequent inflammation observed in organ xenografts other than the lung (6, 16,
33, 40). Indeed, direct manipulation of the eicosanoid balance has been used experimentally to facilitate short-term survival of kidney and lung xenografts in the setting of complement regulation (30, 44). If thromboxane's trigger proves to be independent of complement, pharmacological inhibition of thromboxane, or organ-specific approaches to donor macrophage depletion, may contribute to safe clinical use not only of lung xenografts but of other solid organ xenografts as well.

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