Thromboxane A2 Mediates Increased Pulmonary Microvascular Permeability Following Limb Ischemia

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Lower torso ischemia and reperfusion lead to respiratory dysfunction characterized by pulmonary hypertension and increased lung microvascular permeability. This is associated with lung leukosequestration and thromboxane (TX) generation. This study tests the role of elevated TX levels following muscle ischemia in mediating remote lung injury. Anesthetized sheep prepared with chronic lung lymph fistulae underwent 2 hours of bilateral hind limb tourniquet ischemia. In untreated controls \((n=7)\), 1 minute after reperfusion there was a transient increase in plasma immunoreactive (i)-TXB2 levels from 211 to 735 pg/ml \((p<0.05)\), and at 30 minutes, lung lymph i-TXB2 levels rose from 400 to 1,005 pg/ml \((p<0.05)\). At 1 minute, the mean pulmonary arterial pressure (MPAP) increased from 13 to 38 mm Hg \((p<0.05)\) and pulmonary microvascular pressure \((Pmv)\) from 7 to 18 mm Hg \((p<0.05)\). Lung lymph flow \((QL)\) rose from 4.3 to 8.3 ml/30 min \((p<0.05)\), the lymph/plasma \((L/P)\) protein ratio was unchanged from 0.6, and the lymph protein clearance increased from 2.6 to 4.6 ml/30 min \((p<0.05)\). Two hours after reperfusion, neutrophils were observed sequestered in lung capillaries and proteinaceous exudates were found in alveoli in contrast to sham-operated animals \((n=3)\). To maximize lung vascular surface area and achieve a pressure independent \(L/P\) protein ratio a left atrial balloon was inflated during one group of ischemia-reperfusion experiments \((n=5)\). This resulted in a baseline rise in MPAP to 20 mm Hg \((p<0.05)\); a 4.3-fold increase in QL \((p<0.05)\), a decrease in the \(L/P\) ratio from 0.70 to 0.28 \((p<0.05)\) and a protein reflection coefficient \((cd)\) of 0.72. During reperfusion the \(L/P\) ratio rose to 0.49 \((p<0.05)\) and the \(cd\) decreased to 0.51 \((p<0.05)\), documenting an increase in lung microvascular permeability. In contrast to untreated ischemic controls, inhibition of TX synthetase with OKY 046 \((n=6)\) reduced plasma i-TXB2 levels to 85 pg/ml \((p<0.05)\) but also increased i-6-keto-PGF1a levels to 78 pg/ml relative to 15 pg/ml in untreated controls \((p<0.05)\). OKY 046 prevented the increase in MPAP, Pmv, QL, and lymph protein clearance \((p<0.05)\). Lung histology was normal in distinction to the leukosequestration in untreated ischemic controls. Pretreatment with OKY 046 combined with ibuprofen \((n=5)\) prevented the increase in i-6-keto-PGF1a \((p<0.05)\) but still led to a response unchanged from OKY 046 treatment alone. Pretreatment with the TX receptor antagonist SQ 29,548 \((n=5)\) did not affect the ischemia induced increases in TXB2 levels in plasma and lung lymph to 702 and 789 pg/ml, respectively, but prevented the increase in MPAP, Pmv, QL, lymph protein clearance, and lung leukosequestration \((p<0.05\) for all). These data indicate that the increased lung permeability following lower torso ischemia and reperfusion may be mediated by TX. (Circulation Research 1989;64:1178–1189)

Reperfusion of a large mass of ischemic tissue may pose a threat to the rest of the body, especially to the lungs.1 It has been thought that the respiratory complications of reperfusion were due largely to washout of products of anaerobic metabolism2 or to lung microembolization.2 It is now believed that leukocytes, oxygen-derived free radicals and oxygenation products of arachidonic acid play a central role in mediating the local injury following ischemia and reperfusion.3,4 Further, these

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Supported in part by National Institutes of Health grants GM-24891-10, GM-35141-03, HL-16714-13; The U.S. Navy Office of Naval Research, Contract No. N00014-79-C-0168; The Brigham Surgical Group, Inc; and The Trauma Research Foundation.

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inflammatory mediators appear to be of primary importance in ischemia-induced remote injury to the lungs.5,6 Thus, lower torso or limb ischemia stimulate the synthesis of thromboxane (TX) A2.3,5-7 This vasoactive, chemotactic prostanoid has been shown to be responsible for the pulmonary hypertension and lung leukosequestration following experimental limb ischemia.8 This study tests the possible role of TX in mediating ischemia induced increase in lung microvascular permeability.

Materials and Methods

Sheep Preparation

Female animals (n=31) weighing 25–44 kg underwent cannulation of the lung lymphatic according to a modification of the technique described by Staub.8 During surgery, animals were anesthetized with sodium pentobarbital (15 mg/kg i.v.), paralyzed with 2 mg pancuronium bromide, intubated, and mechanically ventilated with a Harvard respirator (South Natick, Massachusetts) using room air. Through a right posterolateral thoracotomy, the efferent duct of the caudal mediastinal lymph node was transected and ligated, and the diaphragm below the level of the inferior pulmonary ligament was circumferentially cauterized. Systemic lymph tributaries to the proximal portion of the lymph node just below the level of the inferior pulmonary ligament was transected and ligated, and the diaphragm around the lymph node was circumferentially cauterized. Systemic lymph tributaries to the proximal portion of the lymph node were cauterized or ligated to minimize extrapulmonary contamination of collected lymph. The thoracotomy was closed and the lymphatic cannula was exteriorized through the chest wall. A thermistor-tipped pulmonary arterial (Electro-Cath Corp, Rahway, New Jersey) and a central venous catheter were introduced through the right internal jugular vein. The aorta was cannulated via the adjacent carotid artery. After a recovery period of 4 to 5 days, when animals appeared vigorous, were afebrile, and had a steady flow of blood-free lymph, the experiment was conducted. In five of these sheep, 7 days following lung lymph cannulation a left atrial balloon was inserted via a thoracotomy through the left fifth intercostal space. A 16F silicone elastomer coated Foley catheter with a 30-ml inflatable balloon (No 602-155, Dow Corning Corp, Midland, Michigan) was placed in the left atrium along with a small catheter to monitor pressure. Catheters were exteriorized through the chest wall and sewn to the skin. Experiments in these sheep were conducted after an additional 4–6-day period of recovery.

Experimental Protocol

Experiments were conducted in anesthetized sheep, placed supine and ventilated at a tidal volume of 15 ml/kg and rate of 12 to 15 cycles/min, adjusted to keep the Paco2 levels between 30 and 35 mm Hg. Anesthesia was maintained with a continuous infusion of pentobarbital (0.1 mg/kg · min) and pancuronium (30 µg/kg · min). Saline, 7 ml/kg · hr, was infused throughout the experiment. External heat was used to maintain body temperature between 38° and 39°C. After 2 hours of stabilization, baseline measurements were taken. Both hind limbs were then elevated for 2 minutes to drain them of blood, and tourniquets were applied as high on the thighs as possible and inflated to 300 mm Hg. After 2 hours of ischemia, the tourniquets were removed and the animals monitored for another 2 hours.

Cardiopulmonary Function

Stain-gauge transducers (model D-240, Bently Laboratories, Inc, Irvine, California) were used to measure the following pressures: mean arterial (MAP), mean pulmonary arterial (MPAP), pulmonary arterial wedge (PAWP), and mean left atrial (LAP). The pulmonary microvascular pressure (Pmv) was calculated from the Gaar equation, Pmv=PAWP+0.4 (MPAP—PAWP).9 Pulse rate was determined from an arterial pressure trace. Cardiac output was measured in triplicate by thermodilution (model 5000, Electro-Cath Corp) and divided by body weight to obtain the cardiac index (CI). Blood gases, pH, oxygen saturation, and hemoglobin were measured with Clark and Severinghaus electrodes and by spectrophotometry using extinction coefficients specific to sheep blood (model 813 and 282, Instrumentation Laboratory, Lexington, Massachusetts).

Hematology

Circulating platelets and white blood cells (WBC) were counted by means of phase microscopy. Differential counts were made on Wright's stained blood smears.

Radioimmunoassay for TX and Prostacyclin

Plasma and lymph concentrations of immunoreactive (i)-TXB2 and i-6-keto-prostaglandin (PG)F1α, the stable hydrolysis products of TXA2 and prostacyclin, were measured by radioimmunoassay (RIA) as described by Levine et al.10,11 Blood was drawn into cooled syringes containing EDTA and aspirin. The blood was immediately centrifuged at 1,500g at 4°C for 20 minutes, the plasma separated and stored at −20°C until assayed. Lung lymph was collected at 30-minute intervals in cold graduated tubes containing EDTA and aspirin. The lymph was then centrifuged at 1,500g and 4°C for 20 minutes and the supernatant separated and stored at −20°C until assayed for TXB2 and i-6-keto-PGF1α. The RIA for each of the prostanoids studied was performed on two separate aliquots taken from the same volume of plasma or lymph sample. The final result is presented as the average. Specific rabbit TX and PG antiseras (provided by Dr. L. Levine, Brandeis University, Waltham, Massachusetts) were stored in 0.5-ml volumes at −80°C until use when the antiserum was thawed. The TX antiserum was diluted 1:10,000, and the PG antiserum was diluted...
Counts/min. A control assay run in Isogeltris (0.2 ml) added tracer (0.1 ml) had an activity of 6,000–7,000 counts/min. A control assay run in Isogeltris (0.2 ml) was substituted for test plasma. A blank assay was also used in which Isogeltris (0.2 ml), rabbit plasma, or serum (0.1 ml, mixed 9:1 with 0.1 M EDTA) were incubated with the TX or PG tracer (0.1 ml). After 1 hour of incubation, the reaction was stopped by the addition of rabbit plasma (0.1 ml) for the i-TXB2 or rabbit serum (0.1 ml) for the i-6-keto-PGF1α assay. Goat anti-rabbit serum (0.1 ml) (Armel Products, Brooklyn, New York) was added and precipitation was allowed to occur overnight. Then, 0.1 M EDTA (0.5 ml) was added. The precipitate was collected by centrifugation at 1,200 × g for 20 minutes, dissolved in 0.1 ml dihydroxyacetone, and mixed with 2 ml scintillation fluid (Biofluor, New England Nuclear). The scintillation counts from test plasma, control, and blank assays were used to calculate percent inhibition from the following formula:

Percent inhibition = \( \frac{\text{test blank} - \text{control blank}}{\text{test blank}} \times 100\% \)

The acceptable inhibitory range was between 15% and 85%. The lower limit permitted the minimum detectable amount of i-TXB2 and i-6-keto-PGF1α to be 6 or 10 pg, respectively. Ninety-five percent of the assays performed on the pair of aliquots from each plasma and lymph sample yielded data that differed by less than 15%.

Studies were performed to assess the serological specificity of the antisera used in the radioimmunoassays. In the TXB2 system, 28 pg TXB2 inhibited homologous binding by 50%; PGD2, PGF2α, 13,14-dihydro-15-keto-PGF2α, PGF2α, and 6-keto-PGF1α cross-reacted by less than 1%; in the 6-keto-PGF1α system, 140 pg 6-keto-PGF1α inhibited homologous binding by 50%; 6-keto-PGF1α cross-reacted by 3%, whereas PGD2, PGE2, 13,14-dihydro-15-keto-PGE2, PGF2α, and TXB2 cross-reacted less than 1%. Finally, the specificity of the antisera was indicated by the fact that levels of TXB2 and 6-keto-PGF1α measured by RIA were almost identical before and after separation by high-pressure liquid chromatography.\(^{11}\) However, this comparison was done in samples obtained from culture media and not whole plasma.

**Lung Fluid Balance**

Lymph (L) and plasma (P) total protein concentrations were determined in duplicate by the spectrophotometric protein dye method described by Bradford.\(^{12}\) The L/P protein ratio was calculated and multiplied by lymph flow (QL) to obtain the lymph protein clearance. The osmotic reflection coefficient (σd) for total protein was calculated using the minimum L/P protein ratio, achieved at a steady state, during balloon inflation when LAP is increased and QL is high. At this point, the L/P protein ratio becomes independent of the filtration rate and approaches \( (1-\sigma d) \).\(^{13}\)

**Histological Examinations**

At the end of the experiment animals were euthanized with an overdose of pentobarbital and potassium chloride. Glutaraldehyde (2%) was instilled into the lungs through an endotracheal tube at a pressure of 25 cm H2O. After 20 minutes, the hilum of the left lung was clamped. The lung was removed and immersed in glutaraldehyde for 72 hours before sampling. Tissue sections were taken from the dorsolateral area of the lower lobe. All microscopic sections were stained with hematoxylin-eosin and were interpreted by a pulmonary pathologist (L.K.) in a blinded fashion. Lung sequestration of neutrophils was quantitated by counting alvelor septal wall neutrophils in 10 randomly chosen high-powered fields \( (×1,000) \). Microscopic fields in proximity to bronchial structures, pleura, and large vessels were excluded.

**Experimental Groups**

Sheep \( (n=12) \) were pretreated with saline placebo or the TX synthetase inhibitor OKY 046, sodium \( (E)-3-[p-(1H-imidazol-1-methyl) phenyl]z-propanolate \( (n=6) \) (Ono Pharmaceutica, Osaka, Japan). This imidazol derivative acts in a noncompetitive fashion to inhibit TXA2 synthetase without a direct effect on other enzymes related to the arachidonic acid cascade.\(^{14,15}\) OKY 046 was administered 2 mg/kg by intravenous bolus starting at the beginning of the baseline period and then repeated every 2 hours. This dosage regime effectively inhibits TXA2 synthesis in a variety of species, including sheep.\(^{5,7,15}\) Because OKY 046 not only inhibits TX generation but also increases baseline i-6-keto-PGF1α levels through redirection of endoperoxides toward prostacyclin synthesis,\(^{14,15}\) a third group of animals \( (n=5) \) was pretreated with the combination of OKY 046 2 mg/kg every 2 hours and the cyclooxygenase inhibitor ibuprofen (The Upjohn Co, Kalamazoo, Michigan) 12 mg/kg given as a single intravenous bolus at the start of the baseline period. Inhibition of cyclooxygenase was designed to prevent OKY 046-induced PG synthesis.

Since ibuprofen may have actions unrelated to cyclooxygenase inhibition,\(^{15,17}\) further studies were conducted with the TXA2-receptor antagonist SQ 29,548 \([15-(1α,2β(IZ),3β,4α)]-7-[3-2-(phenylamino) carbonyl] hydrazino] methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid \( (n=5) \) provided as a gift by Dr. M. Ogletree, Squibb Pharmaceuticals, Princeton, New Jersey.\(^{16,17}\) This compound has been shown to com-
Table 1. Effect of Thromboxane-Receptor and Synthetase Inhibitors on Systemic Hemodynamics

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HR, heart rate; LAP, left atrial pressure; MAP, mean arterial pressure; CI, cardiac index; PAWP, pulmonary artery wedge pressure. Daggers refer to significant difference relative to untreated ischemic controls (N=7), increased LAP (n=5), OKY 046 (n=6), OKY 046+ibuprofen (n=5), and SQ 29,548 (n=5).

Petitively inhibit the receptor activated by stable analogues of TXA2 and PG endoperoxides. It is highly selective, with only weak antagonistic activity to PGF1α and PGE2. SQ 29,548 was prepared by dissolving equimolar amounts with Tris in 95% ethanol and then evaporating the ethanol under nitrogen. The Tris salt of the drug was then dissolved in distilled water. Administration of the drug was started after 1 hour of baseline measurements. The dose was 2.2 mg/kg as an intravenous bolus followed by a continuous infusion of 2.2 mg/kg • hr throughout the experiment. This amount is more than 10-fold higher than that necessary to blunt the pulmonary pressor response to infusion of a TX mimic in vivo. It was also found to be an amount effective in blocking the endotoxin-induced rise in MPAP associated with a fivefold increment in urinary 2,3-dinor-TXBr generation in sheep.

In five out of the 12 untreated ischemic sheep, after a baseline period of 1 hour, the left atrial balloon was inflated to increase LAP to 18–20 mm Hg. This is well tolerated in sheep but does affect systemic hemodynamics leading to a mild decrease in MAP. This study was designed to maximize and stabilize pulmonary vascular surface area and achieve a pressure independent L/P protein ratio in order to interpret lymph data. The left atrial balloon was kept inflated throughout the experiment. By the time of reperfusion, after 3 hours of increased LAP, a steady state of high QL and filtration independent L/P protein ratio had been achieved.

Sham sheep (n=3) were subjected to 6 hours of anesthesia in a supine position after which they were killed and the lungs removed for histological examination.

Results are expressed in the text and figures as mean±SEM. Differences between means were tested by an analysis of variance, paired and nonpaired t tests, and by the Wilcoxon's and Mann-Whitney nonparametric tests. When multiple comparisons were done, the Bonferroni procedure was applied. Significance was accepted if p<0.05.

Animals used in this study were maintained in accordance with the guidelines of the Committee on Animals of the Harvard Medical School and those
Figure 1. Release of tourniquets following 2 hours of bilateral hind-limb ischemia (n=7) led to an immediate rise in mean pulmonary arterial pressure (MPAP) that was prevented by pretreatment with either the thromboxane synthetase inhibitor OKY 046 alone (n=6), or in combination with ibuprofen (n=5), or with the thromboxane-receptor antagonist SQ 29,548 (n=5). Asterisks and daggers refer to significant differences relative to baseline values and between groups respectively.

Results

Untreated Ischemic Controls

During the 2 hours of tourniquet ischemia, there were no alterations in hemodynamics (Table 1), blood counts or prostanoid levels. One minute after tourniquet release MPAP rose from 13±1 to 38±4 mm Hg (p<0.05) and returned to baseline levels within 30 minutes (Figure 1). There was a transient rise in Pmv from 7±1 to 18±2 mm Hg (p<0.05). This occurred while the PAWP was unchanged at 4±1 mm Hg. Also unaffected were blood gases, MAP and CI.

Lung QL increased from a baseline value of 4.3±0.6 to 8.3±1.8 ml/30 min (p<0.05) 30 minutes after ischemia (Figure 2). The L/P protein ratio was unchanged from 0.6±0.03 and the lymph protein clearance increased from 2.6±0.4 to 4.6±0.8 ml/30 min (p<0.05) (Figure 2). This occurred while the PAWP was unchanged at 4±1 mm Hg. Also unaffected were blood gases, MAP and CI.

Untreated Ischemic Controls With Increased Left Atrial Pressure

Left atrial balloon inflation led to mild but significant decreases in MAP and CI (Table 1). There

Figure 2. During reperfusion QL rose, the lymph-to-plasma (L/P) protein ratio was unchanged, and lymph protein clearance increased. These changes are consistent with increased pulmonary microvascular permeability. OKY 046 alone or combined with ibuprofen as well as SQ 29,548 prevented the rises in lung lymph flow (QL) and lymph protein clearance. Asterisks and daggers refer to significance relative to baseline and between groups, respectively.

The white blood cell count fell during reperfusion in each of the untreated ischemic sheep. The average decline during the first hour of reperfusion was from 6,853±1,149 to 3,796±874/mm³ (p<0.01). After the second hour, white blood cell count had returned to 6,583±1,749/mm³. Platelet counts were unchanged by ischemia and reperfusion from their baseline values of 305,000±106,000/mm³. Histological examination of the lungs revealed accumulations of polymorphonuclear leukocytes (PMN) within alveolar capillaries throughout the lung parenchyma (Figure 5A). Foci of proteinaceous exudate were present within alveolar spaces (Figure 5B). Neither platelet nor thrombin microaggregates were seen. Quantitative PMN counts in the lungs were 62±3 PMN/10 high powered fields (HPF). In contrast, the lungs of sham animals had 22±3 PMN/10 HPF (p<0.05) (Figure 5D).

Untreated Ischemic Controls With Increased Left Atrial Pressure

Left atrial balloon inflation led to mild but significant decreases in MAP and CI (Table 1). There
were baseline increases in MPAP from 12±1 to 20±2 mm Hg (p<0.05) and LAP from 4±1 to 19±2 mm Hg (p<0.05) (Table 1). There was a further transient increase in MPAP to 36±4 mm Hg after 1 minute of reperfusion while the LAP remained unchanged at 19±2 mm Hg. During balloon inflation there was a progressive rise in QL and fall in the L/P protein ratio (Figure 6). Before reperfusion QL had stabilized at a high flow, 4.3 times that of baseline QL (p<0.05) while the L/P protein ratio had decreased from 0.70±0.01 to 0.28±0.02 (p<0.05) (Figure 6). The calculated protein reflection coefficient (σd) was 0.72±0.02. During reperfusion, the L/P protein ratio increased to 0.49±0.05 (p<0.05) (Figure 6) while the σd decreased to 0.51±0.05 (p<0.05). These data indicate an increase in pulmonary microvascular permeability to protein.

**Treatment With OKY 046**

Pretreatment with the TX synthetase inhibitor led to baseline plasma i-TXB2 levels of 85±20 pg/ml, a value lower than in untreated sheep (p<0.05) (Figure 3). In contrast, plasma i-6-keto-PGF1α levels increased to 78±31 pg/ml, higher (p<0.05) than untreated ischemic control animals (Figure 4). The administration of OKY 046 did not affect systemic or pulmonary hemodynamics (Table 1, Figure 1). Compared with untreated ischemic controls, OKY 046 prevented, after tourniquet release, the increases in MPAP, Pmv, QL, lymph protein clearance, and i-TXB2 levels in both plasma and lung lymph (p<0.05 for all) (Figures 1–3). The circulating WBC counts during reperfusion were unchanged from baseline values of 5,329±1,179/mm³. Lung histology was normal (Figure 5C). The neutrophil count in alveolar capillaries was 24±6 PMN/10 HPF, a value lower than in untreated ischemic controls (p<0.05) and unchanged from sham-operated animals.
Treatment With OKY 046 and Ibuprofen

This combination did not alter baseline systemic or pulmonary hemodynamics (Table 1, Figure 1). Baseline plasma i-6-keto-PGF$_{1a}$ levels were 21±13 pg/ml, lower than in OKY 046 treated sheep ($p<0.05$) (Figure 4). Values for MPAP, Pmv, QL, L/P, lymph protein clearance, i-TXB$_2$ levels in plasma and lymph, WBC counts during ischemia and reperfusion as well as lung histology were similar to OKY 046 treated animals (Figure 1-3).

Treatment With SQ 29,548

Pretreatment with the TX receptor antagonist did not alter systemic or pulmonary hemodynamics (Table 1, Figures 1 and 2). The baseline concentration of plasma and lymph i-TXB$_2$ was similar to untreated ischemic animals. SQ 29,548 blunted the rise in MPAP at 1 minute of reperfusion from 10±1 to 13±1 mm Hg ($p<0.05$), a value lower than in untreated ischemic controls ($p<0.05$) (Figure 1). The Pmv was unchanged from 6±1 mm Hg. The
Figure 5. In untreated animals (n=7) neutrophil sequestration (arrows) was found in alveolar capillaries following 2 hours of limb ischemia (A). In addition, foci of proteinaceous exudates (arrows) were present in alveolar spaces (B). Pretreatment with the thromboxane synthetase inhibitor OKY 046 (C) (n=6) or the thromboxane receptor antagonist (n=5) prevented pulmonary leukosequestration, and maintained normal histology similar to lungs from sham animals (D) (n=3).

Reperfusion-induced changes in QL and lymph protein clearance were also prevented (Figure 2). Plasma levels of i-TXB$_2$ increased at 1 minute of reperfusion from 228±35 to 702±227 pg/ml ($p<0.05$) similar to untreated ischemic controls (Figure 3). Lung lymph i-TXB$_2$ concentration increased at 30 minutes in all animals from 395±87 to 789±273 pg/ml but this was not statistically significant (Figure 3). Plasma and lymph i-6-keto-PGF$_{1a}$ levels were unchanged from 14±8 and 43±5 pg/ml, respectively, values similar to those obtained in untreated ischemic controls. The circulating WBC count was unchanged during reperfusion from a baseline value of 8,825±1,393/mm$^3$ and lung histology was normal with 17±3 PMN/10 HPF.
FIGURE 6. Relative to baseline values (○), 3 hours of left atrial balloon inflation and increase in lung capillary filtration pressure (n=5) led to a 4.3-fold increase in lung lymph flow (QL) and a fall in the lymph-to-plasma (L/P) protein ratio (○). With reperfusion, under the same conditions of pressure independent L/P ratio, the ratio rose significantly indicating increased lung microvascular permeability. ●, lymph data at 90 minutes of reperfusion. The dagger indicates significant difference between the L/P protein ratio obtained during reperfusion to that achieved by balloon inflation alone.

Discussion
Reperfusion following 2 hours of lower torso ischemia led to pulmonary hypertension and leukocyte entrapment in the lungs. Further, QL doubled and remained elevated for the two monitored hours of reperfusion while the L/P protein ratio was not significantly altered. These changes are consistent with increased microvascular permeability to protein. However, interpretation of pulmonary lymph data where QL rises but the L/P ratio is unchanged is clouded by the possibility that the rise in QL can be caused by two factors in addition to permeability: an increase in vascular surface area or an increase in filtration pressure secondary to the pulmonary hypertension.13 These two alternative explanations where excluded by using sheep in which increases in surface area and in MPAP were achieved by inflation of a left atrial balloon. After 3 hours of balloon inflation and before reperfusion, an L/P protein ratio of 0.28, which is considered filtration independent,13,19 was achieved. Reperfusion then led to increases in the L/P ratio and a decline in σd, documenting increased lung microvascular permeability. The pulmonary vascular barrier in this preparation does not appear to be affected by the slight decreases in MAP and CI since the L/P ratio declines rapidly to a pressure independent value that is accepted as reflecting normal barrier function under the influence of maximal surface area and high filtration pressure. Finally, since MAP and CI were not further altered during reperfusion the significant increase in L/P during this period can only be attributed to the effects of ischemia and reperfusion.

Tourniquet release led to increases in plasma and lung lymph i-TXB2 levels. These levels are much higher than anticipated from the use of other methodology, such as measuring urinary dinor TXB21 and therefore may not reflect an accurate assay of TXB2 levels. The overestimate is most likely secondary to the sampling process that activates platelets and WBC and leads to artifactual TXA2 production. However, the relative changes in the immunoreactive TXB2 concentrations likely reflect the same magnitude of change in endogenous i-TXB2 synthesis.22

Thromboxane generation following ischemia and reperfusion has been demonstrated in various other clinical and experimental settings.5-7,23,24 The site of TXA2 synthesis is not clear. Although platelets are known to be a potentially rich source of TX,3 previous data in humans and dogs indicate that ischemia does not activate platelet synthesis.7 Recent experiments indicate that neutrophils may play a role directly or indirectly in causing the generation of TX in the ischemic setting.25 Thus, rendering sheep neutropenic prevents the rise in plasma i-TXB2 during reperfusion. Extravascular sources of TXA2 are suggested by the findings of high concen-
tations of i-TXB₂ in popliteal lymph from the reperfused ischemic limb as well as high and sustained i-TXB₂ levels in lung lymph. In addition, after aortic cross-clamping during aneurysm surgery, the observation that systemic arterial i-TXB₂ levels are higher than levels in pulmonary arterial blood₂³ substantiates a pulmonary site of synthesis. The entrapped neutrophils in the lungs may be the direct source of TX, or they may stimulate the generation of TX from lung tissue by releasing mediators such as oxygen free radicals or leukotriene B₄.²⁶

The vasoconstrictive effects of TX³⁻²² appear responsible for the brief pulmonary hypertension following ischemia. This is strongly supported by the observation of a temporal relation between the rise in plasma i-TXB₂ and increase in MPAP and secondly by the fact that inhibition of TX synthesis or blocking TX receptors prevents the pulmonary hypertension. Although this magnitude of pulmonary hypertension does not directly affect lung microvascular permeability¹³⁻¹⁹ it may enhance transvascular fluid filtration through an already damaged microvascular barrier. Thus, by mediating this pulmonary hypertension, TXA₂ indirectly affects lung transvascular fluid filtration.

TXA₂ is also believed to directly alter microvascular permeability. Thus, the rise in lung lymph i-TXB₂ concentration was at least temporarily related to an increase in lung permeability (Figure 7). A causal relationship is suggested by the observation that pretreating sheep with the TX synthetase inhibitor or the TX receptor antagonist prevented the reperfusion-induced increase in lymph flow and lymph protein clearance. Since the pulmonary hemodynamic changes that could affect lung capillary filtration pressure and surface area were also prevented by TX blockade, that is, the MAP and Pmv remained unchanged during reperfusion, it is likely that permeability was unaltered in these treatment groups. The possibility that the action of OKY 046 in modifying permeability was due to its ability to redirect endoperoxide precursors to increase prostacyclin synthesis was partially excluded by the combined treatment with OKY 046 and ibuprofen. Interestingly, although ibuprofen inhibited the OKY 046-induced increase in i-6-keto-PGF₁₀ levels it did not affect the baseline levels of this prostanoïd. A downward trend occurred only after 4 hours of ibuprofen administration (Figure 4). The combination therapy was as effective as OKY 046 alone in preventing the pulmonary hypertension and altered permeability after ischemia. Nevertheless, the possibility that OKY 046 exerts its effect by other actions such as blocking oxygen free radicals generation cannot be excluded. The same argument holds true for ibuprofen, which has been reported to exert effects in addition to inhibition of the cyclooxygenase pathway.¹¹⁻¹⁸ This could have accounted for some of the protective effects of the combination, OKY 046 and ibuprofen. However, the protection provided by the TX receptor antagonist strongly suggests that TXA₂ mediates increased lung permeability following lower torso ischemia.

TX may modulate permeability in two ways. This prostanoïd has been observed to directly modulate the microvascular barrier by altering endothelial cell architecture and cytoskeleton.²⁷ The mechanism is by the disassembly of actin microfilaments. These elements appear to regulate endothelial cell motility, structural relations to adjacent cells, and barrier function.²⁸ Agents, such as phalloidin, that promote the cytoskeleton by microfilament assembly, enhance barrier function by tightening of interendothelial junctions.²⁹ TX, as well as other permeability-promoting agents such as histamine and cytochalanin B²⁸ lead to disassembly and disruption of cytoskeletal actin microfilaments. This is associated with widening of interendothelial tight junctions and increased permeability to protein. In vivo studies in WBC-free preparations document that TX mediates nicotine- and α-thrombin-induced lung permeability.³⁰⁻³¹

TXA₂ may affect microvascular permeability indirectly through interaction with neutrophils. Thus, pretreatment with a TX synthetase inhibitor prevents ischemia-induced leukopenia and pulmonary leukosequestration.³² The TX-receptor antagonist is also effective in preventing these events.

The mechanism whereby TX induces leukosequestration is not certain. In the first place, TX can lead to increased PMN-endothelial interaction and adhesion.³³ Secondly, TX enhances PMN diapedesis through an endothelial cell monolayer,³³ presumably by widening interendothelial junctions. In any case, activation of these neutrophils sequestered in the lungs allows a number of toxic agents to be released, which may increase permeability. These agents include oxygen free radicals, lysosomal enzymes and arachidonic acid metabolites.²⁶

The simple finding of an elevation in plasma levels of TX does not document its role in permeability. Thus, in some experimental settings such as bacteremia³⁴ and endotoxemia³⁵ in which increased plasma TX concentrations are found, other mediators of permeability are of paramount importance. In other settings, inhibition of TX synthesis prevents increased microvascular permeability. This is true of the lungs of sheep following complement activation³⁶ and lung microembolization³⁷; in rat skin after a local burn³⁸ and in dog lungs following acid aspiration.³⁹

TXA₂ cannot account for the entire ischemia-induced sequence of injury since therapies directed at other mediators such as lipoxygenase products and oxygen free radicals effectively limit lung permeability.⁴⁰ Since the lungs appear to be a major site of TX synthesis, we postulate that tissue ischemia stimulates the local or pulmonary release of other inflammatory mediators such as lipoxygenase products and/or oxygen free radicals, which then
lead to TX synthesis in the lungs. These agents probably induce neutrophil lung entrapment but require TX to complete this activity.

Finally, the possibility that the pulmonary sequelae of limb ischemia are secondary to lung microembolization and thrombin activation is unlikely since neither platelet aggregates nor thrombi were seen in the lung microvasculature. Further, in distinction to sheep infused with thrombin who developed thrombocytopenia, this phenomenon was not observed during ischemia. In addition, the pulmonary hypertension and increased QL seen with thrombin was only attenuated and not prevented with thromboxane inhibitors.37

In summary, perfusion following limb ischemia leads to i-TX synthesis and respiratory dysfunction. These data suggest that the pulmonary hypertension, lung leukosequestration, and increased pulmonary microvascular permeability are mediated by TX.

References


KEY WORDS • thromboxane • ischemia • reperfusion • pulmonary permeability
Thromboxane A2 mediates increased pulmonary microvascular permeability following limb ischemia.

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*Circ Res.* 1989;64:1178-1189
doi: 10.1161/01.RES.64.6.1178

*Circulation Research* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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