Thromboxane A_2 Moderates Permeability after Limb Ischemia

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Reperfusion after limb ischemia results in muscle edema as well as excess secretion of thromboxane A_2 (TxA_2), an agent associated with permeability increase in other settings. This study tests whether TxA_2 moderates the permeability following limb ischemia. A tourniquet inflated to 300 mmHg was applied for 2 hours around the hind limb of four groups of dogs. In untreated animals (N = 25), 2 hours following tourniquet release, plasma TxB_2 values rose from 320 pg/ml to 2416 pg/ml ($p < 0.001$), and popliteal lymph values rose from 378 pg/ml to 1046 pg/ml ($p < 0.001$). Platelet TxB_2 was unaltered and plasma 6-keto-$PGF_{1\alpha}$ levels did not vary. Following ischemia, lymph flow ($\dot{Q}_L$) increased from 0.07 to 0.37 ml/h ($p < 0.05$), while the lymph/plasma (L/P) protein ratio was unchanged at 0.41. These measurements indicate increased permeability since increase in hydrostatic pressure in a second group by tourniquet inflation to 50 mmHg (N = 7) led to a rise in $\dot{Q}_L$ from 0.07 to 0.22 ml/h, but a fall in the L/P ratio to 0.32, a value lower than the ischemic group ($p < 0.05$). Pretreatment with the imidazole derivative ketoconazole (N = 11) reduced platelet Tx synthesis from 42 ng to 2 ng/10^9 platelets, but lymph TxB_2 levels rose to 1703 pg/ml after ischemia, indicating an extravascular or vessel wall site of synthesis not inhibited by ketoconazole. Pretreatment with a lower molecular weight imidazole derivative OKY 046 (N = 9) inhibited all Tx synthesis after ischemia. Prior to tourniquet inflation, both OKY 046 and ketoconazole lowered plasma TxB_2 levels as well as the L/P ratio ($p < 0.05$). After ischemia, OKY 046, but not ketoconazole, maintained the L/P ratio at 0.33, a value below that of untreated animals ($p < 0.05$). These results indicate that nonplatelet-derived TxA_2 modulates both baseline and ischemia-induced increases in microvascular permeability in the dog hind limb.

LIMB ISCHEMIA appears to be relatively well tolerated for periods of 2 hours.[1] A reversible sequence of morphologic alterations involving sarcoplasmic reticulum and mitochondria occurs during this period.[2] Ischemia of just 1 hour results in a significant fall of intracellular adenosine triphosphate (ATP) and creatinine phosphate. Anaerobic glycolysis leads to a progressive rise in lactate and fall in transmembrane potentials.[3] These biochemical events that occur during the ischemic period are prominent, but reversible. Functional changes following ischemia are also prominent. The most commonly described response is reperfusion hyperemia. Other events are also of consequence, although less apparent. Thus, following 2½ hours of tourniquet ischemia in monkeys, despite the observation that muscle metabolites and tissue gas tensions return to normal, muscle compartment pressures rise, suggesting edema.[4] Following 3 hours of ischemia, the first signs of capillary injury are observed and presage the appearance of permanent damage.[5]

Previous reports of limb ischemia have shown that periods as short as 10 minutes will lead to the synthesis of thromboxane (Tx) A_2 during the period of reperfusion.[6] This prostanoid has been associated with increased microvascular permeability in the setting of granulocyte activation,[7] acid aspiration,[8] and infusion of zymosan-activated plasma.[9] This study was designed to test the thesis that the permeability that accompanies limb ischemia is moderated by TxA_2.

Methods

Fifty-two dogs weighing 21 to 26 kg were anesthetized with pentobarbital, 25 mg/kg I.V. Supplementary doses of anesthesia were administered as necessary. A femoral venous catheter was inserted for blood sampling to measure total protein, TxB_2 and 6-keto-$PGF_{1\alpha}$. The popliteal lymphatic was then cannulated.

Five minutes following two intradermal injections, each 0.1 ml methylene blue dye, into the medial and lateral regions of the foot, a vertical incision was made in the lateral popliteal space. Afferent lymphatics, which characteristically run parallel to the lateral saphenous vein, were identified. An encircling ligature was tightened around the lymphatic, just before entry of the lymphatic into the popliteal lymph node to cause distention of the

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Supported in part by the National Institute of Health, Grants No. GM24891-06, HL16714-08; 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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Submitted for publication: March 1, 1985.

channel. Intubation was accomplished with a 20 to 22 gauge cannula. In 10 dogs, bilateral lymphatic cannulations were performed. Lymph free of blood was collected for the measure of flow ($\dot{Q}_L$), total protein,[10] TxB_2 and 6-keto-$PGF_{1\alpha}$.

Radioimmunoassay was used to measure TxB_2 and 6-keto-$PGF_{1\alpha}$, the stable hydrolysis products of TxA_2 and prostacyclin. Blood was collected in plastic syringes containing ethylene diamine tetraacetic acid (EDTA) and aspirin in a final concentration of 50 μg/ml. It was immediately centrifuged at 4 C, the plasma separated and stored at −20 C until assayed. The method that utilizes antibody supplied by Dr. L. Levine has been described.[11-13] In addition to plasma levels of TxB_2, platelet measurements were also performed. Centrifugation at 260 × g for 10 minutes was used to separate platelet rich plasma. Platelets were counted using phase microscopy and then subjected to freeze thaw lysis, as described by Utsunomiya et al.[14] Following centrifugation, the supernatant was assayed for TxB_2.

Initially, three groups of animals were studied. Animals were used in only one experimental protocol. A hind limb tourniquet was inflated to 300 mmHg in a group of untreated control dogs (N = 25), as well as in a second group pretreated with a Tx synthase inhibitor ketoconazole (kindly supplied by Janssen Pharmaceutica), a 1-substituted imidazole derivative with a molecular weight of 531 (N = 11). Ketoconazole was given by mouth in a dosage of 400 mg 10 hours and 2 hours before ischemia. In a third group (N = 7), the hind limb cuff was inflated to 50 mmHg to produce an increase in capillary hydrostatic pressure.

Ketoconazole was found not to prevent the ischemia-induced rise in lymph TxB_2. Therefore, along with additional control dogs (total N = 25), a fourth group (N = 9) of animals was studied. These were pretreated with another imidazole derivative OKY 046 (kindly supplied by Ono Pharmaceutica) with a lower molecular weight of 275. This drug was administered by bolus intravenous infusion, 1 mg/kg, 1 hour before ischemia.

The tourniquet was applied as high in the hind limb as possible after a 2 minute period of limb elevation to minimize the amount of retained blood. Lymph was collected over a 2-hour period before cuff inflation. During this time interval, a sufficient volume of lymph was available to assay proteins and prostanoids. Following cuff deflation lymph was collected for another 2 hours and the experiment ended.

Results are presented as the mean ± standard error. Differences between means were tested by an analysis of variance, paired, and nonpaired t- tests. 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 prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW publication No. (NIH) 78-23, revised 1978).

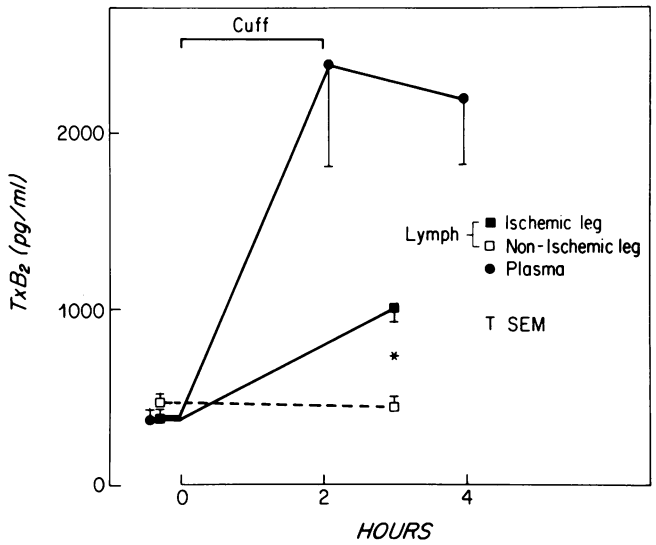

FIG. 1. Inflation of a tourniquet for 2 hours to 300 mmHg about the hind limb, led to a rise in plasma TxB_2 levels within 5 minutes after cuff deflation ($p < 0.05$). TxB_2 concentrations remained elevated for the next 2 hours. TxB_2 concentration in lymph collected for the 2 hour period after cuff deflation was higher in the ischemic than nonischemic limb. The latter value remained unchanged from baseline. An asterisk indicates $p < 0.05$ between the limbs.

Results

Reperfusion for 5 minutes, after 2 hours of tourniquet ischemia resulted in a significant increase in plasma TxB_2 concentration from baseline value of 320 ± 50 pg/ml to 2416 ± 563 pg/ml. These levels remained elevated for the 2-hour period of postischemic monitoring (Fig. 1). Lymph TxB_2 concentration in the ischemic limb also rose significantly from 378 ± 42 pg/ml to 1046 ± 108 pg/ml (Fig. 1). The latter values were lower than TxB_2 in plasma ($p < 0.05$). There were no changes in lymph TxB_2 levels in the nonischemic limb. Concentration of 6-keto-$PGF_{1\alpha}$ was unchanged from an undetectable baseline level in plasma and 38 ± 4 pg/ml in lymph.

Pretreatment with ketoconazole lowered baseline plasma TxB_2 levels compared to untreated dogs ($p < 0.025$), but did not prevent the significant rise in plasma TxB_2 following ischemia (Fig. 2). This was noted despite the ability of ketoconazole substantially to reduce platelet TxB_2 concentration from 42 ± 8 ng/10^9 platelets to 2.2 ± 1.5 ng/10^9 platelets (Fig. 3). A parenchymal source in the limb for the increased plasma TxB_2 is indicated by the large rise in lymph TxB_2 from 342 ± 36 pg/ml to 1703 ± 340 pg/ml ($p < 0.05$) (Fig. 4).

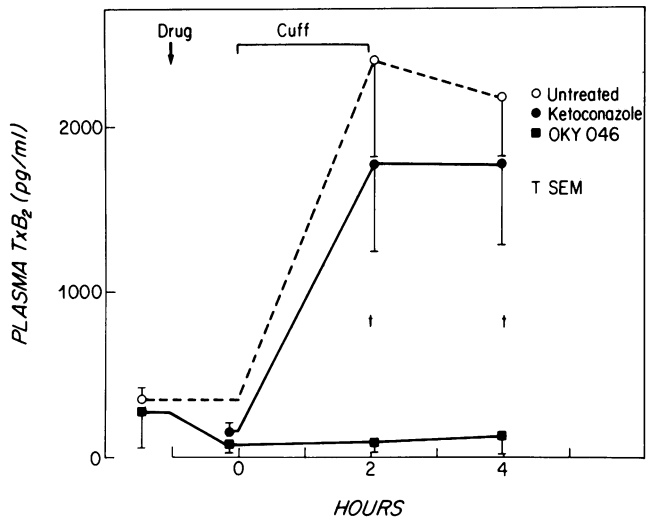

FIG. 2. Prior to cuff inflation both ketoconazole and OKY 046 led to significant reductions in plasma TxB_2 concentration relative to the untreated group ($p < 0.05$). In the case of OKY 046, the reduction in plasma TxB_2 levels was significant when compared to levels of TxB_2 measured before drug infusion. Following cuff inflation, only OKY 046 led to reduced TxB_2 levels relative to untreated animals. A cross indicates $p < 0.05$ between OKY 046 and untreated control animals. Data from the untreated animals are repeated from Figure 1.

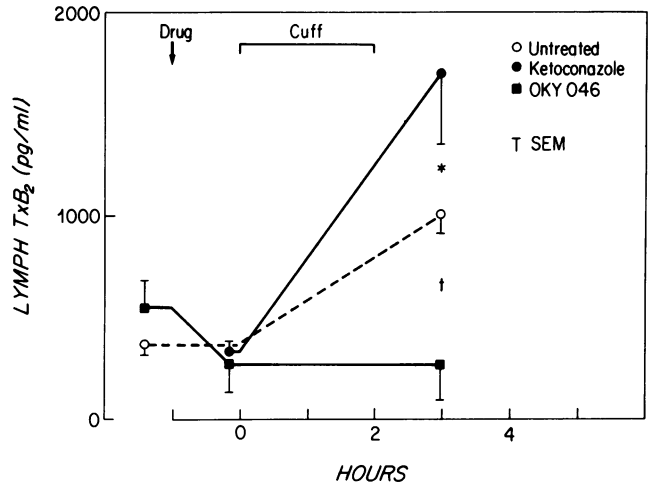

FIG. 4. Ketoconazole did not prevent a rise in lymph TxB_2 levels after ischemia. Indeed, for unexplained reasons, the rise in TxB_2 levels was greater than in untreated ischemic animals. In contrast, OKY 046 caused a fall in baseline levels of TxB_2 in lymph ($p < 0.05$) and maintained these levels below those in the untreated group. The asterisk and cross indicate $p < 0.05$ comparing ketoconazole and OKY 046 pretreated animals to the untreated group. The latter data are duplicated from Figure 1.

Pretreatment of dogs with OKY 046 led to a lowering of baseline plasma TxB_2 concentration from 301 ± 213 pg/ml to 62 ± 36 pg/ml ($p < 0.05$). In contrast to ketoconazole-pretreated animals, this drug effectively limited the ischemia induced increase in plasma and lymph TxB_2 concentration (Figs. 2 and 4).

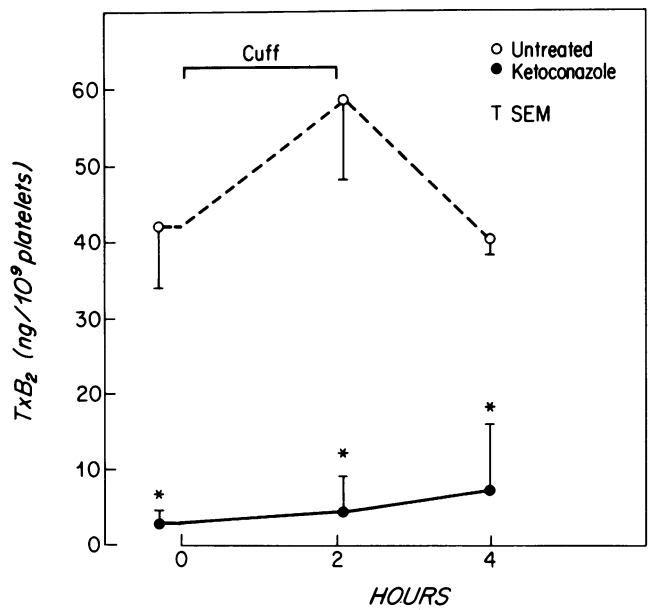

FIG. 3. In untreated animals, deflation of the cuff led to a nonsignificant rise in platelet levels of TxB_2. Ketoconazole inhibited platelet Tx synthesis before and after ischemia. An asterisk indicates $p < 0.05$ between groups.

Cuff inflation to 50 mmHg did not stimulate prostanoid synthesis. Plasma TxB_2 concentrations were unchanged from baseline values of 388 ± 73 pg/ml and 6-keto-$PGF_{1\alpha}$ levels remained undetectable. Lymph TxB_2 levels fell from 685 ± 161 pg/ml to 348 ± 64 pg/ml.

During venous occlusion by cuff inflation to 50 mmHg, there was a significant increase in $\dot{Q}_L$ from 0.07 ± 0.01 ml/h to 0.22 ± 0.06 ml/h. This was associated with a fall in the lymph-to-plasma (L/P) protein ratio from 0.43 ± 0.06 to 0.32 ± 0.07 ($p < 0.05$). Following tourniquet ischemia, $\dot{Q}_L$ rose from 0.07 ± 0.01 ml/h to 0.37 ± 0.07 ($p < 0.05$), but the L/P ratio was unchanged from 0.41 ± 0.06 (Figs. 5 and 6).

Pretreatment with ketoconazole or OKY 046 led to significant declines in baseline L/P ratios with no changes in $\dot{Q}_L$ (Figs. 5 and 6). Following 2 hours of ischemia, $\dot{Q}_L$ rose in all groups, while the L/P ratio in the OKY 046, but not ketoconazole, group remained significantly below the ratio in untreated animals (Figs. 5 and 6).

Discussion

Reperfusion following 2 hours of tourniquet ischemia as well as simple venous occlusion leads to increases in $\dot{Q}_L$. The fact that the L/P ratio did not fall with ischemia but did fall with a rise in capillary hydrostatic pressure indicates that there was an enhanced flux of protein or increase in microvascular permeability in the former state. Pretreatment with the Tx synthase inhibitor OKY 046 prevented ischemia-induced increases in plasma and lymph TxB_2 concentration. OKY 046 did not inhibit the

rise in $\dot{Q}_L$, but did lower the L/P ratio below that observed in untreated ischemic animals to a value similar to that noted following venous occlusion (Fig. 6).

Ketoconazole was observed to lower platelet levels of TxB_2 before and after ischemia. Despite this action of ketoconazole on platelets, ischemia led to increases in TxB_2 concentrations in plasma and lymph to levels even higher than those of untreated dogs demonstrating that the site of Tx synthesis is other than platelets. A number of cells have the capacity to synthesize TxA_2, including white blood cells,[7] endothelial cells,[15] fibroblasts,[16] and perhaps other parenchymal cells.[17] The ability of ketoconazole to inhibit platelet Tx synthesis suggests that it may also come into contact with, and act on, white blood cells and endothelial cells, although data to substantiate this postulate is not available. However, ketoconazole's molecular size, and poor solubility except in acid, may have limited its penetrance into the extravascular space and therefore limited its action in reducing ischemia induced Tx synthesis in the parenchyma of the limb.

The majority of the TxB_2 assayed in lymph from the reperfused ischemic limb is likely to have been locally synthesized by extravascular parenchyma. The failure to observe a rise in TxB_2 levels in lymph from the nonischemic limb suggests that plasma TxB_2, even in high concentration, cannot diffuse through a normal microvasculature into the interstitial space and appear in lymph. However, it remains possible that the increased permeability of the ischemic limb provides a unique setting in which TxB_2 can diffuse into the interstitium and account at least in part for the rise in lymph TxB_2 concentration.

Reperfusion following prolonged ischemia stimulates formation of select oxygenation products of arachidonic acid. This presumably relates to differential cell sensitivity to ischemia. Thus, levels of prostacyclin were unchanged after 2 hours of ischemia, indicating that endothelial synthesis was not stimulated. Similar findings have been reported in other settings such as 30 minutes of abdominal aortic clamping and 45 minutes of renal pedical clamping.[18,19] However, shorter periods of cuff inflation about a limb, less than 5 minutes, have been reported to result in the appearance of vasodilating prostaglandins which have functional importance. Thus, pretreatment with cyclo-oxygenase inhibitors limited the period of reactive hyperemia following several minutes of ischemia.[20]

One hour following a 10 minute period of tourniquet ischemia applied to the arm in man, plasma TxB_2 levels, which had risen two-fold, had returned to baseline. In contrast, the present data show that a 2-hour ischemic stimulus results in levels of plasma TxB_2 that remain elevated as long as 2 hours following reperfusion (Fig. 1). This implies a continued stimulus to Tx synthesis. Studies reported by Miller et al. of ischemic periods of 2½ hours suggest that following cuff deflation the ischemic stimulus

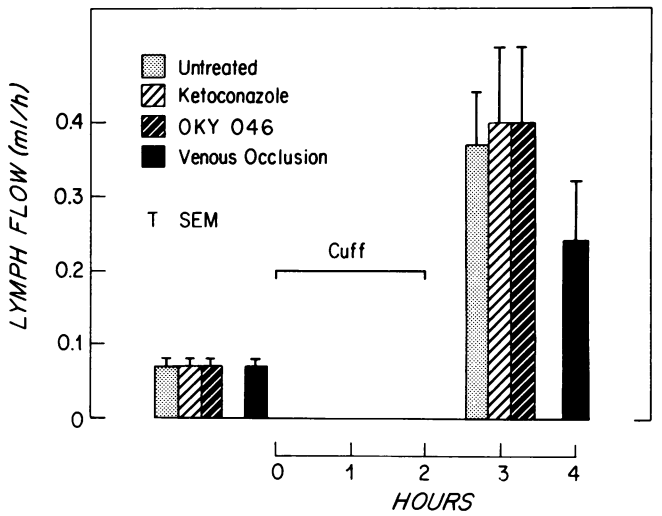

FIG. 5. Inhibition of Tx synthase with ketoconazole or OKY 046 did not influence baseline lymph flow, nor did these drugs moderate the rise in lymph flow following ischemia. For comparison, lymph flow increase during venous occlusion is shown as a black bar. It should be noted that the venous occlusion group was not subjected to ischemia.

may persist, as a result of a nonuniform restoration of flow.[4]

Baseline reductions in the L/P ratio without changes in $\dot{Q}_L$ occurred following either ketoconazole or OKY 046 pretreatment (Figs. 5 and 6). This reduction in permeability to protein may be due to a basic modulation of endothelial cell architecture. Thus, endothelial cell

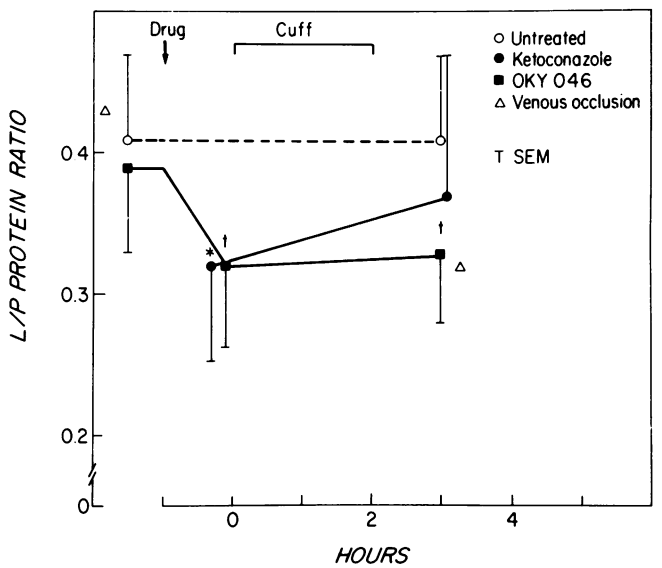

FIG. 6. Treatment with ketoconazole or OKY 046 led to significant reductions in pre-ischemic L/P ratios. Following ischemia, the L/P ratios remained lower than in untreated animals only in the OKY 046 group. The two triangles indicate the L/P ratio before and during venous occlusion. These latter animals were not subjected to ischemia. The asterisk and crosses indicate $p < 0.05$ between the ketoconazole, OKY 046 groups, and untreated animals.

motility and structural relationships to adjacent cells are thought to be determined at least in part by contractile proteins, especially actin microfilaments.[21,22] Agents that stimulate assembly of actin into stress fibers increase structural integrity. Stress fibers may contribute isometric contraction to cells, thereby maintaining apposition of plasma membranes allowing control of junctional integrity and permeability.[23] TxA_2 is thought to cause a disassembly of stress fibers, and thereby an increase in permeability. The drugs OKY 046 and ketoconazole block Tx synthase in platelets and probably also in endothelium and circulating white blood cells. In the nonstimulated state, this appears to be sufficient to reduce permeability (Fig. 6). However, after ischemic stimulation, the accentuated parenchymal contribution to TxA_2 synthesis is likely to bathe endothelium in concentrations of TxA_2 sufficient to increase their permeability, even though the endogenous Tx synthetic capability of endothelium is blocked.

Similar considerations regarding Tx synthesis and permeability apply in other organs. Thus, in normal sheep an infusion of imidazole will inhibit Tx synthesis and reduce permeability. Without altering lung lymph flow, the lung L/P ratio falls from 0.81 ± 0.01 to 0.74 ± 0.03 ($p < 0.05$), a value lower than 0.77 ± 0.03, the level reported in a group of untreated control sheep ($p < 0.03$).[9]

It is possible that the failure of limb ischemia to induce prostacyclin synthesis is an important determinant of the observed permeability increase. Thus, after renal ischemia, the prostacyclin/TxA_2 ratio, but not TxA_2 itself, appears to be of critical importance in determining kidney weight gain and tubular necrosis.[19] This is consistent with the thesis that stress fibers modulate permeability, that prostacyclin stimulates and TxA_2 inhibits assembly of these elements that determine cytoskeletal structure of endothelium.

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