Thromboxane Mediates Diapedesis After Ischemia by Activation of Neutrophil Adhesion Receptors Interacting With Basally Expressed Intercellular Adhesion Molecule–1

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Ischemic injury is characterized by neutrophil (PMN)–endothelial cell adhesion and diapedesis associated with thromboxane (TX) generation. Neutrophil–endothelial cell interaction is regulated in part by the leukocyte adhesion receptor CD 18 glycoprotein complex and the endothelial intercellular adhesion molecule–1 (ICAM-1). This study tests the role of TX in ischemia-induced diapedesis and evaluates whether the diapedesis is regulated by neutrophil or endothelial adhesion receptors. Plasma derived from rabbit hind limbs made ischemic for 3 hours (n=6) and reperfused for 10 minutes had increased levels of TXB₂ 3,450 pg/ml, which was higher than sham rabbit (n=6) values of 653 pg/ml (p<0.05). When introduced into abraded skin chambers placed on the dorsum of normal rabbits (n=6), this ischemic plasma induced 1,000 pg/ml of new TX synthesis and diapedesis of 1,235 PMN/mm³. The total TX concentration and PMN accumulations in blister fluid were correlated (r=0.88, p<0.05). In contrast, sham rabbit plasma induced no TX synthesis and diapedesis of only 77 PMN/mm³ (p<0.05). Administration of 50 ng/ml of authentic TXB₂ into blisters induced an accumulation of 453 PMN/mm³, which was higher than that in saline controls (18 PMN/mm³) (p<0.05). Pretreatment of normal rabbits used for the diapedesis assay (n=4) with the TX synthetase inhibitor OKY 046 (2 mg/kg/hr) limited ischemic plasma and authentic TXB₂ induced diapedesis to 142 and 76 PMN/mm³, respectively (both p<0.05). Pretreatment of other normal rabbits (n=4) with the TX receptor antagonist SQ 29,548 (2 mg/kg) by intravenous bolus and then 0.2 mg/kg/min also reduced diapedesis induced by ischemic plasma and authentic TXB₂ to 48 and 8 PMN/mm³ (both p<0.05). In yet other rabbits (n=9), antagonists and agonists were locally administered into the abraded skin chambers. With the saline control antagonist, ischemic plasma or authentic TXB₂ led to 927 and 404 PMN/mm³. The CD 18 monoclonal antibody R 15.7 lowered ischemic plasma and authentic TXB₂ induced diapedesis to 21 and 22 PMN/mm³, respectively (both p<0.05). R 15.7 was shown to diffuse from blister and progressively bind to circulating PMNs over the 3-hour monitoring period. Local administration of the anti–ICAM-1 antibody RR 1.1 also reduced diapedesis induced by ischemic plasma and authentic TXB₂ to 119 and 93 PMN/mm³, respectively (both p<0.05). In contrast, local application of the protein synthesis inhibitor actinomycin D (3 ng) was without inhibitory effect, suggesting a lack of synthesis of new endothelial adhesion proteins. The data indicate that TX mediates diapedesis after ischemia through regulation of neutrophil but not endothelial cell adhesion receptors. (Circulation Research 1991;68:1013–1019)

Inhibition of TX synthesis prevents neutrophil accumulation and protects the vascular barrier. Neutrophil adhesion is probably a key step in any neutrophil-mediated injury. The local injury is characterized by accumulation of polymorphonuclear leukocytes (PMNs) in the microcirculation associated with increased permeability.

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1 Ischemia and reperfusion lead to thromboxane (TX)- and neutrophil-dependent injury. The local injury is characterized by accumulation of polymorphonuclear leukocytes (PMNs) in the microcirculation associated with increased permeability.
The CD 18 complex consists of three α subunits: CD 11a, CD 11b, and CD 11c, each linked to CD 18, the common β subunit. CD 11a and CD 11b, but not CD 11c mediate early adhesion induced by chemoattractants in unstimulated endothelium. In contrast, CD 11c mediates delayed adhesion to endothelium 24 hours after its activation with cytokines. The CD 11a is basally expressed in the plasma membrane and is not upregulated by stimulation. However, chemoattractants such as leukotriene (LT) B4 or formylmethionyl-leucyl-phenylalanine (FMLP) increase the function of CD 11a and CD 11b, perhaps by inducing conformational changes. In addition, these chemoattractants upregulate CD 11b from intracellular granules, an event associated with increased adhesion. Finally, the function of each α subunit can be antagonized by CD 18 mAbs directed to the common β subunit.

The endothelial determinants of adhesion consist of the intercellular adhesion molecule-1 (ICAM-1) and the endothelial leukocyte adhesion molecule-1 (ELAM-1). The former serves as the ligand for CD 11a and CD 11b, but the receptor for ELAM-1 on PMN is unknown. It has been shown that it is not the CD 18 complex. The endothelial ligand for CD 11c is also unknown.

Sufficient evidence has accumulated to suggest that there are at least two major pathways for PMN–endothelial cell adhesion and diapedesis. The first is rapidly invoked by PMN stimulation with chemoattractants such as LTB4, C5a, or FMLP and is associated with increased activity of the CD 18 complex but not with increased activity of endothelial cell adhesion molecules. The second pathway relates to endothelial activation with expression of ICAM-1/ELAM-1. This process requires cytokines and is dependent on protein synthesis. The mechanism by which neutrophils migrate into a site of inflammation without endothelial cell activation is not clear. Theoretically, it is possible that local diffusion of the chemoattractant from the abluminal to the luminal side of the microvasculature will activate marginating PMNs to increase CD 11/CD 18 activity and interact with basally expressed ICAM-1.

The observations that ischemia and reperfusion lead to the generation of TX in ischemic tissue as well as in plasma, that the TXA2 mimic and the TXA2 hydrolysis product TXB2 increased neutrophil adhesion and diapedesis, and that ischemic plasma was chemotactic resulted in the present study. Two hypotheses are examined: TXA2 and TXB2 mediate ischemia-induced diapedesis, and neutrophil and endothelial adhesion receptors are involved. The results indicate that TX is a mediator of neutrophil diapedesis through regulation of neutrophil but not endothelial cell adhesion receptors.

Materials and Methods

Thirty-six New Zealand White male rabbits weighing approximately 3 kg each were used. The rabbits were initially anesthetized with ketamine (35 mg/kg i.m.) and xylazine (5 mg/kg i.v.) and maintained with xylazine (2 mg/kg) every 30 minutes. Saline (0.3 ml/kg/hr) was infused through a carotid cannula placed aseptically by way of a small neck incision on the day of the experiment. All animals were placed on 37°C heating pads.

Hind Limb Ischemia and Reperfusion

Bilateral hind limb tourniquet ischemia was induced in the anesthetized rabbits for 3 hours. These rabbits were treated with saline (n=6) or OKY 046 (n=2) (2 mg/kg i.v.) 30 minutes before reperfusion. Before the completion of the ischemic period, the vena cava was ligated just above the iliac confluence. During the first 10 minutes of reperfusion, the venous return of both hind limbs (approximately 0.5 ml/min) was collected from the vena cava distal to its point of ligation and discarded. A similar volume of saline was simultaneously replaced by way of the carotid artery catheter. After this 10-minute washout, 10 ml of blood was collected for the next 20 minutes, a period during which perfused blood is known to contain high levels of eicosanoids. Samples of plasma were collected in cooled (4°C) heparinized syringes containing 0.07 M EDTA and centrifuged immediately at 4°C, 1,500g for 20 minutes (PR-2, International Equipment Co., Needham Heights, Mass.). Aliquots of 0.5 ml plasma were frozen at −20°C for later diapedesis or TXB2 assay.

Sham Ischemia

The preparation was the same for the ischemic group but without application of tourniquets (n=6). Plasma from the inferior vena cava of these animals was collected and used in the chemotactic and TXB2 assays.

Dermabrasion Skin Chambers

This diapedesis bioassay was based on the methods of Otani and Palder. Briefly, clear plastic chambers of 0.25-ml capacity (Rexhaus Corp, Westfield Industrial Park, Westfield, Mass.) were affixed to dermabraded areas of the backs of anesthetized rabbits (n=24). There were 18–25 blisters on each rabbit. Injections into the blisters were made with a 27-gauge needle. One antagonist/agonist combination was tested in six to 12 blisters per rabbit in three to six rabbits. After 3 hours fluid was withdrawn from the chambers, PMNs were counted with a hemocytometer, and TXB2 was assayed.

Preparation of Drugs

OKY 046. The anhydrous crystal (Ono Pharmaceuticals, Osaka, Japan) of this TX synthesis inhibitor was dissolved in 2 ml of normal saline. It was infused...
as a bolus of 2 mg/kg for 5 minutes, starting 30 minutes before ischemia or blister treatment and then being repeated every hour.

SQ 29,548. This compound (Squibb Pharmaceuticals, Princeton, N.J.), provided as a gift by Dr. M. Ogletree, competitively inhibits TXA$_2$ receptors as well as the precursor prostaglandin endoperoxide. It is unknown whether TXB$_2$ has receptors on the PMN plasma membrane or whether SQ 29,548 can block TXB$_2$ activity. SQ 29,548 is a highly selective inhibitor with weak antagonistic activity to prostaglandin F$_{1a}$ (PGF$_{1a}$) and prostaglandin D$_2$ (PGD$_2$). The drug was prepared by dissolving equimolar amounts with Tris buffer in 95% ethanol and then evaporating the ethanol with nitrogen. The Tris salt of the drug was then dissolved in distilled water. Administration of the antagonist started 30 minutes before blister treatment. It was given as 2.0 mg/kg i.v. bolus and was followed by a continuous infusion of 0.2 mg/kg/min throughout the experiment. This amount is greater than 10-fold that necessary to blunt the pulmonary hypertensive response from infusion of the TX mimic in vivo.

Authentic TXB$_2$. Authentic TXB$_2$ (Sigma Chemical Co., St. Louis) in a stock solution of 1 mg/ml was diluted with saline to a final concentration of 50 ng/ml.

R 15.7. This purified mAb (Boehringer Ingelheim Pharmaceuticals Inc., Ridgefield, Conn.) was kindly provided by Dr. Robert Rothlein. It recognizes an antigen on the common β subunit of CD 11a, CD 11b, and CD 11c and was prepared as a stock solution of 5 mg/ml. Flow cytometric analysis documented binding of R 15.7 to unstimulated rabbit neutrophils and enhanced binding to phorbol myristate acetate (10$^{-7}$ M)–stimulated ones. To be certain that the abraded skin chamber allowed flux of R 15.7 from the abluminal section to the intravascular compartment, we measured its binding to circulating neutrophils by the use of flow cytometry. In preliminary experiments, we found that maximum inhibition of PMN diapedesis into blisters in response to ischemic plasma or authentic TXB$_2$ was obtained with 2,000 μg/ml of R 15.7 injected locally into the dermabration blister chamber.

RR 1.1. This purified mAb (Boehringer Ingelheim Pharmaceuticals Inc.), which was also provided by Dr. Robert Rothlein, recognizes ICAM-1. RR 1.1 was prepared in a final concentration of 10 mg/ml. We found that maximum inhibition of PMN diapedesis into blisters in response to ischemic plasma or authentic TXB$_2$ was obtained with 2,000 μg/ml RR 1.1 given locally.

Nonspecific immunoglobulin G. The immunoglobulin G (IgG) isotype IgG$_1$ (Cappel, Organon Teknika Corp., West Chester, Pa.) (2,000 μg/ml) was given locally as a control antibody.

Actinomycin D. Actinomycin D (Sigma Chemical Co.) in a stock solution of 5 mg/5 ml was dissolved in phosphate-buffered saline to a final concentration of 0.12 μg/ml.

Tumor Necrosis Factor-α. Tumor necrosis factor-α (TNFα) (Amgen Biologicals, Thousand Oaks, Calif.) in a stock solution of 10$^7$ units/ml was dissolved in saline to a final concentration of 10$^6$ units/ml.

TXB$_2$ Assay

The concentration of this prostaglandin in plasma and blister fluid was measured with a double radioimmunoassay by the use of an antibody whose cross-reactivity with heterologous prostanooids was less than 1%.

Skin Capillary Blood Flow

To be certain that variations in the accumulation of neutrophils were not a result of alterations in blood flow, this was measured by Doppler laser (Laserflo Model BPM, No. 3, TSI Inc., St. Paul, Minn.). The Doppler signal produced an output proportional to blood flow expressed as milliliters per 100 g per minute.

Flow Cytometry

To detect flux of R 15.7 from the blisters into the local circulation, we created 30 blisters into which ischemic plasma and 2,000 μg/ml mAb was introduced. Flow cytometry was performed with whole blood in one rabbit before and after blister treatment with R 15.7. Blood samples were taken in a heparinized syringe. The samples were washed two times with phosphate-buttered saline, centrifuged at 4,000g for 3 minutes (model 59, Fisher, Springfield, N.J.), and fluorescein isothiocyanate–IgG (Cappel) was added for 10 minutes at 4°C. Red cells were lysed with an immunolyzing reagent (Coulter Corp., Hialeah, Fla.), and the leukocytes were fixed (Clone Kit, Coulter Corp.). Analysis was performed on an Ortho Diagnostics System 2151 Cytov fluorograph flow cytometer after electronic gating on a 3,000–5,000-neutrophil sample. Data were obtained as mean channel fluorescence converted to a linear scale. Fluorescence values of the baseline controls were expressed as arbitrary units after subtracting the fluorescence values of the negative controls (nonspecific matched isotype IgG$_1$ and fluorescein isothiocyanate–IgG).

Protocol

Rabbits used for the chemotactic assay (n=16) were given intravenous saline, OKY 046, or SQ 29,548. Their blisters were treated with sham plasma, ischemic plasma, saline, or authentic TXB$_2$. In other rabbits (n=9), antagonists and agonists were injected locally into the blisters. The agents were premixed before their introduction. Thus, 0.1 ml of the antagonists (saline, 200 μg R 15.7, 200 μg RR 1.1, 200 μg IgG control, or 0.012 μg actinomycin D) were mixed with 0.9 ml of the agonists (ischemic plasma, 50 ng of authentic TXB$_2$, or TNF-α 10$^6$ units). The total volume of 1 ml was divided into four aliquots and 0.25 ml was injected into each of four blisters. Two rabbits were included where saline as an antagonist.
control was added to the agonists. Similar values were obtained and pooled.

All data are expressed as mean±SEM. When analysis of variance showed significance between groups, further examination was done by a nonpaired Student's t test. Statistical significance was accepted at p<0.05.

Animals in this study were maintained in accordance with the guidelines of the Committee of Animals of Harvard Medical School and those prepared by the Committee of Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (Department of Health, Education, and Welfare Publication No. 78-23 [National Institutes of Health], revised 1978).

Results

Three hours of ischemia and 10 minutes of reperfusion increased TXB₂ levels to 3,450±467 pg/ml, which was higher than sham ischemic values of 653±105 pg/ml (both p<0.05). Introduction of ischemic plasma into blisters resulted after 3 hours in de novo TX generation, with concentrations of 4,550±219 pg/ml (Figure 1) associated with neutrophil accumulations of 1,235±58 PMN/mm³ (Figure 2). These values were higher than sham plasma values, in which there was a slight, nonsignificant increase in TXB₂ to 727±97 pg/ml and accumulation of 77±6 PMN/mm³ (both p<0.05). A correlation was noted between the total concentration of TXB₂ and PMN accumulations in the blisters treated with ischemic plasma (Figure 3). Administration of authentic TXB₂ into blisters induced diapedesis of 453±51 PMN/mm³, which was higher than saline values of 18±3 PMN/mm³ (p<0.05, Figure 2).

Pretreatment of ischemic animals with intravenous OKY 046 30 minutes before reperfusion prevented a rise in plasma TXB₂ (425±105 pg/ml). However, this plasma was still chemotactic and induced diapedesis of 550±50 PMN/mm³ (p<0.05 relative to ischemic plasma). Pretreatment of nonischemic animals with intravenous OKY 406 limited ischemic plasma--and authentic TXB₂--induced diapedesis to 142±18 and 76±5 PMN/mm³, respectively (p<0.05). Pretreatment of other rabbits with SQ 29,548 reduced diapedesis induced by ischemic plasma and authentic TXB₂ even further to 48±7 and 8±3 PMN/mm³, respectively (p<0.05, Figure 4).

Introduction of ischemic plasma or authentic TXB₂ into the blister together with antagonists resulted in accumulations of 927±67 and 404±36 PMN/mm³, respectively, with saline as the antagonist control. These values were similar to those obtained when IgG control was added to ischemic plasma (940±60 PMN/mm³) and authentic TXB₂ (490±30 PMN/mm³); to lower values of 21±7 and 22±7 PMN/mm³ (p<0.05) with the anti-CD 18 mAb R 15.7, which progressively bound to circulating PMNs (Table 1); and to 119±17 and 93±11 PMN/mm³ (p<0.05) with
the anti–ICAM-1 mAb RR 1.1. The R 15.7 values were significantly lower than RR 1.1 (p<0.05). In contrast, treatment with actinomycin D did not alter PMN accumulations induced by ischemic plasma or authentic TXB$_2$ 96±186 (PMN/mm$^3$ and 400±53 PMN/mm$^3$, respectively) (Figure 4). In the same concentration, actinomycin D limited diapedesis induced by TNF-α 10$^4$ units in the blister from 15±8 PMN/mm$^3$ (with saline control) to 13±4 PMN/mm$^3$ (p<0.05).

OKY 046 and SQ 29,548 did not affect skin blood flow of the abraded skin region as measured by the Doppler method. Baseline blood flow, defined as 100±3%, was not significantly increased by OKY 046 (105±4%) or SQ 29,548 (101±3%) (both n=4) 1 hour after drug administration.

Discussion

The data of this study indicate that both TXA$_2$ and TXB$_2$ are cofactors in mediating ischemic plasma–induced diapedesis. This conclusion is based on several findings. First, TXB$_2$ levels in ischemic plasma were high and rose even higher when this plasma was introduced into abraded skin chambers at a time that diapedesis was prominent (Figures 1 and 2). Second, the total concentration of TXB$_2$ correlated closely with PMN accumulations in blister fluid (Figure 3). Third, inhibition of TX synthesis or TX receptors prevented ischemic plasma–induced diapedesis (Figure 4). Fourth, authentic TXB$_2$ induced diapedesis in the same dermabrasion bioassay setting (Figure 2). The following results also indicate that TXA$_2$ is the active agent modulating the action of TXB$_2$. Inhibition of TXA$_2$ synthesis prevented authentic TXB$_2$-induced diapedesis; there were increased levels of TXB$_2$ in the blister fluid after 3 hours (derived from neosynthesized TXA$_2$); and the TX receptor antagonist was more effective in preventing diapedesis than the TX synthetase inhibitor (Figure 4). This latter argument accepts the unfounded possibility that SQ 29,548 will inhibit TXB$_2$ receptors, thereby blocking TXB$_2$-stimulated TXA$_2$ synthesis.

Ischemic plasma contained approximately one tenth the concentration of TXB$_2$ that was added to the blisters. It was almost threefold more effective in inducing PMN accumulations, suggesting that other chemoattractants exist in ischemic plasma. Although abolishing TX synthesis, this plasma was still chemoattractive and resulted in accumulation of 550±50 PMN/mm$^3$ in the treatment of ischemic animals with OKY 046 before reperfusion. TXA$_2$ itself probably was not present in ischemic plasma because this agent has a brief 30-second half-life at 37°C. A previous study has shown that LTBr levels rise in ischemic plasma and significantly contribute to diapedesis. However, an LTBr receptor antagonist limited but did not abolish diapedesis induced by ischemic plasma in the same setting, again suggesting other chemoattractant action. The ability of TX inhibitors to prevent

Table 1. Transcutaneous Flux of R 15.7 Across Abraded Skin Shown by Progressive Labeling of Circulating Neutrophils

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<th>Minutes after blister treatment</th>
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diapedesis induced by ischemic plasma containing high levels of LTB₄ is not surprising. Previous observations have shown that inhibition of TX synthesis attenuated diapedesis induced by authentic LTB₄ placed in a blister.¹⁵

The cellular source of TX in the ischemic animal has not been defined. PMNs are probably important because rendering animals neutropenic before ischemia prevents the rise in TX.² However, sites of synthesis such as platelets and microvascular endothelium, as well as tissue macrophages and mast cells, are possible.²⁵ The origin of newly synthesized TX in the blister chamber is also not defined. We speculate that migrating PMNs are the source.

The mechanism of TXB₂-induced diapedesis is unknown. The biological activity of this TXA₂-hydrolysis product has been previously demonstrated.¹⁵ In the present study, we postulated that slow diffusion of TXB₂ from the extravascular space in the dermabrasion chambers into the skin microcirculation (the analogy of TX synthesized in ischemic tissue that gains access to the local circulation) may induce marginating PMNs to activate/upregulate their adhesion receptors, which in turn will promote local adhesion and diapedesis. These activated PMNs synthesize one or both of the eicosanoids LTB₄ and TXA₂ that consequently induce further synthesis by the same or adjacent cells. These eicosanoids function as autocoids that show extraordinary synergism in mediating local diapedesis.¹⁰,²²

The importance of adhesion receptors in ischemia-related diapedesis is evident from two specific observations: Locally administered anti–CD 18 mAb prevented PMN accumulations, and the anti–ICAM-1 mAb substantially, though not completely, prevented diapedesis (Figure 5). The reduction of diapedesis with the anti–ICAM-1 mAb RR 1.1 supports an involvement of CD 11a/CD 11b because ICAM-1 may serve as their ligand.²⁶ The lesser inhibition of diapedesis afforded by RR 1.1 than the anti–CD 18 mAb could be explained by a lower affinity of RR 1.1 to its target. The lesser effect of the anti–ICAM-1 mAb relative to the anti–CD 18 mAb probably indicates interaction of a non–ICAM-1 ligand with the CD 18 integrin complex. Indeed, CD 11b–mediated adhesion is only partially related to ICAM-1,²⁶ and CD 11b might interact with another endothelial ligand.⁶

The importance of the endothelial adhesion molecule ICAM-1 suggests that ischemic plasma and TX might have induced endothelial expression of this adhesion protein. The following findings indicate that in this setting TX increases activity of the neutrophil adhesion receptor CD 18, that this receptor interacts with basally expressed ICAM-1, and that de novo synthesis of ICAM-1 or another endothelial adhesion molecule (ELAM-1) is unimportant. First, TX and LTB₄ cannot induce ICAM-1 or ELAM-1 expression in vitro.²⁷ Second, the short period of reperfusion (10 minutes) before collection of ischemic plasma probably suggests that there had been sufficient time for cytokine synthesis. These agents are required for endothelial activation and are not stored.¹⁴ Furthermore, reversal of ischemic plasma–induced diapedesis by TX inhibition is inconsistent with cytokine action because other studies have also shown that the cytokine effect on endothelium is not modified by cyclooxygenase or lipooxygenase inhibition.²⁷,²⁸ Third, the protein synthesis inhibitor actinomycin D did not affect diapedesis induced by ischemic plasma or authentic TXB₂ (Figure 5), suggesting a lack of synthesis of new endothelial adhesion proteins.¹³ Thus, intravenous administration of actinomycin D in ischemic animals was without any inhibitory effect on PMN accumulation in response to ischemic plasma in dermabrasion chambers. Moreover, this protein synthesis inhibitor, when given intravenously in nonischemic animals, did not alter diapedesis induced by ischemic plasma.²² These results are consistent with our in vitro findings. Thus, PMN treated with TX mimicking demonstrated increased adhesion to an endothelial monolayer within minutes, an event blocked with an anti–CD 18 mAb.²⁹ These data do not deny the possibility that cytokines could be involved in later endothelial activation and recruitment of PMN in the intact animal. Lung leukosequestration after lower-torso ischemia occurs over several hours,³⁰ and the PMN accumulations after myocardial ischemia continue throughout a 24-hour period,³¹ time courses that are more in accord with cytokine activation of endothelial adhesion molecules rather than chemoattractant-induced PMN adhesion receptor expression.

In summary, the data of this study indicate that TX is a cofactor in mediating early neutrophil diapedesis in reperfused ischemic tissue by activation of neutrophil adhesion receptors (CD 18) that interact with basally expressed intercellular adhesion molecules (ICAM-1).

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