Myocardial Ischemia: Platelet and Thromboxane Concentrations in Cardiac Lymph and the Effects of Ibuprofen and Prostacyclin

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SUMMARY Blood platelets have been implicated in several mechanisms leading to and/or modifying myocardial ischemia. Cardiac lymph examination allows insight into the extracellular fluid that is in equilibrium with the capillary blood. In order to obtain an index of platelet activation during coronary artery events in the awake chronic animal, we wished to ascertain whether evaluation of cardiac lymph would detect changes in platelet activation resulting from a vascular occlusion. The study used conscious dogs in which cardiac lymph vessels had been previously cannulated by open-chest surgical protocol. The concentrations of immunoreactive thromboxane B₂ and platelet counts were assessed in the cardiac lymph during the control period, the 10–60 minute occlusions, and the reperfusion periods. The same protocols were effected on another series of dogs after infusion of ibuprofen or prostacyclin. Initially, immunoreactive thromboxane B₂ concentrations in the systemic blood and cardiac lymph were identical. A three-fold increase in immunoreactive thromboxane B₂ concentrations occurred in untreated animals and was accompanied by a fall in platelet count in the lymph. The infusion of ibuprofen or prostacyclin, which inhibit platelet aggregation by different mechanisms, prevented both the decrease in platelets and the increase in immunoreactive thromboxane B₂. In this study, intravascular events resulting from coronary occlusion invoke a rapid rise of immunoreactive thromboxane B₂ in the extravascular fluid. A decrease in platelet escape into the extravascular compartment is interpreted as a result of intravascular aggregation promoting decreased platelet numbers. Thus, examination of continuously flowing cardiac lymph allows rapid detection of intravascular activation of platelets in the awake animal in the absence of surgical trauma. (Circulation Research 1986;59:49-55)

KEY WORDS platelets • myocardial ischemia • thromboxane • ibuprofen • prostacyclin

According to current concepts, an intravascular event such as endothelial disruption or flow perturbation can promote platelet reactivity and thereby increase the production of thromboxane. Therefore, monitoring thromboxane production or platelet aggregation may be useful in evaluating the onset of vascular spasm and/or thrombosis in various models of ischemia. However, the continuous measurement of these parameters is technically difficult. Sampling by coronary sinus or distal coronary artery has only limited value. In each case, the presence of a chronically indwelling catheter may represent a vascular injury. Drawing blood through such a catheter at varying velocities and with alterations in catheter geometry, which result from changes in body position, clearly affects platelet activation. In addition, in relatively acute studies the implantation of such a foreign body within the vasculature, which is rich in platelets, can lead to variability in baseline values. Thus, direct study of platelet aggregation by measurement of immunoreactive thromboxane B₂ concentration has been limited to short-term experiments in open-chest, unconscious animals.

Our laboratory has developed a method of cannulating the cardiac lymph duct so that cardiac lymph can be continuously sampled in conscious dogs for periods of weeks. While others have demonstrated that blood elements are found in the cardiac lymph, their concentration is considerably lower than in plasma. In addition, the flow rate of cardiac lymph through the short cannula is very slow and virtually constant and continuous (thus requiring no special sampling procedure such as drawing blood into a syringe). These factors lessen potential sources of artifact and platelet activation associated with coronary sinus sampling in the chronic animal described above. We thus began the current study to evaluate whether coronary vascular events resulting from coronary occlusion would be reflected by changes in thromboxane concentrations in the cardiac lymph. Studies were performed in conscious animals to avoid various components of surgical trauma that are potent effectors of platelet activity. The animals were subjected to no medications other than those associated with the experiments and Talwin (pentazocine lactate), 0.5 mg/kg, to reduce pain during coronary occlusion. We examined the effects of coronary occlusion for 10–60 minutes, then the effects of reperfusion in the presence and absence of 1)
prostacyclin, an inhibitor of platelet aggregation that functions primarily by stimulating adenylate cyclase, resulting in an increase in cAMP, reduced platelet calcium concentration, and impaired reactivity and 2) ibuprofen, a potent cyclooxygenase inhibitor that prevents thromboxane formation, which is important in amplification of the platelet aggregatory response. The results suggest that platelet aggregation, subsequent to a coronary occlusion, results in marked changes in cardiac lymphatic thromboxane concentration that can be easily detected after a 10-minute occlusion. These changes are attended by a striking fall in cardiac lymph platelet numbers, suggesting that intravascular aggregation has lowered the platelet numbers and thereby reduced the quantity that escape across the capillary into the extracellular fluid. Both ibuprofen and prostacyclin completely inhibit this response. Thus, this is the first report of platelet aggregation changes in response to coronary occlusion in an awake unanesthetized animal. The results also suggest that the conscious animal with a cannulated cardiac lymph duct may be a useful model by which to assess platelet reactivity in ischemia.

Materials and Methods
Preparation of Materials
The preparation of conscious dogs with cardiac lymph ducts cannulated has been described previously. This study is based on 24 healthy, mongrel dogs of either sex weighing 12-24 kg. During a median thoracotomy, a hydraulically activated occluding device and a Doppler flow probe were secured around the circumflex coronary artery in animals prepared for ischemic studies. The occluding device was activated at will in the conscious animal to create partial or full occlusions for different time intervals. Cardiac lymph was collected before, during, and after coronary artery occlusions.

The cardiac lymph was collected in ethylenediaminetetraaceta-cetate and 20 μM indomethacin-treated 12 × 75 mm plastic tubes on ice. The samples were centrifuged at 8,000g for 10 minutes at 4°C, and the supernatant was decanted to a second tube. Each tube was immediately frozen on dry ice-acetone and stored at −70°C until analysis. Radioimmnoassay was performed by the method of Levy et al and alternately by the New England Nuclear thromboxane B2 (T2) RIA kit. The former method relies on 3H-thromboxane B2 and competitive binding of anti rabbit thromboxane B2 antiserum, precipitation with saturated ammonium sulfate, and liquid scintillation (LKB model 1217) counting of the supernatant after centrifugation; the latter method achieves separation of the antibody-antigen complexes from free antigen by precipitation of the antibody-bound tracer with polyethylene glycol in presence of carrier IgG and subsequent gamma counting (LKB model 1282) of the precipitate after centrifugation. Standard curves were generated in presence of an assay buffer containing 0.9% NaCl, 0.01 M EDTA, 0.3% bovine γ-globulin, 0.0005% Triton X-100, and 0.05% sodium azide in 50 mM phosphate buffer, pH 6.8. These immunoreactive thromboxane B2 concentrations will subsequently be referred to as *TXB2.

Platelets were monitored by gamma counting either 111In- or 3Cr-labelled platelets or by direct counting. The labelling of platelets followed the procedures specified by the "Panel on Diagnostic Application of Radioisotopes in Hematology" and Scheffel et al. Dog blood, 0.3-1 l, was collected in anticoagulant citrate platelet dextrose (ACD) sterile packs and centrifuged at 220g for 15 minutes. The platelet-rich plasma was acidified to pH 6.5-6.7 with 0.15 M citric acid and centrifuged at 1,000g for 15 minutes. The pellet was resuspended in 5 ml of platelet-poor plasma (PPP) and 500 μl of sodium chromate, 2Cr (>3000 Ci/g) or 11In-8-hydroxyquinoline was added for 30 minutes incubation at 25°C. At this time, 20 ml of PPP was added with subsequent centrifugation at 1000g for 15 minutes. This step was performed twice, with re-suspension in 5 ml PPP, and centrifuged at 150g for 5 minutes. The resultant supernatant contained the labelled platelets and was adjusted to final volumes ranging from 5 to 40 ml (50-500 μCi) with approximately 1 × 109 platelets/ml.

Platelet determination by visual enumeration was performed using the unopette (Becton Dickinson) in vitro diagnostic reagent system and reservoirs. A diluent of ammonium oxalate and Sorenson's phosphate buffer allowed preservation of the platelets while lysing the red blood cells. Platelets were subsequently counted in a Neubauer hemocytometer according to standard technique using phase-contrast microscopy at 400× magnification. This system compares favorably with the results using the Coulter counter (Coulter Electronics Inc) counting method. Specimens of cardiac lymph were obtained directly from the cardiac lymph cannulae via capillary pipettes. All measurements were made within 3 hours at room temperature.

Prostacyclin (Prostaglandin LI) (Upjohn Diagnostics) was made in 1 M Tris buffer at pH 9.4; it was diluted with normal saline at 0°C and immediately used at an infusion rate of 0.05 μg/kg/min. The left atrial infusion was begun during control periods at least 1 hour prior to the CFX occlusion-reperfusion protocol; the infusion was continued for at least 2 hours of reperfusion.

Ibuprofen was given at 12.5 mg/kg iv during a 5-minute injection and repeated at 1-hour intervals.

All control animals for drug studies were given vehicular controls at the appropriate rate and time.

Experimental Design
The first protocol involved collection of cardiac lymph and plasma in control states in the conscious dogs for determination of platelets and *TXB2 levels. This protocol was carried out with and without a bolus of labelled platelets injected via the femoral vein. The next protocol included CFX occlusion for 10, 20, 30, or 60 minutes with subsequent reperfusion. This was also done with or without labelled platelets injected 1 hour prior to occlusion. The last two protocols in-
volved either infusion of prostacyclin or ibuprofen starting at least 30 minutes prior to occlusion. Both drugs were continued for at least 2 hours after start of reperfusion.

During each protocol cardiac lymph was collected at 30-minute intervals with the exception of occlusion intervals, which lasted only 10 or 20 minutes. Systemic blood samples were taken from a short left atrial cannula exteriorized to the posterior thorax to avoid collecting blood from a chronically occluded vessel. Samples were then analyzed for platelet quantity and *TXB2 concentration.

Data Analysis

All values are expressed as the mean ± SEM, and the differences between the various parameters were determined by either paired or nonpaired t test; differences and correlations were considered significant at p < 0.05.

Results

The control count of platelets in each dog remained relatively constant either measured by the chromium labelling technique or the visual microscopic method. The control count of platelets varied from 8,000/mm3 to 45,000/mm3 in 28 dogs. Counts of leukocytes ranged from 50 to 500 mm3 and did not vary during the experimental paradigms herein reported. Platelet counts in blood varied from 220,000/mm3 to 560,000/mm3 and did not significantly vary within each dog during the course of the experiment. In the experiments in which labelled platelets were infused into the left atrium, there was an initial oscillation in numbers of platelets in the cardiac lymph and return to plateau level as shown for a representative dog in Figure 1 (Day 1); in this particular dog on the next day (Day 2), during anesthesia and an open-chest protocol, an injection of Evan’s Blue (0.1 ml) into the subepicardium created a sharp increase in numbers of platelets gaining entrance to the cardiac lymphatic space. This indicated that our method of monitoring the platelets was at a sensitivity sufficient to detect such platelet appearance after physical damage to a very small area. The average counts of platelets in cardiac lymph was 2–10% of those found in blood.

The concentration of *TXB2 in cardiac lymph during control, occlusion, and reperfusion is shown in Table 1. The concentration of *TXB2 in the lymph is the same as that in systemic plasma (Table 1 legend), demonstrating that the lymphatic thromboxane concentration was in equilibrium with the vascular space. With coronary occlusion of 10–60 minutes there is no significant change in *TXB2 concentration in cardiac lymph. However, immediately on the onset of reperfusion, regardless of the length of time of occlusion, the concentration and appearance of *TXB2 are markedly elevated (p < .001). It will be seen that the length of time of occlusion did not influence the *TXB2 appearance rate during reperfusion over this time course (Figure 2). *TXB2 concentrations in plasma were not significantly (p > .05) altered: control 725 ± 180 pg/min; occlusion 805 ± 195; and reperfusion 870 ± 210.

The amount of platelets and *TXB2 in cardiac lymph as a function of occlusion and reperfusion are shown in Figure 3A and 3B for a representative dog with a 20-minute occlusion–reperfusion protocol. Within the occlusion periods examined (10–60 minutes), the pattern shown here was consistent. There was no rise in *TXB2 concentration until reperfusion and the highest *TXB2 concentration was found in the first reperfusion sample.

Although this may appear to suggest that reperfusion induces *TXB2 formation, it is more likely a function of markedly reduced lymphatic drainage from the ischemic area during ischemia (see “Discussion”).
Note that the lymphatic platelet count decreases from control state and that the \( \text{TXB}_2 \) concentration is strikingly elevated on reperfusion. When either ibuprofen or prostacyclin is given prior to the occlusion–reperfusion protocols, the decrease in platelets is not seen (Figure 3B) and the concentration of \( \text{TXB}_2 \) is not increased from control to reperfusion. The data for all dogs are summarized in Table 2 (platelets) and Table 3 and Figure 2 \( \text{TXB}_2 \). Both ibuprofen and prostacyclin significantly \( (p < .05) \) increased platelet counts in cardiac lymph from control; subsequently, platelets did not significantly change with occlusion or reperfusion. In control experiments the vehicles (Tris diluted in saline to concentrations the same as used to dissolve PGI, or ethanol–saline used to dissolve ibuprofen) did not significantly alter the levels of thromboxane or platelets.

**Discussion**

Platelet activation and aggregation has been suggested as mechanistically important in promoting or altering the magnitude of angina pectoris and vasospasm, myocardial ischemia and necrosis, and ventricular arrhythmias. Platelets adhere to physically damaged endothelium and promote contractile activity presumably by releasing agents such as thromboxane \( \text{A}_2 \), which is synthesized from arachidonic acid by the enzyme cyclooxygenase and thromboxane synthetase. Thromboxane \( \text{A}_2 \) is immediately metabolized to \( \text{B}_2 \). In addition to direct physical damage of the endothelium, it is likely that platelets adhere to the endothelial membranes when ischemic injury results from reduced or no coronary blood flow. Preliminary studies by Coker et al showed that acute coronary artery ligations of 2, 10, and 30 minutes in dog promoted appearance of both \( \text{TXB}_2 \) and the metabolic product of prostacyclin (6-keto-PGF\(_{1\alpha}\)) into venous drainage from the ischemic region in acute animals. Bush et al showed in the distal coronary artery that thromboxane increased in a vessel that had a partial proximal constriction and cyclic reduction of coronary blood flow. This latter phenomenon may be associated with acute endothelial cell damage resulting from occlusive events and thrombus formation. In our studies, the first appearance of elevation in cardiac lymph \( \text{TXB}_2 \) occurs during the initial reperfusion period regardless of occlusion time. We have noted a similar phenomenon (ie, first appearance of elevated levels during initial reperfusion) with enzyme release from the myocardium (creatine kinase and phosphorylase) during the first

**FIGURE 2.** Peak cardiac lymph \( \text{TXB}_2 \) appearance rate under all experimental conditions. \( C \) = control, \( D \) = drug, \( O \) = occlusion, and \( R \) = reperfusion. \( * \) = 10-minute occlusion, \( \triangle \) = 20-minute occlusion, \( \Delta \) = 30-minute occlusion, and \( \diamond \) = 60-minute occlusion. I --- = mean ± SEM.

**FIGURE 3.** Cardiac lymph platelets (o) and \( \text{TXB}_2 \) (*) in 2 representative dogs: A. One which had no drug given prior to a 20-minute CFX occlusion followed by reperfusion. B. One which had ibuprofen IV prior to the 20-minute occlusion followed by reperfusion. The control (c) is at 0 time followed by either CFX occlusion (OCC) or ibuprofen (I) and then occlusion.
hour of occlusion. Lymph drainage (as well as coronary sinus drainage) from the microvascular distribution of an occluded artery is very low and represents a very small component of total flow during early ischemia. Reperfusion allows the formerly ischemic bed to be well represented in the lymph drainage.

The use of inhibitors prevented the immediate cardiac lymphatic \( \ast \text{TXB}_2 \) concentration elevation that was seen during reperfusion without the inhibitors. Simultaneously, these inhibitors prevented the decrease in platelets in cardiac lymph that was observed in their absence.

It has been reported that ibuprofen exerted its main beneficial effect on the ischemic myocardium by preventing leukocyte mobilization. The experiments presented here suggest that platelet aggregation is also altered by ibuprofen. The experimental protocol and time course of the two experiments do not allow dissection of the different findings. In another study prostacyclin prevented blockage of partially obstructed coronary arteries and this correlated to its inhibition of platelet aggregation.

From our studies the following are suggested:

1. There is a buildup of \( \ast \text{TXB}_2 \) in the blood vascular space and, by diffusion, in the interstitial space resulting from acute coronary occlusion.
2. The \( \ast \text{TXB}_2 \) production is primarily of platelet origin at the site of endothelial wall and vascular space obstruction and equilibrates with the interstitial space in the ischemic area where lymph drainage is low.
3. Reperfusion restores lymph flow to the area supplied by the formerly occluded coronary vessel and results in the prompt appearance of elevated \( \ast \text{TXB}_2 \) in the lymph.
4. The aggregation of platelets induced by the vascular occlusion (endothelial damage?) lowers platelet number distally and thereby decreases the number of platelets that escape to the extravascular space.
5. Antiplatelet agents prevent the \( \ast \text{TXB}_2 \) production and prevent the reduction in platelet number observed in the lymph from the formerly ischemic area.
6. The levels of thromboxane and platelets remain abnormal for several hours, suggesting that the acute occlusion may result in a chronic process (endothelial damage?).

Prostaglandin \( I_2 \) is synthesized by blood and lymphatic vessels, but \( \ast \text{TXB}_2 \) appears derived from extra lymphatic sources. Thromboxane \( A_2 \) is synthesized mainly by platelets after contact with subendothelial collagen of the vessel walls. Therefore, it seems tenable that the \( \ast \text{TXB}_2 \) is released from activated platelets attached to either blood or lymphatic vessel walls that have presented injured sites resulting from the ischemia.

It should be emphasized that platelets appear to be the major producers of thromboxane but not the sole producers. Polymorphonuclear neutrophils also have the potential for arachidonic acid metabolism with alteration in vascular permeability, stimulation of further leukocyte migration, and aggregation, which is most certainly involved in the inflammatory response in myocardial infarction. The possibility that these cells are involved in free radical formation and involvement in cell injury has also been suggested. The time course of neutrophil activity was reported for 90-minute occlusion periods followed by 24 hours of reperfusion, and this activity was inhibited by ibuprofen. In addition, the work of Engler has also demonstrated a role for leukocyte plugging after 1–5 hours of ischemia as a cause of “no reflow” phenomena. These studies did not measure leukocyte metabolites so that a direct comparison would be difficult even if the time courses were similar. However, in our studies using much shorter times of occlusion, reflow is not impaired; and, had it been impaired, the cardiac lymph flow from the “no reflow” area would not have been represented in the total lymph flow. The involvement

<table>
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<th>Table 2. Platelets in Cardiac Lymph</th>
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<td>No drug (n = 8)</td>
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<td>Control</td>
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<tr>
<td>Drug</td>
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<td>CFX occlusion</td>
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<td>Reperfusion</td>
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Platelets measured in cardiac lymph during control state before CFX occlusion; with ibuprofen or prostacyclin IV; during CFX occlusion; and during the first 2 hours of reperfusion. Values (× 10^9) are means ± SEM; n = 4 for each drug. There was one dog at each occlusion time of 10, 20, 30, and 60 minutes, respectively.

*Significantly different from control (p < 0.05).
†Not significantly different than pre-occlusion drug level (p > 0.1); control plus drug vehicle was not significantly (p > 0.1) different from control.

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<th>Table 3. Effect of Ibuprofen and Prostacyclin on Thromboxane B2 Levels in Cardiac Lymph</th>
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<td>pg/ml</td>
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<td>Ibuprofen</td>
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<td>Control</td>
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<td>Ibuprofen</td>
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<td>Occlusion (10–60 min)</td>
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<td>Prostacyclin</td>
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<td>Prostaglandin G12</td>
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<td>Occlusion (10–60 min)</td>
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<td>Reperfusion (1st 1 hour)</td>
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Means ± SEM are given for control state prior to drug, after infusing ibuprofen or prostacyclin, during occlusion, and during the first 1 hour of reperfusion.

Values are picograms per milliliter corrected for cardiac lymph flow and, hence, picograms per minute. Cardiac lymph flow averaged 2.04 ± 1.0 ml/hour in control state, decreased below control an average of 15% during occlusion, and increased above control an average of 18% during reperfusion. There was one dog at each occlusion time of 10, 20, 30, and 60 minutes in presence of ibuprofen (n = 4) and another series with prostacyclin (n = 4). No significant differences (p > 0.1) comparing control to control plus drug vehicle, occlusion, or reperfusion states.
of leukocytes in modifying the ischemic injury resulting from the "shorter occlusions" used in this study with changes seen at 10 and 20 minutes has not been reported. It is important to realize that occlusions of 10 and 15 minutes do not usually result in tissue necrosis. As described above, in more chronic models with extensive cellular damage, leukocyte aggregation may become very prominent. Our laboratory has recently shown significant complement fixation and leukotaxis after a 45-minute occlusion.

Thus, while leukocytes may contribute to some extent to the experimental findings described herein, the data suggest a primary role for platelets. The leukocyte counts in the cardiac lymph were unchanged by the experimental paradigms. This is in contrast to the dramatic changes in platelet number, which correlate inversely with \( \text{TXB}_2 \) concentration in the cardiac lymph. The effects of the cyclooxygenase inhibitor, ibuprofen, do not allow distinction between a leukocyte or platelet origin of the increased concentration of \( \text{TXB}_2 \). However, the extremely potent effects of prostacyclin also tends to favor a platelet origin for a major part of the generated \( \text{TXB}_2 \) since its affect as a potent stimulator of adenylyl cyclase markedly alters platelet activation. While leukocyte metabolism is also altered by prostacyclin in a way that has been linked to cyclic adenosine monophosphate levels, the effect on leukocytes is 1) not as dramatic, 2) limited to effects on adherence and chemokinesis, and 3) appears to be mitigated by multiple factors such as the extremely active leukocyte lipoxygenase pathway.

Thus, we have demonstrated that short duration coronary occlusions induce onset of platelet aggregation and \( \text{TXB}_2 \) formation in awake unanesthetized dogs with no other trauma. The use of the chronic cardiac lymph cannula enables detection of these changes in a sensitive and consistent way that will allow evaluation of platelet activation in experimental chronic models of coronary artery flow reduction. The combination of low lymphatic flow rates (and, therefore, low shear stress) and low platelet counts in lymph (2–10% of blood levels) reduces the problem of \( \text{TXB}_2 \) formation as an artifact of collection methods and allows stable repeatable baselines and measurements. The \( \text{TXB}_2 \) concentrations reported here are similar to data of others using standard radioimmunoassay procedures. It is anticipated that development of new prostaglandin assay techniques (negative ion chemical ionization coupled with gas chromatography–mass spectrometry) may allow detection of more subtle changes in prostaglandin concentrations and lower basal levels.

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