Thromboxane Synthetase Inhibitors Reduce Infarct Size by a Platelet-Dependent, Aspirin-Sensitive Mechanism

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Platelets are suggested to exacerbate ischemia-induced myocardial injury, which has led to the study of various antiplatelet therapies including thromboxane synthetase inhibitors (TXSI). Two such agents, benzylimazole and OKY-046, reduce infarct size commensurate with a diminution in serum thromboxane B, formation in anesthetized dogs subjected to 90 minutes of coronary artery occlusion followed by 5 hours of reperfusion. In contrast, platelet depletion with specific antiserum does not reduce infarct size but prevents the cardioprotection afforded by the TXSI. Platelet-derived prostaglandin endoperoxides (PGG, and PGH,), which cannot be converted to thromboxane A, in the inhibited platelet, can be transformed to PGE, and PGD, in plasma and to PGI, by the blood vessel wall. These prostaglandins are considered "cardioprotective." Consequently, a low dose of aspirin (3-5 mg/kg) given 24 hours before coronary occlusion was used to selectively block the platelet cyclooxygenase enzyme. Aspirin, by itself, does not reduce infarct size, but it suppresses the myocardial salvage induced by OKY-046. Thus, TXSI reduce infarct size by platelet-dependent, aspirin-sensitive mechanism that depends on the redirection of platelet-derived PGG, and PGH, to protective metabolites, rather than inhibition of thromboxane A, per se. Moreover, myocardial salvage induced by the TXSI is accompanied by a reduction in neutrophil accumulation in the myocardium, as indicated by the levels of the neutrophil-specific myeloperoxidase enzyme. Platelet depletion or pretreatment with aspirin prevents the TXSI-induced suppression of neutrophil accumulation. Consequently, it is proposed that the prostaglandin-mediated protective effects of TXSI can be resolved, at least in part, in terms of a braking action of neutrophil activation to prevent leukocyte-dependent tissue injury.

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Platelets accumulate rapidly in infarcted myocardium as demonstrated by the use of radiolabeled platelets and by histological studies at autopsy. The formation of microthrombi and the release of vasoactive mediators, in particular thromboxane A, (TXA,) and serotonin, are suggested to exacerbate ischemia-induced myocardial injury, promoting infarct expansion and fatal ventricular arrhythmias. Consequently, the use of antithrombotic therapy, such as aspirin, to decrease the incidence and severity of acute myocardial infarction and associated sudden death has been the basis of a large number of studies. While studies with aspirin in patients with unstable angina have yielded significant protection, other studies examining the incidence of reinfarction have observed a more limited benefit. This marginal protection is attributed to inhibition of both the proaggregatory vasoconstrictor TXA, within the platelet and its physiological antagonist prostacyclin (PGI,) synthesized by the vascular wall. It appears that a low dose of aspirin may selectively inhibit platelet TXA, formation, requiring a reexamination of this antithrombotic drug.

An alternative approach is provided by the development of selective inhibitors of TXA, formation that are devoid of effects on PGI, synthesis. A variety of such drugs have now been synthesized and are of benefit in experimental, acute myocardial ischemia as assessed by various indexes of myocardial injury including creatine kinase release, lactate extraction, and the incidence of arrhythmias and ventricular fibrillation. While the beneficial effects of these thromboxane synthetase inhibitors (TXSI) are attributed to the inhibition of TXA, neither the mechanism of TXA,-mediated damage nor the source of the TXA, has been defined. TXA, induces coronary vasoconstriction in vitro and may have cytolytic actions. The platelet is the presumed source of this mediator in myocardial ischemia, although leukocytes also accumulate in the ischemic myocardium, some of which can synthesize and release TXA,. However, platelet depletion does not appear to afford the same protection as that observed with TXSI. Like the platelets, the leukocytes should also be considered as a major source of mediators that can exacerbate tissue injury. Inhibitors of neutrophil-derived lipoxygenase metabolites of arachidonic acid or oxygen-derived free radicals reduce infarct size. As neutropenia induced with specific antineutrophil serum suppresses platelet accumulation in the ischemic myocardium,
there is a potential for a leukocyte-platelet interaction that contributes to the myocardial damage. The aims of this study are 1) to assess the abilities of two TXSI, benzylimidazole (BZI) and OKY-046, to reduce infarct size commensurate with a reduction in serum thromboxane B, (TXB₂) production; 2) to examine the effects of platelet depletion with specific antiserum on the cardioprotection afforded by these inhibitors to determine whether the platelets are the source of the TXA₂; 3) to determine whether the cardioprotective effects of BZI and OKY-046 are the result of TXA₂ inhibition per se or are mediated through diversion of platelet-derived prostaglandin endoperoxides to cytoprotective metabolites. If the cardioprotection is due to this latter mechanism, low-dose aspirin, which selectively blocks the platelet cyclooxygenase and thus the formation of the prostaglandin endoperoxides, should prevent the beneficial effects of the TXSI; and finally 4) to explore a possible mechanism of prostaglandin-mediated protection involving the activated neutrophils.

An occlusion-reperfusion model of ischemic injury in the anesthetized open-chest dog was used for these studies because we and others have shown that there is a rapid and substantial platelet accumulation in the damaged myocardium in this model.

Materials and Methods

Ten groups of animals (72 dogs) divided into three series of experiments were used in this study (Figure 1). Animals were initially randomized within each series. However, when an increased incidence of ventricular fibrillation occurred in a particular group, additional animals were included at the end of that series. The first series of experiments addressed the abilities of two TXSI, BZI (5 mg/kg i.v. dissolved in 0.9% saline) or OKY-046 (1 mg/kg i.v. in saline plus 5% NaHCO₃) to reduce infarct size in an occlusion-reperfusion model of injury commensurate with a diminution in serum TXB₂ levels when compared with a control, saline-treated group of dogs. The drugs were administered 30 minutes before coronary occlusion, with supplemental doses of 2 mg/kg and 0.5 mg/kg for BZI and OKY-046, respectively, given intravenously 30 minutes after reperfusion.

In the second series of experiments, the effects of thrombocytopenia induced with specific anti-dog platelet serum (0.25 ml/kg i.v.) on the cardioprotective effects of BZI or OKY-046 were examined and compared with the effects of platelet depletion alone. For the thrombocytopenia controls, the data are taken from a previous study with the addition of three extra dogs, one treated with the antiplatelet serum and two controls that received nonimmune serum. Thus, the results from an additional 14 dogs (eight platelet-depleted and six controls) are also included for completeness.

Finally, the effects of low-dose aspirin (3-5 mg/kg i.v., 24-26 hours before surgery) on infarct size and serum TXB₂ and vascular PGI₂ production, given alone or in combination with OKY-046 (administered 30 minutes before occlusion), were studied.

Samples of myocardium were retained from all groups for determinations of myeloperoxidase (MPO) activity.

![Figure 1. Diagrammatic representation of the protocols used in the three series of in vivo experiments.](http://circres.ahajournals.org/lookup/suppl/doi:10.1161/01.CIR.69.4.669/-/DC1/figure1.jpg)
Preparation of Dogs

Male dogs weighing 12.5–20.6 kg were fasted overnight, then anesthetized initially with Biotal (thiamylated sodium, 25 mg/kg i.v.) and maintained with chloralose (80 mg/kg i.v.), supplemented as required. The dogs were intubated and ventilated artificially with room air. Catheters were inserted into a femoral artery and vein for the measurement of arterial pressure and administration of drugs, respectively. Heart rate was monitored from the electrocardiogram, limb lead II. A left thoracotomy was performed at the fifth intercostal space to expose the heart, which was suspended in a pericardial cradle. A micromanometer-tipped catheter (7F, Gaeltec, Medical Instruments, Hackensack, New Jersey) was inserted through a stab wound in the apex of the heart and anchored with a purse-string suture for the measurement of left ventricular pressure and its first derivation (dP/dt). A portion of the left anterior descending (LAD) coronary artery was dissected free just below the first major diagonal branch, and blood flow was measured with an electromagnetic cuff-type flow probe (Carolina Medical Electronics, King, North Carolina). A screw clamp and an occlusive snare ligature were placed on the vessel proximal to the flow probe. Myocardial injury was produced by occluding the coronary artery for 90 minutes followed by 5 hours of reperfusion, as described previously. No attempt was made to revive dogs undergoing ventricular fibrillation, and they were excluded from the study.

Quantification of Area at Risk and Infarct Size

Infarct size is related to the size of the hypoperfused zone upon coronary occlusion. This zone was delineated at the end of the experiment by cannulating the LAD coronary artery at the site of occlusion and infusing heparinized blood into the LAD coronary artery at mean blood pressure while simultaneously injecting Evans blue dye intravenously, which rendered all parts of the heart blue except for the area at risk. The dog was killed, and the heart was removed and sliced (0.5 cm thick) from apex to base. The slices of myocardium were then incubated with triphenyltetrazolium chloride in phosphate-buffered saline, pH 7.4, for 20 minutes, which causes the viable tissue to stain brick red while the infarcted area remains colorless. The upper surface of each slice and the boundaries of the infarct zone, risk area, and normal tissue were traced onto a clear acetate sheet, and the areas of each zone were measured by planimetry.

Preparation of Antiplatelet Serum

Anti-dog platelet serum was a generous gift of Dr. S. Kunkel, University of Michigan, and was made in the following manner. Canine platelets (5 x 10^9), isolated by gel filtration chromatography with Sepharose 2B equilibrated with a calcium-free phosphate buffer (100 mM, pH 7.0), were mixed in incomplete Freund's adjuvant and injected intradermally into a sheep. Twenty-five days later, the sheep was bled, and the serum was heat inactivated and stored at -70° C until use.

Myeloperoxidase Assay

The neutrophil-specific MPO enzyme was measured by an adaptation of the method of Bradley et al., as we have previously described. Briefly, segments of myocardium of 0.1–0.2 g wet wt were frozen in liquid nitrogen, pulverized, suspended in 0.5% hexadecyltrimethylammonium bromide in 50 mM potassium phosphate buffer, blended for 1.5 minutes in a Waring blender, further disrupted in an Oxwell 3000 mechanical mortar and pestle, sonically agitated for 10 seconds (model 300, Fisher Sonic Desembrator, Fisher Scientific, Springfield, New Jersey), subjected to freezing and thawing in liquid nitrogen three times, and finally sonicated for a further 10 seconds to liberate the MPO enzyme. Enzyme activity was determined spectrophotometrically with diaminidine–hydrogen peroxide as substrate and monitoring changes in absorbance at 460 nm. The results were expressed in units of MPO per milligram tissue, where one unit of MPO activity is defined as that degrading 1 µmol of peroxide per minute at 25° C.

Eicosanoid Radioimmunoassay

TXB2 levels in serum and plasma were measured by specific radioimmunoassay with a TXB2 antibody kindly provided by Dr. P.V. Halushka, University of South Carolina. Serum was harvested from samples of peripheral venous blood (3 ml), which was left to clot at 37° C for 2–3 hours. Control "TXB2-free" serum was prepared by charcoal-dextran purification of serum samples and used to construct the appropriate standard curve. The serum samples were diluted 1:5,000 and 1:1,000 with Tris-polyvinylpyrrolidone buffer (pH 7.4), and 100-µl aliquots were assayed. Plasma TXB2 was assayed directly with serial dilutions of 1:25, 1:50, and 1:100 to confirm the reproducibility of the TXB2 concentration. The free [3H]TXB2 was separated by a charcoal-dextran procedure, and the amount of radioactivity was determined by liquid scintillation counting. The TXB2 antibody shows less than 0.7% cross-reactivity with other prostaglandins, and the lower limit of sensitivity of the assay was approximately 10 pg/tube with an interassay variation of <10%.

Canine platelet-rich plasma stimulated with collagen (10 µg/ml) in the absence or presence of OKY-046 (5 µg/ml) was used to demonstrate inhibition of TXB2 formation and transformation of the platelet-derived prostaglandin endoperoxides to PGE2 and PGD2 in plasma. Plasma TXB2 was assayed directly in 0.1 ml, with serial dilutions of 1:25, 1:50, and 1:100 to confirm the reproducibility of the TXB2 concentration by the method described previously. Plasma samples were extracted for radioimmunoassay of PGE2 and PGD2. [3H]PGD2 was added to each sample before extraction to determine the extraction efficiency, which averaged 67.9 ± 1.7% (± SEM). As the extraction efficiency was similar in all samples, the values given represent uncorrected prostaglandin levels.

For extraction, 1 ml high-performance liquid chromatography–grade water was added to 1 ml plasma, and the mixture was acidified to pH 3.5 with formic acid.
Platelet Aggregation Ex Vivo

Blood samples (3 ml) were collected into 0.3 ml of 3.15% sodium citrate, gently inverted, and then centrifuged for 2 seconds in an Eppendorf fixed-speed microcentrifuge (Brinkman Instruments, Westbury, New York). The platelet-rich plasma was collected, and 0.5 ml was transferred to a Payton dual-channel aggregometer (Payton Associates, Buffalo, New York), incubated at 37°C and stirred at 900 rpm. Aggregation was induced with adenosine diphosphate (ADP) (10-20 μM) or arachidonic acid (0.5-1.0 mM).

Vascular PGI₂ Formation

The formation of PGI₂ by segments of carotid and femoral arteries taken from control and aspirin-treated dogs was determined by two separate methods. The formation of bioassayable PGD₂, was measured by inhibition of platelet aggregation, and the conversion of [¹⁴C]arachidonic acid to [¹⁴C]6-keto PGF₁α was also assessed.

Bioassay of PGI₂ formation. Rings of femoral arteries weighing approximately 50-100 mg wet wt were suspended in Krebs buffer and agitated for 30 seconds with a pair of forceps. An aliquot of buffer (20-100 μl) was then transferred to a cuvette containing rabbit platelet-rich plasma stirred at 900 rpm and warmed to 37°C. Aggregation, induced by ADP (5-10 μM), was monitored in a Payton light aggregometer. The inhibitory effects of the buffer from the vessel and serum TXB₂ levels were made by a one-way analysis of variance with the Bonferroni statistic. Changes in hemodynamics between groups and over time were analyzed by a two-way analysis of variance and a Bonferroni modified t statistic. Changes in hemodynamics between groups and over time were analyzed by a two-way analysis of variance.

Statistical Analysis

All data are expressed as the mean ± SEM. Multi-group comparisons of infarct size data, MPO activity, and serum TXB₂ levels were made by a one-way analysis of variance. 

HARVESTING OF CANINE NEUTROPHILS AND SUPEROXIDE ANION GENERATION

Citrated canine blood was mixed (1:9) with 6% dextran (mol wt 500,000), and the erythrocytes were allowed to sediment for 2 hours. The leukocyte-rich fluid was centrifuged at 180g for 5 minutes. Contaminating erythrocytes were removed by hypotonic lysis, and the leukocytes were washed with 0.9% saline before layering onto a Ficoll-Hypaque density gradient (Histopaque 1077, Sigma Chemical, St. Louis, Missouri), and centrifuged at 200g for 20 minutes. Histological analysis revealed the final cell pellet to be composed of 80-85% polymorphonuclear leukocytes (PMNs), with 95% viability as assessed by Trypan blue exclusion. The PMN pellet was resuspended in Hank's buffer supplemented with Tris HCl (0.01 M, pH 7.4) at a concentration of 5 x 10⁶ PMNs/ml.

The generation of superoxide anions (O₂⁻) was measured spectrophotometrically by the superoxide dismutase (SOD)–inhibitable reduction of cytochrome C. Briefly, 100 μl neutrophil suspension (10⁷/ml) was mixed with 400 μl cytochrome C (3.75 mg/ml) and 50 μl either Krebs-Ringer phosphate (KRP) or SOD (1 mg/ml). Where appropriate, the cells were preincubated for 5 minutes at 37°C with OKY-046, PGE, or PGD₂. The neutrophils were then stimulated with either phorbol myristate acetate (PMA, 0.5 μg, from 0.2 mg/ml stock in dimethyl sulfoxide or serum-activated zymosan (0.5 mg in KRP), and the reaction was allowed to continue for periods up to 20 minutes. The reaction was stopped by the addition of 2 ml ice-cold KRP containing 1 mM N-ethylmaleimide, the cells were pelleted by centrifugation at 10,000g for 2 minutes in an Eppendorf fixed-speed centrifuge, and the absorbance of the supernatant was measured at 550 and 468 nm. Results are expressed as nanomoles O₂⁻ produced per 10⁶ cells, with an extinction coefficient of 0.0245 μmol/l for cytochrome C.

Statistical Analysis

All data are expressed as the mean ± SEM. Multi-group comparisons of infarct size data, MPO activity, and serum TXB₂ levels were made by a one-way analysis of variance and a Bonferroni modified t statistic. Changes in hemodynamics between groups and over time were analyzed by a two-way analysis of variance. Vascular PGI₂ formation and platelet aggregation were compared by a Student's t test for unpaired samples, while eicosanoid levels in collagen-stimulated platelet-rich plasma before and after OKY-046 treatment were compared by a paired t test. The inhibition of neutrophil-derived superoxide anion production by PGE₂ or PGD₂ was analyzed over time by a one-way analysis of variance with the Bonferroni correction factor and after a fixed 20-minute incubation by a paired t test. In all cases, a probability of less than 0.05 was considered significant.
Results
Salvage of Ischemic Myocardium by Thromboxane Synthetase Inhibition

The cardioprotective effects of the TXSI in the occlusion-reperfusion model of myocardial injury in the anesthetized dog are depicted in Figure 2. Occlusion of the LAD coronary artery distal to the first major diagonal branch renders approximately one third of the left ventricle ischemic. The infarct zone within this hyperperfused area amounts to 57 ± 4% after 90 minutes occlusion followed by 5 hours reperfusion in control dogs (n = 7). Dogs pretreated with OKY-046 (1 mg/kg i.v., n = 7) or BZI (5 mg/kg i.v., n = 11) before coronary occlusion had significantly smaller infarcts of 19 ± 4% and 39 ± 5% of the risk area (p < 0.01 and p < 0.05, respectively) despite comparable areas at risk of 30 ± 2 versus 33 ± 2% for OKY-046- and BZI-treated animals, respectively, compared with 32 ± 2% for the control group. The cardioprotective effects of the two TXSI were accompanied by reductions in serum TXB2 formation, which is an index of the degree of TX inhibition (Figure 2). Consequently, OKY-046 and BZI reduce infarct size commensurate with the inhibition of thromboxane synthesis.

Contribution of Platelets to Thromboxane Synthetase Inhibitor-Induced Myocardial Salvage

Reduction of infarct size by two "antiplatelet" drugs that inhibit the formation of a specific mediator, TXA2, implies that platelets and platelet-derived TXA2 exacerbate myocardial injury, yet removal of the platelets with a specific antiserum does not reduce infarct size. This apparent anomaly could be explained if, for example, the source of TXA2 produced during myocardial ischemia was not the platelet but rather some other cell. Indeed, Jolly and coworkers reported that thrombocytopenia did not attenuate the release of immunoreactive TXB2 into the coronary sinus blood during myocardial ischemia and reperfusion. If the TXA2 promoting myocardial injury is extraplatelet in origin, then platelet depletion would not impair the ability of TXSI to reduce infarct size. Consequently, dogs rendered thrombocytopenic with antiplatelet serum were treated with OKY-046 (n = 5) or BZI (n = 5) before coronary occlusion and compared with our previous results with platelet-depleted and control nonimmune serum-treated dogs, supplemented with three additional animals. Treatment with antiserum evoked a rapid and sustained reduction in circulating platelets from 295 ± 43 to 21 ± 4 x 10^9/ml (p < 0.001, n = 8) and suppressed serum TXB2 formation to undetectable levels (<10 ng/ml). Platelet depletion prevented the myocardial protective actions of the two TXSI (Figure 3). Platelet-depleted dogs had an infarct size of 52 ± 3% risk area, where the risk area equaled 33 ± 2% left ventricle. In dogs given antiserum plus OKY-046, the area of necrosis was 46 ± 11% risk area, while antiserum and BZI treatment resulted in an infarct of 57 ± 3% risk area, with risk areas of 32 ± 2 and 34 ± 2% left ventricle, respectively. Thus, the...
presence of platelets is a prerequisite for producing myocardial salvage with TXSI.

**Cyclooxygenase Inhibition and Myocardial Salvage Induced by Thromboxane Synthetase Inhibitors**

The requirement for platelets in the protective action of TXSI compared with the lack of effect of platelet depletion suggests that the platelet must be the source of an agent that acts beneficially to impede myocardial necrosis. The platelet-derived prostaglandin endoperoxides may be redirected to form prostaglandins that are cardioprotective. This possibility was investigated with a low dose of aspirin to selectively inhibit the platelet cyclooxygenase enzyme, thereby preventing formation of the prostaglandin endoperoxides. Aspirin (3–5 mg/kg i.v., 24–26 hours presurgery) reduced serum TXB₂ formation by 80% at the time of coronary occlusion (from 630 ± 50 to 80 ± 10 ng/ml, p<0.01; Figure 3), and it attenuated ex vivo platelet aggregation induced by ADP or arachidonic acid. This dose of aspirin did not significantly impede PGI₂ generation by extracardiac blood vessels taken at the time of myocardial ischemia. The bioassay of PGI₂-like activity released from femoral arterial rings was 2.1 ± 0.4 and 1.3 ± 0.24 ng/100 mg tissue for control (n = 6) and aspirin-treated (n = 4) vessels, respectively (p = 0.10). The conversion of [14C]arachidonic acid by segments of carotid arteries was 1.7 ± 0.29% for control (n = 4) versus 1.3 ± 0.26% (n = 4) for the arteries from aspirin-treated dogs (p = 0.10). Dogs administered aspirin showed no reduction in infarct size when compared with vehicle-treated controls (62 ± 5 versus 56 ± 4% risk area, with risk areas of 32 ± 3 and 32 ± 3% for aspirin and control groups, respectively). Aspirin pretreatment abolished the myocardial protection afforded by OKY-046 (Figure 4) with the combination therapy resulting in an infarct size of 54 ± 6% risk area (n = 9). Thus, the integrity of the platelet cyclooxygenase is required for TXSI to reduce infarct size and implies that the transformation of platelet-derived prostaglandin endoperoxides to other products is principally responsible for the myocardial salvage, rather than inhibition of TXA₂ formation per se.

**Mechanism of the Indirectly Mediated Myocardial Salvage by Thromboxane Synthetase Inhibitors**

**Hemodynamics.** Changes in oxygen supply and demand are an important determinant of the extent of ischemia-induced myocardial injury. BZI increased blood pressure and heart rate (Figure 5A) and, consequently, the rate-pressure product (considered a crude index of oxygen consumption), presumably by a mechanism unrelated to thromboxane inhibition since it is not observed with OKY-046 but is with some other imidazole derivatives, including imidazole itself. This hemodynamic profile may offset the myocardial salvaging ability of the drug and may account, in part, for the limited protection obtained when compared with OKY-046. However, BZI only produced a 50% reduction in serum TXB₂ formation (Figure 2), which probably accounts for the partial reduction in infarct size. Higher doses of BZI could not be used to achieve greater inhibition of TXA₂ production because of these adverse hemodynamic effects. Consequently, most additional studies focused on OKY-046, which had no demonstrable hemodynamic activity.

Aspirin, OKY-046, antiplatelet serum, or combinations thereof induced no significant changes in mean arterial blood pressure, heart rate, the rate-pressure product, peak left ventricular pressure, peak negative left ventricular dP/dt, and LAD coronary blood flow (Figures 5A and 5B). Thus, the TXSI-induced reduction in the area of necrosis and its prevention by platelet depletion or aspirin pretreatment cannot be ascribed to hemodynamic alterations. The distribution of coronary blood flow was not measured in this study and an increase in collateral flow may contribute to the smaller infarcts obtained with BZI and OKY-046.

**Antiplatelet activity.** Samples of platelet-rich plasma were taken from control and OKY-046–treated dogs for ex vivo studies of aggregation, induced by either ADP (5–10 μM) or arachidonic acid (0.25–1 mM). Treatment with OKY-046 reduced platelet aggregation to ADP by 45.8 ± 7.7% (n = 6) and to arachidonic acid by 40.7 ± 4% (n = 4; both p<0.05; Student's t test). This partial inhibition is compatible with studies indicating that prostaglandin endoperoxides themselves can provoke aggregation without conversion to TXA₂.

**Metabolism of platelet-derived prostaglandin endoperoxides.** The foregoing results in vivo suggest that the conversion of platelet-derived prostaglandin endo-
peroxides to other metabolites may contribute to the cardioprotective effects of TXSI. Consequently, the inhibition of immunoreactive (i) TXB₂ in collagen-treated, platelet-rich plasma by OKY-046 and the appearance of iPGD₂ and iPGE₂ were examined (Figure 6). The addition of collagen (10 μg/ml) to platelet-rich plasma increased iTXB₂, from 1.1 ± 0.14 to 10.7 ± 2.7 ng/ml. This increase in iTXB₂ was prevented by treatment of the platelet-rich plasma with OKY-046 (5 μg/ml) and was associated with significant increases in iPGD₂ (p < 0.05, n = 4) and iPGE₂ (p < 0.02, n = 3; see Figure 6).

Myeloperoxidase activity in the ischemic myocardium. Measurements of cardiac MPO activity quantitatively reflect the degree of neutrophil accumulation since the MPO is a neutrophil-specific enzyme not normally found in cardiac tissue. Nitric oxide of coronary occlusion and 5 hours of reperfusion resulted in an increase in cardiac MPO activity in the ischemic region from 0.018 ± 0.009 to 0.4 ± 0.08 units MPO/mg tissue (p < 0.001, Figure 7). Treatment with BZI or OKY-046 resulted in a significant reduction in cardiac MPO activity at the end of the 5-hour reperfusion period to 0.19 ± 0.035 (p < 0.05) and 0.08 ± 0.2 U MPO/mg tissue (p < 0.01), respectively. Thrombocytopenia with a specific antiserum did not reduce MPO activity (0.48 ± 0.1 U MPO/mg tissue) suggesting the reduction in neutrophil accumulation is not secondary to an antiplatelet effect of these drugs. Aspirin also had no effect on neutrophil accumulation (0.52 ± 0.16 U MPO/mg tissue) and prevented the OKY-046-induced reduction in MPO activity (0.5 ± 0.07 U MPO/mg tissue for aspirin plus OKY-046, p < 0.01 compared with OKY-046 alone).

Inhibition of superoxide anion formation in canine neutrophils. Incubation of canine neutrophils with PMA (0.5 μg) resulted in a time-dependent increase in O₂⁻ generation to 22.3 ± 6 nmol O₂⁻/10⁶ cells at 20 minutes (n = 8). OKY-046 (1–5 μM) had no effect on PMA-induced O₂⁻ formation. In contrast, PGD₂ (0.05–0.1 μM) or PGE₂ (0.1–1 μM) produced a dose-related inhibition of O₂⁻ release, where PGD₂ was approximately 10-fold more potent than PGE₂ (Figure 8A). Similar results were obtained when the neutrophils were stimulated with serum-activated zymosan instead of PMA, although the amount of O₂⁻ generated was less, 6.8 ± 1.8 nmol O₂⁻/10⁶ cells at 20 minutes (n = 6). Both PGD₂ and PGE₂ attenuated zymosan-induced O₂⁻ formation, with PGD₂ again being approximately one log unit more potent than PGE₂ (Figure 8B).

Discussion

Ninety minutes of coronary artery occlusion followed by 5 hours of reperfusion results in a 12-fold increase in the accumulation of ¹¹¹In-labeled platelets in the ischemic myocardium. The active accumulation of platelets implies that platelet aggregates or the release of platelet-derived mediators could participate in the process of myocardial injury. This study indicates that two inhibitors of a specific platelet mediator, TXA₂, reduce infarct size commensurate with a reduction in
serum TXB₂, thereby apparently supporting the concept of platelet-mediated injury. Beneficial effects of various TXSI have been described previously in other models of myocardial ischemia. However, the mechanism of this protective effect, presumed to be due directly to inhibition of platelet TXA₂, has not been determined. The present results demonstrate that the ability of TXSI to reduce infarct size is dependent on

the presence of platelets and can be prevented by pretreatment of the dogs with aspirin, implying an indirectly mediated cardioprotection.

The beneficial effects of OKY-046 or BZI in the ischemic myocardium cannot be attributed to any apparent hemodynamic effects of these drugs, or the prostaglandins that may be formed, to improve the oxygen supply/demand ratio. Although regional myocardial blood flow was not measured in this study, Huddleston and coworkers found that another TXSI, RO-22-4679, did not improve collateral flow to the ischemic zone. However, whether OKY-046 or BZI can increase blood flow to the ischemic region remains to be resolved.

The cardioprotective effects of TXSI cannot be ascribed to their weak antiaggregatory activity observed ex vivo or to an ability to suppress platelet accumulation in vivo. The limited antiaggregatory properties of this group of compounds is attributed to the platelet endoperoxides themselves being able to evoke platelet aggregation independent of conversion

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**Figure 6.** Immunoreactive (i) eicosanoid levels in canine platelet-rich plasma before and after stimulation with collagen (10 μg/ml) in the presence or absence of OKY-046 (5 μg/ml). Collagen increased iTXB, levels without altering iPGD₂ and iPGE₂. OKY-046 abolished the increase in iTXB, and produced an increase in iPGD₂ and iPGE₂. Data were analyzed by a Student's t test, where *p<0.05, **p<0.01 (n = 4).

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**Figure 7.** Myocardial levels of neutrophil-specific myeloperoxidase (MPO) enzyme, an index of tissue neutrophil content. BZI and OKY-046 significantly reduced (*p<0.05, **p<0.01, ANOVA) the accumulation of neutrophils in ischemic regions of the heart, as determined by MPO activity.

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**Figure 8.** PGD₂ and PGE₂-induced inhibition of superoxide anion formation stimulated by (A) PMA or (B) serum-activated zymosan (n = 4–6 for each point). A, PGD₂ at 0.1 and 0.5 μM, and PGE₂ at 1 μM significantly reduced PMA-induced superoxide anion formation (all p<0.02, ANOVA). B, PGD₂ and PGE₂ inhibit zymosan-induced superoxide anion formation. Solid bar, control. Results compared by a Student's t test, where *p<0.05, **p<0.01.
phil activation is the generation of oxygen-derived free radicals, such as O$_2^-$, which can provoke tissue injury. OKY-046 (1–5 μM) had no effect on PMA-induced O$_2^-$ generation or on neutrophil aggregation provoked by the calcium ionophore A23187 (results not shown). In contrast, nanomolar concentrations of PGE$_2$ and, in particular, PGD$_2$ produced a dose-dependent inhibition of O$_2^-$ formation. Kuehl and coworkers have previously demonstrated that prostaglandins can suppress neutrophil activation, attributed to their ability to stimulate adenylyl cyclase and increase cyclic adenosine monophosphate levels in the neutrophil.

It is difficult to assess the relative importance of the different prostaglandins in attenuating neutrophil activation or contributing to the salvage of the ischemic myocardium. In these dynamic processes, the concentration and potency of the particular prostaglandin have to be considered as well as any additive or synergistic interactions. While similar quantities of PGE$_2$ and PGD$_2$ are produced in the canine platelet-rich plasma, the presence of vascular tissue would also lead to PGI$_2$, the formation of the potency, and hence the contribution of PGI$_2$, is difficult to estimate because of its lability. Because PGI$_2$, PGE$_2$, and PGD$_2$ can all modulate neutrophil behavior (present results and Kuehl et al), it is not necessary to invoke a role for any one particular prostaglandin.

A suggested sequence of events culminating in salvage of the ischemic myocardium induced by TXSI is the release and transformation of platelet-derived prostaglandin endoperoxides to PGD$_2$, PGE$_2$, and PGI$_2$, all of which, in turn, can inhibit neutrophil activation, and their accumulation in the ischemic heart, thereby preventing neutrophil-mediated tissue injury. Direct-acting free radical scavengers, including SOD and catalase, and N-2 mercaptopropionylglycine have also been shown to reduce infarct size in an occlusion-reperfusion model of myocardial injury. It should be stressed that this sequence of events is compatible with a platelet-dependent, aspirin-sensitive mechanism, but it represents just one potential pathway of protection. Pinane-TXA$_2$ is a TXSI and receptor antagonist that also reduces infarct size. Whether this dual activity confers any added benefit that can be attributed to blockade of TXA$_2$ per se has yet to be determined.

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Indirectly Mediated Cardioprotection by Inhibitors of Thromboxane A₂


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Myeloperoxidase activity as a quantitative marker of polymorphonuclear leukocyte accumulation into an experimental myocardial infarct — the effect of ibuprofen on infarct size and polymorphonuclear leukocyte accumulation. *J Cardiovasc Pharmacol* 1985;7:1154—1160


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Thromboxane synthetase inhibitors reduce infarct size by a platelet-dependent, aspirin-sensitive mechanism.
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