
Platelet Preservation During Simulated Cardiopulmonary Bypass Via Phosphodiesterase Inhibition

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ABSTRACT

Inhibition of platelet function reduces adverse platelet alterations during cardiopulmonary bypass (CPB) but agents most efficacious must be given intravenously. Consequently, we evaluated a new type III phosphodiesterase inhibitor (PDEI), CK2130 (100 μ M), comparing it with the most commonly prescribed oral PDEI, dipyridamole (100 μ M). 450 ml of fresh heparinized (5U/ml) blood, drawn from aspirin-free volunteers, was recirculated for 2 hrs at 37°C in a polypropylene circuit (1.0m²) containing a spiral-coil membrane oxygenator. In control saline circuits (N=5), platelet counts fell to 10 \pm 3% (mean + standard error of the mean) of initial levels within 5 min and sensitivity to ADP disappeared. Plasma levels of a platelet-specific protein platelet factor 4 (PF4) rose from 2780 \pm 222 ng/ml at five minutes to 6338 \pm 767 ng/ml after 2 hrs ($p < .05$), indicating extensive platelet release. In contrast, with CK2130 (N=3), although platelets were similarly insensitive to ADP, platelet counts dropped to only 63 \pm 11% at five minutes and PF4 rose from 1012 \pm 510 ng/ml at five minutes to only 3689 \pm 898 ng/ml after 2 hrs ($p < .05$). With dipyridamole (N=4) platelet counts fell to 66 \pm 7% and PF4 rose from 861 \pm 122 ng/ml at five minutes to only 2157 \pm 346 ng/ml after 2 hrs ($p < .05$) but platelets remained responsive to ADP. In conclusion, both CK2130 and dipyridamole significantly preserved the initial circulating platelet count and reduced release of PF4 with CK2130 producing more pronounced inhibition of function as well. Thus, PDEIs may play a promising role as platelet inhibitors during CPB.

INTRODUCTION

Platelet alterations which occur during contact between blood and synthetic surfaces contribute to thrombohemorrhagic complications following cardiopulmonary bypass (CPB).¹⁻³ Indeed, activation of platelets by synthetic surfaces has limited successful long-term mechanical cardiopulmonary support.^{4,5} Since a truly non-thrombogenic surface has yet to be developed, pharmacologic inhibition of platelets holds the greatest promise as a means of preventing surface-mediated thrombosis.^{6,7,8}

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Effective platelet inhibition during in vitro CPB has been successful using prostacyclin and its stable analogue, iloprost.^{9,10} Potent adenylyl cyclase agonists, these prostanoids exert their inhibitory effect by raising the intracellular level of cyclic adenosine monophosphate (cAMP).^{11,12} However, the vasoactive and extremely unstable nature of prostacyclin is unacceptable for even routine cardiopulmonary bypass.^{13,14} In addition, though iloprost exhibits limited vasoactive effects, it must be administered intravenously and requires continuous hemodynamic monitoring.¹⁵

Phosphodiesterase inhibitors (PDEI), by preventing the hydrolysis of cAMP, are capable of maintaining the level of cAMP.^{11,16} CK2130 is a recently developed type III phosphodiesterase inhibitor which we have shown can inhibit platelet function in vitro to both weak and strong agonists (manuscript in preparation). Moreover, the type III PDEIs can be administered orally, which may make them the ideal agents with which to achieve long-term platelet inhibition. Therefore, we tested the efficacy of CK2130 towards prevention of adverse platelet alterations during simulated CPB, a well-defined model to assess antiplatelet agents, and compared it with that of dipyridamole, the most commonly clinically used phosphodiesterase inhibitor.

MATERIALS AND METHODS

PERFUSION SYSTEM

Perfusion circuits with a surface area of 1.0 m² were assembled from standard medical grade polyvinylchloride tubing,^a polycarbonate connectors,^b 400 ml venous reservoir^c and an 0.8 m² infant spiral-coil membrane oxygenator.^d Blood and gas compartments were flushed with 100% carbon dioxide for fifteen minutes prior to priming. Tubing and oxygenators were primed by permitting the blood to enter the circuit by gravity flow. Blood was recirculated by a precisely shimmed, barely occlusive, calibrated double-roller pump^e for two hours at a rate of two times the circulating volume in milliliters per minute. An external heat exchanger^f attached to a warming pads wrapped

- a. Intersept, J&J Cardiovascular, Anaheim, CA
- b. Cobe Laboratories, Lakewood, CO
- c. Terumo Corp., Piscataway, NJ
- d. SciMed Life Systems Inc., Minneapolis, MN
- e. Sarns, Inc., Ann Arbor, MI
- f. Radiometer, Copenhagen, Denmark

around the venous reservoir maintained blood temperature at 37°C. The oxygenator was ventilated with a 95% oxygen/5% carbon dioxide mixture at a rate of one liter per minute.

DRUG PREPARATIONS

CK2130^h and dipyridamoleⁱ were made fresh on the day of the study, dissolved in 0.9% saline solution, and adjusted to a pH of 2.0 to 3.0 for stock concentrations of 10,000 μ M.

SAMPLE ACQUISITION

These studies were approved by the University of Pennsylvania Committee on Human Investigations and the National Institutes of Health. Written and verbal informed consent were obtained from each donor. Blood for all recirculation trials was drawn from donors who abstained from all medications for at least two weeks prior to donation. 450 ml of human blood was drawn directly into venous reservoirs that contained 2,250 units of beef lung heparin, 1.65 gm of glucose, and an equivalent volume of either dipyridamole (100 μ M, n=4), CK2130 (100 μ M, n=3), or 0.9% saline solution (control, n=5). The concentrations of CK2130 and dipyridamole employed were determined from in vitro dose response trials in our laboratory (manuscript in preparation). All blood samples were drawn into syringes containing 3.8% trisodium citrate (9:1V/V). For all recirculation trials, three 10 ml aliquot control samples were obtained from the reservoir bag after priming but prior to the start of recirculation. For all PDEI recirculation trials, an additional 10 ml aliquot control sample was obtained directly from the donor. All control aliquots were incubated at 37°C and assessed at five, 60 and 120 minutes for platelet function. Twenty milliliter aliquots were withdrawn from the circuit at five minutes, one hour and two hours of recirculation for platelet functional and release studies. Additionally, 1 ml samples were withdrawn at 15, 30 and 45 minutes for platelet counts. Samples to be assayed for platelet release products were processed immediately and frozen at -70°C for later analysis.

PLATELET STUDIES

PLATELET COUNTS

Whole blood platelet counts were obtained with a Coulter Z-F cell counter and checked when necessary by phase microscopy.¹⁷

PLATELET AGGREGATION

From 10 ml aliquots of citrated whole blood, platelet-rich plasma (PRP) was prepared as previously described.¹⁸ Briefly, samples were centrifuged at 150g for 10 min at 25°C. Following gentle aspiration of the PRP (350,000 \pm 50,000 platelets/ μ l), the remaining blood was centrifuged at 12,000g for five min at 25°C in a microcentrifuge to obtain platelet-poor plasma (PPP) with a platelet count less than 1000/ μ l. Platelet aggregation studies were performed as

described previously.¹⁹ From previous studies, we determined that the platelet release reaction is complete at the 95% confidence limit when ADP-induced aggregation exceeds 62%.²⁰ Consequently, we defined complete second wave aggregation as more than 62% light transmission through PRP at five minutes. The threshold concentration of aggregating agent is defined as the lowest concentration of aggregating agent necessary to produce second wave aggregation. Normal reactivity was demonstrated in control samples for all studies using 1-5 μ M ADP. Maximal concentration of ADP tested was 50 μ M.²¹

PLATELET FACTOR 4

The appearance of the platelet-specific protein platelet factor 4 (PF4) in plasma was used to indicate the occurrence of the platelet release reaction. 4.5 ml aliquots of citrated whole blood were transferred to plastic tubes containing 10% disodium ethylenediaminetetraacetic acid (EDTA), 5.4 mg/ml theophyllin,^e and 3 x 10⁻³ M PGE₁ and immediately centrifuged at 2000g for 20 minutes at 4°C to obtain platelet-poor plasma. The PPP was again centrifuged at 12,000g at room temperature for two minutes in a microcentrifuge.¹⁰

Plasma levels of PF4 were quantitated by radioimmunoassay with a specific antibody^j as previously described.²² The sensitivity of this assay is 1 ng/ml.

THROMBOXANE B2

Thromboxane B2 (TxB2), the stable end-product of thromboxane A2, is a potent platelet activator and vasoconstrictor. 4.5 ml aliquots of citrated whole blood were transferred to a plastic tube containing EDTA for a final concentration of 10 mM and spun for PRP. Prior to high speed centrifugation (2000g at room temperature for 10 min) to prepare PPP, enough indomethacin was added to give a final concentration of 10 μ M. The indomethacin was dissolved in 100% ethyl alcohol.¹⁰

Plasma levels of TxB2 were measured by radioimmunoassay with a specific antibody^k as previously described.²³ The sensitivity of this assay is 25 pg/ml.

STATISTICAL ANALYSIS

Mean, standard deviation and standard error of the mean were calculated for all groups. Differences within each group were compared utilizing a one-way analysis of variance. Duncan's Multiple Range t-test was used to make comparisons among sampling periods within each recirculation group. A two-way analysis of variance was used to compare differences between saline control and drug-treated circuits. Post hoc contrasts (F-tests) were used to make comparisons among sampling periods between these treatment groups. A p value < .05 was considered statistically significant.

RESULTS

PLATELET COUNT

In control saline circuits (n=5), mean platelet counts, expressed as a percentage of the initial platelet count, fell to 10 \pm 3% (mean + standard error of the mean) within five minutes of recirculation but rose to 61 \pm 9% after two hours (p < .05) (Figure 1). In circuits with dipyridamole (n=4), mean platelet counts

g. Cobe Laboratories, Lakewood, CO

h. Berlex Laboratories, Cedar Knolls, NJ

i. Sigma Chemical Company, St. Louis, MO

j. Abbott Laboratories, North Chicago, IL

k. Dupont-New England Nuclear, Boston, MA

FIGURE 1: Whole Blood Platelet Count - Platelet count is plotted against time and expressed as a percentage of the initial platelet count obtained from the reservoir bag after priming but prior to the start of recirculation. The symbol *i* represents the initial control sample in this and subsequent figures. The points represent means and the error bars represent one standard error of the mean in this and subsequent figures. o - - o Incubated controls (n=12) ○ - - ○ Blood recirculated with saline (n=5) ▲ - - ▲ Blood recirculated with 100 uM CK 2130 (n=3) ▲ - - - ▲ Blood recirculated with 100 uM dipyridamole (n=4)

FIGURE 2: Platelet Function - Platelet responsiveness to adenosine diphosphate (ADP) is plotted against time and expressed as the lowest logarithmic concentrations of aggregating agent necessary to produce second wave aggregation. Otherwise legend is identical to that of Figure 1.

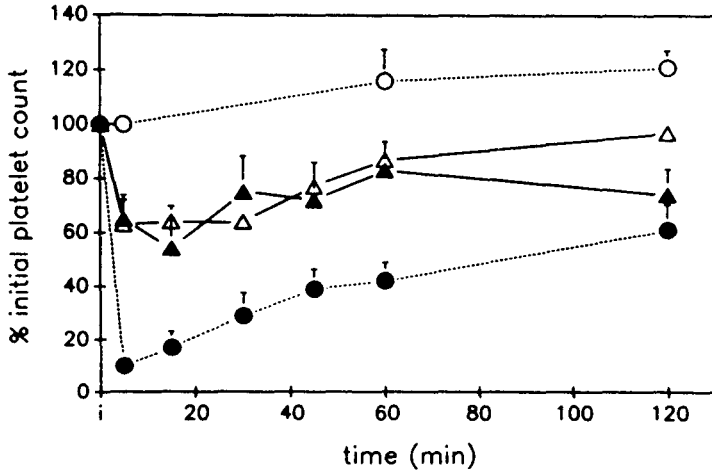


FIGURE 1

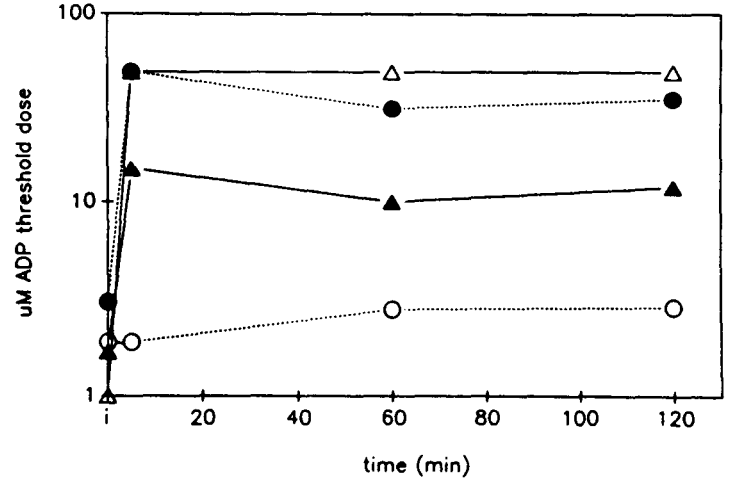


FIGURE 2

FIGURE 3: Platelet Release - Plasma level of platelet factor 4 is plotted against time and expressed in nanograms per milliliter (ng/ml). Otherwise legend is identical to that of Figure 1.

FIGURE 4: Thromboxane Release - Plasma level of thromboxane B₂ is plotted against time and expressed in picograms per milliliter (pg/ml). Otherwise legend is identical to that of Figure 1.

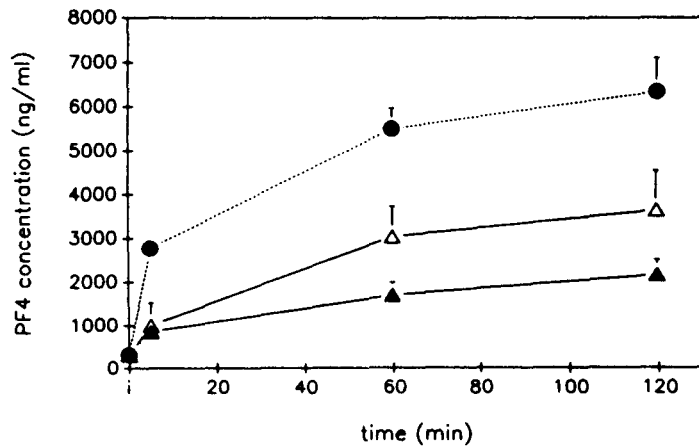


FIGURE 3

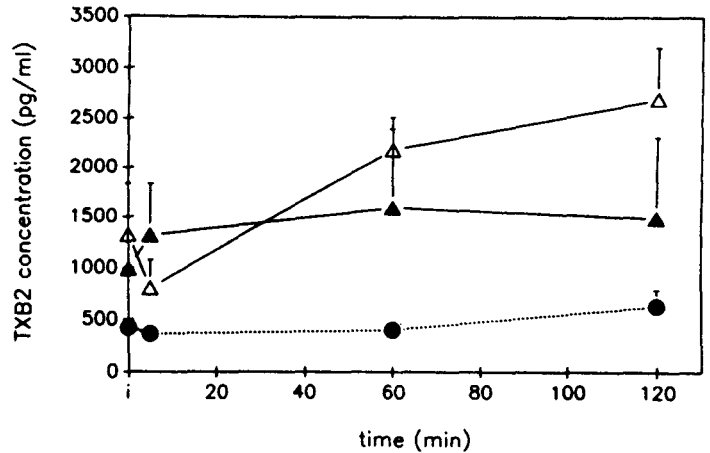


FIGURE 4

only declined to $66\pm 7\%$ with five minutes of simulated CPB and rose to $74\pm 10\%$ at two hours ($p > .05$) (Figure 1). Finally, in circuits with CK2130 ($n=3$), mean platelet counts dropped to $63\pm 11\%$ at five minutes but rose to $97\pm 4\%$ after two hours of recirculation ($p > .05$) (Figure 1). Both dipyridamole and CK2130 significantly reduced initial adhesion to the circuit when compared to the saline count.

PLATELET AGGREGATION

Aggregation of platelets obtained from control samples that were merely incubated at 37°C for two hours ($n=12$) demonstrated essentially normal reactivity when challenged with ADP (3 μM) (Figure 2). Platelets prepared from blood recirculated in control saline circuits immediately lost their ability to react to ADP which persisted throughout recirculation (Figure 2). Platelets from dipyridamole-treated circuits demonstrated a reduction (15 μM) in ability to respond to threshold doses of ADP within five minutes of simulated CPB but significant reactivity persisted even after two hours (12 μM) (Figure 2). As expected, platelets from circuits treated with CK2130 exhibited complete inhibition throughout recirculation even when challenged by the maximal concentration of ADP (50 μM) (Figure 2).

PLATELET RELEASE PLATELET FACTOR 4 LEVELS

After five minutes of recirculation in control saline circuits, plasma levels of PF4 rose from 318 ± 102 ng/ml to 2780 ± 222 ng/ml ($p < .05$) and continued to increase to 6338 ± 767 ng/ml at two hours ($p < .05$) (Figure 3) indicating extensive release of platelet granule contents. In contrast, in dipyridamole treated circuits PF4 plasma levels rose from 335 ± 85 ng/ml to only 861 ± 123 ng/ml at five minutes ($p > .05$) and 2157 ± 346 ng/ml after two hours ($p < .05$) of simulated CPB (Figure 3). In CK2130-treated circuits, plasma levels of PF4 increased from 307 ± 140 ng/ml to only 1012 ± 510 ng/ml after five minutes ($p > .05$) of simulated CPB, and rose to only 3689 ± 898 ng/ml after two hours ($p < .05$) (Figure 3). During the entire recirculation period, both dipyridamole and CK2130 significantly reduced release of platelet granule contents when compared to the saline control.

THROMBOXANE B2 LEVELS

In control saline circuits, plasma levels of TxB2 rose from initial levels of 410 ± 81 pg/ml to only 632 ± 157 pg/ml after two hours ($p > .05$) of recirculation (Figure 4). In contrast, dipyridamole-treated circuits rose from initial plasma levels of 991 ± 268 pg/ml to 1493 ± 825 pg/ml after two hours ($p > .05$) (Figure 4). Circuits treated with CK2130 showed more pronounced initial plasma levels of 1322 ± 513 pg/ml rising to 2700 ± 506 pg/ml after two hours ($p < .05$) of simulated CPB (Figure 4). At two hours of recirculation, neither the dipyridamole-treated nor the CK2130-treated circuits significantly prevented generation of thromboxane B2.

DISCUSSION

The exposure of blood to the non-biologic surface encountered during both in vitro and in vivo cardiopulmonary bypass results in adverse platelet alterations.²⁴ In the laboratory,

these changes are reflected in decreased platelet counts and reduced sensitivity to aggregating agents.² Clinically, these platelet changes contribute to prolonged post-operative bleeding times and excessive blood loss.^{1,3} Indeed, the thrombohemorrhagic complications of surface-mediated platelet activation has severely limited the successful use of long-term mechanical cardiopulmonary support, including the total artificial heart.^{4,5} Since no synthetic surface can duplicate normal endothelium, an alternative method of preventing surface-mediated platelet activation is required.

Following initial exposure of blood to an artificial surface, a layer of plasma proteins, consisting predominantly of fibrinogen, is immediately deposited on the surface.^{24,25,26} This protein layer functions as a bridge to the platelets' site of attachment, which appears to be a fibrinogen receptor. Following initial adhesion to the surface, platelets release granule constituents, a process which coincides with platelet recruitment as aggregates form. In fact, platelet activation by the fibrinogen coated artificial surface mimics that of very strong soluble agonists.²⁷

Since synthetic surfaces mimic strong agonists, we have attempted to prevent surface-induced platelet activation with agents which are known to prevent activation by strong agonists.^{6,7,8} The ideal agent required to prevent platelet reactivity in the clinical setting should be reversible, limited in its side effects and relatively specific to platelets. In addition, an oral route would vastly improve its applicability for long-term administration.

Aspirin, the most commonly used oral antiplatelet agent, prevents thromboxane-dependent platelet activation via its irreversible action on cyclooxygenase.²⁸ Acetylation of this enzyme prevents conversion of arachidonic acid to cyclic endoperoxides, thereby blocking production of thromboxane A₂, a potent aggregatory agent, for the life of the platelet.^{28,29} Aspirin, however, is a weak inhibitor and will not prevent activation by strong agonists, such as thrombin.²⁷ Consistently, our laboratory has previously shown that although aspirin completely abolished thromboxane formation in platelets undergoing simulated CPB, it was unable to prevent platelet adhesion, preserve platelet number, or prevent release of platelet granule constituents.²⁷ Thus, aspirin is not only irreversible but ineffective. An alternative to aspirin are the prostanoids, i.e., prostaglandin E₁ (PGE₁), prostacyclin (PGI₂) and iloprost.

The mechanism of action of the prostanoids as inhibitors of platelet reactivity is the elevation of intracellular levels of cyclic adenosine monophosphate (cAMP) through stimulation of adenylate cyclase.³⁰ By raising intracellular cAMP, prostanoids can prevent platelet activation by weak or strong agonists. Fortuitously, prostanoids are completely reversible in their effects on platelets. Data from our laboratory comparing iloprost, PGI₂ and PGE₁ has shown a direct correlation of the measured efficacy in raising intracellular levels of cAMP with inhibition of platelet aggregation.³⁰ Unfortunately, the vasoactive nature of both PGE₁ and PGI₂ is dramatic, rendering their use for even routine cardiopulmonary bypass difficult.^{13,14} In addition, prostacyclin is extremely unstable. Iloprost, a potent stable analogue of prostacyclin, has

demonstrated an excellent ability to preserve platelet number and function in both animal and human studies with limited vasoactive effects.^{6,7,15} However, iloprost must be administered intravenously and requires continuous hemodynamic monitoring. Its use, therefore, is limited to short-term administration in a critical care setting.

Phosphodiesterase inhibitors are a group of compounds which, like prostanoids, modulate the concentration of intracellular cAMP. However, rather than stimulate synthesis, they prevent the enzymatic hydrolysis of the cyclic nucleotide, thereby maintaining intracellular levels of cAMP.¹¹ We previously have shown that phosphodiesterase inhibitors are capable of inhibiting platelet aggregation in vitro in response to both strong and weak agonists, with type III inhibitors several orders of magnitude more effective than standard agents (manuscript in preparation). This implies that the basal level of cAMP production alone may be sufficient to inhibit platelet activation if the cyclic nucleotide is not degraded. Furthermore, since certain agents in this class can be taken orally, we hypothesized that if they are demonstrably efficacious in preventing surface-induced platelet activation, they would have a most useful clinical role.

As a type III PDEI, CK2130 belongs to the same family of compounds as milrinone and amrinone.³¹ Interestingly, we have shown that CK2130 is a more potent antiplatelet agent than milrinone (manuscript in preparation). Consistently, we have also shown that CK2130 is at least 100 times more potent as an antiplatelet agent than either dipyridamole or theophylline in both untreated and aspirinized platelets (manuscript in preparation).

This work demonstrates that platelet inhibition via phosphodiesterase inhibition does mitigate adverse platelet alterations which occur during simulated extracorporeal circulation. Both CK2130 and dipyridamole preserve the circulating platelet count and reduce platelet granule release. Furthermore, dipyridamole preserved platelet reactivity as well. These salutary effects should translate into improved post-operative bleeding times and reduced blood loss after acute cardiopulmonary bypass. Finally, our work demonstrates that functional inhibition with CK2130 persists beyond that induced by dipyridamole. This is consistent with previous in vitro work in our laboratory which documented the added potency of CK2130 compared to dipyridamole which was a relatively weak platelet inhibitor (manuscript in preparation). However, further work is necessary to prove that CK2130 is reversible in its effects on platelet function.

In summary, phosphodiesterase inhibitors reduce the surface-mediated alterations in platelet function which accompany extracorporeal circulation. Furthermore, agents within this class can be taken orally and are thus useful for long-term antithrombosis. Indeed, by inhibiting phosphodiesterase, these agents would be expected to act synergistically with prostanoids which also act through the cAMP system. It is hoped that a combination of intravenously administered prostanoids and orally administered phosphodiesterase inhibitors will provide effective control of platelet reactivity during synthetic surface contact. This should extend horizons for long-term mechanical

support up to and including extracorporeal membrane oxygenation and the total artificial heart.

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