Role of Platelet-Prostaglandin Synthesis in Shear-Induced Platelet Alterations

By D. E. Stevens, J. H. Joist, and S. P. Sutera

Exposure of citrated human platelet-rich plasma (C-PRP) to mechanical shear stress has been shown to induce platelet aggregation (PAG), release from platelets of dense granule contents, and platelet lysis. The mechanisms involved in these shear-induced alterations have not been defined. To examine the possible role of prostaglandin (PG) synthesis in shear-induced platelet reactions, C-PRP was sheared in a concentric cylinder viscometer for 5 min at stresses ranging from 50 to 460 dyn/sq cm in the presence or absence of acetylsalicylic acid (ASA), a potent inhibitor of platelet thromboxane-A$_2$ synthesis. With increasing shear stress, a progressive loss of platelets from the sheared C-PRP was observed due to PAG and adherence of the aggregates to the viscometer surfaces. PAG was associated with a progressive release from platelets of serotonin and $\beta$-thromboglobulin ($\beta$-TG) and formation of malondialdehyde (MDA) a stable end-product of platelet cyclooxygenase-mediated arachidonate metabolism reflecting thromboxane-A$_2$ synthesis. At stresses above 150 dyn/sq cm, a progressive loss of lactic dehydrogenase (LDH) was observed, indicating platelet damage. ASA (0.1 mM) completely abolished shear-induced MDA formation and caused a 50% reduction of shear-induced release of serotonin and $\beta$-TG. However, ASA did not cause an appreciable reduction of shear-induced PAG and did not alter the extent of or threshold for shear-induced platelet lysis. The findings indicate that (1) shear-induced release from platelets of dense and $\alpha$-granule contents is associated with and potentiated by stimulation of thromboxane-A$_2$ synthesis and (2) thromboxane-A$_2$ synthesis appears to play no appreciable role in shear-induced PAG or platelet lysis in this system.

THE FACTORS and mechanisms involved in the development of thrombocytopenia, thrombembolic events, and platelet dysfunction that complicate the use of extracorporeal circulatory devices and implanted cardiovascular prostheses are still incompletely understood. Whereas an impressive body of recent information exists in regard to the nature of the factors involved in platelet interaction with the injured vessel wall and foreign surfaces,$^1$ relatively little is known about the mechanisms and factors involved in in-bulk shear stress-induced platelet–platelet interactions. Recent experimental evidence$^5$ seems to indicate that platelet aggregation (PAG), release of platelet granule contents, and platelet lysis induced by shearing of citrated human platelet-rich plasma (C-PRP) in a rotational viscometer are predominantly related to in-bulk shear stress effects rather than to platelet–surface interactions. Platelet–platelet interaction or PAG induced by chemical (e.g., adenosine diphosphate [ADP], epinephrine, thrombin) or surface (e.g., collagen) stimuli is thought to be mediated by at least two mechanisms, i.e., the release from platelets of ADP and the liberation from platelets of thromboxane-A$_2$ formed by stimulation of platelet prostaglandin (PG) synthesis.$^7$ The role of ADP release and PG-endoperoxide formation in shear-induced platelet reactions is not known.$^9$ The aim of the present study was (a) to investigate whether shear stress may induce stimulation of the platelet cyclooxygenase-mediated arachidonate pathway that leads to the formation of the powerful platelet-aggregating intermediate, thromboxane-A$_2$, and (b) to assess the importance of platelet thromboxane-A$_2$ synthesis for shear-induced platelet alterations.

MATERIALS AND METHODS

Preparation of Platelet-Rich Plasma

Blood was obtained from healthy appearing male and female volunteers by clean puncture of an antecubital vein and collected into plastic syringes containing 1/10 volume of 3.8% sodium citrate solution. All donors had signed an informed consent form and had not taken aspirin-containing medications for at least 10 days before blood collection. The citrated blood was gently mixed and centrifuged at 180 g for 10 min at room temperature and the supernatant C-PRP was carefully transferred to a plastic tube by plastic pipette. The platelet concentration in C-PRP was adjusted to approximately 500,000/ïl by addition of appropriate amounts of platelet-poor plasma (C-PPP) prepared by centrifugation of the remainder of the blood at 5000 g for 8 min at 5°C. Platelet concentrations in C-PRP were determined by phase-contrast microscopy.$^{14}$ The final C-PRP was stored in a capped plastic syringe at room temperature to avoid a rise in plasma pH due to loss of CO$_2$ from the mixture, which may affect platelet reactivity.

Shear Experiments

Samples of C-PRP were subjected to a uniform shear stress in a concentric cylinder (Couette-type) viscometer (Fig. 1) consisting of a stationary inner cylinder (stator) and a rotating outer cylinder (rotor). Both cylindrical surfaces are made of stainless steel and were freshly siliconized with Prosil-28 (PRC Research Chemicals, Inc., Gainesville, Fla.) before each experiment. The rotor has a transparent lucite bottom. The gap between cylinders (0.013 cm...
circulating constant temperature water bath (24°C, surface area in contact with sheared C-PRP) form the top and bottom boundaries of the sheared C-PRP. The rotor is encased in a water jacket, and the primary flow in the gap is always laminar (maximum Reynolds number of 20-500 dyn/sq cm). The duration of shear was standardized at 5 min. Because the platelets comprise such a small volume fraction in the C-PRP (<0.3%), they contribute negligibly to the suspension viscosity, and μ may be treated as constant regardless of PAG or platelet lysis. The working stress range of the viscometer is 20-500 dyn/sq cm. The shear stress, \(\tau\), is calculated from the formula:

\[
\tau = \frac{\mu R_i S_i}{R_e - R_i}
\]

where \(\mu\) is the dynamic viscosity of plasma (0.015 poise), and \(R_e\) and \(R_i\) are the radii of the rotor and stator (1.930 and 1.917 cm), respectively. The amount of \(^{14}\)C-serotonin-related radioactivity in the supernatant plasma was determined following exposure of labeled C-PRP to shear and expressed as percentage of the total radioactivity in an aliquot of sonicated C-PRP after subtraction of that present in the supernatant plasma of intact C-PRP.

Platelet Release

**Release of serotonin.** Serotonin release was studied by incubating C-PRP with \(^{14}\)C-hydroxytryptamine creatinin sulfate (55 mCi/mM, 0.03 \(\mu\)Ci/ml C-PRP) (Amersham/Searle, Inc., Arlington Heights, Ill.) for 30 min at room temperature, which resulted in the uptake of more than 90% of the label by the platelets. The amount of \(^{14}\)C-serotonin-related radioactivity in the supernatant plasma reflects the uptake of more than 90% of the label by the platelets. The results obtained with sheared C-PRP were expressed as a percentage of that determined in the same C-PRP before shear exposure.

**Platelet Thromboxane-A\(_2\) Synthesis**

This was assessed in intact and sheared C-PRP by measuring malondialdehyde (MDA), a stable end-product of the cyclooxygenase-mediated pathway of platelet-arachidonate metabolism, which reflects thromboxane-A\(_2\) synthesis, using a colorimetric assay as described in detail elsewhere.\(^{12}\) The results were compared with those obtained in C-PRP exposed to ADP (3 \(\mu\)M) (Sigma Chemical Company, St. Louis, Mo.), acid-soluble collagen (2.5 \(\mu\)g/ml), and bovine thrombin (1 U/ml) (Parke, Davis Company, Detroit, Mich.) in an aggregometer for 5 min at 37°C.

**Platelet Lysis**

This was measured by determining the activity of the cytoplasmic constituent, lactate dehydrogenase (LDH), lost from the platelets into the plasma according to a method previously described.\(^{13}\) The results obtained with sheared C-PRP were expressed as percentage of the activity measured in an aliquot of sonicated C-PRP after subtraction of the activity measured in intact C-PRP.

**Electron Microscopy**

Aliquots (1.0 ml) of intact and sheared C-PRP were added to plastic tubes containing 3.0 ml of cold 1% glutaraldehyde in cacodylate buffer, incubated at 5°C for 60 min and centrifuged at 2000 g for 10 min. The sediment was postfixed in 2% osmium tetroxide, dehydrated in ethanol, and imbedded in epon-araldite. Thin sections were stained with uranylacetate and lead citrate, and examined with a Phillips 300 electron microscope.

**Effect of ASA on Shear-Induced Platelet Alteration**

Acetylsalicylic acid (ASA) (Mallinckrodt, Inc., St. Louis, Mo.) was dissolved in distilled water with the addition of equimolar concentrations of NaHCO\(_3\) to give a final concentration of 1 mM. The pH was adjusted to 7.35 and the osmolarity to 280 mosmole by addition of 30% NaCl. Aliquots of a freshly prepared solution were
added to C-PRP 5 min before each shear experiment to give a final concentration of 100 μM. This concentration of ASA completely inhibited PAG induced by a collagen suspension (2.5 μg/ml) as measured by a turbidimetric technique described previously.14

RESULTS

When C-PRP was subjected to increasing levels of shear for a standard period of 5 min, there was an initial progressive reduction in the platelet concentration up to a shear stress level of 270 dyn/sq cm (Fig. 2). This was found to be due predominantly to PAG and sedimentation of the aggregates to the bottom of the viscometer immediately following the termination of each shear experiment. Thus, virtually all of the platelets could be recovered from the viscometer (mostly in the form of small and medium-sized aggregates) by repeated washing of the cylinder with a 1% EDTA-saline solution. At shear stresses above 270 dyn/sq cm, the reduction in platelet concentration became progressively less (Fig. 2) and smaller aggregates were found in the sheared C-PRP, presumably due to prevention of the formation of larger aggregates or partial disruption of previously formed aggregates by the higher shear forces.

Shear-induced PAG was associated with a progressive release from platelets of both 14C-serotonin and β-TG up to approximately 20% of total at a shear stress level of 180 dyn/sq cm (Fig. 2). At higher shear stress, there was further accumulation in the plasma of 14C-serotonin, as well as β-TG, which could be due at least in large part to platelet damage since there was a simultaneous progressive increase in the plasma of the platelet cytoplasmic constituent LDH. Electron microscopic examination of intact (Fig. 3A) and sheared C-PRP revealed that at sublytic shear stress (90 dyn/sq cm) platelet aggregates contained many platelets that had retained all or most of their organelles (Fig. 3B). At higher shear stress (365 dyn/sq cm), at which a 10%–15% loss of LDH was observed, platelets were markedly distorted with irregular surfaces and partial or complete loss of organelles and an enlarged open canaliculic system. In addition, swollen and spherical platelets with more or less complete loss of normal intracellular structures were seen, some of which showed peripheral displacement of granules and mitochondria along the plasma membrane (Fig. 3C).

When thromboxane-A2 synthesis was examined in C-PRP exposed to a high (lytic range) shear stress (365 dyn/sq cm) for 5 min in three separate experiments, a mean MDA of 0.95 ± 0.15 nmole/109 platelets was observed (Table 1). This value was slightly less than those observed with ADP, collagen, and thrombin in an aggregometer at 37°C. ASA added to C-PRP 5 min before exposure to the same shear stress completely abolished shear-induced MDA formation, as well as MDA-formation induced by ADP, collagen, and thrombin.

Figure 4 shows the results of a series of paired experiments designed to assess the effect of inhibition by ASA of thromboxane-A2 synthesis on shear-induced platelet alteration. ASA caused an approximately 50% reduction in the release of both 14C-serotonin and β-TG over the entire shear stress range examined. However, ASA had no significant effect on shear-induced PAG nor did it modify the extent of or threshold for shear-induced loss of LDH from platelets.

DISCUSSION

The data reported in this article confirm and extend previous observations5,15,16 on the response of human platelets to laminar shear stress in a Couette viscometer. Platelets in C-PRP subjected to sublytic shear stress in this device were previously shown to undergo aggregation and release of dense and lysosomal granule constituents.5,15,16 These platelet alterations were observed at shear stress levels similar to those encountered by platelets in circulating blood under physiologic and some pathologic (prosthetic devices) conditions.17 In this paper, it is shown that shear-induced...
PAG in this system is also associated with the release of the platelet-specific protein, β-TG, an α-granule constituent. In addition, evidence in regard to the morphological alterations associated with exposure of C-PRP to sublytic and lytic stress is presented.

The main purpose of this study was to examine whether thromboxane-A$_2$ synthesis is induced by shear, and if so, whether and to what extent thromboxane-A$_2$ synthesis affects shear-induced PAG, release and lysis. The findings presented show that shear-induced PAG and release are associated with formation of MDA, an indicator of stimulation of the
thromboxane-A2-related pathway of platelet arachidonate metabolism.\textsuperscript{13} ASA completely suppressed shear-induced MDA formation and caused a marked inhibition of shear-induced release of dense and α-granule constituents. However, ASA was found to have no appreciable effect on shear-induced PAG over a wide range of sublytic shear stress levels. Thus, thromboxane-A2 appears to play no significant role in shear stress-induced PAG in the system used. Strikingly similar results have recently been reported in experiments using simulated extracorporeal circulation.\textsuperscript{19} Since shear-induced release from dense granules, the storage site for releasable ADP, was not completely suppressed by ASA, ADP release could be responsible for shear-induced PAG. Alternatively, a third pathway as previously invoked for thrombin-

induced PAG\textsuperscript{7} may be involved in shear-induced PAG. Finally, the findings of our study seem to indicate that ASA-induced partial inhibition of shear-induced platelet release does not affect the extent of or threshold for shear-induced platelet damage. Whether complete suppression of platelet secretion by pharmacologic elevation of platelet cyclic AMP\textsuperscript{19} can inhibit shear-induced platelet lysis in our system is currently under study.

**ACKNOWLEDGMENT**

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**REFERENCES**

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**Table 1. Platelet Malondialdehyde Formation Induced by Shear Stress as Compared to ADP, Collagen, and Thrombin and its Inhibition by ASA**

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Number of Experiments</th>
<th>MDA (n mole/10(^6) Platelets)</th>
<th>Saline</th>
<th>ASA (100 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shear stress (365 dyn/cm(^2))</td>
<td>3</td>
<td>0.95 ± 0.15</td>
<td>0.04 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>ADP (3 μM)</td>
<td>4</td>
<td>1.45 ± 0.22</td>
<td>0.03 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>Collagen (2.5 μg/ml)</td>
<td>4</td>
<td>2.35 ± 0.29</td>
<td>0.02 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>Thrombin (1 U/ml)</td>
<td>4</td>
<td>1.78 ± 0.21</td>
<td>0.05 ± 0.04</td>
<td></td>
</tr>
</tbody>
</table>

Malondialdehyde (MDA) was measured in C-PRP after a 5-min exposure to shear in the viscometer (24°C) or to ADP, collagen, and thrombin in an aggregometer (37°C). The means ± 1 SEM of experiments performed in C-PRP preincubated with either ASA or saline for 5 min are shown.

**Fig. 4. Effect of acetylsalicylic acid on shear-induced platelet alterations. Human C-PRP prelabeled with 14C-serotonin was subjected to varying shear stress in the absence (■—■) or presence (○—○) of acetylsalicylic acid (ASA) (100 μM), and changes in platelet concentration and accumulation of 14C-radioactivity, β-TG, and LDH were determined. The means ± 1 SEM of 4–16 individual experiments are presented. The data were found to be normally distributed, and the differences were analyzed for statistical significance using a paired Student’s t test (p < 0.06).**
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