Duration of the Effect of Aspirin on the Synthesis of Thromboxane by Density Subpopulations of Rabbit Platelets Stimulated With Thrombin

By Marian A. Packham, Maria A. Guccione, and Kathryn M. O'Brien

The controversy concerning the relationship between platelet buoyant density and platelet age is unresolved. Our earlier results with rabbit platelets indicate that the most-dense subpopulations are enriched in young platelets and that some platelets become less dense as they age. Other investigators have concluded that platelets either do not change in density upon aging or become more dense. In the present experiments, rabbit platelets were separated on discontinuous gradients of Stractan. Most-dense platelets synthesized significantly more thromboxane B₂ (TXB₂) (1.27 ng per 10⁶ platelets) in response to thrombin (0.75 U/ml) than did least-dense platelets (0.70 ng per 10⁶ platelets), indicating that the arachidonic pathway in most-dense platelets is more active than in least-dense platelets. After aspirin administration to rabbits, most-dense platelets recovered their ability to synthesize thromboxane B₂ significantly more quickly than did least-dense platelets. Because the platelet cyclooxygenase that is responsible for TXB₂ formation is permanently inhibited by aspirin, it is only the new platelets entering the circulation that will be able to form TXB₂. These results indicate that, at least in rabbits, the most-dense platelets are enriched in young platelets, and that platelets decrease in density as they age in the circulation.

© 1985 by Grune & Stratton, Inc.

Materials

Stractan 2 was obtained from the St Regis Paper Co. Tacoma, Wash, and was prepared as described previously.² Bovine albumin (Pentex, fraction V) was obtained from Miles Laboratories, Elkhart, Ind. Bovine thrombin (Topical) was obtained from Parke, Davis and Co. Detroit. Apyrase was prepared as described by Molnar and Lorand.³ Aspirin (Bayer), flavored, children's size was obtained from the St Regis Paper Co. Tacoma, Wash, and was prepared as described previously.⁴ Bovine thrombin (Topical) was obtained from Parke, Davis and Co. Detroit.

Materials

© 1985 by Grune & Stratton, Inc.

Address reprint requests to Dr M.A. Packham, Department of Biochemistry, University of Toronto, Toronto, Ontario, Canada M5S 1A8.

From the Department of Biochemistry, University of Toronto.

Supported by grant No. MT 2629 from the Medical Research Council of Canada.

Submitted Nov 8, 1984; accepted Jan 24, 1985.

From www.bloodjournal.org by guest on October 3, 2017. For personal use only.
**Methods**

Preparation of suspensions of washed platelets. Blood samples (4.3 mL) were withdrawn from the marginal ear veins of New Zealand white rabbits (2.5 to 3 kg) into 0.7 mL of acid–citrate–dextrose (ACD). Platelets were isolated in high yield as described previously and were suspended in 3 mL of calcium-free Tyrode's solution containing 0.35% albumin and 0.02% EGTA, pH 6.5. The platelet count varied between 400,000 and 700,000/μL, as determined with a Coulter Counter, model ZB. A C-1000 Coulter Channelyzer was used to measure platelet size distributions.

Isolation of subpopulations. Three platelet subpopulations were isolated by centrifugation (6,456 g for 45 minutes at 23°C) on discontinuous gradients of Stractan, as described earlier. The least-dense and most-dense subpopulations were removed with Pasteur pipettes and diluted with three volumes of calcium-free Tyrode's solution containing 0.35% albumin and 0.02% EGTA, pH 6.5. The platelets were recovered by centrifugation at 2,660 g for 25 minutes at 23°C and were resuspended in 0.5 mL Tyrode's–albumin containing apyrase, pH 7.35. The platelet count was adjusted to 200,000/μL. It was established that pretreatment of platelets with aspirin did not affect the distribution of platelets on the density gradients.

Aspirin administration. Blood samples were taken before aspirin administration and 1, 24, 48, 72 and 96 hours later, and, in some experiments, at 120 or 144 hours when recovery from the effects of aspirin was complete. Each rabbit received one tablet (65 mg).

Assay of TXB2. Samples (0.495 mL) of suspensions of platelet subpopulations were mixed by inversion with thrombin (5 μL of 75 U/mL) to give a final concentration of 0.75 U/mL and were incubated for ten minutes at 37°C. The platelets were removed by centrifugation at 12,000 g for two minutes (Eppendorf centrifuge, Brinkmann, Rexdale, Ontario). The supernatants were diluted with Tyrode's–albumin solution so that concentrations of TXB2 would be on the standard curve and were assayed in duplicate for TXB2, using the RIA kit from New England Nuclear. Assays were also performed on control samples in which thrombin solution was replaced with Tyrode's–albumin solution. Tests were done to ensure that Stractan did not affect the values in the TXB2 assays.

**RESULTS**

Rabbit platelets were separated into three subpopulations of different densities by centrifugation on discontinuous density gradients of Stractan. The percentages of platelets in the least-dense and most-dense subpopulations varied to some extent from rabbit to rabbit, but the mean values were 26.2% ± 6.8% in the least-dense and 27.8% ± 9.3% in the most-dense (mean ± SD, N = 34).

The concentration of thrombin (0.75 U/mL) used to stimulate the platelets to produce TXB2 was chosen on the basis of earlier experiments in which this concentration released more than 90% of the dense granule contents of rabbit platelets in one minute, and on the basis of preliminary experiments with the total platelet population, which showed that maximum stimulation of TXB2 production occurred with 0.75 U/mL of thrombin and that higher concentrations did not cause more TXB2 formation.

Before aspirin treatment, or with platelets from control rabbits that were not given aspirin, the platelets in the least-dense subpopulation synthesized significantly less TXB2 in response to thrombin (0.70 ± 0.19 ng per 10^9 platelets) than the platelets in the most-dense subpopulation (1.27 ± 0.29 ng per 10^9 platelets) (mean ± SD, N = 11). By paired difference analysis, P < .001.

Although the size distributions of least-dense and most-dense platelets overlapped considerably, the median size of least-dense platelets (4.25 ± 0.46 μm^2) was less than that of most-dense platelets (4.78 ± 0.51 μm^2) (mean ± SD, N = 33): P < .001 by paired difference analysis.

Residual cyclooxygenase activity one hour after aspirin administration was similar in both subpopulations, but during the next three days, the most-dense platelet subpopulations recovered their ability to synthesize TXB2 in response to thrombin more rapidly than did the least-dense platelet subpopulations (Table 1).

**DISCUSSION**

These results show that most-dense platelets from rabbits synthesize more TXB2 in response to thrombin than do least-dense platelets. The difference is greater than can be accounted for by the larger size of most-dense platelets. These observations are in accord with the observations of several other investigators that large, most-dense platelets are functionally more active than small, least-dense platelets and are capable of synthesizing more MDA in response to thrombin. However, Martin et al reported that although most-dense platelets formed more TXB2 in response to arachidonic acid than did least-dense platelets, the two subpopulations responded to thrombin by forming similar amounts of TXB2.

After aspirin administration to rabbits, residual activity of cyclooxygenase at one hour was similar in both subpopulations, but during the next three days, the most-dense platelets recovered their ability to synthesize TXB2 more rapidly than did the least-dense platelets. This observation leads to the conclusion that most-dense platelets are enriched in young platelets and least-dense platelets are enriched in old platelets. This conclusion agrees with our results from earlier experiments with rabbit platelets labeled in vivo with H3S, in which the label appeared first in the most-dense subpopulation and reached a maximum before the relative specific radioactivity of the least-dense platelets reached a maxi-

### Table 1. Thrombin-Induced TXB2 Production by Least-Dense and Most-Dense Platelet Subpopulations After Aspirin Administration

<table>
<thead>
<tr>
<th>Time After Aspirin Administration (h)</th>
<th>n</th>
<th>Least-Dense Platelets (Mean ± SD)</th>
<th>Most-Dense Platelets (Mean ± SD)</th>
<th>Pf</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre</td>
<td>6</td>
<td>100.0</td>
<td>100.0</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>6</td>
<td>8.4 ± 6.7</td>
<td>9.9 ± 11.4</td>
<td>NS</td>
</tr>
<tr>
<td>24</td>
<td>6</td>
<td>16.1 ± 8.6</td>
<td>26.4 ± 21.7</td>
<td>NS</td>
</tr>
<tr>
<td>48</td>
<td>6</td>
<td>38.3 ± 13.9</td>
<td>70.4 ± 13.7</td>
<td>&lt;.005</td>
</tr>
<tr>
<td>72</td>
<td>4</td>
<td>85.1 ± 10.5</td>
<td>100.7 ± 14.6</td>
<td>&lt;.05</td>
</tr>
</tbody>
</table>

TXB2, thromboxane B2; NS, not significant.

* The platelets were stimulated with 0.75 U/mL of thrombin for ten minutes.

†Paired difference analysis.
mum, indicating that most-dense platelets become less dense as they age. The results of these experiments in which TXB₂ production was measured also agree with our experiments, in which the survival of most-dense platelets was longer than that of least-dense platelets, indicating that the most-dense platelet subpopulation is enriched in young platelets. All our results are also in agreement with those of Karpatkin’s group, who used human and rabbit platelets, and with those of Corash and his co-workers, who used human and monkey platelets. Both groups reached the same conclusion regarding the relation between platelet density and platelet age.

The time required for the most-dense platelets to recover completely their ability to form TXB₂ corresponds to the mean survival time of rabbit platelets (65.7 ± 7.2 hours).²⁷

However, our observations concerning recovery of the ability to form TXB₂ after aspirin administration do not agree with most of the results reported for human platelets from experiments in which similar techniques were used. Using N-ethylmaleimide to stimulate thromboxane formation, Mezzano et al. showed no significant difference in the abilities of most-dense and least-dense platelets to synthesize TXB₂ at 24 and 48 hours after aspirin administration; however, at 72 hours, least-dense platelets produced significantly more TXB₂ than did most-dense platelets. They concluded that the ability to synthesize thromboxanes was restored more rapidly in least-dense platelets than in most-dense platelets and that platelets increase in density as they age. Boneu and his associates reached a similar conclusion, measuring the reappearance of the ability of human platelets to form MDA upon addition of arachidonic acid. McDonald and Ali also used arachidonic acid in this type of experiment and observed that least-dense platelets regained their ability to synthesize TXB₂ more rapidly than did most-dense platelets. A somewhat different result was obtained by Leone and his co-workers. They measured MDA production in response to thrombin and found a parallel recovery of the ability of platelet subpopulations to form MDA after aspirin ingestion. However, unlike other investigators who used density gradients of Stractan, they separated platelets by repeated centrifugation of platelet-rich plasma at increasing concentration of the discontinuous Stractan density gradients would be attributable to activation of human platelets during centrifugation, or to the species difference. Our results are consistent with our earlier observations obtained in experiments in which a-SO₄ was administered to rabbits, and in experiments in which the survival of a-Cr and a-In labeled most-dense and least-dense platelets were measured. They are also in agreement with those of Karpatkin and Corash et al. who postulate that young platelets emerge from the bone marrow with a range of sizes, densities, and other properties that are determined during thrombocytopoiesis, but whose mean values are greater than those of the average-aged circulating platelet; however, with time spent in the circulation, these initially higher mean values could decline.

The concept that at least some platelets decrease in density as they circulate is supported by the observations that thrombin, ADP, and plasmin decrease platelet buoyant density; these are aggregating and release-inducing agents to which platelets may be exposed in vivo. In addition, several investigators have shown that a-granule contents are released from human platelets under in vivo conditions in which platelets may be activated; that least-dense platelets have fewer a-granules than do most-dense platelets; that least-dense platelets release less platelet factor 4 in response to ADP, collagen, or thrombin than do most-dense platelets; and that least-dense platelets are deficient in the granule glycoprotein, platelet factor 4.

REFERENCES

6. Corash L, Shafer B: Use of asplenic rabbits to demonstrate that platelet age and density are related. Blood 60:166, 1982
9. Rand ML, Packham MA, Mustard JF: Survival of density populations of rabbit platelets: Use of a-Cr or In-labeled platelets...
to measure survival of least dense and most dense platelets concurrently. Blood 61:362, 1983
33. Packham MA, Perry DW, Kinlough-Rathbone RL, Rand ML, Guccione MA, Evans RM, Mustard JF: Effects on the buoyant density of rabbit platelets of ADP and agents that increase the concentration of cyclic AMP. Blood 65:564, 1985
Duration of the effect of aspirin on the synthesis of thromboxane by density subpopulations of rabbit platelets stimulated with thrombin

MA Packham, MA Guccione and KM O'Brien