The Effect of Cold on Platelets. III. Adenine Nucleotide Metabolism After Brief Storage at Cold Temperature

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With the technical assistance of Dorothy Mormino

The effect of cold on platelet adenine nucleotide (PAN) metabolism was studied. Spontaneous aggregation which occurs when chilled platelet-rich plasma (PRP) is simultaneously warmed and stirred was not accompanied by the changes in adenine nucleotides associated with the release reaction. Connective tissue caused the release of the same amount of ADP and conversion of equal amounts of ATP to IMP and hypoxanthine in cold-stored platelets as it did in room temperature stored platelets. However, cold did have an important effect on PAN. In PRP stored at cold (0°C, 3°C) temperatures and warmed up to 37°C in the presence of ³H adenine, there was an increase in the conversion of adenine to its metabolites and ultimately to hypoxanthine as compared to PRP stored at warmer temperatures. This effect could not be prevented by ouabain, prostaglandin E₁, antibody to immunoglobulin M or adenosine.

Cold-temperature storage affects human platelets in several ways. Some of these are as follows: (1) Chilled platelets spontaneously aggregate when warmed and stirred.¹ (2) After prolonged warming without stirring, at which time this spontaneous aggregation disappears, the previously chilled platelets respond better to aggregating agents than unchilled platelets.² (3) When cold-stored platelets are transfused their in vivo survival is shorter than room-temperature-stored platelets.³ In this study we have determined whether these effects are due to cold-induced changes in adenine nucleotide metabolism.

MATERIALS AND METHODS

Chemicals

Tritiated adenine (8-³H, specific activity 19 Ci/m mole, 0.5 mCi/ml), dissolved in water, was obtained from Schwartz-Mann (catalog No. 1633-11 Van Nuys, Calif.). Nonradioactive chemicals were obtained from Sigma Chemical Company, St. Louis, Mo. except for prostaglandin E₁ (PGE₁) which was obtained from Upjohn Company, Kalamazoo, Mich. Antiserum to human IgM was obtained from Miles Laboratories, Inc., Kankakee, Ill. (catalog #61-021).
Platelet-Rich Plasma (PRP)

PRP was prepared by differential centrifugation of blood obtained from normal volunteers and anticoagulated with either 1/10 volume of 3.8% sodium citrate or NIH formula A ACD (1.5 ml ACD to 10 ml blood). For one study 0.01 M EDTA, and sodium heparin 2.5 U/ml were also used.

Connective Tissue Suspension (CT)

CT was prepared by a modification4 of the method of Zucker and Borrelli.5

Platelet Suspensions

Platelet suspensions were prepared from 10 ml of blood anticoagulated with 1.5 ml of ACD and 0.5 ml of 0.1 M EDTA, pH 7.4. PRP was prepared and centrifuged 10 min at room temperature in a Clay-Adams Serofuge to sediment the platelets. The platelet button was resuspended in the buffer described by Gaintner et al.6 containing 0.01 M EDTA. This was repeated twice. The final resuspension was in the same buffer containing 0.002 M EDTA.

Platelet counts were performed electronically (Coulter Counter, Model FN). Extracts of PRP or suspensions were prepared with an equal volume of a mixture of 9 parts ethanol and 1 part 0.1 M EDTA. pH 7.4.

Chemical content of ATP and ADP of the extracts was determined by the luciferin-luciferase technique recently modified by Holmsen et al.7 Platelet metabolites of radioactive adenine were measured after separation by high-voltage electrophoresis,8 on Whatman 3 MM chromatography paper. Internal standards were used to identify the metabolites. The appropriate spots were cut out and their radioactivity measured by liquid scintillation counting.

RESULTS

CT-Induced Release Reaction After Cold Storage

The adenine nucleotide response to CT was similar in PRPs stored at either 0°C or 22°C. Similar amounts of ADP and ATP were released and similar amounts of ATP were converted to IMP and hypoxanthine (Table I). These re-

<table>
<thead>
<tr>
<th>Expt No</th>
<th>Plasma 22°C</th>
<th>ADP 22°C</th>
<th>ADP 0°C</th>
<th>ATP 22°C</th>
<th>ATP 0°C</th>
<th>Change in Radioactive Compounds</th>
<th>IMP 22°C</th>
<th>IMP 0°C</th>
<th>HYP 22°C</th>
<th>HYP 0°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.2</td>
<td>2.0</td>
<td>1.3</td>
<td>2.0</td>
<td>-11.0</td>
<td>+9.3</td>
<td>+7.0</td>
<td>-8.0</td>
<td>+5.4</td>
<td>+3.0</td>
</tr>
<tr>
<td>2</td>
<td>1.5</td>
<td>1.8</td>
<td>1.6</td>
<td>1.8</td>
<td>-9.5</td>
<td>+6.2</td>
<td>+5.8</td>
<td>-15.0</td>
<td>+4.8</td>
<td>+3.2</td>
</tr>
<tr>
<td>3</td>
<td>0.14</td>
<td>0.16</td>
<td>0.14</td>
<td>0.27</td>
<td>-3.0</td>
<td>+2.0</td>
<td>+1.4</td>
<td>-5.0</td>
<td>+2.5</td>
<td>+3.1</td>
</tr>
</tbody>
</table>

*Expressed as μmoles/1011 platelets in the original PRP.
†Expressed as change in percent radioactivity for each compound (e.g., -8% ATP means a fall from 78% to 70% in the fraction of total radioactivity represented by ATP).

* Diluted CT used

PRP stored 4 hr at either 0°C or 22°C. Warmed at 37°C 1 hr in presence of 0.5 μM 3H adenine and aggregated with 1/10 volume of CT suspension for 2 min at which time 1/10 volume 0.1 M EDTA was added. The mixture was chilled and centrifuged at 10,000 g at 4°C. Supernatant was extracted and platelets resuspended in Tris buffered saline, pH 7.4 containing 5 mM EDTA and extracted. Radioactive compounds measured in cell suspensions. Chemical ATP and ADP measured in plasma. Experiments performed in duplicate and results averaged.
Table 2. Measurement of Release Reaction in Cold-Induced Aggregation

<table>
<thead>
<tr>
<th>Expt No</th>
<th>ATP</th>
<th>IMP</th>
<th>HYP</th>
<th>ATP + ADP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+1.0</td>
<td>-0.15</td>
<td>+0.2</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>-2.0</td>
<td>+0.70</td>
<td>-0.3</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>+4.0</td>
<td>+0.30</td>
<td>+1.0</td>
<td>0</td>
</tr>
<tr>
<td>Mean</td>
<td>+1.0</td>
<td>+0.28</td>
<td>+0.3</td>
<td></td>
</tr>
</tbody>
</table>

Sodium citrate PRP labeled with 0.5 μM ³H adenine was stored at 0°C for 4 hr after which it was warmed at 37°C during agitation. A sample from the same PRP was chilled and processed without agitation as a control. Both were processed as in Table 1. Results expressed as in Table 1. Experiments performed in duplicate and averaged.

Experiments were not affected by diluting the CT to overcome a possibly overwhelming effect of the undiluted CT.

Adenine Nucleotide Change During Cold-Induced Platelet Aggregation

Cold-stored ³H adenine labeled PRP was spontaneously aggregated by agitation at 37°C. The release reaction was not observed. There was no release of ADP or ATP into the plasma nor conversion of ATP to IMP or hypoxanthine (Table 2).

Adenine Metabolism After Cold-Temperature Storage

PRP was stored at cold temperatures and warmed at 37°C in the presence of ³H adenine. During this warming more adenine was converted to metabolites in cold stored PRP than in RT or freshly prepared (control) PRP. This is reflected by a more rapid decrease in adenine in the 0°C stored PRP and increase in radioactive ATP and hypoxanthine (Fig. 1) as well as ADP (not shown). A similar increase in adenine conversion and its metabolites occurred in PRP stored at 3°C and warmed at 37°C (not shown). Even room-temperature-stored platelets took up more adenine and converted more to ATP and hypoxanthine than did control platelets. After storage of PRP 4 hr at 37°C, conversion of adenine to metabolites was the same as in freshly prepared PRP (not shown).

Fig. 1. The effect of storage of ACD PRP at 22°C and 0°C on adenine conversion to its metabolites (in this figure adenine uptake by platelets is equated with decrease of adenine in PRP). After 4 hr storage at these temperatures the PRP was incubated with 5 μM (10 x previous amount) ³H adenine at 37°C and extracted at intervals. The specific activity had been reduced to 5 Ci/mmole by addition of nonradioactive adenine. The control was incubated with ³H adenine without storage. Mean ±1 SD of three experiments.
Table 3. Adenine Decrease and Hypoxanthine Production After 2 and 4 Hr Storage at 0°C and 22°C

<table>
<thead>
<tr>
<th></th>
<th>Adenine Decrease</th>
<th>Hypoxanthine Production</th>
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<tbody>
<tr>
<td></td>
<td>22°C</td>
<td>0°C</td>
</tr>
<tr>
<td>2 hr</td>
<td>-311 ± 17</td>
<td>-408 ± 82</td>
</tr>
<tr>
<td>4 hr</td>
<td>-366 ± 23</td>
<td>-588 ± 26</td>
</tr>
</tbody>
</table>

ACD PRP stored 2 and 4 hr at 0°C and 22°C, warmed at 37°C in presence of 3H adenine and extracted after 1 hr. Results expressed in nmoles/10^11 platelets. Mean ± 1 SD of three experiments. Decrease in adenine and increase in hypoxanthine (the end product of adenine metabolism) are used to represent adenine conversion to its metabolites.

Storage of ACD PRP for periods of time at 0°C shorter than 4 hr also increased adenine conversion to its metabolites (Table 3). Significant differences were detected after 2 hr of storage.

This cold-induced increase in adenine conversion to its metabolites also occurred in PRP prepared from blood anticoagulated with EDTA or heparin (Table 4). There were some differences with these anticoagulants. In particular, adenine conversion was diminished in PRP prepared from heparin. This may reflect the decreased yield of platelets obtained when we prepare PRP from heparinized blood.

Since the increased adenine conversion may reflect increased Na-K ATPase activity upon rewarming due to imbibing of sodium and water during cold storage,9 chilled ACD PRP was incubated with 1 mM ouabain before the addition of 3H adenine. However, this did not prevent the increased adenine conversion in cold-stored PRP (Table 5). In fact, adenine decrease and hypoxanthine production was increased in both RT and cold-stored PRP in the presence of ouabain as compared with ACD PRPs incubated without ouabain (e.g., Table 4).

It has been suggested that cold temperatures fix IgM and complement onto the platelet membrane.10 To avoid this possible cause for cold-induced increase in adenine conversion to metabolites, platelet suspensions were chilled in the presence of goat antiserum to human IgM. Increased adenine conversion to metabolites, as reflected by increased hypoxanthine production, still occurred in cold-stored PRP (Table 6). Adenine conversion to metabolites was much increased in these suspensions even after room-temperature storage and increased

Table 4. Effect of Cold on Adenine Conversion to its Metabolites in PRP Collected in Various Anticoagulants

<table>
<thead>
<tr>
<th></th>
<th>Adenine Decrease</th>
<th>Hypoxanthine Production</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>22°C</td>
<td>0°C</td>
</tr>
<tr>
<td>ACD</td>
<td>46</td>
<td>81</td>
</tr>
<tr>
<td>Sodium citrate</td>
<td>48</td>
<td>100</td>
</tr>
<tr>
<td>EDTA</td>
<td>48</td>
<td>71</td>
</tr>
<tr>
<td>Heparin</td>
<td>38</td>
<td>59</td>
</tr>
</tbody>
</table>

ACD PRP stored at 22°C and 0°C 4 hr then 37°C 1 hr in presence of 5 μM 3H adenine (5 Ci/mmole). Results expressed as per cent of total radioactivity. Mean of two experiments each performed in duplicate. Decrease in adenine and increase in hypoxanthine are used to represent conversion of adenine to its metabolites.
Table 5. Effect of Ouabain on Increased Hypoxanthine Production Caused by Cold

<table>
<thead>
<tr>
<th></th>
<th>22°C</th>
<th>0°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>21.5±4</td>
<td>32.3±7.2</td>
</tr>
</tbody>
</table>

Hypoxanthine production in ACD PRP stored at 22°C or 0°C and then incubated in presence of 1 mM ouabain 30 min prior to adding 5 μM [3H] adenine (5 Ci/m mole) at 37°C. Adenine uptake not shown since it was complete (99%) even at 22°C. Results expressed as percent of total radioactivity. Mean ± 1 SD of three experiments.

Table 6. Effect of Cold in Platelet Suspensions With and Without Antibody to Human Immunoglobulin M

<table>
<thead>
<tr>
<th></th>
<th>Adenine Decrease</th>
<th>Hypoxanthine Production</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>22°C 0°C</td>
<td>22°C 0°C</td>
</tr>
<tr>
<td>Suspension only</td>
<td>76 99</td>
<td>9 ± 1.7 16 ± 4.0</td>
</tr>
<tr>
<td>Suspension + antibody</td>
<td>99 99</td>
<td>13 ± 2.2 26 ± 4.5</td>
</tr>
</tbody>
</table>

Platelet suspension incubated with and without antibody to IgM 4 hr at 22°C and 0°C and then warmed 30 min at 37°C in presence of 5 μM (5 Ci/m mole) [3H] adenine. Results expressed as per cent of total radioactivity. Mean ± 1 SD of three experiments. Decrease in adenine and increase in hypoxanthine production are used to represent conversion of adenine to its metabolites.

even more in the presence of the antiserum. For this reason the warmed PRP was extracted at 30 min instead of the 1 hr as in the previous studies.

Adenosine inhibits adenine uptake. Therefore 5 μM adenosine was added to PRP which had been stored 4 hr at cold or room temperature. Upon rewarming at 37°C, increased adenine conversion to metabolites still occurred in the cold-stored PRP but at a slightly lower level (results not shown).

PGE1 has been suggested as a tool to use in resuspending platelet concentrates prepared at cold temperatures. We added this compound to PRP, 200 ng/ml, prior to storage of the PRP at cold temperatures or room temperature. However, this did not prevent an increase of adenine conversion to its metabolites in cold-stored PRP (results not shown).

Fig. 2. The effect of storage temperature on PRP labeled with [3H] adenine before storage. ACD PRP was incubated with 0.5 μM [3H] adenine (19 Ci/mM) 1 hr at 37°C (all adenine converted to metabolites). The PRP was then placed at either 22°C or 0°C 4 hr. rewarmed at 37°C an additional 4 hr and the change in radioactive ATP and hypoxanthine during this rewarming period measured. Results, expressed as in Table 1, represent mean ± 1 SD of six experiments. p < 0.01 for differences in both ATP and hypoxanthine when 22°C versus 0°C storage are compared.
Effect of Cold on Prelabeled Platelets

The increased adenine conversion to metabolites in cold-stored PRP reflects in large part increased platelet adenine uptake, since adenine is not metabolized by platelet poor plasma. To determine if there is increased ATP hypoxanthine conversion, PRP was labeled with $^3$H adenine and then kept 4 hr at either 0°C or 22°C and rewarmed at 37°C. After 4 hr at 37°C there was a decline in ATP and increase in hypoxanthine in both specimens but this was much greater in the cold-stored PRP (Fig. 2).

DISCUSSION

Cold-stored platelets aggregate when warmed and stirred. This is not due to the extrusion of ADP from the platelets during storage since this compound was not present in the plasma prior to stirring and therefore is not responsible for the initiation of aggregation. In this study we have found that ADP is not released during aggregation nor is platelet ATP converted to IMP and hypoxanthine. Thus cold-induced aggregation is not associated with the release reaction after the onset of aggregation.

Recently we have found that cold-stored platelets aggregate better in response to connective tissue than room-temperature-stored platelets. In that study serotonin release was not increased in the connective tissue aggregated cold-stored platelets and thus we postulated that the mechanism whereby cold storage improved the response to connective tissue was not due to better preservation of the release reaction. In this study we have specifically measured adenine nucleotide changes and confirmed this hypothesis since adenine nucleotide release and ATP conversion to IMP hypoxanthine was the same in connective tissue aggregated cold-stored platelets as in connective tissue aggregated room-temperature-stored platelets.

Perhaps the significant effect of cold on platelet adenine nucleotide metabolism is the increased conversion of adenine to metabolites in cold-stored platelet rich plasma warmed to 37°C. One factor responsible must be increased adenine uptake since adenine is not metabolized in platelet free plasma. Recently, Sixma, et al. found that adenine uptake is increased in platelets exposed to ADP in the presence of EDTA, which suggests that the increased adenine uptake is due to disc-sphere transformation caused by ADP. A similar mechanism, i.e., sphering of cold-stored platelets may be responsible for the increased adenine uptake in this situation.

However, we also observed a disproportionate increase in hypoxanthine formation in cold-stored platelets warmed in the presence of adenine. Hypoxanthine cannot be derived directly from adenine. Platelets convert adenine to nucleotides which in turn are catabolized to hypoxanthine. No direct pathway of adenine hypoxanthine conversion exists in platelets. Therefore, increased hypoxanthine formation must be due to increased adenine nucleotide catabolism. This is confirmed by our studies with platelets whose adenine nucleotides were labelled before storage. There was significantly greater ATP hypoxanthine conversion in cold-stored PRP upon warming at 37°C than in room-temperature-stored PRP.

The cause of the increased ATP hypoxanthine conversion in cold-stored PRP
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which has been rewarmed is not clear. One point of interest is that when 3H adenine labeled PRP is incubated in the presence of metabolic inhibitors such as 2-deoxy-D-glucose and KCN, increased ATP hypoxanthine conversion occurs. This similarity to the effect of cold suggests that a metabolic defect may develop in cold-stored platelets. This is conceivable in light of several studies which have demonstrated an adverse effect of cold on metabolism. For example, Heldt and Klingenberg have found that the access of adenine nucleotides to mitochondria is limited by low temperature. Perhaps more important is a demonstration by Penefsky and Warner that the mitochondrial protein Fl, which is responsible for the coupling of oxidation to phosphorylation, is irreversibly inactivated by cold storage. Cold may also effect glycolysis; for example, phosphofructokinase, which may be a rate-limiting enzyme in platelet glycolysis, is unstable in the cold. Finally, it is also possible that cold somehow activates AMP deaminase, which is the enzyme responsible for AMP-hypoxanthine conversion. However, although many compounds can activate or inhibit the enzyme, the effect of cold has not to our knowledge been described. Whatever its mechanism of action, cold storage appears to affect platelet adenine nucleotide metabolism. In particular, the increased ATP hypoxanthine conversion may be an adverse effect since, if it is not reversible, the platelets could become depleted of ATP. Whether this effect on adenine nucleotides accounts for the shortened in vivo survival of transfused cold-stored platelets is not answered directly by these studies, but it would appear to be one possible mechanism.

ACKNOWLEDGMENT

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REFERENCES

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The Effect of Cold on Platelets. III. Adenine Nucleotide Metabolism After Brief Storage at Cold Temperature

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