In Vitro Hypothermia Enhances Platelet GPIIb-IIIa Activation and P-Selectin Expression

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Background: A clinical bleeding diathesis is associated with hypothermia. Inhibition of platelet reactivity is the purported cause of this coagulopathy despite inconsistent evidence to support this hypothesis. To clarify the effect of temperature on intrinsic platelet function, platelet GPIIb-IIIa activation and P-selectin expression were assessed under normothermic and hypothermic conditions in vitro.

Methods: Blood was obtained by venipuncture from healthy volunteers. Platelet activation was assessed by aggregometry and by cytometric analysis of platelet binding of fibrinogen, PAC-1, and P-selectin antibodies. Measurements were made at normothermia (37°C), moderate hypothermia (33°C), and profound hypothermia (22°C) after stimulating samples with adenosine diphosphate (ADP), collagen, or thrombin receptor activating peptide.

Results: Agonist-induced platelet aggregation and fibrinogen binding were significantly greater at 22°C and 33°C than at 37°C. Platelet fibrinogen binding values to 20 μM ADP were 23,400, 14,300, and 9,700 molecules/platelet at 22°C, 33°C, and 37°C, respectively. The aggregation responses of platelets that were cooled and rewarmed were indistinguishable from those of platelets maintained at 37°C throughout the study. Platelet binding of PAC-1 and P-selectin antibodies was greater under hypothermic conditions.

Conclusions: Aggregation, fibrinogen binding, PAC-1 binding, and P-selectin antibody binding studies showed that platelet GPIIb-IIIa activation and α-granule release were enhanced at hypothermic temperatures. Thus hypothermia appears to increase the ability of platelets to respond to activating stimuli. The coagulopathy associated with hypothermia is not likely to be the result of an intrinsic defect in platelet function. (Key words: Aggregation; coagulopathy; GPIIb-IIIa; hypothermia; platelet; P-selectin; temperature.)

A clinical bleeding diathesis is associated with both deliberate and inadvertent hypothermia. However, the complex clinical settings in which coagulopathy and hypothermia have been observed simultaneously has made it difficult to determine the specific coagulation defect associated with hypothermia. This is particularly evident in cases involving extracorporeal circulation and trauma, in which coagulopathy can result from exposure of blood to artificial surfaces, to massive volume replacement, or both, instead of hypothermia per se.

Many investigators have tried to identify the hemo-static defect caused by hypothermia using various in vivo and in vitro techniques. In a baboon model of in vivo hypothermia, Valeri et al. described prolonged bleeding time and reduced platelet thromboxane production. Similar results have been described in humans. Using P-selectin and glycoprotein Ib (GPIb) expression as in vitro measures of platelet activation, Michelson et al. reported that hypothermia reversibly inhibits platelet activation. Despite the fact that bleeding time is not a measure specific for platelet function, it is generally accepted that hypothermia inhibits platelet reactivity.

The conclusion that hypothermia inhibits platelet function is particularly troublesome because it is at odds with much of the literature on platelet function. Hypothermia has been shown to induce morphologic changes in platelet structure suggestive of activation and to induce platelet protein phosphorylation, which is a sensitive measure of activation. In vitro platelet aggregation also has been shown to increase at hypothermic temperatures. Importantly, aggregation is the result of activation-induced binding of fibrinogen to its platelet receptor GPIIb-IIIa. However, the ef-
fect of temperature on GPIIb-IIIa activation has not been assessed. The importance of GPIIb-IIIa activation is underscored by the fact that patients with absent or dysfunctional GPIIb-IIIa receptors demonstrate a clinical bleeding diathesis,10 and that blockers of GPIIb-IIIa have clinical utility in inhibiting thrombus formation.17

Although some of the literature suggests that platelet activation occurs at hypothermic temperatures, many of the reported observations have been made at temperatures not clinically encountered. To clarify the effect of temperature on intrinsic platelet function, we measured the physiologically critical platelet function of GPIIb-IIIa activation. We also measured platelet P-selectin expression under hypothermic conditions. We hypothesized that in vitro hypothermia would enhance GPIIb-IIIa activation and that this could be measured as an increase in platelet aggregation, fibrinogen binding, and PAC-1 binding (an antibody specific for the activated conformation of GPIIb-IIIa18) at cold temperatures.

Methods

Volunteers

Healthy men and women (21 men, 12 women) between the ages of 20 and 45 yr volunteered to participate in our study. After informed consent and medical histories were obtained, we excluded anyone with acute or chronic medical illness or who had ingested any drugs in the past 10 days. The use of aspirin and nonsteroidal anti-inflammatory drugs was specifically sought.

Platelet Aggregation Studies

Blood was drawn by venipuncture through a 21-gauge needle. After discarding the initial 5 ml. blood was collected and anticoagulated with 3.8% sodium citrate (9:1). Immediately after withdrawal, 500-μl aliquots of blood were placed into plastic cuvettes and maintained for 30 min at 33°C and 37°C (for the moderate hypothermia experiments), or at 22°C and 37°C (for the profound hypothermia experiments). Platelet-poor plasma was prepared from a portion of the whole blood by centrifugation, and whole-blood samples were diluted 1:1 with platelet-poor plasma maintained at either 22°C, 33°C, or 37°C. In the moderate hypothermia experiments, whole-blood aggregation was measured with the aggregometer temperature maintained at 35°C or 37°C in concordance with the sample temperature. In the profound hypothermia experiments, whole blood aggregation was performed at 22°C or 37°C. Aggregation in response to 1, 5, and 20 μM adenosine diphosphate (ADP) and to 0.1, 1, and 10 μg/ml collagen was measured by impedance aggregometry using two Chrono-Log Lumi-aggregometers (Havertown, PA), as previously described.19 Results are reported for peak impedance response in ohms. The maximal slope of aggregation was defined as the peak increase in impedance per minute.

In 10 experiments, blood was cooled and rewarmed to normothermic temperature before analysis. In these experiments, samples were maintained in a water bath at either 22°C, 33°C, or 37°C for 30 min and then all samples were allowed to equilibrate at 37°C for an additional 10–15 min. The temperature of cooled samples was verified to reach 37°C in <10 min. Aggregation in all samples was then analyzed with the aggregometer temperature set at 37°C. Control studies did not detect a difference in platelet aggregation responses for samples run between 30–60 min of blood drawing.

Platelet–Fibrinogen Binding Studies

Fibrinogen binding to platelets in a plasma-free medium was quantified essentially as described previously.20 Briefly, blood obtained by venipuncture was anticoagulated in acid-citrate-dextrose anticoagulant. Platelets were isolated from whole blood by differential centrifugation and then resuspended in Tyrode’s buffer maintained at 22°C, 33°C, or 37°C. Fibrinogen labeled with fluorescein isothiocyanate (FITC-FGN) was added to the platelet suspensions at a final concentration of 100 μg/ml. Platelets were stimulated with ADP (0, 1, 5, 10, and 20 μM) for 20 min at the designated temperatures. Platelet samples were examined for FITC-FGN binding using flow cytometric analysis, and the number of fibrinogen molecules bound per platelet was measured as previously described.20 All samples were maintained at the designated temperature until the time of cytometric analysis using a portable water bath. Cytometric acquisition of data was complete within 30 s. For analysis, platelets were gated based on their characteristic scatter profile, and these gates were used for all samples in each experimental set to ensure that platelet aggregates were excluded.

PAC-1 and P-selectin Binding Studies

Blood obtained by venipuncture was anticoagulated with heparin and centrifuged at 180g for 15 min to obtain platelet-rich plasma. This plasma was then diluted 1:15 in Tyrode buffer with heparin (final heparin...
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collection, 1 U/ml) that was maintained at either 22°C or 37°C. The FITC-labeled PAC-1 antibody (4 μg/ml) or FITC-labeled P-selectin antibody (2.5 μg/ml) were added to platelet-rich plasma before stimulation with ADP (0, 2, or 20 μM) or 50 μM thrombin receptor activating peptide (TRAP). All samples were incubated in a water bath maintained at either 22°C or 37°C for 20 min before flow cytometric analysis. The quantification of FITC-PAC-1 and FITC-P-selectin antibody binding to platelets was similar to that described for FITC-FGN; except data are reported as fluochrome equivalents per platelet (instead of molecules per platelet) because a fluorometric determination of the FITC to protein ratio was not available for these antibodies.

Statistical Analysis

Results are reported as means ± SEM. Data from aggregation studies and PAC-1 and P-selectin binding assays were analyzed using paired Student's t-tests. Data from fibrinogen binding studies were analyzed using one-way analysis of variance for repeated measures and post hoc Newman-Keuls analysis was used to determine the significance between individual means. Probability values <0.05 were considered significant.

Results

To determine the effect of moderate hypothermia on platelet function, we compared aggregation responses at 33°C and 37°C. Platelet aggregability in whole blood was greater at 33°C than at 37°C for each of the ADP concentrations tested (fig. 1A, table 1). Results showed that both the magnitude and rate of aggregation in response to ADP were greater at the hypothemic temperature. Changes in temperature appeared to have no effect on collagen-induced aggregation (tables 1 and 2). As expected, the magnitude of aggregation depended on the concentration of agonist and was higher for collagen (strong platelet agonist) than for ADP (weak agonist). Thus moderate in vitro hypothermia did not adversely affect platelet function, as previously described, but tended to increase platelet aggregability.

To determine the effect of profound hypothermia on platelet function, we compared aggregation responses at 22°C and 37°C. Once again, platelets demonstrated greater reactivity at the lower temperature. Specifically, platelet aggregation in whole blood was greater at 22°C than at 37°C in response to 1 and 5 μM ADP (fig. 1B). A dose response to increasing ADP concentrations was

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Fig. 1. Adenosine diphosphate-induced peak platelet aggregation in response to (A) moderate in vitro hypothermia (33°C), (B) profound in vitro hypothermia (22°C), and (C) in vitro platelet cooling to 22°C with subsequent rewarming to 37°C. Results are means ± SEM. *P < 0.05 versus 37°C. **P < 0.01 versus 37°C. n = 10 in each set of experiments.
Table 1. Rate of Aggregation in Response to ADP and Collagen

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<th>33°C</th>
<th>37°C</th>
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<tr>
<td>ADP 1 μM</td>
<td>7.1 ± 2.0*</td>
<td>2.8 ± 1.2</td>
</tr>
<tr>
<td>ADP 5 μM</td>
<td>14.8 ± 1.0*</td>
<td>9.8 ± 1.9</td>
</tr>
<tr>
<td>ADP 20 μM</td>
<td>16.0 ± 0.9*</td>
<td>12.4 ± 1.5</td>
</tr>
<tr>
<td>Collagen 0.1 μg/ml</td>
<td>1.2 ± 0.2</td>
<td>0.7 ± 0.2</td>
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<tr>
<td>Collagen 1.0 μg/ml</td>
<td>18.2 ± 1.8</td>
<td>18.0 ± 2.0</td>
</tr>
<tr>
<td>Collagen 10.0 μg/ml</td>
<td>27.7 ± 1.1</td>
<td>29.1 ± 1.6</td>
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Results are for maximum slope of aggregation reported as change in impedance units per minute; values are mean ± SEM.
ADP = adenosine diphosphate.
* P < 0.01 by paired t tests for 33°C versus 37°C (n = 10 for ADP experiments and n = 10 for collagen experiments).

again noted at 37°C. However, this relation was lost at 22°C, with maximum aggregation occurring with the lowest dose tested. Thus it appeared that lower concentrations of ADP induced maximal platelet aggregation at profoundly hypothermic temperature but produced only submaximal stimulation under normothermic conditions.

Next we tried to determine the effect of a short period of rewarming on platelets that had been cooled to hypothermic temperatures. Figure 1C shows aggregation responses of platelets maintained under these conditions. Platelets maintained at 22°C or 33°C and then warmed to 37°C exhibited aggregation responses that were indistinguishable from those maintained continuously at normothermic temperature. Thus the cold-induced increase in platelet reactivity appeared to be reversed rapidly when normothermic temperature was reestablished.

To determine if results obtained in whole blood were specific for changes intrinsic to platelet function, we isolated platelets in a medium free of erythrocytes, leukocytes, and plasma. We measured the ability of platelets to bind fibrinogen by quantifying agonist-induced binding of FITC-FGN to GPIIb-IIIa at different temperatures. Figure 2 shows results from cytometric studies and greater platelet fibrinogen binding at lower temperature (P < 0.01 by one-way analysis of variance). Post hoc analyses confirmed that platelets bound more fibrinogen at 22°C than at 33°C or 37°C when stimulated with 1, 5, and 20 μM ADP. Therefore, it appeared that intrinsic platelet reactivity was greater at hypothermic temperature than at 37°C as assessed by the ability of individual platelets to bind fibrinogen.

Greater platelet fibrinogen binding to hypothermic platelets implied greater GPIIb-IIIa activation with lower temperature, because only the activated conformation of GPIIb-IIIa is capable of high-affinity fibrinogen binding. To verify this, we measured platelet binding of PAC-1 antibody, which recognizes only the activated conformation of GPIIb-IIIa, at normothermic and hypothermic temperatures. PAC-1 binding was greater under hypothermic conditions in response to both ADP and TRAP stimulation (fig. 3). There was a small but statistically significant increase in PAC-1 binding in hypothermic unstimulated samples, suggesting the possibility of spontaneous activation.

Table 2. Peak Aggregation in Response to Collagen

<table>
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<tr>
<th>Collagen Concentration (μg/ml)</th>
<th>33°C</th>
<th>37°C</th>
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<tbody>
<tr>
<td>0.1</td>
<td>2.3 ± 0.4</td>
<td>1.3 ± 0.4</td>
</tr>
<tr>
<td>1.0</td>
<td>35.4 ± 2.7</td>
<td>33.6 ± 2.8</td>
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<tr>
<td>10.0</td>
<td>68.0 ± 2.9</td>
<td>63.4 ± 2.9</td>
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Results are peak aggregation responses (in ohms) to the indicated collagen concentrations reported as mean ± SEM (n = 10).

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Fig. 3. Platelet PAC-1 binding at hypothermic temperature. Platelet-rich plasma was incubated with fluorescein-labeled PAC-1 antibody (4 µg/ml) and adenosine diphosphate or thrombin receptor activating peptide for 20 min at the indicated temperatures. Platelet PAC-1 binding was determined using flow cytometric analysis. Results are means ± SEM. *P < 0.05 versus 37°C, **P < 0.01 versus 37°C. n = 6.

of some platelets at 22°C. Thus PAC-1 binding studies confirmed that hypothermia enhanced activation of platelet GPIIb-IIIa receptors.

To determine if platelet α-granule release was similarly affected by hypothermia, we measured P-selectin expression (P-selectin is present on platelet α-granules) in response to ADP and TRAP. It has been reported previously that modest increases in P-selectin expression in response to ADP stimulation can be detected by flow cytometric analysis. We found no difference in P-selectin expression between platelets at 22°C and 37°C when stimulated with ADP (fig. 4). However, TRAP-stimulated platelets expressed more P-selectin at hypothermic temperatures than did platelets at normothermia (fig. 4). Again, there was a small but statistically significant increase in P-selectin expression in hypothermic unstimulated samples suggestive of spontaneous platelet activation at 22°C.

Discussion

Using three different methods to assess platelet GPIIb-IIIa activation—platelet aggregation, platelet fibrinogen binding, and PAC-1 binding—we found that moderate and profound degrees of in vitro hypothermia enhanced activation of this important adhesive receptor. P-selectin binding studies also showed that hypothermia enhanced agonist-inducible platelet α-granule release, although to a lesser degree than GPIIb-IIIa activation. These results are in stark contrast to the conclusions drawn by some investigators based on nonspecific measures of platelet function (e.g., bleeding time, shed blood thromboxane release) but correspond with those of other researchers who describe an activating effect of hypothermia on platelets. These results do not change the observation that hypothermia is associated with clinical bleeding, but they suggest that inhibition of intrinsic platelet function is not the cause of this coagulopathy. Importantly, these results provide a potential mechanism to explain pathologic platelet activation in hypothermic patients.

Although both platelet GPIIb-IIIa activation and P-selectin expression were enhanced at hypothermic temperatures, these effects appeared to depend on the platelet agonist used. Increased aggregability was clearly demonstrated for ADP but was not apparent for collagen. Unlike collagen activation, platelet stimulation with another potent agonist, TRAP, dramatically increased GPIIb-IIIa activation at hypothermic temperature. Hypothermia also en-

Fig. 4. Platelet P-selectin expression at hypothermic temperature. Platelet-rich plasma was incubated with fluorescein-labeled P-selectin antibody (2.5 µg/ml) and adenosine diphosphate or thrombin receptor activating peptide for 20 min at the indicated temperatures. Platelet P-selectin antibody binding was determined using flow cytometric analysis. Results are means ± SEM. *P < 0.05 versus 37°C. n = 6.
hanced P-selectin expression in response to TRAP but not to ADP. Thus the potency of an agonist at inducing platelet degranulation (both collagen and thrombin are considered strong in this regard, but ADP is weak) does not appear to define platelet susceptibility to the effects of temperature. The disparity in results obtained with ADP, collagen, and TRAP likely reflects a fundamental difference in their mechanisms of platelet activation. Importantly, however, there was no decrease in platelet reactivity at hypothermic temperature in any of our assays or with any agonist.

Fibrinogen and PAC-1 binding experiments confirmed that hypothermia increased intrinsic platelet reactivity by enhancing the exposure of activated GPIb-IIIa receptors. The number of fibrinogen binding sites we observed in this study closely approximated results reported before. The mechanism by which hypothermia augmented surface expression of activated GPIb-IIIa receptors is not clear. Our results suggest that both enhanced receptor activation and increased receptor number participated in this process, because both PAC-1 and P-selectin antibody binding were increased under hypothermic conditions. (GPIb-IIIa receptors are present on platelet α-granules.) An alteration in receptor affinity with hypothermia does not explain the increased number of PAC-1 and P-selectin binding sites we observed, because receptor affinities for our antibodies were not affected by temperature (data not shown). Regardless of the mechanism by which hypothermia enhanced platelet activation, it appeared that this process was rapidly reversible with restoration of normothermia.

Our findings of enhanced platelet aggregation and P-selectin expression at hypothermic temperatures contrast sharply with those of Michelson et al., who reported that in vitro hypothermia reduced platelet aggregability and P-selectin expression. However, there are many methodologic differences between these studies that could explain the disparate results. Aggregation, in Michelson and colleagues’ studies, was performed in a washed platelet preparation instead of the whole blood assay that we used. Although the washed platelet preparation allowed isolated platelets to be studied, this preparation had many disadvantages that made it less physiologic. In addition to the prolonged delay required for platelet isolation, the washed platelet preparation was performed in buffer that lacked exogenous fibrinogen. Under those conditions, platelet aggregation depends on fibrinogen release from α-granule stores. The finding by Michelson et al. that washed platelet aggregation proceeded more slowly at hypothermic temperature was consistent with their other finding that P-selectin expression was delayed with hypothermia (fibrinogen is contained in α-granules). However, it may not accurately reflect in vitro aggregation events.

Results of whole blood aggregation assays can be more easily applied to the in vitro setting because all the cellular and plasma elements that participate in aggregation are present. Our whole blood aggregation assays confirm the results of other investigators in which hypothermia augmented agonist-induced aggregation in platelet-rich plasma. Furthermore, because aggregation depends on GPIb-IIIa activation and subsequent fibrinogen binding, results of PAC-1 and fibrinogen binding assays provide a mechanistic explanation for hypothermia’s effect on aggregation that is internally consistent. The finding that hypothermia modestly increased TRAP-induced P-selectin expression neither confirms nor refutes the results found by Michelson et al. The reduction in agonist-induced P-selectin expression that those authors observed was limited to the first few minutes after agonist stimulation (and was absent at 10 min), and all our studies were performed after a 20-min incubation period (equilibrium conditions). The clinical relevance of a brief delay or prolonged increase in P-selectin expression is not well characterized. However, an increase in P-selectin expression has been associated with some clinical thrombotic states.

There is no doubt that hypothermic patients are at risk for a clinical bleeding diathesis to develop and that prudent perioperative management includes maintenance of normothermia. However, our results cast doubt on the purported mechanism of this coagulopathy (i.e., that hypothermia inhibits platelet function). Thrombus formation is the result of a complex series of biochemical and biophysical events that is initiated by exposure of platelets to activating stimuli. Our results suggest that hypothermia enhances the intrinsic capacity of platelets to respond to these activating stimuli. Furthermore, our results lead us to hypothesize that hypothermia results in coagulopathy by reducing the availability of platelet activators. This hypothesis is supported by the following observations: (1) The generation of thrombin, a potent platelet agonist, decreases under hypothermic conditions; and (2) hypothermia results in the release of a circulating anticoagulant with heparin-like effects. Kestin et al. similarly concluded that prolongation of the bleeding time in patients after cardiopulmonary bypass is likely the result of a lack of available platelet activators rather than an intrinsic platelet defect.

Indeed, our results suggest that hypothermia might
promote pathologic platelet aggregation in the presence of platelet activators. This suggestion may have clinical relevance under conditions in which hypothermic blood is exposed to platelet activators such as extracorporeal circuits or atherosclerotic plaque. It is important to note that under our experimental conditions platelets were exposed to hypothermia for only a brief period (30-45 min). Therefore, it is difficult to extrapolate to the clinical setting, where more prolonged periods of hypothermia might be encountered. Nonetheless, there is evidence to support the hypothesis that clinical hypothermia may promote pathologic platelet activation. It has been reported that normothermic cardiopulmonary bypass preserves platelet function better than hypothermic bypass, and that hypothermia promotes the development of myocardial ischemia in patients having vascular surgery. It is tempting to speculate that thrombogenic surfaces, such as extracorporeal circuits, vascular graft material, or atherosclerotic plaque, allow elaboration of platelet activators in sufficient quantity to stimulate hypothermic/hyperactivatable platelets. A better understanding of the interaction of platelets with normal endothelium and diseased or artificial surfaces at different temperatures is required to address this hypothesis.

References

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