Effects of Low Temperature on Shear-Induced Platelet Aggregation and Activation

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Background: **Hemorrhage is a major complication of trauma and often becomes more severe in hypothermic patients. Although it has been known that platelets are activated in the cold, studies have been focused on platelet behavior at 4°C, which is far below temperatures encountered in hypothermic trauma patients. In contrast, how platelets function at temperatures that are commonly found in hypothermic trauma patients (32–37°C) remains largely unknown, especially when they are exposed to significant changes in fluid shear stress that could occur in trauma patients due to hemorrhage, vascular dilation/constriction, and fluid resuscitation.**

Methods: **Using a cone-plate viscometer, we have examined platelet activation and aggregation in response to a wide range of fluid shear stresses at 24, 32, 35, and 37°C.**

Results: **We found that shear-induced platelet aggregation was significantly increased at 24, 32, and 35°C as compared with 37°C and the enhancement was observed in whole blood and platelet-rich plasma. In contrast to observation made at 4°C, the increased shear-induced platelet aggregation at these temperatures was associated with minimal platelet activation as determined by the P-selectin expression on platelet surface. Blood viscosity was also increased at low temperature and the changes in viscosity correlated with levels of plasma total protein and fibrinogen.**

Conclusion: **We found that platelets are hyper-reactive to fluid shear stress at temperatures of 24, 32, and 35°C as compared with at 37°C. The hyperreactivity results in heightened aggregation through a platelet-activation independent mechanism. The enhanced platelet aggregation parallels with increased whole blood viscosity at these temperatures, suggesting that enhanced mechanical cross-linking may be responsible for the enhanced platelet aggregation.**

Key Words: **Hypothermia, Shear stress, Platelet activation, Aggregation.**

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Bleeding arrest is the fundamental function of human hemostasis and is accomplished by two basic systems: platelets and coagulation/fibrinolysis. Both systems are constantly regulated to maintain a proper balance. Disruption of this balance will inevitably result in abnormalities, ranging from bleeding to thrombosis. Such a disruption is best illustrated in the pathophysiological process of trauma.

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Trauma is the most common cause of death in young people from age 1–45. Bleeding and its complications contribute significantly to the trauma morbidity and mortality.[1] The majority of deaths occurring during the first 24 hours following traumatic events result from uncontrolled hemorrhage.[2] In response to trauma, the hemostatic system is first mobilized locally to stop bleeding. Platelets play a critical role in this early response by adhering to the injured vessel wall to form a hemostatic plug.[3] The whole body response that follows includes systemic hypotension, vasoconstriction to ensure blood supply to vital organs, mobilization of blood storage, and increased production of coagulation factors and platelets.[3] These responses, however necessary, may produce complications, which become even more severe when traumatic hemorrhage is associated with hypothermia.,[3–6] resulting in a very poor prognosis.[7,8] It has been shown that a core body temperature of 32°C or less is associated with a mortality of near 100%, even in mildly injured patients.[9,10,23,24] This hypothermia-related coagulopathy may be caused by three distinct, but overlapping abnormalities in coagulation enzymes, fibrinolytic system, and platelets.[5,11–14]

Circulating platelets adhere to blood vessel subendothelium exposed by injuries to the blood vessel wall. The adhesion and subsequent aggregation stop bleeding by forming a plug at the site of injury. This process is initiated by an interaction between the platelet glycoprotein (GP) Ib-IX-V complex and the subendothelial von Willebrand factor (VWF), and completed with fibrinogen cross-linking platelets through its

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interaction with the platelet GP IIb-IIIa complex (integrin αIIbβ3). In addition, platelets can also aggregate similarly in vivo when the blood fluid shear stress is significantly elevated such as it may be found in significantly constricted arteries. Interestingly, in contrast to other cells, low temperatures (4°C) often render platelets hyperreactive.[15,16] Studies conducted nearly half a century ago demonstrated that exposure of platelets to 4°C results in changes of platelet shape from the resting discoid state to activated spherical cells with pseudopods induced by rearrangement of the platelet cytoskeleton.[13,15,17,18] and an increase in intracellular calcium concentrations.[15,19] Recent studies indicate that even prolonged storage of platelets in 22°C causes the activation of GP IIb-IIIa.[20–22] Although these studies are critical for understanding the mechanism through which platelets are activated in the cold, temperatures used in these studies are far lower than that observed in hypothermic trauma patients. In contrast, platelet function at sub-body temperatures of 32–37°C remains largely unknown.

Here, we report that platelets are hyperreactive to fluid shear stress at clinically relevant hypothermic temperatures (32–35°C) and the hyperreactivity of platelet aggregation at these temperatures is likely due to increased mechanical cross-linking rather than enhanced platelet activation.

MATERIALS AND METHODS

Blood Sample Collection and Platelet Preparation

Blood was collected from 38 healthy donors, who were medication-free for at least two weeks before blood draw, under a strict protocol of using human blood that was approved by the Institutional Review Board for Human Subject Research for Baylor College of Medicine and Affiliated Hospitals. All donors signed consent forms before the blood draw. Blood was collected into vials containing 0.38% sodium citrate (final concentration). Blood samples were either used for the experiments directly or processed to obtain platelet-rich plasma (PRP). The latter was obtained by centrifuging whole blood at 150 $\times$ *g* for 15 min at 24°C.

Shear-Induced Platelet Aggregation

The method for inducing shear-induced platelet aggregation has previously been described.[25,28] Briefly, platelets (500 μL of PRP or whole blood) were exposed to various levels of shear stress for 60 seconds on a fully computerized cone-and-plate viscometer (RS1, HAAKE Instrument Inc., Paramus, NJ). Shear-induced platelet aggregation was induced under temperatures of 24, 32, 35, or 37°C.

For platelet aggregation in PRP, 10 μL of the sheared PRP sample was immediately mixed with 10 mL of Isoton II solution (Beckman-Coulter, Miami, FL) containing 0.5% glutaraldehyde. The number of platelets was then counted by a Z2 Coulter Counter (Beckman-Coulter). Shear-induced platelet aggregation was defined as the percent reduction of single platelets as compared with the unsheared control from the same donor. Platelet aggregation in whole blood was measured by flow cytometry. For this, 10 μL of the sheared sample was mixed with 1 mL of phosphate-buffered saline (PBS) containing 1% paraformaldehyde. The fixed samples were analyzed on a Coulter Epic XL MCL flow cytometer (Beckman-Coulter) by first identifying the platelet population based on the particle size on forward scatter and then collecting data for 30 sec from the defined platelet gate. Platelet aggregation was again defined as the reduction of single platelets in the platelet gate.[28,39]

To mimic the effects of fluid resuscitation on platelet aggregation, blood samples were also diluted one to one with lactated Ringer's solution.

Blood Viscosity Measurement

Fluid viscosity of whole blood was determined real-time on the cone-and-plate viscometer under different temperatures and shear stresses by measuring the torque force required to rotate the cone at a defined speed. The viscosity was expressed as centapoise (cp).

Measurement of Platelet Activation

Expression of P selectin on the platelet surface was the most commonly used marker for platelet activation[25,28,40] and therefore used here to evaluate platelet activation under different fluid shear stresses. For this, 10 μL of PRP that was exposed to shear stress was mixed with 90 μL of Ca^{++} and Mg^{++} free Tyrode's buffer (137.0 mmol/L NaCl, 2.0 mmol/L $MgCl_2$, 0.4 mmol/L NaH_2PO_4, 11.9 mmol/L $NaHCO_3$, 2.9 mmol/L KCl, 5.5 mmol/L Glucose, pH 7.35) containing 1% bovine serum albumin and 5 μg/mL of a R-Phycoerythrin-conjugated monoclonal anti–CD62P antibody (BD-PharMingen, San Diego, CA) for 20 min at room temperature. After incubation, 1 mL of PBS containing 1% paraformaldehyde was added and the samples were analyzed for CD62P expression on a flow cytometer (Beckman-Coulter). Unsheared PRP was used as the negative control.

In addition to comparison among samples sheared at different temperatures, levels of platelet activation induced by shear were also compared with those activated by 20 μmol/L of ADP. For this, PRP was first treated with 20 μmol/L of ADP and then stained for the surface P-selectin in the same way as that of sheared platelets. The P-selectin expression was quantitated as the artificial units of geometrical mean fluorescence on FL2 scale.

Statistical Analysis

The experimental data were all presented as mean $\pm$ SE. The unpaired 2-tailed Student's *t* test was used for all data analysis and a *p* value less than 0.05 was considered to be statistically significant.

RESULTS

Shear-Induced Platelet Aggregation was Enhanced at Low Temperatures

We tested shear-induced platelet aggregation at three different shear rates. These shear rates were chosen because

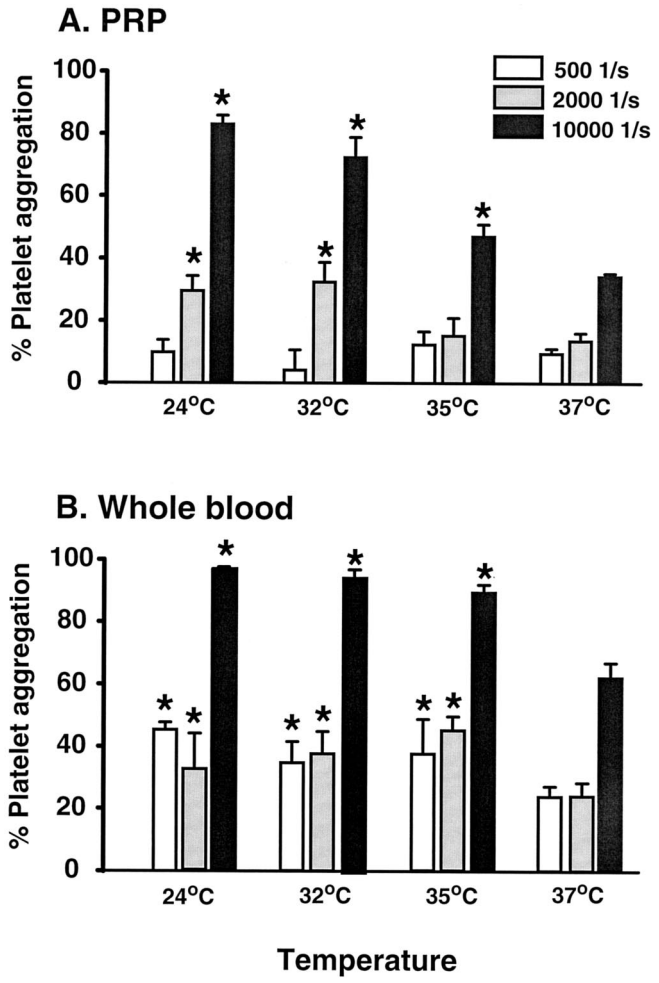

Fig. 1. *Shear-induced platelet aggregation at different temperatures. Citrated whole blood or PRP was sheared at rates of 500, 2000, or 10,000^{-s} at different temperatures for 1 min and platelet aggregation determined by the reduction of single platelets. The percentage of platelet aggregation was higher at low temperatures for both PRP (A) and whole blood (B) samples at arterial shear rates of 2000 and 10,000^{-s} (ANOVA test, n = 24 for each group, * p < 0.01 compared with control at 37°C). The percentage of platelet aggregation was also higher at a low shear rate of 500^{-s} in the whole blood sample (ANOVA test, n = 24 for each group, * p < 0.01 compared with control at 37°C).*

their relevance to hydrodynamic conditions that may be found in patients with trauma hemorrhage. For example, a low shear rate of 500^{-s} mimics that in veins or hypovolemic blood flow induced by traumatic bleeding. A 2000^{-s} shear rate is commonly encountered in arteries, whereas a high shear rate of 10,000^{-s} may be found in constricted vessels.[28] When platelets in PRP were sheared at a high rate of 10,000^{-s} at 24, 32, 35, and 37°C, the percentage of platelet aggregation significantly increased with decreases in temperatures (Fig. 1A). The difference was also observed at 2000^{-s} shear rate, where the enhanced platelet aggregation was found at both 24 and 32°C, but not at 35°C as compared with that at 37°C. There was no significant difference in the levels of aggregation at the low shear rate of 500^{-s}. Using PRP allows us to directly investigate the effects of low temperature on platelets, but this approach fails to consider the indirect effects brought on by other cells such as erythrocytes, which contribute significantly to the blood viscosity and are the major source of the platelet agonist ADP. To address this concern, whole blood was used for shear-induced platelet aggregation. As shown in Figure 1B, the percentage of platelet aggregation in whole blood was also increased at low temperatures. Compared with PRP, levels of platelet aggregation in whole blood were significantly higher at all shear rates tested including a low shear rate of 500^{-s}.

Fluid Shear Stress has Minimal Effects on Shear-Induced Platelet Activation

We have previously demonstrated that shear-induced platelet aggregation requires minimal platelet activation.[25] To test if platelets are more shear-activated at lower temperatures, we measured platelet activation on samples that were subjected to various shear rates at different temperatures using P-selectin as the platelet activation marker. In whole blood, P-selectin expression increased in samples sheared at different shear rates and at different temperatures, with the highest levels found in samples sheared at a high shear rate of 10,000^{-s} (Fig. 2A). There was no significant increase in P-selectin expression for samples sheared at 24, 32, and 35°C. Similarly, there was no difference for PRP samples sheared at 24 and 37°C (Fig. 2B). Consistent with our previous observations, P-selectin expression in samples exposed to shear stress was significantly less than that by 20 μmol/L of ADP, a common platelet agonist.

Blood Viscosity Increased at Low Temperature

As shown in Figure 1, the levels of platelet aggregation in whole blood is greater than that in PRP, suggesting that, in addition to the direct effects on platelets, low temperature may affect platelet function indirectly through other cells, such as erythrocytes. Since red blood cells are the major contributor of blood viscosity and low temperature is known to increase viscosity of other viscous fluids,[26,27] we measured whole blood viscosity at 24, 32, 35, and 37°C with fluid shear rates of 500, 2000, and 10,000^{-s}. We found that blood viscosity was lower in samples sheared at higher rates (Fig. 3). The viscosity of whole blood was higher at low temperatures as compared with those at 37°C (Fig. 3). At a shear rate of 500^{-s}, blood viscosity increased 43.4% and 26.2% at 32 and 35°C, respectively as compared with that at 37°C (1.75 ± 0.01, 1.54 ± 0.02, and 1.22 ± 0.04 at 32, 35 and 37°C, respectively, ANOVA test, n = 33 for each group, $p < 0.01$). In comparison, the percent increases in blood viscosity at a pathologic high shear of 10,000^{-s} were only 15.3% and 13.5%, respectively as compared with that at 37°C (1.36 ± 0.01 at 32°C, 1.34 ± 0.03 at 35°C, and 1.18 ± 0.02 at 37°C, ANOVA test, n = 33 for each group, $p < 0.05$). As found in

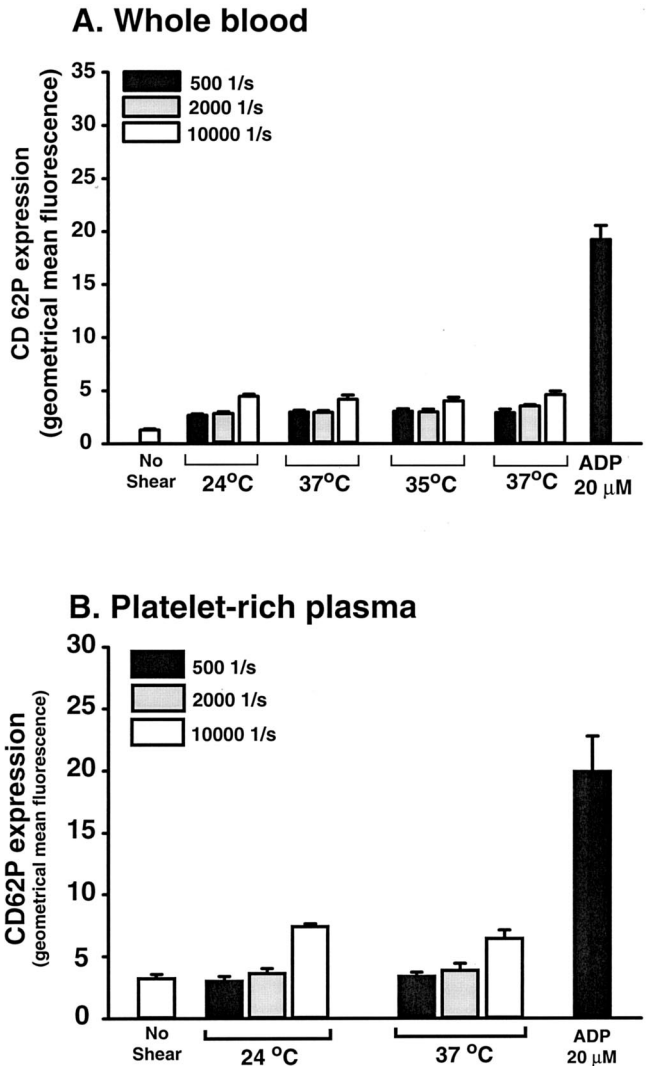

Fig. 2. *Shear-induced platelet activation at different temperatures. Citrated whole blood or PRP was sheared at rates of 500, 2000, or 10,000^{-s} at different temperatures for 1 min and platelet activation measured by the P-selectin expression on platelet surface as determined by flow cytometry. The levels of P-selectin expression were significantly higher in platelets sheared at a high shear rate of 10,000^{-s} in both whole blood (A) and PRP (B) samples. For both whole blood and PRP, no significant difference in P-selectin expression was observed among samples sheared at different temperatures as compared with those at 37°C (ANOVA test, n = 22 for each group).*

platelet aggregation, the viscosity increase was most significant at 24°C (Fig. 3).

Changes in Viscosity Correlated with Concentrations of Blood Proteins

Blood is a viscous fluid and its viscosity, which is constituted by blood proteins and largely by erythrocytes, is one of the determinants of shear stress. To determine how blood proteins contribute to the blood viscosity, we measured levels

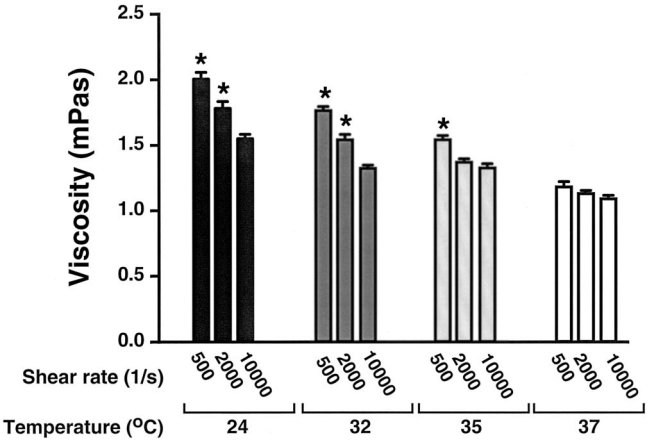

Fig. 3. *Whole blood viscosity at different temperatures and shear rates. Citrated whole blood was sheared at rates of 500, 2000, or 10,000^{-s} at different temperatures for 20 sec and viscosity measured in real-time on a cone-and-plate viscometer. Viscosity increased at low temperature and the increase was more significant at low shear (Student's* t *test, n = 12 in each group, * p < 0.001 compared with those at 37°C). Viscosity decreased with increased shear rates and the changes were again more significant at low temperatures.*

of total blood proteins and fibrinogen, one of the most abundant blood proteins that is critical for platelet aggregation, and correlated these measurements to that of whole blood viscosity. As shown in Figure 4, plasma protein levels correlated significantly with blood viscosity at both low and high shear stresses. Furthermore, plasma level of fibrinogen also correlated with blood viscosity (Fig. 4C). In contrast, the whole blood viscosity showed no correlation with hematocrits measured in 14 donors ($r^2 = 0.038$).

Effects of Hemodilution on Shear-Induced Platelet Aggregation

Shear-induced platelet aggregation is caused by platelet collision so that numbers of cells in blood play a critical role in the process, especially in whole blood that contains significant amount of erythrocytes.[28] Blood dilution, which can result from fluid resuscitation following trauma, may reduce the frequency of platelet-platelet collision and therefore decrease platelet aggregation. To test this hypothesis, we mixed whole blood or PRP with Ringer's lactate solution at a ratio of 1:1 and measured shear-induced platelet aggregation of these diluted samples. For whole blood, the amount of platelet aggregation in diluted samples at 32°C were reduced significantly as compared with the undiluted samples at shear rates of 2000 and 10,000^{-s} (Fig. 5A). The reduction in platelet aggregation was also observed at 37°C (Fig. 5B). In comparison, platelet aggregation in PRP samples was largely unaffected except at a high shear rate of 10,000^{-s}. Diluted samples aggregated significantly less at 32°C, but not 37°C (Fig. 6A & B).

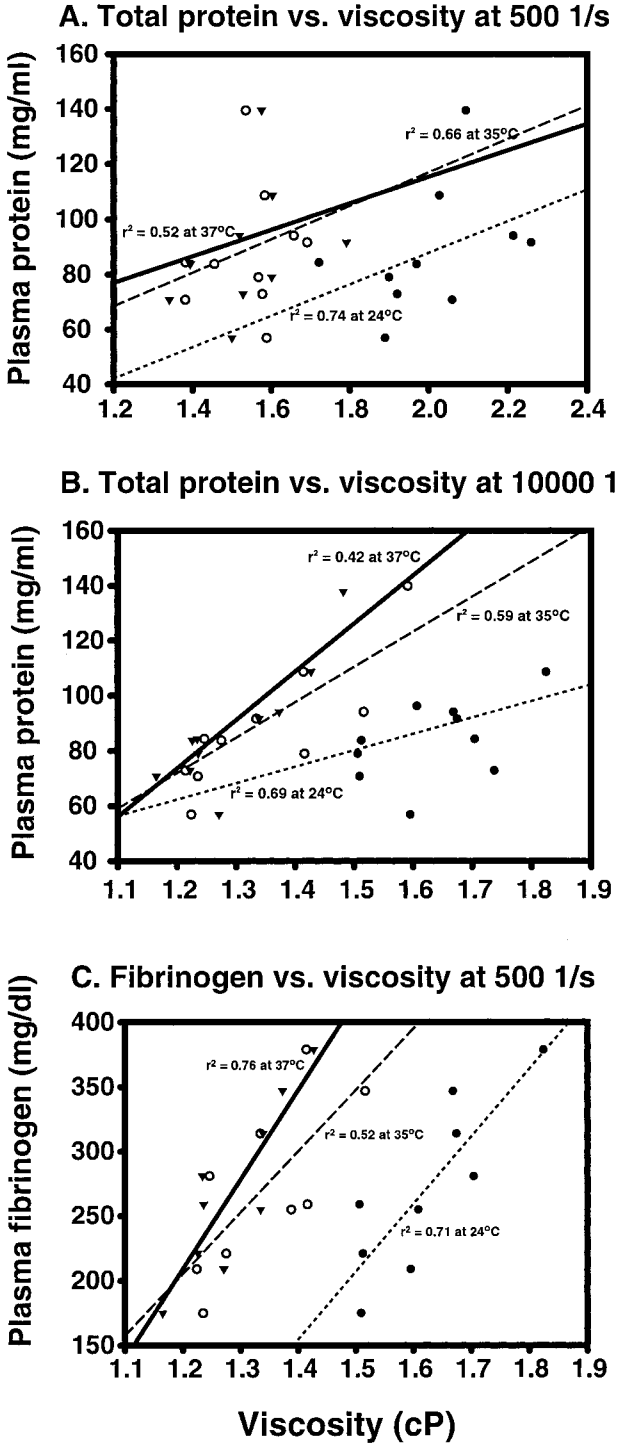

Fig. 4. *Correlation between whole blood viscosity and plasma proteins. Levels of total plasma proteins and fibrinogen were quantitated and correlated with whole blood viscosity by a leaner regression model. Viscosity correlation with total proteins was determined at both 500 (A) and 10,000^{-s} (B) shear rates, whereas that with fibrinogen at a low shear rate of 500^{-s} (C).*

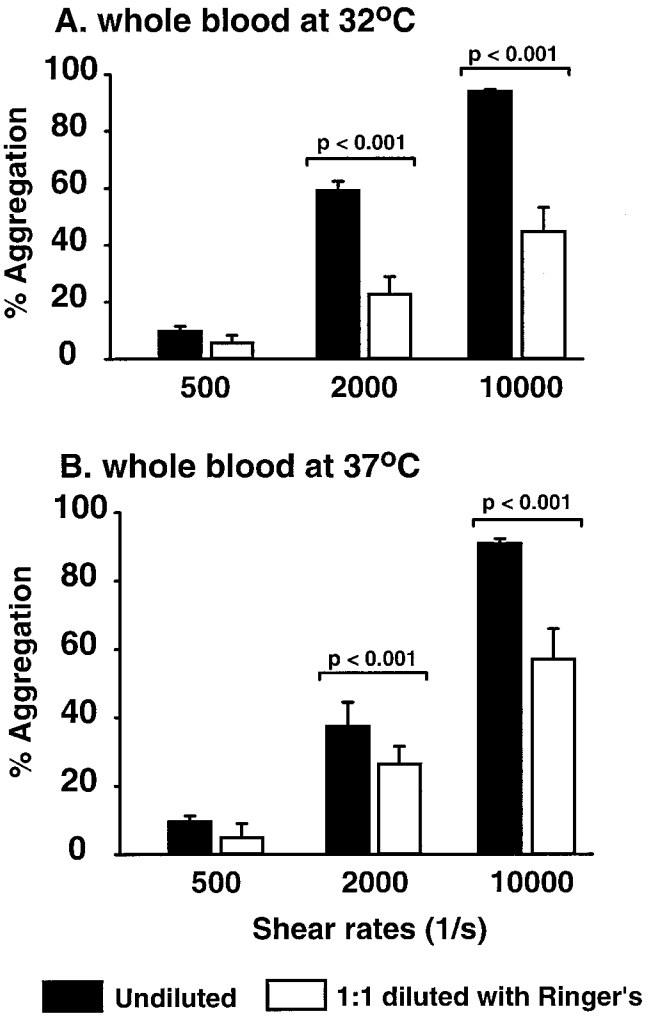

Fig. 5. *Shear-induced platelet aggregation in diluted whole blood at low temperatures. Citrated whole blood was first diluted 1:1 with the Ringer's solution and then exposed to different shear stresses at either 32°C (A) or 37°C (B). Shear-induced platelet aggregation was determined by flow cytometry and calculated as the percentage reduction of single platelets. Hemodilution significantly reduced the amount of shear-induced platelet aggregation at the arterial shear rates of 2000 and 10,000^{-s} for both temperatures (Student's* t *test, n = 4 in each group). The reduction in shear-induced platelet aggregation was 74.2% and 52.8% for 2000 and 10,000^{-s} shear rates at 32°C (A) as compared with 32.2% and 35.8% reduction at 37°C (B).*

DISCUSSION

We have demonstrated that shear-induced platelet aggregation is significantly enhanced at clinically relevant temperatures of 32 and 35°C as compared with that at 37°C in both whole blood and PRP samples (Fig. 1). For PRP, the enhanced platelet aggregation is demonstrated at arterial shear rates of 2000 and 10,000^{-s}, but not at a venous shear rate of 500^{-s}, whereas for whole blood, it is found in all three-shear rates tested (500, 2000, and 10,000^{-s}). The enhanced platelet

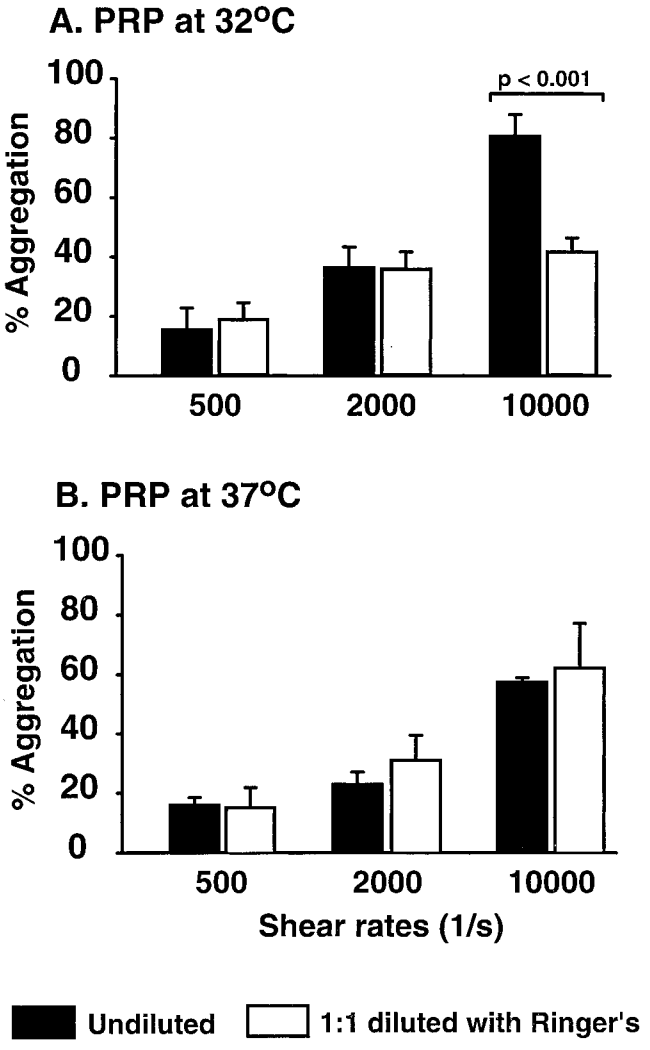

Fig. 6. *Shear-induced platelet aggregation in diluted PRP at low temperatures. Citrated PRP was first diluted 1:1 with the Ringer's solution and then exposed to different shear stresses at either 32 (A) or 37°C (B). Shear-induced platelet aggregation was determined by particle counts and calculated as the percent reduction of single platelets. Shear-induced platelet aggregation at 32°C was similar between undiluted and diluted samples at shear rates of 500 and 2000^{-s}, whereas it was significantly less in diluted samples at a high shear rate of 10,000^{-s} (Student's* t *test, n = 4 in each group). There was no significant difference in shear-induced platelet aggregation at all temperatures and shear rates at 37°C (B, Student's* t *test, n = 4 in each group).*

aggregation appears to be activation independent because P-selectin expression, a commonly used marker for platelet activation, is not increased (Fig. 2). This suggests that alternative mechanisms are involved in the observed platelet hyperreactivity at low temperatures.

Low temperature may affect shear-induced platelet aggregation by at least two ways: directly affecting platelets and indirectly altering properties of other cells and blood viscosity.[5] We find that platelet aggregation in PRP, which excludes erythrocytes and leukocytes, is greatly enhanced, suggesting that low temperature indeed has direct effects on platelets. However, the aggregation enhancement induced by low temperature is more significant in whole blood, especially at low shear, indicating that the enhancement may also result from non-platelet factors such as blood viscosity. Consistent with this possibility, we find that the viscosity of whole blood is significantly higher at low temperatures (32–35°C) (Fig. 3). The viscosity change is closely correlated with levels of total blood proteins as well as fibrinogen, the adhesion protein that is responsible for cross-linking platelets by shear stress, but not with hematocrits. Increase in whole blood viscosity is more apparent at a low shear rate, which also results in significantly enhanced platelet aggregation for whole blood, indicating that, at low shear, low temperature may enhance shear-induced platelet aggregation by increasing blood viscosity. One direct consequence of high blood viscosity is to produce higher shear stress on platelets without significant changes in blood flow velocity. Since the relationship between the wall shear stress (shear stress found near the vessel wall, where platelets are concentrated) and blood viscosity is expressed as:

$$\text{Shear stress } (\tau_w) = 4\,\mu Q/\pi r^3 \qquad (1)$$

(where μ is the blood viscosity, Q is the volumetric flow rate, and r is radial diameter of a vessel[28]), one can assume that any change in viscosity, when other parameters remain constant, will be amplified four-fold in shear stress.

Previous studies have shown that platelets are spontaneously activated at 4°C as demonstrated by calcium influx and platelet shape changes.[13,15,16,18,19,29,30] The latter is caused by the reorganization of platelet cytoskeleton.[17] Winokur and Hartwig[15] demonstrated that actin filaments undergo rearrangements by uncapping the barbed end to elongate actin filaments in chilled platelets.[15] In addition, microtubules, which are critical in maintaining the discoid shape of resting platelets, depolymerize and dissolve in the cold.[13,30,31] These studies provide valuable insight into the physiology of platelets in the cold (4°C) and are important for developing efficient ways to the long-term storage of platelets. However, whether platelets undergo similar changes at clinically relevant temperatures of 32–35°C remains largely unknown. For example, the surface density of the GP Ib-IX-V complex has been found to be reduced at 4°C, but not at 22°C,[21,22] a temperature at which platelets were recently found to be partially activated.[20] One potential mechanism may be through changes in the lipid structures of platelet membrane. It has been known that low temperature clusters lipid rafts of platelet membrane.[19,32,33] The cluster may be caused by the platelet membrane lipid transition from a liquid-crystalline to gel phase as demonstrated by the Fourier transform infrared spectroscopy.[13,18,32,34] The transition occurs at 15–18°C for membrane phospholipids, but at about 30°C for lipid rafts.[33,35] Since shear-induced platelet aggregation is initiated by the GP

Ib-VWF interaction,[36] potential changes in either VWF or its receptor, the GP Ib-IX-V complex could contribute to the enhanced platelet aggregation observed at low temperature. The GP Ib-IX-V complex is translocated to the lipid rafts[37] so that the cluster of the lipid domains may result in aggregation of the GP Ib-IX-V complex. As a result, the locally concentrated GP Ib-IX-V complex may increase the binding avidity for VWF by providing more binding sites for the multimeric VWF. This possibility is consistent with our finding that the levels of platelet activation are not increased at low temperature (Fig. 2) and is further supported by our previous studies showing that shear-induced platelet aggregation requires minimal platelet activation.[25] The rearrangement of the GP Ib-IX-V complex may also alter the conformation of the GP Ib-IX-V complex, resulting in interaction of the complex with other molecules. Indeed, Hoffmeister et al.[16] have recently shown that platelets in the cold rearrange the surface configuration of GP Ibα, the ligand binding subunit,[36] to bind $\alpha_{m\beta2}$ integrin (CD11b/CD18, MAC-1). Since $\alpha_{m\beta2}$ integrin is the major mediator of antibody-independent clearance by hepatic macrophages, the labeled platelets are phagocytosed in hypothermia mice and cleared from blood.

Finally, low temperature or higher shear stress brought by high viscosity may also change the conformation of adhesion ligand such as VWF to enhance its binding to platelets as suggested by previous studies showing that shear stress changes the VWF conformation from a globule structure to a linear one.[38]

Our results suggest that platelets may have a heightened response to mechanical stress at low temperatures (32–35°C) in patients with trauma-associated hypothermia. As a result, they may form microaggregates in response to changes in blood hydrodynamics induced by trauma-associated hemorrhage and the body's stress responses, which will change blood flow rate, shear stress, and viscosity. These platelet microaggregates may then be rapidly cleared from blood, resulting in consumptive thrombocytopenia and severe bleeding such as it is found in DIC. Furthermore, the formation of platelet microaggregates may also contribute to the development of multiple organ failure in hypothermia patients with severe trauma. These results will allow us to understand how platelets function under hypothermic conditions (combined with hemodilution) and to develop strategies to treat hypothermic coagulopathy and other complications.

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