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ORIGINAL PAPER

Temperature effects on the activity, shape, and storage of platelets from 13-lined ground squirrels

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Abstract The objective of this study is to determine how a hibernating mammal avoids the formation of blood clots under periods of low blood flow. A microfluidic vascular injury model was performed to differentiate the effects of temperature and shear rate on platelet adhesion to collagen. Human and ground squirrel whole blood was incubated at 15 or 37 °C and then passed through a microfluidic chamber over a 250-μm strip of type I fibrillar collagen at that temperature and the shear rates of 50 or 300 s^{-1} to simulate torpid and aroused conditions, respectively. At 15 °C, both human and ground squirrel platelets showed a 90–95% decrease in accumulation on collagen independent of shear rate. At 37 °C, human platelet accumulation reduced by 50% at 50 s^{-1} compared to 300 s^{-1}, while ground squirrel platelet accumulation dropped by 80%. When compared to platelets from non-hibernating animals, platelets from animals collected after arousal from torpor showed a 60% decrease in binding at 37 °C and 300 s^{-1}, but a 2.5-fold increase in binding at 15 °C and 50 s^{-1}. vWF binding in platelets from hibernating ground squirrels was decreased by 50% relative to non-hibernating platelets. The source of the plasma that platelets were stored in did not affect the results indicating that the decreased vWF binding was a property of the platelets. Upon chilling, ground squirrel platelets increase microtubule assembly leading to the formation of long rods. This shape change is concurrent with sequestration of platelets in the liver and not the spleen. In conclusion, it appears that ground squirrel platelets are sequestered in the liver during torpor and have reduced binding capacity for plasma vWF and lower accumulation on collagen at low shear rates and after storage at cold temperatures, while still being activated by external agonists. These adaptations would protect the animals from spontaneous thrombus formation during torpor but allow them to restore normal platelet function upon arousal.

Keywords Von Willebrand factor · Hypothermia · Fibrinogen · Collagen · Tubulin

Introduction

Blood clotting or hemostasis is a critical process in animals with a closed circulatory system. In primary hemostasis, circulating anucleated cells called platelets are activated, causing them to adhere to the vessel wall and aggregate with each other (Fig. 1). Platelet adherence to the vessel wall is mediated by surface receptor glycoprotein Ibα (GPIbα) binding to von Willebrand Factor (vWF) which in turn binds to collagen exposed at sites of vascular injury (Weiss et al. 1977; Lenting et al. 2012). Under high shear stress, vWF becomes elongated and is more likely to bind to platelets (Fuchs et al. 2010), but this is blocked at temperatures below 17 °C (Hewlett et al. 2011). Platelets also respond to shear stress, with GPIbα receptors clustering in lipid rafts and increasing binding to vWF (Gitz et al. 2013). Platelets aggregate with each other through binding of fibrinogen and other ligands to glycoprotein IIb/IIIa

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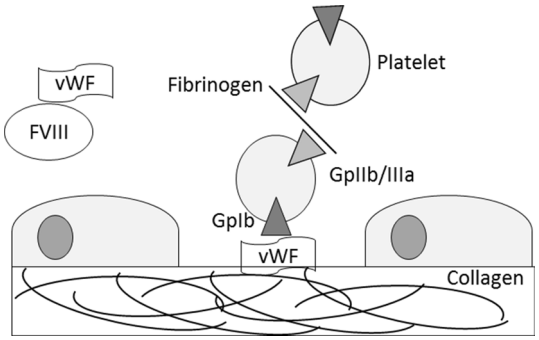

Fig. 1 Adhesion and aggregation of platelets. Platelets adhere to a damaged vessel wall through binding of their glycoprotein Ib (GpIbα) receptor to von Willebrand Factor (vWF) which in turn binds to exposed collagen. Platelet aggregation occurs when two platelets bind to a single molecule of fibrinogen through their glycoprotein IIb/IIIa receptors (GpIIb/IIIa). Glycosylated proteins on the surface of the platelets also contain sialic acid caps, and the removal of these leads to clearance through Ashwell–Morell receptors on hepatocytes

(GPIIb/IIIa) receptors (Marguerie et al. 1979). This binding is stimulated by various platelet agonists including thrombin and ADP (Zucker and Nachmias 1985).

When most mammalian platelets are chilled, they undergo both structural and functional changes termed cold storage lesions (Michelson et al. 1994; Vostal and Mondoro 1997; Hoffmeister et al. 2001; Thon et al. 2008; Springer et al. 2009; Van Poucke et al. 2014). For example, platelet aggregation, vWF and fibrinogen binding, and P-selectin expression are enhanced under hypothermic temperatures (Michelson et al. 1994; Berger et al. 1998; Faraday and Rosenfeld 1998; Engelfriet et al. 2000; Frelinger Iii et al. 2003; Xavier et al. 2007; Hogberg et al. 2009; Scharbert et al. 2010; Gitz et al. 2013). Platelets have a dynamic circumferential microtubule ring (Patel-Hett et al. 2008) and this depolymerizes in the cold, causing platelets to change from a discoid shape to a sphere (White and Krivit 1967; White and Rao 1998; Italiano et al. 2003). When platelets from mice are chilled, the GPIbα subunit of the vWF receptor clusters and is recognized by complement receptor type 3 (CR3) on Kupffer cells, and terminal sialic acid residues on glycoproteins are cleaved creating a ligand for Ashwell–Morell receptors on hepatocytes. Both events contribute to platelet phagocytosis, clearance, and apoptosis (Hoffmeister et al. 2003; Rumjantseva et al. 2009; van der Wal et al. 2010). Because of this, units of human platelets cannot be stored in the cold and can only be stored for 3–4 days at 22 °C, increasing the risk of bacterial contamination and resulting in half of all units being discarded (Engelfriet et al. 2000; Jacobs et al. 2001). The characteristics of cold storage lesions are observed in several mammalian species, but do not appear to occur in mammalian hibernators (Cooper et al. 2012; de Vrij et al. 2014).

Hibernating mammals routinely sustain body temperatures below 10 °C for weeks at a time. Hibernation can be broken into two phases: prolonged periods of torpor with decreased body temperature and heart rate, and brief 12–24 h periods of interbout arousal (IBA) with a rapid restoration of euthermia and heart rate (Carey et al. 2003). During the torpor phase of hibernation, 13-lined ground squirrels (*Ictidomys tridecemlineatus*), hereafter referred to as ground squirrels, reduce their body temperatures from 35–38 to 4–8 °C, heart rates from 200–300 to 3–5 beats/min, and respiration from 100–200 to 4–6 breaths/min (Lechler and Penick 1963; Reddick et al. 1973; Zatzman 1984). Under these conditions of low flow, hibernating mammals could be at increased risk of forming stasis clots or deep vein thrombi. Stasis is thought to promote thrombosis (i) by causing local hypoxia leading the endothelial cell activation and subsequent recruitment of platelet, leukocytes, and microparticles to the vessel wall via adhesive ligands (P- and E-selectin, vWF), and (ii) in the absence of dilution by flow, coagulation products can accumulate, leading to fibrin formation (Mackman and Davis 2011; Rana and Neeves 2016). The hibernators will have some protection because their lower body temperatures decrease both primary and secondary hemostasis by reducing the activity of the enzymes and receptors involved in both processes (Van Poucke et al. 2014). However, hibernators have also adapted to this extreme decrease in blood flow by reversibly decreasing platelet, monocyte, neutrophil, vWF, FVIII, and FIX levels (Suomalainen and Lehto 1952; Svihla et al. 1952a, b, 1953; Smith et al. 1954; Lechler and Penick 1963; Pivorun and Sinnamon 1981; Cooper et al. 2012, 2016; de Vrij et al. 2014).

During torpor, ground squirrel platelets go through reorganization of their circumferential microtubule ring to form long rods and circulating platelet levels drop by 90%, returning to normal levels within 2 h post arousal (Reddick et al. 1973; Reznik et al. 1975; Cooper et al. 2012; de Vrij et al. 2014). This rapid release is not consistent with new synthesis of platelets, but rather release of platelets that had been sequestered (Cooper et al. 2012). Initial models proposed that the rod shape may cause platelets to become trapped in the spleen (Reddick et al. 1973); however, splenectomy in Syrian hamsters (*Mesocricetus auratus*) did not affect sequestration during hibernation (de Vrij et al. 2014). New platelet synthesis by megakaryocytes in the bone marrow of hibernating ground squirrels lags 24–48 h behind arousal, indicating that platelet synthesis is also decreased during hibernation (Cooper et al. 2012). However, most proteins known to be involved in platelet function did not show differential expression in the bone marrow of hibernating squirrels including the surface glycoprotein receptors (*GPIBA, GPIBB, GPIIB, GPIIIB*) and proteins found

in secretory granules (*vWF*, P selectin) (Cooper et al. 2016).

Ground squirrels are adapted to spending several months each year with a body temperature of 4–8 °C, and their platelets are resistant to the clearance seen in non-hibernating mammals (Cooper et al. 2012). In this study, we measured binding of vWF and fibrinogen to platelets and platelet adhesion to collagen at different temperatures and shear rates to determine if the decrease in blood clotting during hibernation is a property of the squirrel's platelets, plasma, body temperature, or blood flow rate. In addition, the kinetics of the temperature-dependent microtubule rearrangements and location of platelets during torpor were examined.

Materials and methods

Animals

Ground squirrels were born in captivity and housed at the University of Wisconsin-La Crosse following protocols approved by the institutional IACUC. Non-hibernating animals were housed individually in rooms with a Wisconsin photoperiod (9 h in December gradually increasing to 15.5 h in June and then decreasing again). Animals were implanted with a temperature transponder (IPTT-300, BioMedic Data Systems) and body temperatures monitored with a hand-held reader. In October when an animal's body temperature dropped to 25 °C (ambient), they were moved into a 4 °C hibernaculum. Blood and organs were collected from hibernating animals in the torpid state in January and February at a body temperature of 9.8 ± 2.1 °C. Blood samples were collected from anesthetized animals at 2 h post arousal in March (36.4 ± 0.8 °C), and from non-hibernators in June–July (35.5 ± 2.6 °C).

Platelet isolation

Blood was collected in 1/9th volume of acid citrate dextrose (ACD) anticoagulant from the tail arteries of summer non-hibernating or winter IBA ground squirrels while being under anesthesia with isoflurane (1.5–5%). Blood cell counts were performed using a HemaVet HV950 (Drew Scientific, Waterbury, CT). Human blood was collected in ACD from volunteers by venipuncture. Differential centrifugation was performed at 25 °C with a brake to isolate platelets. Blood was centrifuged at 200×*g* for 8 min, and the platelet-rich plasma (PRP), white blood cells, and a limited number of red blood cells were isolated and centrifuged at 100×*g* for 6 min. Prostaglandin E1 (PGE1, final concentration of 6.0 µM) was added to the PRP to prevent platelet activation and centrifuged at 800×*g* for 5 min. Plasma aliquots were stored at −20 °C for incubations, and the platelet pellet was resuspended in 200 µl of Tyrodes-HEPES buffer (12 mM $NaHCO_3$, 138 mM NaCl, 5.5 mM glucose, 2.9 mM KCl, 10 mM HEPES, pH 7.4). Platelets were counted on a hemocytometer and resuspended at 1×10^9 platelets/ml in Tyrodes.

To measure the kinetics of platelet shape change, PRP was incubated at 37 °C for 2 h and then placed in an ice bath and aliquots removed at time points, fixed with 5% glutaraldehyde, and centrifuged onto polylysine-coated slides at 2100×*g* for 4 min in a CytoFuge 2 (StatSpin, Norwood, MA), before Wright staining and counting the percentage of platelets with a rod shape. To determine if the shape changes required tubulin polymerization or depolymerization, platelets were pre-incubated for 2 h at 37 °C, then incubated at 4 or 37 °C for 2 h, and fixed on slides as described. Some aliquots were treated with 1 µM taxol (Life Technologies, Carlsbad CA) or 250 nM nocodazole (Thermo Fisher) for 20 min at 37 °C prior to incubation at 4 or 37 °C. Platelets were visualized with anti-β-tubulin antibodies (Life Technologies, Eugene, OR) and a secondary Alexa Fluor 488 goat anti-mouse antibody (Life Technologies) as described previously (Cooper et al. 2012) and the percentage of platelets in a rod or ring formation was counted.

Fibrinogen binding

Fresh platelets were isolated from non-hibernating ground squirrels and allowed to rest for 30 min at room temperature to reduce isolation-induced platelet activation. PRP samples were stored for 2 days at either 4 °C or room temperature (20–25 °C). Platelets were diluted to 2×10^8/ml in Tyrodes, centrifuged at 300×*g* at 25 °C to remove plasma proteins, and resuspended in Tyrodes at 2×10^8/ml. The washed platelets were mixed with 0.5 µg/µl Alexa-488 fibrinogen (Invitrogen) and then activated with 50 µM ADP, 200 µM thrombin receptor activation peptide (TRAP), or 200 µM protease-activated receptor 4 peptide (PAR4) for 5 min in the dark at 37 °C. Samples were diluted in 1 ml PBS and fixed with 4% paraformaldehyde before counting on a flow cytometer. Mean fluorescence values were analyzed by two-way ANOVA for the human and squirrel TRAP results and three-way ANOVA for the three agonist-treated squirrel samples.

vWF binding

Changes in vWF binding during hibernation could be due to effects of temperature, changes in ground squirrel platelets, or a 10-fold decrease in vWF relative to normothermic ground squirrels (Cooper et al. 2016). To isolate these three variables, a triple-cross-over experiment was designed

in which platelets from non-hibernating and IBA ground squirrels were incubated with shaking in plasma from both non-hibernating and IBA animals at 4 or 37 °C for 48 h. Platelets were pelleted and resuspended twice in Tyrodes buffer as described for the fibrinogen binding assay to remove unbound proteins. 1×10^6 platelets were diluted in an equal volume of 2× sample buffer containing a protease inhibitor cocktail and separated on a 6% acrylamide gel. Proteins were transferred onto a PVDF membrane, blocked, and then probed with a 1:1000 dilution of rabbit anti-human vWF antibody (Dako). The blots were then stripped and probed with a 1:1000 dilution of mouse anti-actin antibody (BD biosciences) as a loading control. After each primary antibody incubation, a 1:10,000 dilution of goat anti-rabbit secondary antibody linked to horseradish peroxidase was added, followed by a chemiluminescent substrate (Thermo Scientific), and exposed to film. Quantification was done by scanning the film, using ImageJ to normalize mean pixels to the actin loading control, followed by a Student's *t* test to analyze the results.

Microfluidics

Blood was collected in PPACK (40 μM) and low-molecular weight heparin (5 U/ml) and incubated at 37 °C for 10–15 min, followed by platelet labeling for 5 min with $DiOC_6$ (3,3′-dihexyloxacarbocyanine iodide, 0.5 μg/ml). PDMS microfluidic devices each containing three channels 500 μm wide and 50 μm high were attached by vacuum to a microscope slide containing a 250-μm-wide strip of type I fibrillar collagen as described previously (Neeves and Diamond 2008; Lehmann et al. 2015). The channels were washed and blocked with HEPES buffered saline (HBS; 7.5 mM $CaCl_2$, 3.75 mM $MgCl_2$, 1 U/ml heparin) containing 5% denatured bovine serum albumin. Flow rates were adjusted using a syringe pump (PHD 2000, Harvard Apparatus) at 0.7 and 3.5 μl/min to produce the shear rates of 50 and 300 s^{-1}, respectively. The blood samples were incubated at 15 or 35 °C for 30 min and passed over the collagen strip for 5 min on a microscope stage heated or cooled to the same temperature, followed by HBS for 1 min and then HBS containing 0.2% glutaraldehyde for 5 min. The microfluidic device was removed from the slide, the slide was fixed for 30 min in 2% gluteraldehyde, and a coverslip was added with anti-fade mounting media. The platelet volume bound to each collagen strip was analyzed by confocal microscopy.

Splenectomy and platelet localization

In July, 10 ground squirrels received a splenectomy and 10 a sham surgery while being under anesthesia with isoflurane (1.5–5%). Pre- and post-surgery blood samples were collected a week before and after the surgery, respectively. Two months after the surgery, a blood sample was taken during entry into hibernation when the animal's body temperature reached ambient temperature (25 °C). The ground squirrels were then moved to a hibernaculum maintained at 4 °C, and additional samples were taken during torpor by treating the animal's tail with lidocaine, pricking the tail and collecting a blood smear. An IBA sample was collected in animals that had spontaneously aroused during the winter. Blood smears from torpid animals were counted manually, while those from alert animals were also counted on a HemaVet. In situ platelet localization was performed on frozen 5-μm liver sections prepared at −30 °C in a cryotome and fixed in cold (4 °C) acetone for 5 min. Endogenous peroxidase activity was inhibited with 0.3% H_2O_2 and 0.1% NaN_3 for 30 min, and blocked with 10% goat serum/PBS for 30 min. Tissue sections were incubated with a 1:200 dilution of polyclonal rabbit anti-GPIbα antibody (Aviva Systems Biology) followed by a horseradish peroxidase-coupled secondary HRP goat anti-rabbit IgG antibody (Thermo Scientific). A negative control with just secondary antibody was performed to ensure that the secondary antibody was not cross-reacting with liver antigens and that endogenous peroxidase activity was blocked. Aminoethylcarbazole (AEC) was used as a substrate and the tissue sections were counterstained with Mayer's hematoxylin. Platelets were counted using ImageJ, adjusted to platelets per μm^2 and compared by *t* test.

Results

Increased fibrinogen binding to GpIIb/IIIa is a measure of platelet activation by various agonists. In fresh human platelets, TRAP caused a 10.8-fold increase in fibrinogen binding when normalized to unstimulated platelets (Fig. 2). After 2 days of storage at room temperature or 4 °C, normalized fibrinogen binding decreased to 62 and 32% of the fresh platelets, respectively (ANOVA, $p < 0.01$). It was unclear which agonists might stimulate ground squirrel platelets, so three different agonists were used. ADP produced a significant increase in fibrinogen binding to freshly collected ground squirrel platelets with a 4.2-fold increase, followed by TRAP with a 3.7-fold increase (ANOVA, $p < 0.01$). PAR4-activating peptide showed a 1.7-fold increase in fibrinogen binding which was not significantly higher than that of controls by ANOVA. After 2 days of storage at room temperature or 4 °C, there was no significant loss in platelet fibrinogen binding with any of the agonists (ANOVA $p = 0.195$) (Fig. 2). Human samples showed a significant interaction between storage conditions and agonist stimulation (ANOVA, $p < 0.001$), while ground squirrel samples did not (ANOVA, $p = 0.968$).

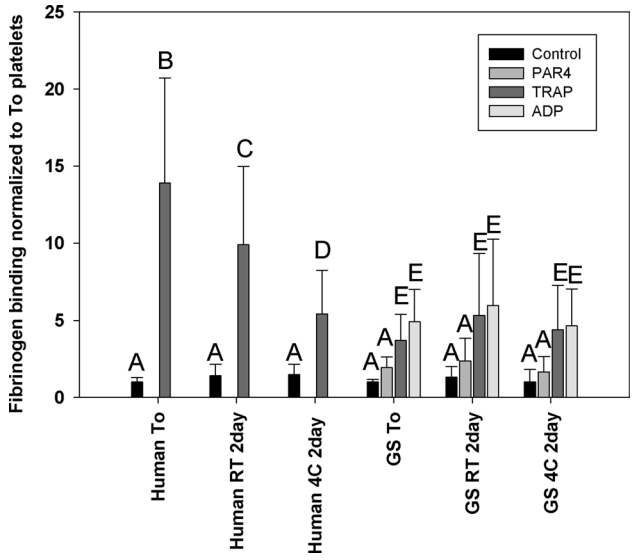

Fig. 2 Platelet fibrinogen binding. Platelets were isolated from non-hibernating squirrels and incubated with Alexa 488 fluorescently labeled fibrinogen. Some samples were activated with different agonists including ADP, TRAP, and PAR4-activating peptide for 5 min. Samples were then fixed with paraformaldehyde, analyzed on a flow cytometer, and normalized to matched unstimulated platelet fibrinogen binding. $N=5$ samples were run in triplicate and analyzed by ANOVA; *bars with different letters* were significantly different, $p<0.05$

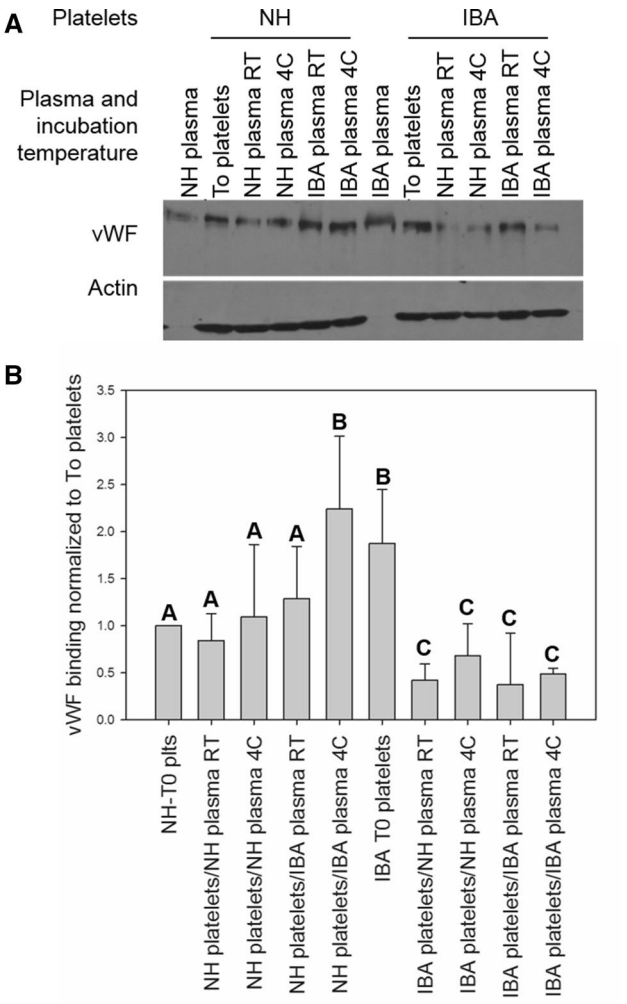

Fig. 3 Effects of temperature and plasma on platelet binding. Platelets were isolated from humans, non-hibernating ground squirrels (NH), and those in an interbout arousal (IBA). Different aliquots of the washed platelets were then incubated for 2 days in either plasma from the non-hibernating or IBA squirrels for 2 days at 4 °C or room temperature. Platelets were washed and immunoblotted for vWF; *size bar* is 50 µm on the large images and 10 µm on the *insets* (**a**). Band densities were quantified and normalized to the To platelets (**b**). $N=5$ samples were analyzed by Student's *t* test; *bars with different letters* were significantly different, $p<0.05$

Glycoprotein GpIbα is a subunit of the vWF receptor on platelets and binds other ligands including thrombospondin, P-selectin, αMβ2 (Mac-1), thrombin, Factor XI, Factor XII, and kininogen (Berndt and Andrews 2011). Non-hibernating ground squirrels have 24% the plasma level of vWF as humans, and this drops an additional 10-fold during torpor (Cooper et al. 2016). Human platelets have been shown to bind increased vWF in the cold (Rumjantseva et al. 2009). Because of the variation in vWF levels, effect of temperature on binding, and potential differences in platelets isolated from hibernating and non-hibernating ground squirrels, all three variables were changed in a triple-cross-over experiment. vWF binding to non-hibernating fresh (T_0) platelets was used to normalize results to the amount of vWF bound under normothermic conditions in vivo (Fig. 3a). Non-hibernating ground squirrel platelet binding of vWF was not influenced significantly by storage for 2 days with the exception of a two-fold increase when stored in IBA plasma at 4 °C. Platelets from IBA animals that had been stored in the cold for weeks in situ bound 2- to 4-fold less vWF than platelets from normothermic animals, regardless of the PRP storage temperature or source of plasma they were stored in for 2 days (Fig. 3b).

In circulation, vWF will bind to collagen exposed by endothelial cell damage. This binding is increased by high shear stress as heart rate increases. To differentiate the effects of temperature, shear rate, and the source of the platelets on their ability to bind to collagen, a microfluidic flow assay was performed. Human and ground squirrel whole blood was incubated at 15 or 35 °C and then passed through a microfluidic chamber over a 250-µm strip of type I fibrillar collagen at the shear rates of 50 or 300 s^{-1} to simulate torpid and aroused conditions, respectively (Fig. 4a). At 15 °C, both human and ground squirrel platelets showed a 90–95% decrease in accumulation on collagen under either of the shear rates compared to 35 °C. At 35 °C, a decrease in shear rate from 300 to 50 s^{-1} reduced human platelet binding by 50%, while ground squirrel platelet

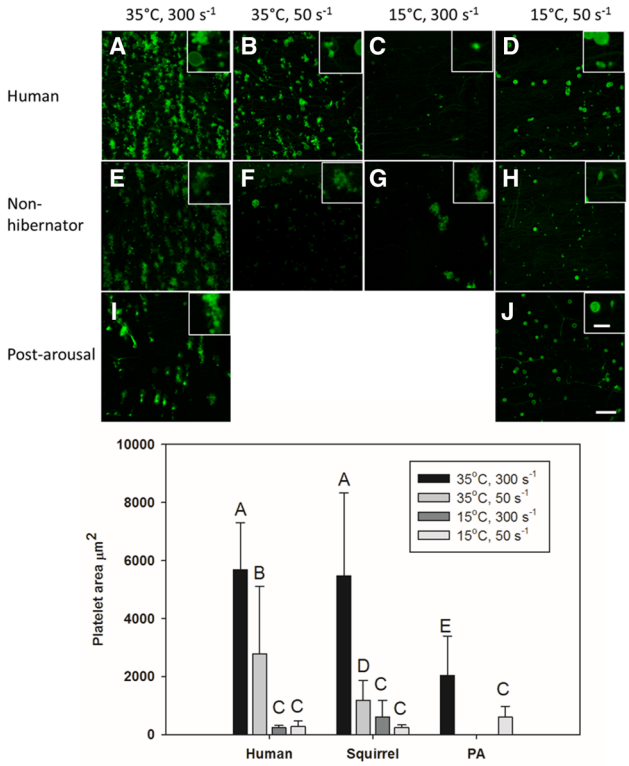

Fig. 4 Effects of temperature and shear rate on platelet binding to collagen. Whole blood samples from humans, non-hibernating ground squirrels, and those in an IBA were incubated at 15 or 35 °C for 30 min. Platelets were labeled with DiOC6 and perfused over a 250-µm strip of type I collagen for 5 min at 15 or 35 °C and the shear stresses of 50 or 300 s^{-1} followed by fixative (**a**). Platelets were then fixed and quantified on a confocal microscope. $N=5$ samples were analyzed by Student's *t* test; *bars with different letters* were significantly different, $p<0.05$. **b** the quantitative analysis of the images shown in panel (**a**)

binding dropped by 80%. When compared to platelets from non-hibernating animals, platelets from animals collected after arousal from torpor showed a 60% decrease in binding at 35 °C and 300 s^{-1}, but a 2.5-fold increase in binding at 15 °C and 50 s^{-1} (Fig. 4b). Half of the ground squirrel platelets bound to collagen at 15 °C showed a ring conformation and half a rod conformation, while human platelets were all round. At 35 °C, all ground squirrel and human platelets were in a ring conformation.

A dramatic conformational change in chilled ground squirrel platelets occurs upon warming or chilling, with a half time of 60 min (Fig. 5a). Rod formation appears to be dependent on polymerization, as blocking depolymerization with taxol (Xiao et al. 2006) at 37 °C leads to increased rod formation and prevents rods that form at 4 °C from returning to rings when the platelets are rewarmed to 37 °C (Fig. 5b). In contrast, blocking polymerization with nocodazole (Vasquez et al. 1997) reduced rod formation (Fig. 5c). One proposed function of this shape change is to sequester platelets in the spleen (Reddick et al. 1973). In splenectomized or sham-operated ground squirrels, there was no significant difference in the sequestration of platelets during torpor or their release during an IBA (Fig. 6). In contrast, significantly more GPIbα staining of platelets was observed in extrasinusoidal regions of the liver in torpid animals ($80.0 \pm 38.1/mm^2$) compared with sinusoidal staining in non-hibernating and IBA animals (13.6 ± 7.0 and $21.8 \pm 16.7/mm^2$, respectively, *t* test $p<0.05$) (Fig. 7).

Discussion

The reduction of both platelets and leukocytes during torpor was first reported over 50 years ago (Lechler and Penick 1963). However, the activity of these platelets has only been assayed at a general level (Cooper et al. 2012; de Vrij et al. 2014). In this study, we examined platelet binding to fibrinogen, vWF, and collagen. Fibrinogen binding can be used as a measure of platelet activation and GpIIb/IIIa activity. Ground squirrel platelet ability to bind fibrinogen after activation with agonists was more resistant to storage at room temperature or in the cold than were human platelets, showing no significant decrease after 2 days of storage at 4 or 25 °C. This is consistent with restored platelet activity measured by thromboelastography on whole blood samples taken from ground squirrels at 2 h post arousal (Cooper et al. 2012). Similarly in humans, both thromboelastography and platelet aggregation were preserved in blood stored up to 21 days at 4 °C compared with room temperature (Pidcoke et al. 2013). Finally, in mice, binding of fluorescent-labeled fibrinogen increases at 34 and 31 °C after TRAP exposure (Lindenblatt et al. 2005). In these three species, cold storage does not appear to decrease the ability of platelets to aggregate and form stable clots; however, all of these assays are done under static conditions and may be different under flow. One caveat in this study was that ground squirrel platelet activation required higher levels of agonist than published for other species (Chung et al. 2002; Nylander et al. 2006; Duvernay et al. 2013); this could be due in part to the addition of PGE1 to samples before the 48-h storage period.

GpIbα is a subunit of the vWF receptor, and its activity can be measured by vWF binding. Plasma vWF is made primarily by endothelial cells and is necessary to support hemostasis at high shear stresses, while platelet vWF can compensate partially for decreased plasma vWF (Kanaji et al. 2012). This is consistent with ground squirrel plasma vWF decreasing in hibernation along with decreased endothelial cell-derived lung vWF mRNA levels (Cooper et al. 2016), but no change in megakaryocyte-derived bone marrow vWF mRNA (Cooper et al. 2016). vWF binding to human platelets increases with cold storage (Rumjantseva

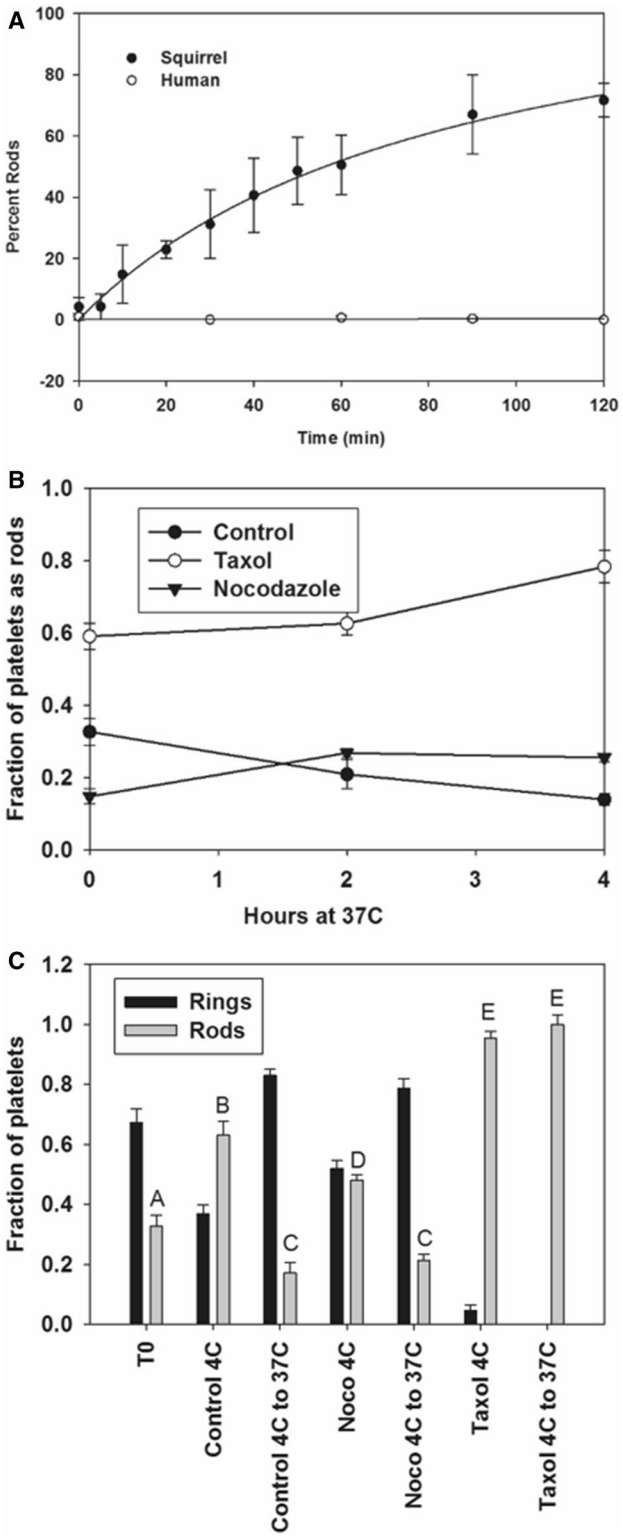

Fig. 5 Platelet microtubule shape changes at 4 °C. **a** Ground squirrel ▶ platelet-rich plasma was incubated at 37 °C for 2 h and then placed on ice. At each time point, aliquots were removed, centrifuged onto polylysine-coated microscope slides, and fixed, and the percentage of platelets in a rod shape was counted. The cold storage curve fit best to a hyperbolic curve, $F=0.312\times x/(14.67+x)$. $N=6$ samples were run in duplicate and at the time points 30, 60, 90, and 120 min compared by Student's t test. **b** Taxol-treated platelets showed increased rod formation even at 37 °C, while no changes were observed in control or nocodazole-treated platelets. **c** Taxol-treated platelets form rods and will not revert to rings upon rewarming to 37 °C unlike control or nocodazole-treated platelets. $N=6$ samples were run in duplicate and analyzed by Student's t test; *bars with different letters* were significantly different, $p<0.05$

et al. 2009), while long-term cold storage decreases surface GpIbα (Reddoch et al. 2014). By storing platelets from hibernating and non-hibernating ground squirrels in each plasma, differences in vWF binding could be attributed to a platelet or plasma effect. Platelets from non-hibernating ground squirrels bound roughly equal to or more vWF regardless of plasma type with only a slight increase in binding when stored at 4 °C. Conversely, platelets from ground squirrels in an IBA bound 2- to 4-fold less vWF than the non-hibernating platelets, regardless of temperature or plasma type. These results suggest that the level of vWF binding is influenced by the source of the platelets and not the plasma or temperature of storage. This could be due to either fewer GpIbα or other vWF receptors on the cell surface, decreased vWF binding affinity, or decreased signal transduction upon binding. Structural changes through tubulin repolymerization or the formation of lipid rafts could also affect vWF binding. The decrease in vWF binding could prevent platelet adhesion to collagen as seen in GpIbα$^{-/-}$ mice and patients with GpIbα deficiency in Bernard–Soulier syndrome (Berndt and Andrews 2011). One drawback of this assay and other clotting assays like thromboelastography is that they are static, while hemostasis occurs under flow. Another drawback of this assay is that it cannot distinguish between internal vWF in alpha granules and externally bound vWF; as a result, direct comparisons in changes in binding were measured with the assumption that intracellular vWF did not change.

Platelet binding to vWF, and subsequently accumulation on collagen, is increased when shear stress stretches vWF, exposing A1 domain binding sites. At a temperature and shear rate simulating the conditions in a non-hibernating ground squirrel (35 °C, 300 s^{-1}), platelets from IBA animals that had been stored in situ at 4–8 °C for weeks showed less accumulation on collagen than platelets from non-hibernating squirrels. Ground squirrel platelets also displayed a greater decrease in binding under low-flow conditions than did human platelets. The binding of all platelets studied was decreased at 15 °C, regardless of the shear rate, possibly because plasma vWF cannot form long cell surface strings at temperatures below 17 °C which would interfere with its ability to bind to platelets (Hewlett et al. 2011). While a body temperature of 15 °C is not physiological for humans, this decrease in collagen or vWF binding could protect hibernating mammals from the activation

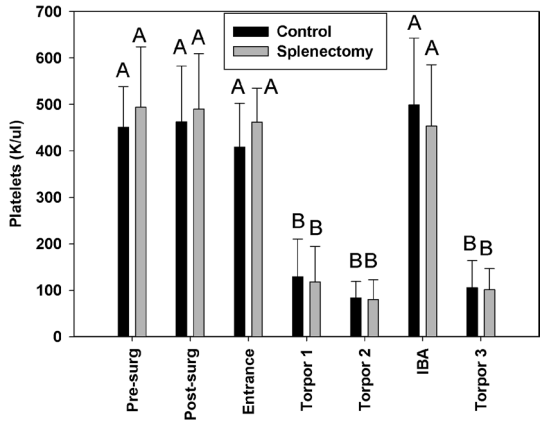

Fig. 6 Effects of splenectomy on platelet levels during hibernation. Ground squirrels received either a splenectomy or sham surgery during the summer. Blood samples were collected pre- and post-surgery, upon entrance into hibernation and during an IBA, and platelets counted on a HemaVet. During torpor, blood smears were collected without waking the animal and counted manually. $N = 10$ animals, analyzed by Student's t test; *bars with different letters* were significantly different, $p < 0.05$

of primary hemostasis as they pass in and out of torpor. Ground squirrels can achieve body temperatures of 4–8 °C, but we could only chill our microscope stage to 15 °C. However, since platelet accumulation on collagen was significantly decreased at 15 °C, no further decrease would be likely at 4 °C. Viscosity is a strong function of temperature, typically with exponential or power law dependence. As a result, exposure of the A1 domain of vWF is reduced and GP1bα–vWF bonds are less likely to form at lower temperatures for the same shear rate because of a reduced shear stress (shear stress = viscosity × shear rate). This reduced force on vWF and platelet is potentially another antithrombotic effect of lowering body temperature. The hematocrit of blood could also affect viscosity, but it does not change in ground squirrels through their hibernal annual cycle (Hampton et al. 2010; Cooper et al. 2016).

Platelets from hibernating ground squirrels go through dramatic shape changes upon chilling. This is due to rearrangement of a circumferential band of microtubules, resulting in long rod-like projections. When this was first reported, one proposed function was mechanical sequestration in the spleen followed by the release of the platelets upon warming. During an IBA, ground squirrels will warm their bodies from 4 to 37 °C in 2 h by metabolic activity of brown adipose tissue deposits, and can cool down again in about 4 h as they go into torpor (Carey et al. 2003). The kinetics of rod formation in platelets indicates that the conformational change could happen within this time frame. However, this study in ground squirrels confirms the work done by de Vrij et al. (2014) in hamsters, which shows that the spleen is not the primary or sole location of platelet sequestration during torpor, as ground squirrels lacking a

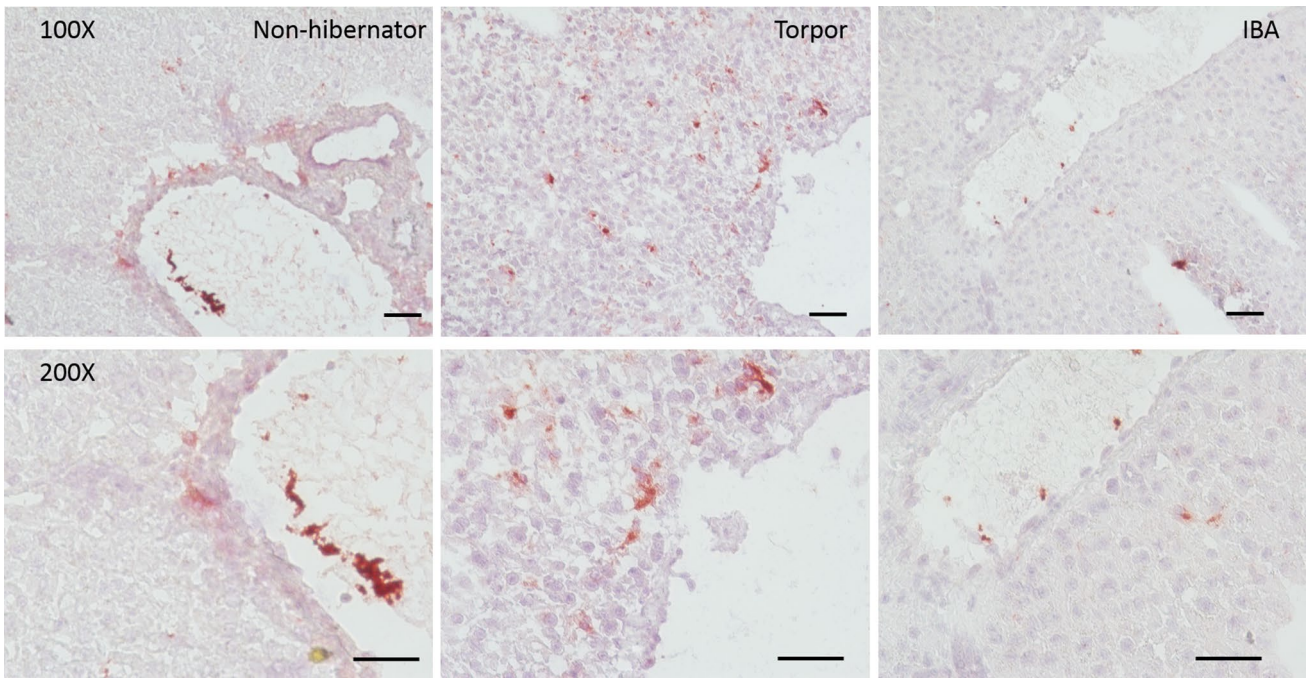

Fig. 7 Platelets are observed in extrasinusoidal regions of liver during torpor. Frozen sections of liver were prepared from non-hibernating, torpid, and IBA animals. The sections were incubated with rabbit anti-GpIbα and goat anti-rabbit HRP antibodies followed by the chromogenic substrate AEC. Sections were then counterstained with hematoxylin; *size bars* are 100 µm ($n = 3$)

spleen could both sequester platelets during torpor and release them in an IBA. In non-hibernating and IBA animals, platelet GPIbα staining is seen in the lumen of liver sinusoids; however, during torpor the staining leaves the sinusoids and becomes diffuse throughout the liver. The staining distribution of GPIbα in the liver is consistent with platelet storage in the liver during torpor and is supported by observations in the hamster liver (de Vrij, personal communication). Liver sinusoids contain Kupffer cells which endocytose chilled platelets (Rumjantseva et al. 2009) and stellate cells expressing ADAMTS-13 which degrades vWF (Vollmar and Menger 2009; Lenting et al. 2012), so sequestration out of the sinusoids may protect platelets from clearance by the liver. A drawback of this study was the use of frozen sections instead of paraffin-embedded sections which would give better resolution of liver structure. However, given the dramatic differences between torpid and IBA or non-hibernating samples, we do not feel that the results would be different with more resolution. Unlike most mammalian tubulins, ground squirrel microtubules grow in the cold, a process stimulated by taxol and blocked by nocodazole. The effects of taxol were more complete than those of nocodazole, and possible explanations could include that depolymerization is faster than polymerization, or that nocodazole is not as an effective inhibitor as taxol. This conformational change could be coincidental with sequestration, but possible physiological roles include the rods forming extensions that trap the platelets in the liver sinusoids or sterically block phagocytosis (Hoffmeister et al. 2003; Rumjantseva et al. 2009). A lack of tubulin depolymerization in the cold could also maintain the overall structure of platelet surface receptors like GPIbα in chilled platelets preventing their clearance (Gitz et al. 2013).

Ground squirrel platelets had reduced adhesion to vWF and thus collagen under low flow and after storage at cold temperatures, while still being activated by external agonists. This should protect the animals from the formation of clots during torpor, but allow them to restore platelet function if injured upon arousal. The decreased binding under low flow could also protect an animal as it rapidly changes both blood flow and temperature going into and out of an IBA. It remains to be determined how platelets are retained in the liver without being activated or cleared by phagocytosis but still retain activity after weeks in the cold.

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Author contributions S.C. and K.N. conceived the ideas and designed the experiments. A.K. and M.Z. performed the platelet vWF and fibrinogen binding assays. S.L., K.D., T. Theisen, and M.L. performed microfluidics. M.G. and T. Tenpas performed immunohistochemistry. X.L., K.B., and S.H. measured microtubule kinetics. S.M. and C.L. performed splenectomy experiments.

Compliance with ethical standards

Conflict of interest No conflicts of interest, financial or otherwise, are declared by the author(s).

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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