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Von Willebrand factor is reversibly decreased during torpor in 13-lined ground squirrels

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Abstract

During torpor in a hibernating mammal, decreased blood flow increases the risk of blood clots such as deep vein thrombi (DVT). In other animal models platelets, neutrophils, monocytes and von Willebrand factor (VWF) have been found in DVT. Previous research has shown that hibernating mammals decrease their levels of platelets and clotting factors VIII (FVIII) and IX (FIX), increasing both bleeding time and activated partial thromboplastin time. In this study, FVIII, FIX and VWF activities and mRNA levels were measured in torpid and non-hibernating ground squirrels (*Ictidomys tridecemlineatus*). Here, we show that VWF high molecular weight multimers, collagen-binding activity, lung mRNA and promoter activity decrease during torpor. The VWF multimers reappear in plasma within 2 h of arousal in the spring. Similarly, FIX activity and liver mRNA both dropped threefold during torpor. In contrast, FVIII liver mRNA levels increased twofold while its activity dropped threefold, consistent with a post-transcriptional decrease in FVIII stability in the plasma due to decreased VWF levels. Finally, both neutrophils and monocytes are decreased eightfold during torpor which could slow the formation of DVT. In addition to providing insight in how blood clotting can be regulated to allow mammals to survive in extreme environments, hibernating ground squirrels provide an interesting model for studying.

Keywords

Factor VIII; Factor IX; Deep vein thrombosis; Hibernation; Neutrophil; DNA NET

Introduction

Hibernation in mammals 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

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(IBA) with a rapid restoration of non-hibernating body temperature and heart rate. 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 °C to 4–8 °C (Lechler and Penick 1963; Reddick et al. 1973), heart rates from 200–300 to 3–5 beats/min (Zatzman 1984), and respiration from 100–200 to 4–6 breaths/min (McArthur and Milsom 1991). As expected, blood pressure also drops during torpor, from 135/90 to 80/40 mmHg, with diastolic values as low as 10 mmHg reported (Lyman and O'brien 1960). Reducing blood flow by partial ligation of the inferior vena cava by 80 % causes formation of deep vein thrombi (DVT) within 2 days in 60 % of mice (von Bruhl et al. 2012), yet ground squirrels survive repeated annual cycles of hibernation and its concomitant reduction in blood flow without the apparent formation of DVT, stroke, or pulmonary embolism. In humans both immobilization that decreases blood flow and tissue damage from a traumatic event increase the risk of DVT by 10- to 20-fold (Heit et al. 1999, 2000; Esmon 2009). An increase in the level of procoagulant proteins (e.g., clotting factors, fibrinogen, von Willebrand factor-vWF) or a reduced level of anticoagulant proteins (e.g., antithrombin, protein C) increases the risk for thrombosis and embolism (Bertina 2003; Kuipers et al. 2009; Bittar et al. 2010; Flinterman et al. 2010; Ribeiro et al. 2012). Platelets, monocytes, and neutrophils also contribute to DVT through the formation of DNA neutrophil extracellular traps (NETS) in baboon and mouse models (Chauhan et al. 2007; Fuchs et al. 2010; Brill et al. 2011).

Hibernating mammals have adapted to this extreme decrease in blood flow and associated risk of DVT by reversibly decreasing platelet, monocyte, neutrophil, 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; de Vrij et al. 2014). In contrast, the levels and activities of prothrombin, fibrinogen, and clotting factors V, VII, X–XII do not decrease significantly in torpor compared with non-hibernating animals. This suggests that the decrease in secondary hemostasis is not due to general suppression of clotting factor activity, but is specific for FVIII and FIX (Lechler and Penick 1963; Pivorun and Sinnamon 1981). Another known protein risk factor for DVT, VWF has not been measured during torpor. VWF is a 250-kDa glycoprotein secreted by endothelial cells (Jaffe et al. 1974) and megakaryocytes (Nachman et al. 1977). VWF is glycosylated and forms disulfide-linked dimers in the endoplasmic reticulum and is then assembled into large thrombogenic multimers of up to 20,000 kDa in the Golgi (Sadler 1998). VWF is stored and then released into plasma from Weibel–Palade bodies in endothelial cells and alpha granules in platelets. Blood VWF stabilizes FVIII in circulation and also binds to GPIIb/IIIa receptors on platelets allowing them to adhere to collagen exposed at sites of vascular injury (Weiss et al. 1977). Under venous flow rates, VWF is required for the formation of stable platelet clots, while the presence of FVIII prevents the clots from breaking free and forming thromboemboli (Chauhan et al. 2007). Under low shear stress, platelets will attach transiently through the surface receptor glycoprotein Iba (GPIIb/IIIa) to VWF on endothelial cells. Without further activation, platelets will be released when the VWF multimers are cleaved by the circulating enzyme ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13) and cleared by the liver through the C-type lectin receptor CLEC4M (Wagner and Frenette 2008; Rydz et al. 2013). In VWF-deficient mice, a

decrease in thrombosis is seen during stasis or stenosis, independent of FVIII levels (Brill et al. 2011). Thus, VWF tethering of platelets to the vessel wall appears to be more important than VWF stabilization of FVIII in the formation of a thrombus. VWF is removed from circulation primarily by spleen and liver macrophages through asialoglycoprotein and Siglec-5 receptors (Casari et al. 2013). Defects in VWF lead to the human bleeding disorder von Willebrand disease (VWD) characterized by a partial quantitative deficiency (type 1), a qualitative deficiency (type 2), or a total deficiency (type 3) of VWF (Sadler 1998).

VWF is also abundant in DNA NETs formed in an in vivo baboon model of DVT along with platelets, histones and granule components secreted from neutrophils (Fuchs et al. 2010). These DNA NETs are formed when neutrophils become activated and release their genomic DNA, forming an extracellular mesh that can trap microbes and stimulate clotting (Fuchs et al. 2012; Kolaczowska and Kubes 2013). Elevated circulating plasma DNA levels are associated with venous thrombi in mice, baboons and humans (Fuchs et al. 2010; Brill et al. 2012; Borissoff et al. 2013; Diaz et al. 2013) while circulating plasma DNase levels negatively correlated with infarct size (Mangold et al. 2015). Neutrophils can also bind directly to VWF in vivo, allowing the neutrophils to adhere to endothelial cells on the vessel wall (Kolaczowska and Kubes 2013). In most animal models, low blood flow conditions are induced artificially to bring about DVT formation. Currently, there are few animal models in which low blood flow occurs naturally without the formation of DVT (Lindenblatt et al. 2005; de Vrij et al. 2014). In this study, we look at known risk factors of DVT in ground squirrels going through torpor to see if these animals have a mechanism to prevent blood clotting during periods of low flow.

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 to December). 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–February at a body temperature of 9.8 ± 2.1 °C. Blood samples were collected from anesthetized animals 2 h post-arousal in March (36.4 ± 0.8 °C), and from non-hibernators in June–July (35.5 ± 2.6 °C). Exsanguination following CO₂ asphyxiation was performed and collected organs were immediately frozen in liquid nitrogen for RNA analysis or fixed in 10 % buffered formalin. Lungs were embedded in paraffin, sectioned, and trichrome stained (Newcomer Supplies, Madison, WI). For the time course post-arousal animals were aroused manually and moved out of the hibernaculum to cages in a room at 25 °C. Blood was collected in 1/9th volume of acid citrate dextrose anticoagulant from the tail arteries of non-hibernating ground squirrels while under anesthesia with iso-furane (1.5–5 %). Blood cell counts were performed using a HemaVet HV950 (Drew Scientific, Waterbury, CT).

Immunoassays

Plasma samples from eight torpid and eight non-hibernating ground squirrels were pooled separately. For the VWF immunoblot, 1 µl of each plasma sample was separated on a 6 % SDS-PAGE and blotted to a PVDF membrane. The blot was blocked with 1 % powdered milk in Tris-buffered saline (TBST, 10 mM Tris, 150 mM NaCl, 0.5 % Tween-20, pH 7.4) and incubated for 1 h each with a 1:1000 dilution sheep anti-human VWF antibody (Hematologic, Essex Junction, VT) followed by a 1:10,000 dilution of donkey anti-sheep secondary antibody conjugated with alkaline phosphatase and stained with NBT-BCIP (Sigma, St. Louis, MO).

For the VWF multimer distribution, plasma containing approximately 1 mU of VWF was loaded on a 0.65 % agarose gel, transferred to a PVDF membrane (Immobilon P, Millipore) and blotted with an anti-VWF antibody (DAKO 1 µg/ml). Detection utilized a goat-anti rabbit HRP-conjugated antibody, followed by a chemiluminescence substrate (Super Signal West Pico, Thermo Scientific) (Flood et al. 2011). Mouse plasma control was pooled from C57Bl6 wild-type mice. Site-directed mutagenesis was used to introduce the 1597W mutation into recombinant full-length human VWF, expressed in HEK293T cells, and supernatants collected at 24 h (Pruss et al. 2012).

Collagen-binding assays were performed by incubating 4 µg/ml type 1 collagen in TBS buffer in a microtiter plate overnight at room temperature. The wells were blocked with 1 % milk in TBST and incubated with dilutions of human pooled normal plasma (PNP, George King Biomedical, Overland Park, KS) or plasmas from individual ground squirrels for 1 h. The plates were then washed and bound VWF detected with anti-VWF antibody (Hematologic, Essex Junction, VT). Alkaline phosphatase activity was measured using *p*-nitrophenyl phosphate (Sigma, St. Louis, MO).

FVIII and FIX assays

APTT assays using human FVIII or FIX-deficient plasma were used to measure FVIII and FIX activity in ground squirrel plasma samples. Equal volumes of FVIII or FIX-deficient plasma were mixed with dilutions of human PNP or pooled ground squirrel plasmas from the different time points, and measured in triplicate. Samples were pooled because the volume required for each assay exceeded the maximum volume we could collect from individual animals through sequential time points approved by our IACUC (100 µl of blood per sample). APTT reagent was added and clotting times were measured on a KC1 Delta analyzer (Trinity Biotech, Bray, Ireland). Clotting times for the ground squirrel samples were compared with the appropriate standard curve to determine the amount of FVIII or FIX activity relative to human PNP.

Quantitative PCR

RNA was isolated from lung and liver of six torpid and six non-hibernating ground squirrels using an Absolutely RNA kit, and cDNA synthesized using random primers and an Affinity Script QPCR cDNA Synthesis kit (Stratagene, La Jolla, CA). Quantitative PCR was performed in triplicate on each sample with ground squirrel-specific FII, FVIII, FIX, VWF and actin primers using a Brilliant II SYBR Green kit (Stratagene) on a Light Cycler 1.5

(Roche, Basel, Switzerland). The Ensembl ground squirrel genome database was used to obtain cDNA sequences for the development of QPCR primers. The following primer sequences were designed using PrimerQuest and purchased from IDT (Coralville, IA): actin forward, CCAGGCATTGCTGATAG-GAT; actin reverse, GCTGGAAGGTGGACAGAGAG; FII forward, TGTGAGCCAGTGAGGAAACCTAGAG; FII reverse, TGTCAGGTTTATGTGGGTAGCGACT; FVIII forward, AAGGGAAGAGCTGGCACTCAGAAA; FVIII reverse, TTCCTGTGGCATCCAATCAGACCT; FIX forward, GAATGTTGGTGTGCGACTTGGATT; FIX reverse, CAGTACAGGAACAAACCACCTTAC; VWF forward, GCCATCATGTATGAGGTCAGATTCC; VWF reverse, ACAGAGGCCATACATTTTGAAGCA.

DNase sensitivity

Liver and lung nuclei were isolated from non-hibernating and torpid samples (Kleinschmidt, AM 2008). The nuclei were treated with increasing DNase concentration (0, 0.025, 0.05, 0.2 $\mu\text{g}/\mu\text{l}$) at 37 °C for 20 min. The remaining DNA was then analyzed by PCR and QPCR using primers designed to bind to the ground squirrel actin and VWF promoters near the transcription start site. Ground squirrel VWF promoter primers forward TTGGTAGTTC-CTGCACCTTCTCTG and reverse TGTAACAATTC-CTGGGAGCCACAC; actin promoter primers forward TCCGACCAGCGTTTGCCTTT and reverse TGGCAAA-GCGGCACAGTGA. Plasma DNase activity was measured using a DNaseAlert™ QC System (Ambion). Plasma DNA levels were measured by mixing 1:10 dilutions of plasma in phosphate buffered saline followed by addition of 2 μM final concentration of SytoxGreen™ (Invitrogen). Fluorescence was measured at excitation and emission wavelength of 485 and 538 nm (Diaz et al. 2013).

Results

Lethal clots during hibernation

Ground squirrels survive repeated bouts of torpor and IBA in a single hibernation season with a low rate of mortality, suggesting some protection against the formation of stasis-induced blood clots. In our lab we have kept animals for up to 6 years through multiple bouts of hibernation (unpublished results). Several ground squirrel species are reported to live for 4–7 years in the wild (Pengelley and Asmundson 1969; Sherman and Morton 1984; Michener 1989). Examination of brain and lung sections showed no evidence of emboli in any of six torpid, non-hibernating, IBA, or spring arousal animals (Supplemental Figs. 1, 2).

Measurement of VWF, FVIII and FIX protein in ground squirrel plasma

Non-hibernating ground squirrels had 25 % the amount of VWF collagen-binding activity found in human PNP (5–10 $\mu\text{g}/\text{ml}$) and VWF collagen binding decreased tenfold during torpor (Table 1). Non-hibernating ground squirrel plasma had 2.3-fold more FVIII and 4.3-fold more FIX activity than human PNP, and both activities dropped threefold during torpor (Table 1, *t* test $p < 0.05$). In the spring, upon arousal from hibernation, ground squirrel body temperatures rose from an average of 9.8 ± 2.1 to 36.4 ± 0.8 °C within 2 h. After spring

arousal, VWF levels increased steadily for 16 days before reaching normal non-hibernating levels of 27 % of human PNP (Fig. 1). In contrast, FVIII and FIX activities increased more rapidly, peaking at 7 and 3 days, respectively, and then equilibrated at non-hibernating levels, 230 and 380 % of human PNP, respectively (Fig. 1).

Ground squirrel VWF monomers had molecular weights of 275 and 300 kDa under reducing and denaturing conditions, and the intensities of both bands decreased during torpor (Fig. 2a). In addition to a quantitative decrease in VWF collagen binding, there was a qualitative change in the loss of high molecular weight VWF multimers during torpor (Fig. 2b). In non-hibernating ground squirrel plasma, the VWF multimer profile (lane 4) was similar to normal mouse plasma (lane 3). During torpor, the large multimers were lost (lane 5), with a VWF multimer profile that resembled the type 2A VWD mutation R1597W (lane 2). Within 2 h of arousal in the spring, large multimers were released into circulation, while the smaller multimers observed during torpor were still present (lane 6). A commercial kit designed to assay mouse ADAMTS13 activity was not functional with the ground squirrel enzyme.

Measurement of VWF, FVIII and FIX mRNA in ground squirrel tissues

Quantitative PCR was performed on cDNA with primers specific for VWF (lung), and FII, FVIII and FIX (liver), and actin (lung and liver). Lung was used to measure VWF expression as it is rich in endothelial cells. The predicted sizes of each PCR product were confirmed by gel electrophoresis; actin and FVIII (142 bp), FIX (130 bp), and VWF (123 bp) (not shown). The actin mRNA levels were not significantly different between the two groups of animals in any tissue tested ($p > 0.05$). During torpor, liver FII and FVIII mRNA normalized to their respective actin mRNA both increased 1.8-fold ($p < 0.05$ for FVIII), while FIX mRNA dropped threefold ($p < 0.05$). Lung VWF mRNA dropped threefold ($p < 0.05$) during torpor (Table 2). To further explore the regulation of VWF expression, a DNase sensitivity assay was performed on the VWF promoters in lung. The actin promoter was used as a control gene and its DNase sensitivity was not affected seasonally. The VWF promoter in lung was 1.9-fold less sensitive to DNase when the animals were in torpor, while the liver samples showed no seasonal change (Fig. 3).

Measurement of ground squirrel blood cell concentrations

Erythrocyte levels did not change from non-hibernating values during torpor or 2 h post-arousal. In contrast, platelet levels dropped tenfold during torpor and rebounded to normal levels within 2 h post-arousal as previously reported (Table 3). Neutrophils were the most abundant leukocyte in non-hibernating blood, and showed the greatest numerical decrease during torpor. Monocytes and eosinophils showed eight and sixfold decreases during torpor, respectively, while lymphocytes and basophils did not change significantly (Table 3). All leukocyte levels returned to non-hibernating levels within 2 h post-arousal. Plasma DNA concentrations did not vary between non-hibernating and torpid squirrels (48.6 ± 3.3 and 53.4 ± 5.2 ng/ml). Plasma DNase activity was increased in non-hibernating relative to torpid squirrels (9.8 ± 1.0 and 4.1 ± 1.6 ng/ml) and this correlated (R^2 0.15, $p < 0.01$) with the neutrophil count in each sample (Fig. 4).

Discussion

Suppression of secondary hemostasis during torpor

In 1963, Lechler and Penick first reported that during torpor FVIII and FIX activities decreased fivefold and twofold, respectively (Lechler and Penick 1963), similar to the threefold decreases we observed. Ground squirrels in the non-hibernating state have twofold more FVIII and fourfold more FIX activities than humans, so the observed threefold drop during torpor brings them down to levels similar to human values (Lechler and Penick 1963). Mice also have twofold more FVIII and 75 % the level of FIX as seen in humans; thus, in a non-hibernating state ground squirrels have similar levels of these clotting factors as a non-hibernating rodent (Sweeney et al. 1990). In previous reports, other clotting factor activities showed little or no change during torpor including; FII, F V, FVII, FX, FXI, and FXII (Lechler and Penick 1963; Pivorun and Sinnamon 1981). Thus, the combined drops in FVIII and FIX activity during torpor contribute to an induced hemophilia-like state, and could account for the prolonged APTT times. Elevated FVIII and FIX are risk factors for DVT, thus their decreased levels could help protect hibernating ground squirrels from stasis-induced blood clots (Bertina 2003; Flinterman et al. 2010; Ribeiro et al. 2012).

VWF was reduced both quantitatively and qualitatively during torpor. Non-hibernating ground squirrels had fourfold less VWF than humans, and VWF activity dropped an additional tenfold during torpor. Comparison of the VWF multimers in ground squirrels to those in humans and mice revealed that ground squirrels formed normal multimers when not in torpor. This suggests that in a non-hibernating state VWF multimers could form in the Golgi, be exported into circulation, and were not rapidly cleaved and removed. In contrast, the VWF multimer profile during torpor could be caused by decreased multimer assembly in the Golgi or increased cleavage of large multimers by ADAMTS13 (Hassenpflug et al. 2006). Future studies using high resolution imaging of endothelial cells by electron microscopy or immunohistochemistry may shed light on this mechanism.

When not torpid, ground squirrels need clotting activity similar to other rodents to avoid bleeding. As expected, the non-hibernator's VWF plasma concentration (Sweeney et al. 1990) and multimer profiles were similar to those of mice. However, while in torpor, blood clotting needs to be suppressed for the animal to not experience massive thrombosis. The combined decrease in VWF protein and selective loss of high molecular weight multimers should reduce primary hemostasis, by preventing platelet adhesion to collagen. Upon arousal, a ground squirrel's body temperature returns to normal within 2 h, requiring rapid restoration of blood clotting activity. This was accomplished in part by release of large VWF multimers back into circulation upon arousal.

Recovery of secondary hemostasis upon spring arousal

The slow rates at which FVIII, FIX and VWF levels returned to normal after arousal in the spring suggests new synthesis instead of release of stored proteins. The differences in activity could also be regulated epigenetically by changes in the stability or clearance of each protein. Both FIX mRNA and protein levels dropped threefold during torpor, consistent with transcriptional regulation of the FIX gene. The tenfold drop in VWF protein was not

completely explained by the 2.5-fold drop in mRNA isolated from lung, suggesting post-translational regulation such as degradation by ADAMTS13 (Hassenpflug et al. 2006), clearance of VWF by CLEC4M receptors in the liver (Rydz et al. 2013), or differential rate of synthesis in non-lung endothelial cells or bone marrow (Sadler 1998). The 2.5 fold decrease in lung VWF mRNA correlated well with the 1.9-fold decrease in DNase sensitivity of the lung VWF promoter during torpor. FVIII mRNA was increased 1.8 fold during torpor while the protein levels were decreased threefold. The discrepancy between FVIII mRNA levels and FVIII activity was likely due to decreases in circulating VWF during torpor, which in turn decreases FVIII stability. This epigenetic regulation is supported by the observation that VWF^{-/-} mice with no detectable VWF have 20 % of normal FVIII, and VWF^{+/-} mice have 60 % of normal FVIII levels (Denis et al. 1998; Pendu et al. 2009). In addition, in humans with type 3 VWD and no detectable VWF, factor VIII activity drops to less than 10 %, in the range of that seen in hemophilia (Sadler 1998). Previous reports showed no significant change in FII activity during torpor (Lechler and Penick 1963; Pivorun and Sinnamon 1981), and we no significant change in FII mRNA, indicating that the observed decreases in FIX and VWF mRNA are not due to a general suppression in transcription of all clotting factors.

Ground squirrels as a model organism to study DVT

Mice deficient in VWF are resistant to DVT induced by stasis, stenosis, and endothelial injury (Chauhan et al. 2007; Brill et al. 2011). This protection appears to be independent of FVIII (Brill et al. 2011), although without FVIII, clots are less stable and more likely to dislodge and form emboli (Chauhan et al. 2007). Under venous flow rates, VWF provides an attachment site on the endothelium for platelets and neutrophils (Badimon et al. 1989; Chauhan et al. 2008; Brill et al. 2011). VWF-deficient mice show the greatest protection from DVT in an in situ assay under low-flow conditions versus stasis (Brill et al. 2011), which would be similar to conditions encountered by torpid ground squirrels. The anti-VWF compound ARC1779 inhibited the formation of occlusive thrombi in cynomolgus monkeys and reduced the risk of thromboembolism and stroke in patients undergoing carotid endarterectomy (Diener et al. 2009; Markus et al. 2011). No link has been reported between thrombocytopenia and DVT in humans. However, in mice antibody-induced thrombocytopenia with a reduction in platelets from 900×10^3 to $11 \times 10^3/\text{ml}$ does protect mice against thromboplastin-induced DVT (Herbert et al. 1992). The recent discovery of a link between DVT and DNA NETs formed by neutrophils and microparticles released by monocytes may also explain the selective advantage of ground squirrels reducing neutrophil and monocyte levels 6- to 9-fold during torpor. Circulating DNA levels are increased in patients who suffer a DVT (Diaz et al. 2013) and in mouse and baboon models of DVT (Fuchs et al. 2010; Brill et al. 2012). No difference in circulating DNA was observed in ground squirrel plasma samples taken from non-hibernating, torpid or post-arousal samples.

There are several limitations to this study. Clotting assays were performed ex vivo using plasma samples rewarmed to room temperature, as is typically done with APTT and PT assays. In vivo the torpid blood would be at 4–8 °C while aroused animals will have body temperatures of 37 °C, so clotting times may be even slower in vivo in torpid animals due to decreased enzymatic activity in the cold. While ground squirrels may have some natural

resistance to the formation of DVT, there are challenges in working with them as a model organism. Unlike experiments done in mice, no published protocols exist to generate knockout ground squirrels to identify the role of specific proteins in the formation of DVT. The visualization of DNA NETS requires in situ fluorescent microscopy in isolated blood vessels. This would be challenging to do in a torpid animal without arousing it.

By maintaining a heart rate of 3–5 beats/min for days at body temperatures of 4–8 °C, torpid ground squirrels should be at increased risk for formation of DVT, pulmonary emboli and stroke. Yet when they arouse in the spring, they must rapidly restore their clotting ability back to normal to avoid bleeding. By decreasing FVIII, FIX, VWF, platelets, monocytes and neutrophils during torpor ground squirrels establish an anticoagulant state that can be reverted to a hemostatic state within a few hours of arousal in the spring. The mechanisms by which the ground squirrels are able to selectively and reversibly regulate FIX and VWF transcription remain to be determined. Neutrophils and platelets marginate to the side of blood vessels during torpor, but the mechanisms of monocyte sequestration and release within hours of arousal still need to be determined (Bouma et al. 2013; de Vrij et al. 2014). In addition to their importance in comparative physiology, understanding the mechanisms responsible for these natural mammalian adaptations to avoid the risk of clot formation under low-flow conditions could lead to clinical therapies to prevent DVT in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Ground squirrel plasma FVIII, FIX, and VWF concentrations after spring arousal. Protein levels were measured in spring post-arousal from hibernation by APTT (FVIII and FIX) or collagen-binding assay (VWF) using human PNP to generate a standard curve. Each time point represents 6–11 samples each measured in triplicate and normalized to non-hibernating plasma levels, *bars* represent standard deviation

Fig. 2.

Immunoblots of ground squirrel plasma VWF. **a** SDS-PAGE of 1 µl of pooled non-hibernating (NH) or torpid (T) ground squirrel plasmas were separated on a 6 % reducing gel and probed with a sheep anti-human VWF antibody. **b** Multimer distribution of VWF from pooled ground squirrel plasmas and controls; 1 human PNP; 2 human recombinant 1597W VWF; 3 murine control plasma; 4 NH squirrel plasma; 5 T squirrel plasma; 6 2 h post-arousal squirrel plasma

Fig. 3.

Relative DNase sensitivity of vWF promoter. Nuclei were isolated from non-hibernating (NH) and torpid (T) lungs and treated with increasing concentrations of DNase ($n = 3$). The VWF promoter sensitivity was measured by QPCR and normalized to the actin promoter in each sample. Slopes of promoter concentration vs. DNase concentration were used to determine relative DNase sensitivity, *bars* represent standard deviation

Fig. 4.

Plasma DNase activity vs neutrophil (*PMN*) counts. Blood was collected from non-hibernating (*NH*) and torpid (*T*) animals and PMN levels measured with a HemaVet. DNase levels were measured relative to a lambda DNA standard curve

Table 1
Plasma levels of FVIII, FIX, and VWF relative to human pooled normal plasma

Protein	Non-hibernating (%)	Torpid	T:NH
FVIII	232 ± 2.0 [#]	68 ± 0.1 [#]	0.29
FIX	425 ± 20 [#]	140 ± 4.0 [#]	0.33
VWF	24.9 ± 3.7 [*]	2.4 ± 0.01 [*]	0.10

^{*} $n = 10$ individual samples measured in triplicate (t test, $p < 0.05$)

[#] $n = 6$ pooled samples measured in triplicate (t test, $p < 0.05$)

Table 2
Levels of FII, FVIII, FIX mRNA in liver, and VWF mRNA in lung relative to actin

mRNA	Non-hibernating	Torpid	T:NH
FII	0.590 ± 0.27	1.075 ± 0.75	1.82
FVIII	0.006 ± 0.001 [#]	0.011 ± 0.004 [#]	1.71
FIX	1.081 ± 0.761 [#]	0.324 ± 0.236 [#]	0.30
VWF	0.065 ± 0.057	0.020 ± 0.028	0.30

n = 5 individual samples measured in triplicate

[#]Significant difference between torpid and non-hibernating animals (*t* test, *p* < 0.05)

Table 3
Blood cell levels in ground squirrels

Cell type	Non-hibernating (<i>n</i> = 34)	Torpid (<i>n</i> = 16)	2 h post-arousal (<i>n</i> = 17)
RBC ($\times 10^6/\mu\text{l}$)	9.57 ± 1.74	9.46 ± 1.89	9.71 ± 0.76
Platelets ($\times 10^3/\mu\text{l}$)	476 ± 112	$45 \pm 27^\#$	486 ± 92
WBC ($\times 10^3/\mu\text{l}$)	4.92 ± 2.02	$1.62 \pm 1.29^\#$	5.33 ± 2.17
Neutrophils	2.83 ± 1.66	$0.34 \pm 0.20^\#$	3.41 ± 1.02
Lymphocytes	1.56 ± 1.73	0.92 ± 1.63	1.05 ± 1.68
Monocytes	0.45 ± 0.37	$0.06 \pm 0.06^\#$	$0.78 \pm 0.39^\#$
Eosinophils	0.06 ± 0.11	$0.01 \pm 0.01^\#$	0.08 ± 0.09
Basophils	0.01 ± 0.04	0.01 ± 0.01	0.01 ± 0.02

[#] Significant difference from non-hibernators (*t* test, *p* < 0.05)