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Graduate Studies

EFFECTS OF HIBERNATION ON BLOOD CLOT FORMATION AND FIBRINOLYSIS IN 13-LINED GROUND SQUIRRELS

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EFFECTS OF HIBERNATION ON BLOOD CLOT FORMATION AND FIBRINOLYSIS IN 13-LINED GROUND SQUIRRELS

By Alison Nicole Bonis

We recommend acceptance of this thesis in partial fulfillment of the candidate's requirements for the degree of Master of Science in Biology

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ABSTRACT

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Thirteen-lined ground squirrels (*Ictidomys tridecemlineatus*) enter hibernation to survive periods of low temperatures and scarce food availability. Typical ground squirrel hibernation is characterized by prolonged periods of torpor with decreased physiological activity, interrupted every few weeks by brief interbout arousals (IBA). Decreased blood flow during torpid states should increase the risk of stasis-induced blood clots. However, ground squirrels have adapted to survive repeated bouts of torpor and IBA without forming lethal blood clots. Although ground squirrels have developed antithrombotic adaptations to avoid lethal blood clots during hibernation, whether these adaptations cease all ability to form blood clots remains unknown. Thrombin-antithrombin complex, was significantly reduced ($p < 0.05$) during hibernation, and D-dimer level remained unchanged throughout the annual cycle. Tissue plasminogen activator complexed with plasminogen activator inhibitor to total plasminogen activator inhibitor ratio, was significantly increased ($p < 0.05$) during hibernation. Protein level of plasminogen activator inhibitor was significantly reduced ($p < 0.05$) during hibernation. These data suggest that ground squirrels do not form blood clots during hibernation, in-part due to suppression of coagulation. Furthermore, hibernators sustain hyperfibrinolytic states even in the absence of blood clots. Although unlikely, any clots formed during hibernation may be less resistant to fibrinolytic degradation

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INTRODUCTION

Ground Squirrels Are Hibernators

To survive seasonal periods in northern environments that include extreme conditions such as below freezing temperatures and scarce food availability, many small mammals have adapted by entering into hibernation. Thirteen-lined ground squirrels (*Ictidomys tridecemlineatus*), hereafter referred to as ground squirrels, are one such example. Typical ground squirrel hibernation is characterized by prolonged periods of torpor with decreased physiological activity and immobilization lasting days to weeks. These bouts of torpor are interrupted by brief interbout arousals (IBA), during which normal physiological activity is restored for 12-24 hours (Cooper et al., 2012).

Annual Ground Squirrel Hibernation and Arousal Cycle

During the spring and summer months, ground squirrels are active with normal body temperatures of 35-38ºC, respirations of 100-200 breaths/min, heart rates of 200-300 beats/min, and a blood pressure of 135/90 mm Hg. Mid-September marks the beginning of the hibernation season, which can last for 6-7 months (Morin and Storey, 2009). Upon entry into hibernation, core body temperature, respiration, heart rate, and blood pressure begin to fluctuate downward for up to 12 hours, until the animal has entered true torpor (Elvert and Heldmaier, 2005). During torpor, core body temperature drops to 4-8ºC, heart and respiratory rates decrease to 3-5 beats/min, and 4-6 breaths/min, respectively. Blood pressure is also significantly reduced to 80/40 mmHg in torpid

Table 1. Physiological changes during hibernation stages in thirteen-lined ground squirrels.

Parameter	Stage of Hibernation Cycle	
	Non-Torpor (NH, IBA)	Torpor
Core Body Temperature (°C)	37	0-8
Respirations (Breaths/min)	100-200	1-6
Heart Rate (bpm)	200-300	3-5
Blood Pressure (mmHg)	135/90	80/40
Metabolic Activity (%)	100	1-5

ground squirrels (Lyman and Obrein, 1960) (Table 1). Periodic arousals from the torpid state occur every few days to weeks, where the nonhibernating physiological state is rapidly restored within 2 hours of arousal (Boyer and Barnes, 1999). Subsequent IBA bouts typically last 22-24 hours before ground squirrels undergo re-entry into their torpid state (Carey et al., 2003). Upon final arousal in the spring, ground squirrels will have endured 10-20 cycles of torpor and IBA throughout the entire 6-7 months of hibernation (Storey and Biggar, 2014) (Figure 1).

Hibernators Avoid Forming Life Threatening Blood Clots

Ground squirrels display similar physiological profiles as nonhibernating mammals during their active phases; yet dramatically alter these profiles upon entering hibernation. Maintenance of a similar hibernating physiological state in nonhibernating mammals, including humans, would be lethal for many reasons (Carey et al., 2003). Looking specifically at the potentially lethal effects of immobilization, a previous study showed

60% of mice subjected to a similar state of decreased blood flow formed deep vein thrombi (DVT), life threatening blood clots in deep veins, within 2 days (Von Bruhl et al., 2012). In humans, a 10- to 20-fold greater risk of DVT is associated with both immobilization and decreased blood flow, as well as tissue damage sustained from traumatic injury (Esmon, 2009). Interestingly, ground squirrels maintain their hibernating state for 6-7 months without apparent formation of lethal clots.

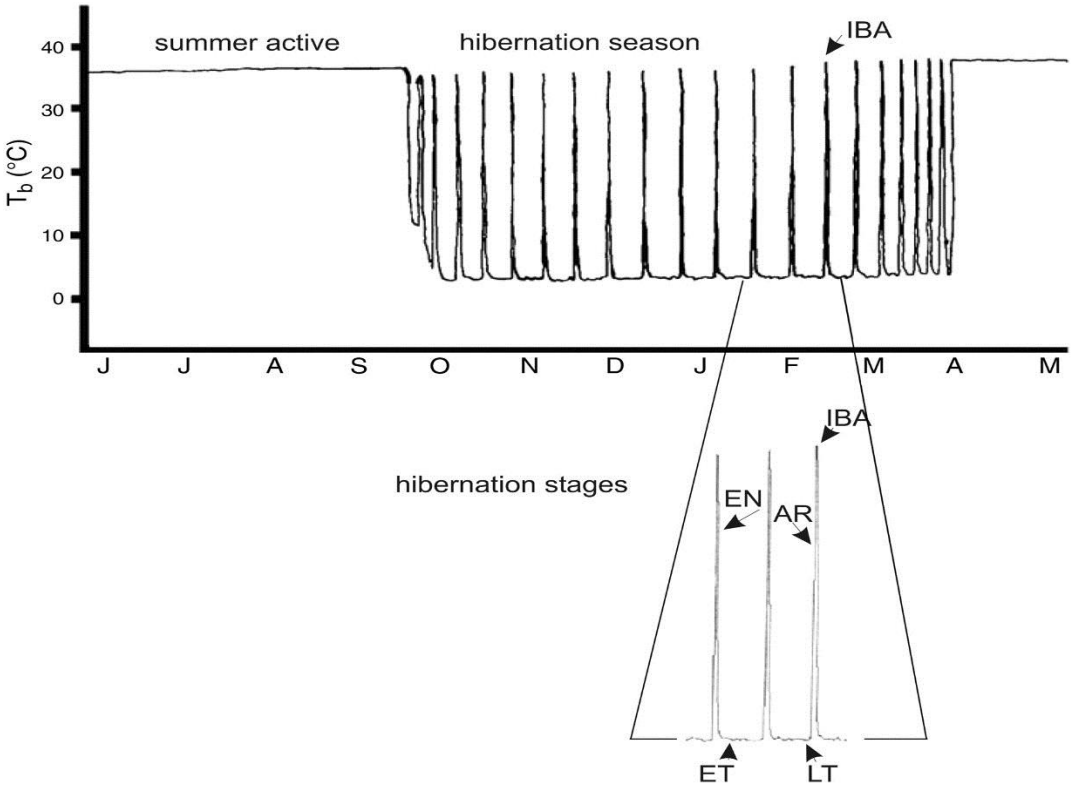

Figure 1. Annual torpor and arousal cycle for the thirteen-lined ground squirrel. Body temperature (ºC) monitored over a year span, from June to May. Various stages of the hibernal cycle noted as: Entrance, EN; Early Torpor, ET; Late Torpor, LT; Arousal, AR; Interbout Arousal, IBA (Carey et al., 2003).

Hemostasis

Hemostasis is a process that causes bleeding to stop, and is the first stage of wound repair (Palta et al., 2014). During primary hemostasis, a soft platelet clot, sometimes referred to as a "platelet plug", is formed. Under normal conditions, disc-shaped platelets circulate freely throughout the blood. Subsequent to vascular endothelial injury, subendothelial collagen becomes exposed. Von Willebrand's Factor (vWF), a large glycoprotein normally inactive in circulation, binds newly exposed collagen and becomes extended. Platelets then bind to the vWF, allowing them to stably bind the subendothelium at sites of initial vascular damage (Nuyttens et al., 2011). Platelets themselves become activated once adhered to collagen bound vWF; transforming from a disc-shape into an irregular sphere-like structure with many pseudopod projections. Activated platelets release factors such as thromboxane A2 (TXA2), and adenosine diphosphate (ADP) that serve to recruit additional platelets to the injury site. Activated platelets aggregate, and bind to one another, thus forming a platelet plug acting as a temporary seal to cover the damaged vessel wall.

One of several adaptations ground squirrels have acquired to prevent clotting during hibernation is the reversible decrease of platelet levels. Upon entering torpor, circulating platelet levels decrease 10-fold (Lechler, 1963), with a majority of platelets becoming sequestered in the spleen. Upon arousal, functional platelets are released back into circulation, reaching normal levels within two hours post arousal (Cooper et al., 2014). In addition, the vWF protein levels in torpid ground squirrels are reduced 10-fold (Cooper et al., 2015). Therefore, the reduced number of platelets remaining in circulation

during torpor would be prevented from adhering to exposed collagen in the absence of vWF; further suppressing primary hemostasis.

Following platelet plug formation during the process of primary hemostasis, the coagulation cascade becomes activated during secondary hemostasis. Both the extrinsic and intrinsic pathways consist of a cascade of coagulation serine proteases, which converge on Factor X activation (Palta et al., 2014) (Figure 2). Activated Factor X

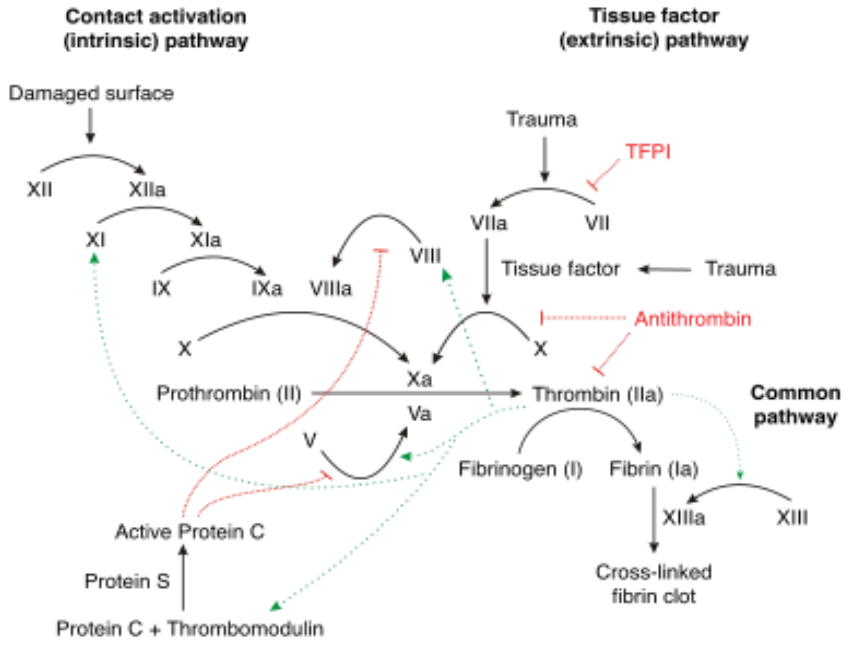

Figure 2. Activation of the coagulation cascade of serine proteases via either the intrinsic or extrinsic pathway ultimately leads to formation of cross-linked fibrin. Schematic diagram retrieved from Wikimedia Commons, 2007.

converts prothrombin to thrombin, which further cleaves soluble fibrinogen to insoluble fibrin. Once insoluble fibrin is covalently cross-linked by thrombin-activated factor XIII, a fibrin mesh is formed and incorporated into the platelet plug, forming a stronger and more stable fibrin clot. A major regulator of the coagulation cascade is the serine protease inhibitor (serpin) antithrombin III (AT). Inhibition of thrombin by antithrombin results in the formation of irreversible thrombin-antithrombin complexes (TAT), and an elevated

TAT level in plasma is a marker of activated coagulation (Boisclair et al., 1990). In ground squirrels, the most dramatic changes to secondary hemostasis during hibernation are an 80% drop in factor VIII and a 50% drop in factor IX activities (Pivorun 1981). Depression of both factor VIII and IX activities, in combination with decreased circulating platelet levels, effectively reduces clotting times in hibernating ground squirrels, and may therefore help to prevent stasis-induced blood clots (Pivorun,1981; Lechler, 1963; Cooper et al., 2015).

Fibrinolysis

Fibrin produced by the coagulation cascade ultimately serves as substrate for fibrinolysis, the process by which fibrin clots are dissolved. Like the coagulation cascade, the fibrinolytic system consists of serine proteases (Figure 3). Fibrinolysis first becomes activated when tissue plasminogen activator (tPA) is released from endothelial cells in

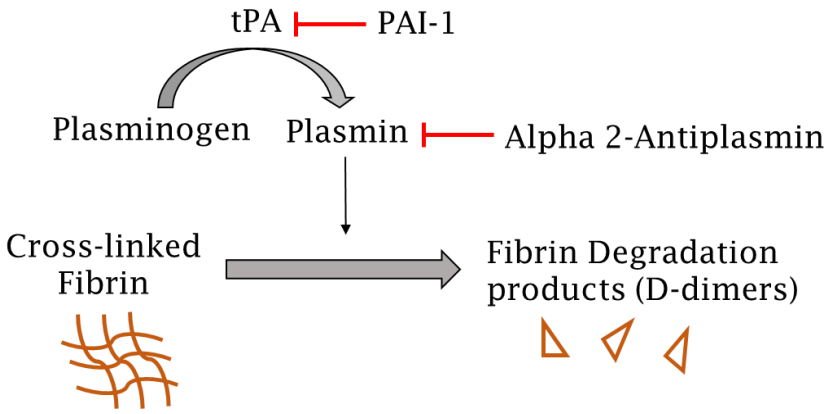

Figure 3. Schematic diagram of the fibrinolytic system of serine proteases leads to the formation of specific fibrin degradation products, including D-dimers. Red bars indicate inhibitory action of serine protease inhibitors.

response to various stimuli such as, thrombin, venous occlusion, and sheer stress. Circulating tPA converts the zymogen plasminogen to active plasmin, which then cleaves

the cross-linked fibrin mesh of clots leading to their dissolution. Cross-linked fibrin is degraded into specific fragments referred to as D-dimers. Activation of fibrinolysis is clinically indicated by elevated blood levels of D-dimers, and used in the diagnosis of venous pulmonary embolism (PE) and DVT.

The fibrinolytic system is tightly regulated by serpins that inhibit individual serine proteases to prevent widespread fibrinolysis. The main inhibitor of fibrinolysis is plasminogen activator inhibitor-1 (PAI-1) which irreversibly binds tPA forming a tPA-PAI-1 complex. The serpin α_2-antiplasmin (α2-AP) directly inhibits the activity of plasmin, forming an irreversible plasmin-α2-AP complex (PAP).

Impaired functioning of fibrinolysis is common in thrombotic diseases. Elevated levels of plasma PAI-1 is a risk factor for vascular ischemic events, such as recurrent DVT. Higher plasma levels of PAI-1 also correlate with an increased resistance to clot lysis by tPA (Zhu 1999). Conversely, human deficiencies of PAI-1, as well as α2-AP are associated with an endogenous hyperfibrinolytic state, as well as a hemorrhagic state (Chapin, 2003). While an elevated tPA-PAI-1 complex level is associated with thrombotic events such as recurrent myocardial infarction (Wiman et al., 2000), tPA-PAI-1 level in relation to total plasma PAI-1 can also indicate activation of fibrinolysis. The tPA-PAI-1 complex to total PAI-1 ratio is elevated in hyperfibrinolytic disease states such as disseminated intravascular coagulation (DIC). This is characterized by widespread activation of the coagulation cascade resulting in formation of many thrombi in the microvasculature (Wantanabe et al., 2001).

One may predict that a hibernating animal would need to have a more active fibrinolytic system to break down any clots that did form. Previous research showed

plasminogen and α2-AP levels are both decreased during torpor in ground squirrels, consistent with activation of plasminogen into plasmin, and then formation of PAP complexes.

Effects of Hibernation on Blood Clot Formation

As evidenced by their survival, ground squirrels do not form lethal clots during hibernation. However, previous research in our lab suggests despite suppression of primary and secondary hemostasis, some clots may still be forming. The degree of histological myocardial ischemic damage significantly increases during entrance into

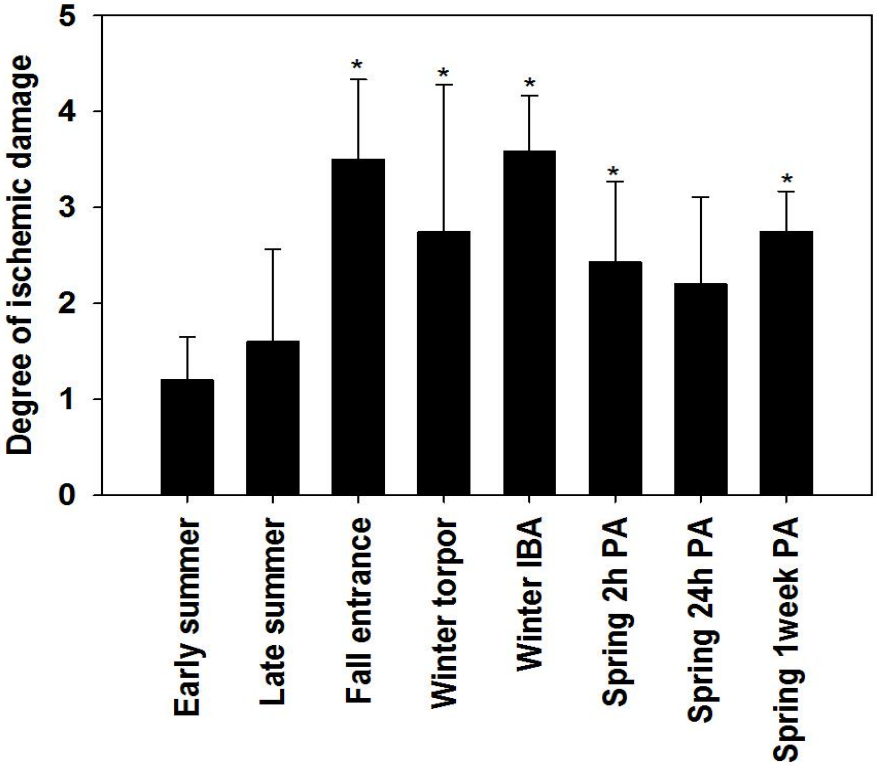

Figure 4. Degree of cardiac ischemia based on histological markers throughout the annual torpor arousal life cycle of the thirteen-lined ground squirrel. Post Arousal, PA. Significance testing used the Student t-test to compare average ischemic score of each group to late summer non-hibernators. *- indicates significant difference, $P < 0.05$ (Morgan, 2013).

hibernation compared to nonhibernating baseline levels, and remains elevated even one week post arousal (Figure 4). This observed histological pattern of myocardial damage

could be due to blood clots forming in coronary circulation during hibernation, or cardiac output not meeting metabolic demand. Lack of pulmonary emboli seen during hibernation, as well as myocardial tissue displaying histological patterns of localized damage, serve as further evidence that ischemic damage sustained during hibernation may be the result of clots forming in coronary circulation.

While previous studies indirectly suggest the possibility of clots forming during hibernation, direct evidence of blood clots forming in hibernating ground squirrels has not been observed. Although clots may form during hibernation, hyper-activity of the fibrinolytic system could result in rapid dissolution of any blood clots. Similarly, clots formed may be less resistant to breakdown by fibrinolysis as well. Therefore, factors such as fibrinolytic capacity, as well as potential clot lysis resistance should be considered when determining the ability of blood clots to cause damage during hibernation.

Significance

Each year there are 350,000 to 600,000 cases of DVT/PE in the United States (Heit 1999). While 100,000-180,000 will die as a result, those who survive will remain at an increased risk for recurrent DVT/PE episodes (Cushman, 2007). Hibernating ground squirrels can survive physiological states of low blood flow that would result in the formation of lethal blood clots in humans. Further understanding the coagulative and fibrinolytic mechanisms behind the antithrombotic adaptations allowing ground squirrels to avoid forming lethal blood clots during hibernation can potentially translate into future novel clinical therapies for prevention of DVT/PE.

Specific Aims

Ground squirrels do not form lethal blood clots during hibernation, as evidenced by their survival throughout the hibernation season. Although ground squirrels have acquired antithrombotic adaptations that prevent lethal blood clots from forming, whether these adaptations result in the complete suppression of all blood clotting activity during hibernation remains unknown. The comprehensive objective of this study was to determine the effect of hibernation on blood clot formation and fibrinolysis in ground squirrels. Activation of coagulation and fibrinolysis was used as indirect evidence of blood clot formation. Coagulation cascade activity was determined by measuring plasma TAT, a hemostatic marker of coagulation activation. Fibrinolysis activity was determined by measuring plasma levels of two hemostatic markers of fibrinolysis activation: tPA-PAI-1 and D-dimer. Furthermore, regulation of fibrinolysis was also accessed by measuring plasma levels of PAI-1 and tPA-PAI-1:PAI-1 ratio, which gave information on clot lysis resistance and fibrinolytic state, respectively.

MATERIALS AND METHODS

Animals and Hibernation Stage Classification

Ground squirrels were collected from the Trempealeau Golf Course (Trempealeau, WI) or born in captivity, and housed at the University of Wisconsin-La Crosse following protocols approved by the Institutional Animal Care and Use Committee (IACUC). Animals were housed individually in controlled light-dark cycle environments mimicking a Wisconsin photoperiod (9 light hours in December gradually increasing to 15.5 light hours in June then decreasing again in December). Animals in the summer active state during June-August, with body temperatures of 37 ºC were classified as nonhibernators. Upon entrance into hibernation in the fall, the animal's temperature dropped to 25 ºC, body assumed a curled position, and activity decreased. Animals were subsequently moved into a 4ºC hibernaculum, and classified as torpid when inactive with body temperatures less than 10ºC. Squirrels were checked daily during hibernation, and IBA ground squirrels were classified as those aroused from the torpid state for 12-24 hours with euthermic body temperatures (37 ºC). In March, hibernators were aroused manually and moved out of the hibernaculum to cages in a room at 25 ºC. These animals were classified as post-arousal (PA) ground squirrels, and time since aroused from hibernation was recorded, up to 10 weeks PA.

Sample Collection

Blood samples were collected from the tail arteries of isoflurane (1.5-5%) anesthetized ground squirrels, or during exsanguination of ground squirrels euthanized by CO_2 asphyxiation. Blood was collected in 150 µl/mL acid citrate dextrose and centrifuged for 5 minutes at 3000 rpm. Plasma was separated into microcentrifuge tubes and stored at -80 ºC until further use in assays was required. Brain, liver, and kidney collected from euthanized animals were immediately frozen in liquid nitrogen. Organ samples were stored at -80 ºC until further use in RT-qPCR or immunoblot analysis was required.

Preparation of Tissue Lysates

Prior to immunoblot analysis, kidney, brain, and liver samples from nonhibernating animals were homogenized. For all samples, approximately 5.0 mg of tissue was homogenized in 300 µl of ice-cold NP-40 lysis buffer (150 mM NaCl, 1.0 % (v/v) NP-40, 50 mM Tris-HCl pH 8.0, 1.0 % (v/v) protease inhibitor) using an electric homogenizer. Thereafter, homogenates were maintained at a constant agitation for 2 hours at 4 ºC, and then centrifuged for 10 minutes at 12000 rpm. Supernatants were aspirated and stored at -80 ºC until further use, while pellets were discarded.

Immunoblots

Immunoblotting was used to confirm cross-reactivity of all polyclonal antibodies used in this study with respective ground squirrel version of immunogen. Homogenized nonhibernating ground squirrel brain (50 µg), kidney (50 µg), and liver (50 µg) were separated on a 12% SDS-PAGE. After transfer to a 0.45 µm PVDF membrane, the blot was blocked with 3% milk powder in Tris-buffered saline Tween-20 (TBST, 10 mM Tris,

150 mM NaCl, 0.5% (v/v) Tween-20, pH 7.4). The blotted bands were immunodetected with 2 µg/ml of a rabbit anti-mouse tPA antibody (ab62763; Abcam, Cambridge, MA) and subsequently probed with 1:10000 diluted goat anti-rabbit IgG antibody conjugated to DyLight™ 649 (611-143-002, Rockland Antibodies & Assays, Limerick, PA). The fluorescent signal was detected using a Typhoon FLA 9500 laser scanner (28996943; GE Healthcare Life Sciences, Pittsburgh, PA).

Homogenized nonhibernating ground squirrel brain (50 µg), and liver (50 µg) were separated on a 10% SDS-PAGE. After transfer to a 0.45 µm PVDF membrane, the blot was blocked with 3% milk powder in TBST. The blotted bands were immunodetected with 1 µg/ml rabbit anti-mouse PAI-1 antibody (ab66705; Abcam, Cambridge, MA) and subsequently probed with 1: 10000 diluted goat anti-rabbit IgG antibody conjugated to DyLight™ 649. The fluorescent signal was detected using a Typhoon FLA 9500 laser scanner.

Homogenized nonhibernating ground squirrel brain (50 µg), and liver (50 µg) were separated on a 10% SDS-PAGE. After transfer to a 0.45 µm PVDF membrane, the blot was blocked with 3% bovine serum albumin (BSA) in TBST. Blots were immunodetected with 1.5 µg/ml rabbit anti-human PAI-1 antibody linked to biotin (ab48366: Abcam, Cambridge, MA) and subsequently probed with 1:5000 diluted goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP) (4030-05; Southern Biotech, Birmingham, AL). Blotted bands were detected using the Super Signal West Pico chemiluminesence substrate (34080, Thermo Fisher Scientific, Waltham, MA) and visualized using a ChemiDoc™ Imaging System (Bio-Rad, Hercules, CA).

Purified human thrombin (0.25 µM), antithrombin (0.5 µM), and thrombin complexed with antithrombin (0.25 µM) were run on a 10% SDS-PAGE. After transfer to a 0.45 µm PVDF membrane, the blot was blocked with 3% milk powder in TBST. Blots were immunodetected with either 1µg/ml sheep anti-human thrombin antibody (PA1-43040; Thermo Fisher Scientific, Waltham, MA), or a 1:1000 diluted rabbit anti-human antithrombin antibody (A9522; Sigma-Aldrich, St. Louis, MO). Species specific secondary antibodies diluted 1:10000 were then added to each respective blot; either rabbit anti-sheep IgG conjugated to alkaline phosphatase (AP) (6010-04; Southern Biotech, Birmingham, AL), or goat anti-rabbit IgG conjugated to AP (A3687;Sigma-Aldrich, St. Louis, MO). Blotted bands were detected with SIGMA*FAST*™ BCIP®/NBT tablets (B5655; Sigma-Aldrich, St. Louis, MO).

Capture Thrombin-Antithrombin (TAT) ELISA

Plasma concentrations of TAT complexes in ground squirrels were determined by capture ELISA. Flat bottom microplates (96 well) were coated for 1 hour at 37ºC with 100 µl of a sheep anti-human thrombin antibody (PA1-43040; Thermo Fisher Scientific, Waltham, MA) diluted 1:500 in Tris-buffered saline (TBS, 10 mM Tris, 150 mM NaCl). Plates were subsequently washed 3 times with TBS, 0.5% (v/v) Tween-20 (TBST), and blocked with 300 µl TBST, 3.0% (w/v) instant nonfat dry milk (TBST-milk) for 1 hour at 37 ºC. After washing plates 3 times with TBST, 100 µl of plasma samples diluted 1:10 in TBST-milk were incubated for 1 hour at 37 ºC. Plates were then washed 5 times with TBST, and incubated for 1 hour at 37 ºC with 100 µl of a rabbit anti-human antithrombin antibody (A9522; Sigma-Aldrich, St. Louis, MO) diluted 1:1000 in TBST-milk. Thereafter, plates were washed 5 times with TBST and incubated for 1 hour at 37 ºC with

100 µl of a goat anti-rabbit IgG antibody linked to alkaline phosphatase (AP) (A3687;Sigma-Aldrich, St. Louis, MO) diluted 1:10000 in TBST-milk. The color was developed with 100 µl of a 1 mg/ml 4-nitrophenyl disodium salt hexahydrate (A12310; Alfa Aesar, Ward Hill, Massachusetts) solution in alkaline phosphatase buffer (AP Buffer, 100 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.5) for 35 minutes at 37ºC. The optical density (OD) was determined at 405 nm. A standard curve for the TAT ELISA was constructed with two-fold serial dilutions of a 120 nM ground squirrel TAT standard sample.

Capture PAI-1 ELISA

Plasma concentrations of PAI-1 in ground squirrels were determined by capture ELISA. Flat bottom microplates (96 well) were coated for 1 hour at 37 ºC with 100 µl of a rabbit anti-mouse PAI-1 antibody (ab66705; Abcam, Cambridge, MA) diluted 1:1000 in Tris-buffered saline (TBS, 10 mM Tris, 150 mM NaCl). Plates were subsequently washed 3 times with TBS, 0.5% (v/v) Tween-20 (TBST), and blocked with 300 µl TBST, 3.0% (w/v) bovine serum albumin (TBST-BSA) for 1 hour at 37 ºC. After washing plates 3 times with TBST, 100 µl of plasma samples diluted 1:10 in TBST-BSA were incubated for 1 hour at 37 ºC. Plates were then washed 5 times with TBST, and incubated for 1 hour at 37 ºC with 100 µl of a rabbit anti-human PAI-1 antibody linked to biotin (ab48366: Abcam, Cambridge, MA) diluted 1:5000 in TBST-BSA. Thereafter, plates were washed 5 times with TBST and incubated for 35 minutes at 37 ºC with 100 µl horseradish peroxidase linked to avidin (HRP-avidin) diluted 1:5000 in TBST-BSA. Plates were subsequently washed 5 times with TBST. The color was developed with 1-Step™ Turbo TMB (34022; Thermo Fisher Scientific, Waltham, MA) for 15 minutes at 21 ºC. The

reaction was stopped with 100 µl of 1 M H_2SO_4, and the optical density (OD) was determined at 450 nm. A standard for the Capture PAI-1 ELISA was constructed with two-fold serial dilutions of platelet free pooled normal human plasma, which has a PAI-1 concentration of 21.0 ± 7.2 ng/ml (Booth et al., 1988).

SERPINE1 RT-qPCR

Total RNA was isolated from ground squirrel livers using the Absolutely RNA Miniprep Kit (400800; Agilent Technologies, Cedar Creek, Texas) according to the manufacturer's instructions. A NanoDrop™ Lite spectrophotometer (ND-LITE; Thermo Fisher Scientific, Rockford, IL) was used to assess the RNA concentration and purity. Total RNA (1 µg) was reverse transcribed into cDNA using the Affinity Script QPCR cDNA Synthesis Kit (600559; Agilent Technologies, Cedar Creek, Texas) according to the manufacturer's instructions. Forward and reverse *SERPINE1* primers were designed using IDT DNA's qPCR primer design program (Integrated DNA Technologies, Coralville Iowa) and were as follows: 5'-CCTTTCTGCCCTCACCAATA-3' and 5'-GAGAACTTGGGCAGAACTAGG-3'. All real-time PCR reactions were performed using the CFX96 Touch™ Real-Time PCR Detection System (1855795; Bio-Rad, Hercules, CA) and cDNA amplifications were performed using the SsoAdvanced™ Universal SYBR® Green Supermix (172-5274; Bio-Rad, Hercules, CA). Briefly, the reactions conditions consisted of 8 µl of cDNA and 2 µM primers in a final volume of 20 µl of Supermix. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous control to normalize each sample. Quantification of *SERPINE1* expression relative to *GAPDH* was determined using the equation: relative expression= $2^{Ct\ GAPDH}$ / $2^{Ct\ SERPINE1}$.

Capture tPA-PAI-1 ELISA

Plasma concentration of tPA complexed with PAI-1 in ground squirrels was determined by capture ELISA. Flat bottom microplates (96 well) were coated for 1 hour at 37 ºC with 100 µl of a rabbit anti-mouse tPA antibody (ab62763; Abcam, Cambridge, MA) diluted 1:1000 in Tris-buffered saline (TBS, 10 mM Tris, 150 mM NaCl). Plates were subsequently washed 3 times with TBS, 0.5% (v/v) Tween-20 (TBST), and blocked with 300 µl TBST, 3.0% (w/v) bovine serum albumin (TBST-BSA) for 1 hour at 37 ºC. After washing plates 3 times with TBST, 100 µl of plasma samples diluted 1:10 in TBST-BSA were incubated for 1 hour at 37 ºC. Plates were then washed 5 times with TBST, and incubated for 1 hour at 37 ºC with 100 µl of a rabbit anti-human PAI-1 antibody linked to biotin (ab48366; Abcam, Cambridge, MA) diluted 1:5000 in TBST-BSA. Thereafter, plates were washed 5 times with TBST and incubated for 35 minutes at 37 ºC with 100 µl horseradish peroxidase linked to avidin (HRP-avidin) diluted 1:5000 in TBST-BSA. Plates were subsequently washed 5 times with TBST. The color was developed with 1-Step™ Turbo TMB (34022; Thermo Fisher Scientific, Waltham, MA) for 15 minutes at 21 ºC. The reaction was stopped with 100 µl of 1 M H_2SO_4, and the optical density (OD) was determined at 450 nm. A standard for the Capture tPA-PAI-1 ELISA was constructed with two-fold serial dilutions of platelet free pooled normal human plasma, which has a tPA-PAI-1 concentration of 2.8 ± 1.6 ng/ml (Fareed et al., 1998).

Competitive D-Dimer ELISA

D-Dimer level in ground squirrel plasma was detected in duplicates with a commercially available competitive ELISA Kit according to the manufacturer's

instructions: Rat D2D (D-Dimer) ELISA Kit (Cat#: E-EL-R0317; Elabscience, Wuhan, China). The optical density was determined at 450 nm. In order to generate a positive and negative control, ground squirrel whole blood was allowed to clot at room temperature for 2 hours. After 2 hours, the resulting top layer of sera was isolated and used as a negative control. The remaining blood was allowed to further clot and breakdown for an additional 20 hours. The resulting top sera layer was collected and used as a positive control.

Data Normalization

For all data, excluding the tPA-PAI-1:PAI-1 ratios, the mean marker value for each animal group was normalized to the nonhibernating group. Therefore, in these data the nonhibernating group resulted in a value of 1.

RESULTS

Immunoblot Analysis

Immunoblot analysis was used to confirm the ability of all antibodies to cross-react with the ground squirrel versions of the human immunogens. Both the sheep anti-human thrombin, and rabbit anti-human antithrombin antibody reacted with ground squirrel thrombin (predicted molecular weight: 36-42 kDa), and ground squirrel antithrombin (predicted molecular weight: 58 kDa), respectively (Figure 5A; Figure 5B). Also, both antibodies were able to react with their immunogen in the complexed form (TAT) (predicted molecular weight: 94-125 kDa). Additional bands seen above 130 kDa for the rabbit anti-human antithrombin blot were most likely due to dimerization of antithrombin at high concentrations.

The rabbit anti-mouse PAI-1 antibody cross-reacted with ground squirrel PAI-1 (predicted molecular weight: 45-55 kDa) from a nonhibernator's liver (Figure 5C), as did the biotinylated rabbit anti-human PAI-1 antibody (Figure 5D). Ground squirrel liver homogenates were used as a positive control, as a majority of PAI-1 is synthesized in the liver. Ground squirrel brain homogenates were expected to produce faint to undetectable bands during immunoblot analysis, as the brain tissue is not a major source of PAI-1. While the non-biotinylated PAI-1 antibody did not detect PAI-1 in ground squirrel brain homogenate, the biotinylated antibody showed a distinct band corresponding to PAI-1. The additional band detected at approximately 31 kDa for both PAI-1 blots were most likely a degradation product of PAI-1 due to cleavage by cellular proteases.

The rabbit anti-mouse tPA antibody was confirmed to cross-react with ground squirrel tPA (Figure 5E). Bands were detected in lanes containing ground squirrel kidney and liver homogenates; both of which are known sources of tPA (predicted molecular weight: 62-75 kDa) in rodents. Banding was not detected in the lane containing brain, which was expected, as tPA is not known to be highly expressed by the brain.

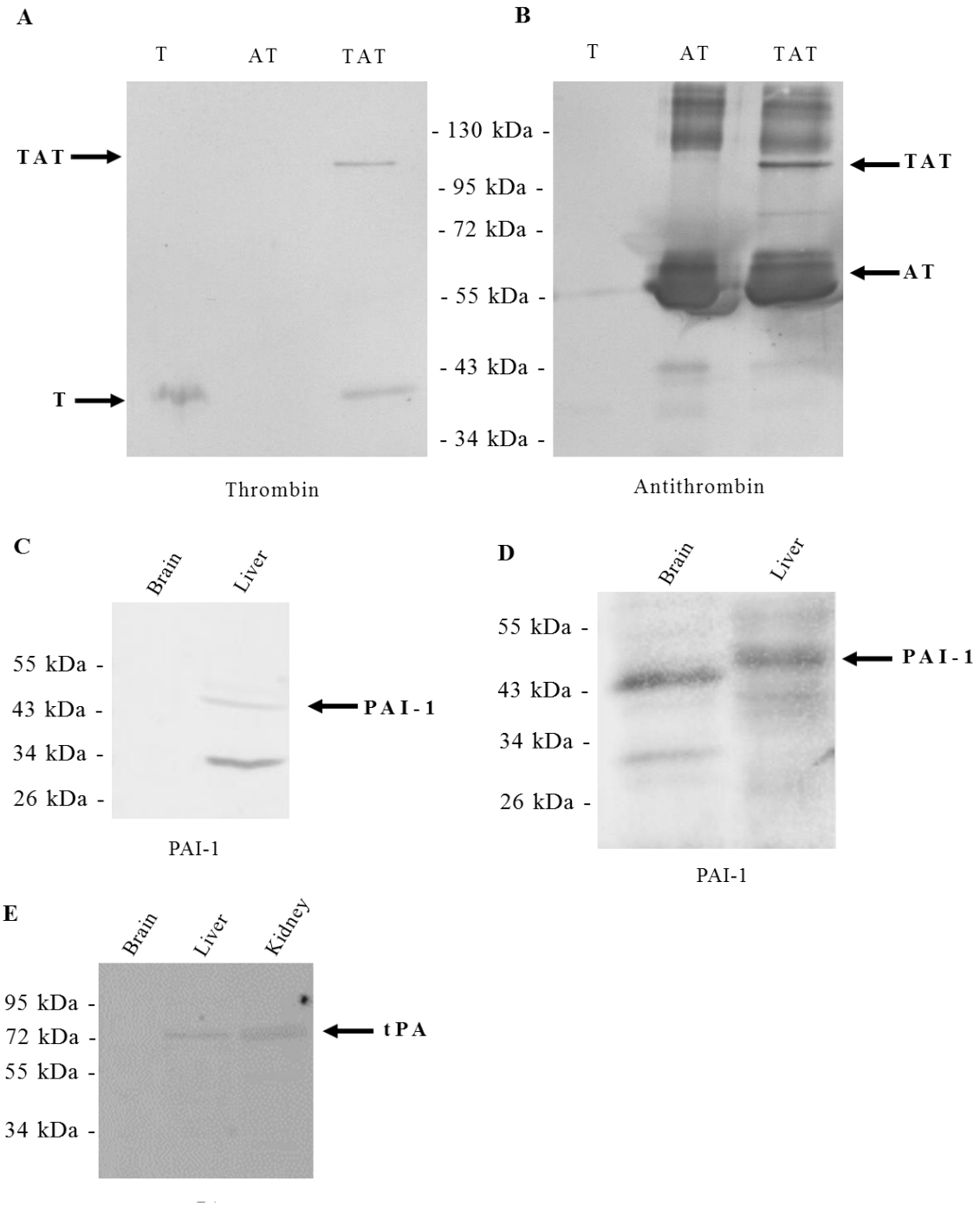

Figure 5. Immunoblot analysis confirms cross-reactivity of antibodies with respective immunogens in ground squirrels. SDS-PAGE of 0.5 µM antithrombin (AT), 0.25 µM thrombin (T), 0.25 µM thrombin-antithrombin complex (TAT), 50 µg ground squirrel brain, 50 µg ground squirrel liver, or 50 µg ground squirrel kidney. Blots were probed with the following primary antibodies: (A) Sheep anti-human thrombin (B) Rabbit anti-human antithrombin (C) Rabbit anti-mouse PAI-1 (D) Rabbit anti-human PAI-1 conjugated to biotin (E) Rabbit anti-mouse tPA. Detected immunogen is labeled under each blot.

Measurement of TAT complexes in ground squirrel plasma

A capture ELISA was performed to determine plasma TAT level in ground squirrels throughout the annual hibernation arousal cycle (Figure 6). For all data, the mean TAT value for each animal group was normalized to the nonhibernating group. The mean TAT was elevated in entrance animals relative to nonhibernators (1.29 ± 0.18 and

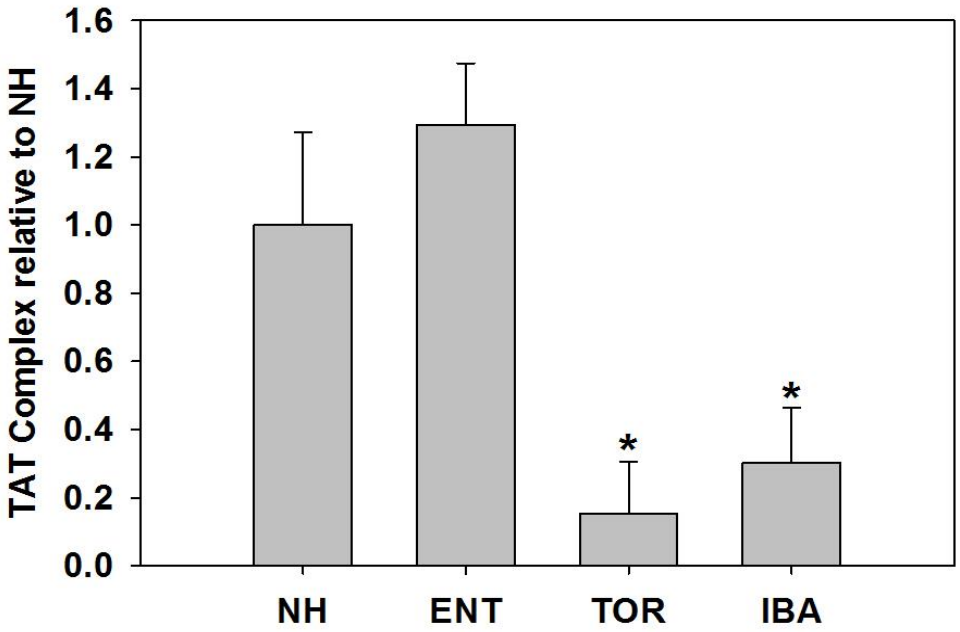

Figure 6. Thrombin-antithrombin Complex (TAT) level relative to nonhibernators throughout annual hibernation arousal cycle in thirteen lined ground squirrels. Nonhibernators, NH (n=8); Entrance into hibernation, ENT (n=4); Torpor, TOR (n=7); Interbout Arousal, IBA (n=7). Significance testing used the student t-test to compare average TAT complex of each group to nonhibernators. *- indicates significant difference, $p < 0.05$.

1.00 ± 0.27, respectively) although this difference was not significant ($p > 0.05$). Both torpid and interbout aroused ground squirrels had mean TAT levels (0.15 ± 0.15 and 0.30 ± 0.16, respectively) differing significantly from nonhibernators ($p < 0.05$). Over a 3-fold drop in IBA TAT was observed, with an even greater 6-fold decrease detected in the torpid group relative to nonhibernators.

Determination of ground squirrel plasma PAI-1 protein and liver mRNA

The serpin PAI-1 is a major inhibitor of fibrinolysis; forming inactive inhibitor complexes with its respective serine protease, tPA. A capture ELISA was performed to detect the level of PAI-1 protein in ground squirrel plasma (Figure 7). For all data, the mean PAI-1 value for each animal group was normalized to the nonhibernating group. Prior to normalization, the total plasma level of PAI-1 in nonhibernators was 1.91 ± 0.99

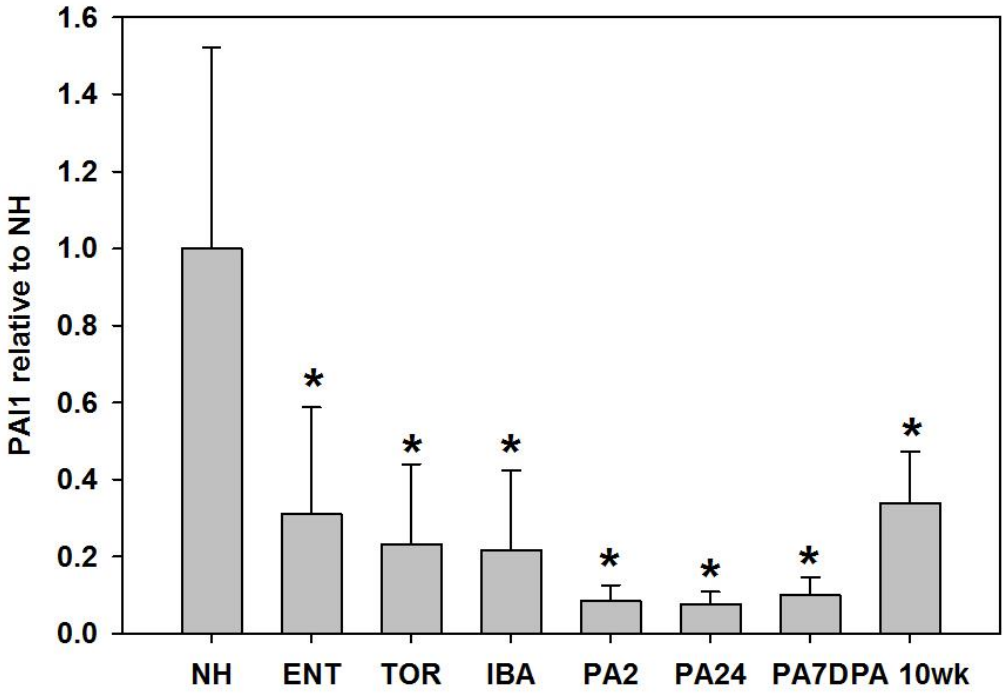

Figure 7. Total PAI-1 plasma levels relative to nonhibernators throughout annual hibernation arousal cycle in thirteen lined ground squirrels. Nonhibernators, NH (n=8); Entrance into hibernation, ENT (n=10); Torpor, TOR (n=10); Interbout Arousal, IBA (n=10); 2 hours post arousal, PA2 (n=5); 24 hours post arousal, PA24 (n=4); 7 days post arousal PA7D (n=5); 10 weeks post arousal, PA10wk (n=5). Significance testing used the student t-test to compare average PAI-1 level of each group to nonhibernators. *- indicates significant difference, $p < 0.05$.

ng/ml; similar to that of other small rodents, such as the mouse. All stages of the annual hibernation arousal cycle had total PAI-1 levels significantly differing from nonhibernators ($p < 0.05$). Upon entry into hibernation, mean PAI-1 levels decreased approximately 3.2-fold (0.31 ± 0.28 ng/ml) and continued to be observed at a reduced

level throughout both the torpid (0.23 ± 0.21) and IBA (0.22 ± 0.21) stage. Lowest mean PAI-1 levels were observed 2 hours (0.08 ± 0.04), 24 hours (0.08 ± 0.03), and 7 days (0.10 ± 0.05) post arousal in the spring, with an approximately 12.5-fold drop compared to nonhibernating total PAI-1. An increasing trend back to nonhibernating levels was observed 10 weeks post arousal (0.34 ± 0.1), yet still reduced 2.9-fold, compared to nonhibernators.

To confirm that fluctuations in PAI-1 plasma protein level throughout the annual hibernation arousal cycle were due to differential production of the protein rather than differential consumption, quantitative PCR was performed on cDNA from ground squirrel liver with primers specific for *SERPINE1* (Figure 8). For all data, the average mRNA level for each animal group was normalized to the nonhibernating group. There was a significant ($p < 0.05$) 9-fold drop in *SERPINE1* mRNA levels, encoding for the PAI-1 protein, in entrance animals compared to nonhibernators (0.11 ± .11 and 1.0 ± 0.34, respectively). Although a 4.2-fold decrease in torpid *SERPINE1* mRNA (0.24 ± 0.18), as well as an over 5-fold decrease in IBA animals (0.19 ± 0.10), neither of these drops in mRNA level were significant compared to nonhibernating animals ($p > 0.05$). The largest drop in *SERPINE1* mRNA levels was observed in animals 7 days post arousal (0.08 ± 0.08), with a significant ($p < 0.05$) 12.5-fold decrease compared to nonhibernators.

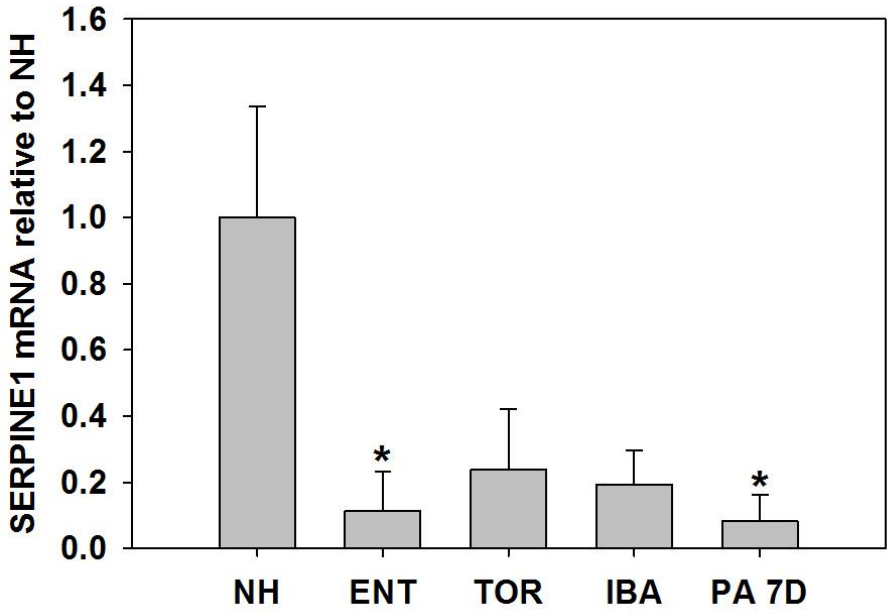

Figure 8. *SERPINE1* mRNA relative to nonhibernators throughout annual hibernation arousal cycle in thirteen lined ground squirrels. Nonhibernators, NH (n=2); Entrance into hibernation, ENT (n=3); Torpor, TOR (n=3); Interbout Arousal, IBA (n=3); 7 days post arousal PA7D (n=3). Significance testing used the student t-test to compare average PAI-1 mRNA level of each group to nonhibernators. *- indicates significant difference, $p < 0.05$.

Measurement of tPA-PAI-1 complexes in ground squirrel plasma

During the events of fibrinolysis tPA cleaves inactive plasminogen into the fibrin-degrading plasmin enzyme. However, PAI-1 inhibitory interaction with tPA results in the formation of irreversible tPA-PAI-1 complexes that prevent tPA functionality. A capture ELISA was performed to quantify plasma tPA-PAI-1 complex level across hibernation stages (Figure 9). For all data, the average tPA-PAI-1 complex value for each animal group was normalized to the nonhibernating group. Also, to examine the fibrinolytic state of animals, the tPA-PAI-1 complex level was compared to total PAI-1 available (Figure 10). For individual animals, the ratio of tPA-PAI-1 complex to total PAI-1 protein (tPA-PAI-1:PAI-1) was calculated, then averaged for each of the major stages assayed.

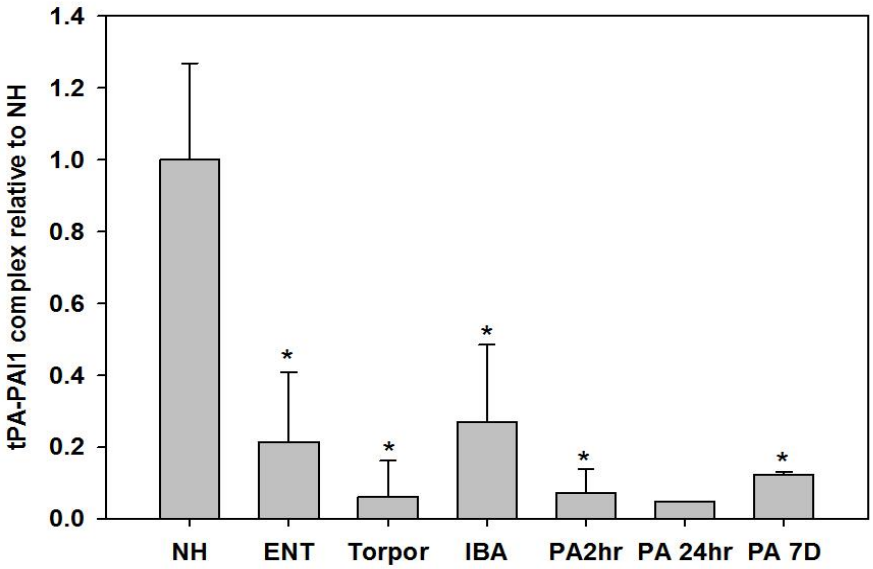

Figure 9. tPA-PAI-1 Complex plasma levels relative to nonhibernators throughout annual hibernation arousal cycle in thirteen lined ground squirrels. Nonhibernators, NH (n=6); Entrance into hibernation, ENT (n=2); Torpor, TOR (n=6); Interbout Arousal, IBA (n=8); 2 hours post arousal, PA2hr (n=3); 24 hours post arousal, PA24hr (n=1); 7 days post arousal, PA7D (n=2). Significance testing used the student t-test to compare average tPA- PAI-1 level of each group to nonhibernators. *- indicates significant difference, $p < 0.05$.

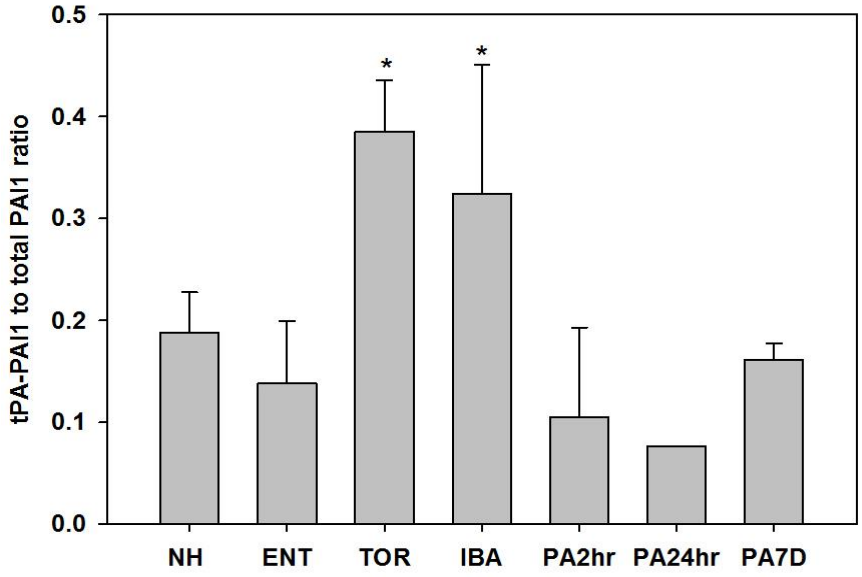

Figure 10. tPA-PAI-1 Complex to total PAI-1 ratio throughout annual hibernation arousal cycle in thirteen lined ground squirrels. Nonhibernators, NH (n=6); Entrance into hibernation, ENT (n=2); Torpor, TOR (n=2); Interbout Arousal, IBA (n=5); 2 hours post arousal, PA2hr (n=3); 24 hours post arousal, PA24hr (n=1); 7 days post arousal, PA7D (n=2). Significance testing used the student t-test to compare average ratio of each group to nonhibernators. *- indicates significant difference, $p < 0.05$.

Entrance ground squirrels had 4.6-fold less tPA-PAI-1 compared to nonhibernators (0.21 ± 0.19 and 1 ± 0.27, respectively), which was a significant difference ($p < 0.05$). Torpid ground squirrels showed one of the most significant ($p < 0.05$) differences in tPA-PAI-1 from nonhibernating levels, as over a 16-fold decrease was observed (0.06 ± 0.10). Interbout aroused animals showed a decrease in tPA-PAI-1 similar to that of entrance animals (0.27 ± 0.21), and differed significantly from the nonhibernating group ($p < 0.05$). Post arousal animals showed significant ($p < 0.05$) drops in the level of tPA-PAI-1, even 1 week after arousal (PA2, 0.07 ± 0.07; PA7D, 0.12 ± 0.006).

When the level of tPA-PAI-1 was compared to total PAI-1 protein, the mean nonhibernating ratio was 0.19 ± 0.04. Both torpid and IBA animals had significantly ($p < 0.05$) elevated ratios (0.39 ± 0.06 and 0.32 ± 0.13, respectively) compared to nonhibernators. Neither entrance (0.14 ± .06), nor post aroused animals from any time period (PA2, 0.10 ± 0.09; PA7D, 0.16 ± 0.016) were observed to have ratios differing significantly from nonhibernators.

Plasma D-dimer level in ground squirrels

The final step of fibrinolysis involves the plasmin-powered cleavage of cross-linked fibrin into fibrin degradation products (FDPs), including D-dimers. A competitive D-dimer ELISA was performed to measure the level of these FDPs in the plasma of ground squirrels in the nonhibernating, torpid, and IBA stage (Figure 11). Average D-dimer values of each animal group were normalized to the nonhibernating group. The D-dimer level of nonhibernators (1 ± 0.55) was not statistically different from either the torpid (0.76 ± 0.22) or IBA (0.80 ± 0.26) stages.

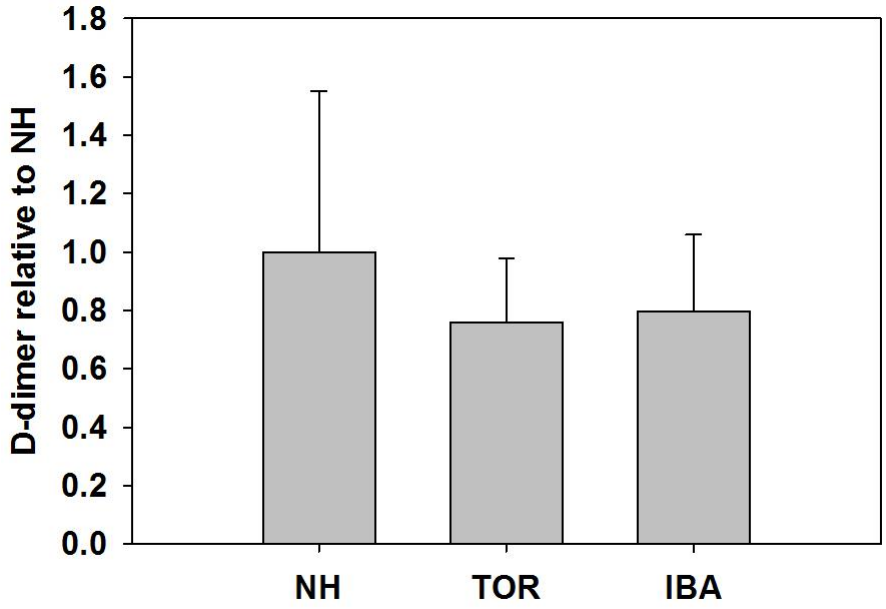

Figure 11. D-dimer level relative to nonhibernators throughout annual hibernation arousal cycle in thirteen lined ground squirrels. Nonhibernators, NH (n=8); Torpor, TOR (n=8); Interbout Arousal, IBA (n=6). Significance testing used the student t-test to compare average D-dimer level of each group to nonhibernators. *- indicates significant difference, $p < 0.05$.

DISCUSSION

Thirteen-lined ground squirrels continually survive consecutive bouts of torpor and interbout arousals throughout their 6-7-month hibernation season. Although ground squirrels have developed antithrombotic adaptations to avoid lethal blood clots during hibernation, whether these adaptations cease all ability to form blood clots remains unknown. Therefore, the main objective of this study was to determine if ground squirrels are still forming blood clots during hibernation despite mechanisms in place to prevent lethal clots.

Blood clots were detected by looking for measurable evidence of their formation- through activation of the coagulation cascade, as well as evidence of their breakdown- through activation of fibrinolysis. Ground squirrels in various stages of the annual hibernation arousal cycle were examined in this study including: summer nonhibernators, fall animals entering hibernation, torpid, IBA, and animals post arousal in the spring.

Elevated plasma TAT complexes are a marker of activated coagulation. Rather than being elevated, ground squirrel TAT was significantly decreased during hibernation compared to summer nonhibernators. This suggested that not only is coagulation not being activated during hibernation, but it is also likely suppressed during this time. Previous studies have also observed suppression of the coagulation cascade during hibernation, and these findings offer further support (Pivorun,1981; Lechler, 1963; Cooper et al., 2015).

D-dimers are specific fibrin degradation products released during the degradation of a fibrin clot. Elevated D-dimers are a widely utilized clinical marker of blood clots and activated fibrinolysis. A steady D-dimer level throughout all stages of the annual cycle, along with an inactivated coagulation cascade during hibernation suggested that blood clots are not forming during hibernation in ground squirrels.

Following observations of a suppressed coagulation cascade during hibernation, it was predicted that the fibrinolytic system would be in an inactivated state as well; however, this was not the case. Hibernating animals were observed to be in a hyperfibrinolytic state compared to nonhibernators. Although the level of tPA-PAI-1 complexes dropped significantly during hibernation, the ratio of tPA-PAI-1 to total PAI-1, a marker of fibrinolytic activity, was significantly elevated in the torpid and IBA state. Normal healthy humans have an average tPA-PAI-1: PAI-1 ratio of approximately 0.22, indicating that about one fifth of their PAI-1 is complexed with tPA. A similar result was seen in nonhibernators. In a previous study the tPA-PAI-1 complex to total PAI-1 ratio was found to be elevated up to 0.38 in hyperfibrinolytic disease states such as disseminated intravascular coagulation (DIC) (Wantanabe et al., 2001). A similar tPA-PAI-1: PAI-1 elevation was seen in hibernating animals, consistent with a two-fold increase in PAI-1 complexed with tPA. These findings would suggest that fibrinolysis is in a hyperactive state during hibernation, even in the absence of blood clots to break down.

Ground squirrels in all assayed stages of the annual cycle had significantly lower plasma PAI-1 levels compared to nonhibernators. The decrease in plasma PAI-1 could have been due to increased consumption of the protein, or decreased production. RT-

qPCR of ground squirrel liver total RNA partially confirmed that the drop in plasma PAI-1 was in-part due to decreased production of the protein by the liver during entrance, and post arousal animals. While a decreasing trend in IBA and torpid *SERPINE1* mRNA was shown, the difference compared to nonhibernating levels was not significant. Therefore, confident conclusions cannot be fully made on the decreased PAI-1 protein in the torpid and IBA stages being due to a decrease in transcription. A recently published study found that *SERPINE1* mRNA levels in ground squirrel bone marrow significantly decreased, approximately 5.2-fold in torpid bone marrow compared to nonhibernators; a reduction strikingly similar to that observed in torpid liver *SERPINE* mRNA levels (Cooper et al., 2016).

Decreased plasma protein levels of PAI-1 correlate with increased susceptibility of clot lysis, whereas an increase of PAI-1 is associated with resistance to clot lysis (Okafor, 2015; Zhu et al., 1999). The data in this study suggest that blood clots formed during hibernation, although unlikely to form in the first place due to suppression of coagulation, may be less resistant to breakdown during fibrinolysis compared to blood clots formed in summer nonhibernating animals. This would help to protect hibernating animals from the formation of potentially lethal blood clots.

In the future more liver samples of all stages should be assayed for PAI-1 mRNA. Having a larger sample size for this assay could allow for a more confident explanation of decreased PAI-1 protein during hibernation. A greater sample size should also be obtained for the tPA-PAI-1: PAI-1 ratio data, with a focus on the torpid and hibernation entrance plasmas in particular. Also, extending the post arousal time frame

for which samples were assayed could be of interest in order to determine when levels of many of the markers returned to their nonhibernating levels.

The results of this study suggest that along with prevention of lethal blood clots during hibernation, ground squirrels likely have adapted to prevent blood clots from forming altogether. This is accomplished primarily through the suppression of hemostasis-including secondary hemostasis (coagulation cascade), as well as sustaining a hyperfibrinolytic state, possibly in place as a "back-up" mechanism to efficiently degrade any blood clots that did form in spite of hemostasis suppression. Furthermore, although unlikely, any clots that did form during hibernation may be less resistant to degradation during fibrinolysis. An increased endogenous fibrinolytic system during hibernation may be another antithrombotic adaptation ground squirrels have acquired to survive hibernation.

DVT/PE remains to be a significant health burden in the United States, exacting a significant human and economic toll each year. Continued research into alternations to hemostasis and fibrinolysis in hibernating organisms like ground squirrels may lead to future development of novel clinical therapies for DVT/PE prevention.

LITERATURE CITED

Biggar, Y., & Storey, K.B. (2014). Global DNA modifications suppress transcription in brown adipose tissue during hibernation. *Cryobiology, 69*(2), 333-338.Boisclair, M.D., Lane, D.A., Wilde, J.T., Ireland, H., Preston, F.E., & Ofosu, F.A. (1990). A comparative evaluation of assays for markers of activated coagulation and/or fibrinolysis: thrombin–antithrombin complex, D-dimer and fibrinogen/fibrin fragment E antigen. *British Journal of Haematology, 74*(4), 471–479.

Booth, N.A., Simpson, A.J., Croll, A., Bennett, B., & MacGregor, I.R. (1988). Plasminogen activator inhibitor (PAI-1) in plasma and platelets. *British Journal of Haematology, 70*(3), 327-333.

Boyer, B.B., & Barnes, B.M. (1999). Molecular and metabolic aspects of mammalian hibernation. *BioScience,49,* 713-724.

Carey, H.V., Andrews, M.T., & Martin, S.L. (2003). Mammalian hibernation: cellular and molecular responses to depressed metabolism and low temperature. *Physiological Reviews, 83*(4), 1153-1181.

Chapin, J.C., & Hajjar, K.A.(2015). Fibrinolysis and the control of blood coagulation. *Blood Review, 29*(1), 17-24.

Coagulation Cascade [Online Image]. (2007). Retrieved February 23, 2017 from https://commons.wikimedia.org/wiki/File:Coagulation_full.svg

Cooper, S.T., Howard, D., Koch, T., Lin, X., Johnston, N., Dobbs, K., Zuberbuehler, M., Cooper, A., Lamont, C., Mortimer, S., Hying, S., Bernhardt, K., Sell, S., Nelson, L,, Hawes, J., Willems, H., Morgan, L., Macapugay, G., Krus, C., Bowman, M., & Llyod, S. (2014). Platelet levels and activity are decreased in hibernating 13-lined ground squirrels which may prevent venous thromboembolism. Journal of the Federation of American Societies for Experimental Biology, 28(1), 879.

Cooper, S.T., Richters, K.E., Melin, T.E., Liu, Z.J., Hordyk, P.J., Benrud, R.R., Geiser, L.R., Cash, S.E., Simon, S.C., Howard, D.R., Ereth, M.H., & Sola-Visner, M.C. (2012). The hibernating 13-lined ground squirrel as a model organism for potential cold storage of platelets. American Journal of Physiology - Regulatory, Integrative and Comparative Physiology, 302, 1202–1208.

Cooper, S.T., Sell, S.S., Fahrenkrog, M., Wilkinson, K., Howard, D.R., Bergen H., Cruz, E., Cash, S.E., Andrews, M.T., & Hampton, M. (2016). Effects of hibernation on bone marrow transcriptome in thirteen-lined ground squirrels. *Physiological Genomics*, *48*(7), 513-525.

Cooper, S.T., Sell, S., Nelson, L., Hawes, J., Benrud, J.A., Kohlnhofer, B.M., Burmeister, B.R., & Flood, V.H. (2015). Von Willebrand factor is reversibly decreased during torpor in 13-lined ground squirrels. *Journal of Comparative Physiology*, *186*, 131-139.

Cushman, M. (2007). Epidemiology and risk factors for venous thrombosis. *Seminars in Hematology*, *44*(2), 62–69.

Elvert, R., & Heldmaier, G. (2005). Cardiorespiratory and metabolic reactions during entrance into torpor in dormice, *Glis glis*. *Journal of Experimental Biology*, *208*, 1373-1383.

Fareed, J., Hoppensteadt, D.A., Leya, F., et al. (1998). Useful laboratory tests for studying thrombogenesis in acute cardiac syndromes. *Clinical Chemistry*, *44*,1845–1853.

Heit, J.A., Silverstein, M.D., Mohr, D.N., Petterson, T.M., O'Fallon, W.M., & Melton, L.J. (1999). Predictors of survival after deep vein thrombosis and pulmonary embolism: a population-based, cohort study. *Archives of Internal Medicine*, *159*(5), 445–53.

Lechler, E., & Penick, G.D. (1963). Blood clotting defect in hibernating ground squirrels (*Citellus tridecemlineatus*). *American Journal of Physiology*, *205*, 985– 988.

Lyman, C.P., & O'brien, R.C. (1960). Circulatory changes in the thirteen-lined ground squirrel during the hibernation cycle. *Bulletin of the Museum of Comparative Zoology*, *124*, 353–372.

Morgan, L. (2013). Effects of hibernation on cardiac ischemia markers; a novel model of cardiac repair in thirteen-lined ground squirrels (*Ictidomys tridecemlineatus*) (Unpublished master's thesis). University of Wisconsin- La Crosse, La Crosse, WI.

Morin, P., & Storey, K.B. (2009). Mammalian hibernation: differential gene expression and novel application of epigenetic control. *International Journal of Developmental Biology*, *53*, 433-442.

Nuyttens, B.P., Thijs, T., Deckmyn, H., & Broos, K. (2011). Platelet adhesion to collagen. *Thrombosis Research*, *127*, 26-29.

Okafor, O.N., & Gorog, D.A. (2015). Endogenous fibrinolysis: an important mediator of thrombus formation and cardiovascular risk. *Journal of the American College of Cardiology*, *65*(16), 1683-1699.

Palta, S., Saroa, R., & Palta, A. (2014). Overview of the coagulation system. *Indian Journal of Anesthesiology*, *58*(5), 515-523.

Pivorun, E.B., & Sinnamon, W.B. (1981). Blood coagulation studies in normothermic, hibernating, and aroused *Spermophilus franklini*. *Crybiology*, *18*(5), 515-520.

Speiser, W., Mallek, R., Koppensteiner, R., Stumpflen, A., Kapiotis, S., Minar, E., Ehringer, H., & Lechner, K. (1990). D-dimer and TAT measurement in patients with deep venous thrombosis: utility in diagnosis and judgement of anticoagulant treatment effectiveness. *Thrombosis and Haemostasis*, *64*(2), 196–20.

Von Bruhl, M.L., Stark, K., Steinhart, A., Chandraratne, S., Konrad, I., Lorenz, M., Khandoga, A., Tirniceriu, A., Coletti, R., Kollnberger, M., Byrne, R.A., Laitinen, I., Walch, A., Brill, A., Pfeiler, S., Manukyan, D., Braun, S., Lange, P., Riegger, J., Ware, J., Eckart, A., Haidari, S., Rudelius, M., Schulz, C., Echtler, K., Brinkmann, V., Schwaiger, M., Preissner, K.T., Wagner, D.D., Mackman, N., Engelmann, B., & Massberg, S. (2012). Monocytes, neutrophils, and platelets cooperate to initiate and propagate venous thrombosis in mice in vivo. *Journal of Experimental Medicine*, *209*, 819–835.

Watanabe, R., Wada, H., Miura, Y., Murata, Y., Watanabe, Y., Sakakura, M., Okugawa, Y., Nakasaki, T., Mori, Y., Nishikawa, M., Gabazza, E.C., Shiku, H., & Nobori, T. (2001). Plasma levels of total plasminogen activator inhibitor-I (PAI-I) and tPA/PAI-1 complex in patients with disseminated intravascular coagulation and thrombotic thrombocytopenic purpura. *Clinical and Applied Thrombosis/Hemostasis*, *7*(3), 229-233.

Wiman, B., Anderson, T., Hallqvist, J., Reuterwall, C., Ahlbom, A., & DeFaire, U. (2000). Plasma levels of tissue plasminogen activator/plasminogen activator inhibitor-1 complex and von Willebrand factor are significant risk markers for recurrent myocardial infarction in the Stockholm Heart Epidemiology Program (SHEEP) study. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *20*(8), 2019-2023

Zhu. Y., Carmeliet, P., & Fay, W.P. (1999). Plasminogen activator inhibitor-1 is a major determinant of arterial thrombolysis resistance. *Circulation*, *99*, 3050-3055.