UNIVERSITY OF CALIFORNIA, SAN DIEGO

Enteral protease inhibition preserves vasopressor resistance and vascular alpha-1 adrenergic receptor expression in hemorrhagic shock

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Bioengineering

by

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2016

The Thesis of Marco Henry Santamaria is approved and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

2016

DEDICATION

In recognition of her support, compassion, love, and every great meal she cooked, this Thesis is dedicated to Dora Santamaria, grandmother of Marco Santamaria.

EPIGRAPH

Facts do not cease to exist because they are ignored.

Aldous Huxley

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

DAMP	Damage Associated Molecular Patterns
DOR	Duration of Response
DU	Digital Units
ENDG	End Goal Directed Therapy
HS	Hemorrhagic Shock
ICC	Immunocytochemistry
IHC	Immunohistochemistry
MABP	Mean Arterial Blood Pressure
SMC	Smooth Muscle Cell
SVR	Systemic Vascular Resistance
TXA	Tranexamic Acid

LIST OF FIGURES

ACKNOWLEDGMENTS

I would like to acknowledge Dr. Geert Schmid-Schönbein, Dr. Frank De Lano, Dr. Rafi Mazor, Dr. Federico Aletti, and all of my students, Joyce Li, Aaron Tan, Diana Munoz, Kevin Chin, and Jessica Leon for their guidance, support, and contributions to my work.

I would to acknowledge Dr. Erik Kistler for his assistance in the editing and revision of this thesis and of the manuscript prepared for publication.

This Thesis is, in full, currently being prepared for submission for publication of the material.

Santamaria, Marco; Aletti, Federico; Li, Joyce; Tan, Aaron; Chang, Monica; Leon, Jessica; Schmid-Schönbein, Geert; Kistler, Erik. The thesis author was the primary investigator and author of this material.

ABSTRACT OF THE THESIS

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Master of Science in Bioengineering

University of California San Diego, 2016

Professor Geert Schmid-Schönbein, Chair

Hemorrhagic shock is characterized by hyporesponsiveness to vasopressor and fluid therapy. Little is known about the contributing mechanisms. We hypothesize that decreased intestinal perfusion in hemorrhagic shock leads to proteolytically-mediated increases in gut permeability, with egress of vasoactive substances. Failure to maintain blood pressure is partially mediated by α_1 adrenergic receptor modulation; we thus hypothesized that decreases

in hemodynamic stability and vasopressor response can be prevented by enteral protease inhibition. Rats were exposed to hemorrhagic shock (35 mmHg mean arterial blood pressure for 2 hrs, followed by 2 hour reperfusion) and challenged with phenylephrine (2 μg/kg) at selected instances to measure responsiveness. A second group of animals received enteral injections with the protease inhibitor tranexamic acid (TXA) (127 mM) along the small intestine and cecum one hour into shock. Blood pressure response to phenylephrine after reperfusion was attenuated in animals subjected to hemorrhagic shock compared to baseline and non-shocked animals, and was restored by TXA. Arteries from shocked animals displayed decreased α_1 receptor density with restoration to baseline density following enteral treatment. This finding was replicated *in vitro*, as rat shock plasma decreased α_1 receptor density of incubated smooth muscle cells, which was also abrogated by TXA treatment. Results demonstrate that hemorrhagic shock leads to decreased vasopressor response to phenylephrine as well as decreased α_1 receptor density via circulating factors. Changes are prevented by enteral TXA with correspondingly improved hemodynamics. Proteolytic inhibition of the small intestine improves outcomes in hemorrhagic shock, partially by restoring α_1 adrenergic activity and maintaining blood pressure.

INTRODUCTION

A. Circulatory Shock

Trauma in the form of burn, hemorrhage, or infection have afflicted humans are a major cause of morbidity and mortality. Shock is the third leading cause of death for the humans and is the leading cause of death for person below the age of 35 (23, 37, 39). Treatment is hampered by an incomplete mechanistic understanding of the condition which perpetuates due to dearth of definitive interventions.

The initial terminology for shock stems from the French “choc”, which in the early 1700s was used to describe the hemodynamic pathology that accompanies severe injury (6). Contemporary medicine deems shock to be a common final pathway resulting from disparate causes that leads to reduced tissue perfusion, multiorgan failure and consequently death. The appellation “circulatory shock” is often used to indicate the inability of the systemic and microcirculation to maintain adequate tissue perfusion and shock is by convention today categorized to distinguish several initial presenting pathologies. In hypovolemic shock there is a diminishment of circulating fluid volume, typically as a result of severe hemorrhage. Distributive shock encompasses sepsis, burns, and anaphylaxis. Its primary characteristic is a drop in vascular perfusion, circulating fluid to leak into the tissue, organ dysfunction and failure. Other forms of shock include obstructive shock, wherein blood flow from the heart is impeded. Instances of this shock type include cardiogenic shock where cardiac output is severely reduced by precipitating insults such as myocardial infarction. The vasculature consists of a set of tubes containing the whole of the fluid volume needed for tissue perfusion with the heart constantly maintaining flow.

It can be surmised that responses to fluid loss would be: fluid replacement, a reduction in tube diameter (via vasopressor agents), or effective volume maintenance through increased flow

rate (inotropic agents and fluid exchange in the tissue). Forms of circulatory shock are accompanied by their corresponding primary treatments. During hemorrhagic shock blood volume is withdrawn from the circulation; thus the circulation compensates by absorbing fluid from the peripheral tissue and constricting (REF). The first treatment response is usually fluid administration. In distributive shock the circulation expands, reducing pressure. Treatments necessitate fluid administrating followed by vasopressor and inotropic agents. Obstructive shock, in particular cardiogenic shock, results from pump deficiency causing fluid available to the circulation to decrease. The treatment focus here is to ameliorate cardiac output through the application of inotropes. Despite the myriad of insult variants, all forms of shock retain the commonality of impaired tissue perfusion.

B. Hemorrhagic Shock – Theories and Treatment

As aforementioned, hemorrhagic shock (HS) is a form of hypovolemia characterized by blood loss from an internal or external origin. Treatment approaches consist of halting blood loss form the site of hemorrhage and restoring the fluid volume lost from the circulation. A number of classifications have been proposed for HS. One classification is comprised of four stages corresponding to increasing large volume deprivations: Stage 1 is defined as a total fluid loss of up to 15%, while Stages 2, 3, and 4 refer to 15-30, 30-40, and greater than 40 percent total blood loss, respectively (11). Additional physiologic parameters include tachycardia, and the drying of the mucous membranes (e.g. the eyes, nostrils, and mouth). These secondary parameters often follow the pressure decrement. Regardless of the classification scheme chosen, the accuracy in predicting patient outcome is consistently low. The heterogeneous nature of the patient's pathology and pathological response result in many

incongruities in patient outcomes even under similar circumstances. Alternatively, HS can be characterized in terms of the hypovolemic response. Under "compensated" circumstances, the patient's vital signs stabilize and survival is highly probable (e.g., after donating blood). Moderate hypovolemic response, or "uncompensated" occurs when physiological parameters are aberrant but reperfusion (fluid return) has a positive effect. Finally, under the "irreversible" context, physiological parameters are abnormal and there is insufficient response to reperfusion, resulting in death (38). From the clinical perspective the "irreversible" condition of shock is most significant as it is beyond the capacity of current therapies to improve. There has yet to be devised a precise definition to describe the point at which "irreversible" shock is reached, generating impetus to establish a reproducible model.

Among the US armed forces the former shock classification regimen of the four stages hypovolemic stages is employed rather than one rooted in physiological responses. In contrast, the most readily repeatable model for HS was proposed and first implemented by Carl Wiggers (43), who wrote a number of books on the subject in the late 1940s-1950s. Dr. Wiggers determined that hemorrhage of experimental animals to a target blood pressure (usually a mean arterial blood pressure (MABP) of 35-40 mmHg) to reproduce ischemia for a known duration followed by resuscitation with shed blood resulted in a reproducible model of HS (18, 34, 43-45). It is the Wiggers model that is used in the present study.

In large part due to insufficient understanding of the mechanisms responsible for the "irreversible" HS condition, patient prognosis is usually poor. In the clinical setting treatment has been centered on advancements in resuscitation. Whole blood is separated into its constitutive elements when used as a treatment (packed-red blood cells, plasma, and platelets) for greater applicability (i.e., treatments can be targeted for element specifically deficient in patients such red blood cells, or platelets, etc.). However any administration of blood

constituent results in a lower hematocrit, and a deprivation of coagulation factors and platelets compared to the original patient blood (33). Moreover, there are adverse effects on oxygen-carrying capacity, as well as issues with potential infection, patient immune responses, and blood constituent functionality (as with platelets) when considering using stored blood for resuscitation. Currently, transfusion protocols mandate a 1:1:1 ratio of packed red blood cells, plasma, and platelets (22) when reperfusion with stored blood is used. These limitations of component therapy have compelled the US military to increasingly implement whole blood transfusion. While results from large, rapid transfusions with whole blood have been successful, this treatment option relies on the availability of whole blood of the matching type.

Albeit advancements in resuscitation have yielded increases in survival in some clinical contexts, there has yet to be developed an effective universal treatment for "irreversible" shock due to a lack of mechanistic understanding. At present there is no accepted theory to account for cardiovascular collapse. Given that HS is characterized by deficient tissue perfusion is it believed that increasing oxygen delivery by elevating cardiac output (oxygen delivery = cardiac output*oxygen carrying capacity) should garner positive results. Shoemaker in the 1990's, first noted that greater oxygen delivery in patients translated to a greater survival percentage (41). This observation was confirmed experimentally by intentionally increasing oxygen delivery (8). Unfortunately these findings failed to be deemed reproducible by other large studies, causing support for this reasoning to subside (20, 21). Shoemaker was able to attain greater oxygen delivery by increasing cardiac output to values beyond control ranges without altering oxygen carrying capacity. In recent research Rivers, et al developed what was dubbed "early goal directed therapy (EGDT)" for septic shock. In his work, Rivers adopted concepts used by Shoemaker to ameliorate oxygen delivery in septic shock patients using a regimen wherein fluids, vasopressors, blood products, and inotropes are introduced sequentially (36). EGDT has become the standard of care for treatment of septic

shock. HS is treated in a similar fashion with the distinction that fluid replenishment through blood products is the primary regimen component.

Beyond consideration of oxygen transport and carrying capacity, treatments have also been concerned with pathologic responses to HS. Radical oxidative species, free fatty acids, cell debris from apoptotic events (e.g., damage associated molecular patterns (DAMPS)), nitric oxide regulation, and cytokines dispensation have all been considered pathology targets for treatment. Addressing these potential effectors has yet to yield an efficacious treatment past preclinical stage and none are currently regarded as therapeutic in a clinical setting.

Another theoretical mechanism is that of "bacterial translocation". During 1960's Jacob Fine posited that, based on the presence of endotoxin-positive organs following resuscitation in his HS rabbit model, posited that this was evidence of bacterial efflux from the intestine as a result of bacterial translocation (35). The theory of bacterial translocation in shock subsequently moved to the forefront of thought in the field, however upon closer scrutiny the theory is susceptible to certain flaws. Among them, is that lymph nodes from which Fine detected endotoxin are designed for pathogen collection. It is therefore expected to be able to derive bacterial culture from excised lymph nodes. Of greater consequence is that treatment of the bowel against bacterial does not result in clinically relevant results (19). Other possible mechanisms are thus required to elucidate pathophysiological events in "irreversible" HS.

C. The Autodigestion Theory

Though bacterial translocation does not appear to be a primary mechanistic driver for "irreversible" HS, there is nevertheless abundant evidence for the gut to be the "motor" of

multiple organ failure and the “engine” of sepsis (27). A widespread hypothesis that the bowel is implicated as a component of “irreversible” HS and participates in hemodynamic dysfunction. Recent experimental evidence suggesting an alternative theory was generated through the observation that pancreatic homogenate caused inflammation hemodynamic dysfunction if administered directly into the circulation. Furthermore, it was noted that enzymes of pancreatic origin were the source of the inflammation. Application of proteolytic enzymes to wild type tissue homogenates also results in inflammation within the homogenate (25). These results in combination with the knowledge that the pancreas released these enzymes into the digestive tract, the hypothesis of native tissue digestion following gut permeability was advanced. This digestion of native tissue was dubbed “autodigestion” and refer to tissue degradation by released intestinal enzymes.

Under non-pathological conditions the intestine is safeguarded from the ingress of digestive enzymes by a protective mucous barrier that coats the surface of the intestinal wall facing the lumen. The mucous boundary is mainly comprised of O-linked oligosaccharides and mucins proteins and is impermeable to proteolytic enzymes (9). It is currently hypothesized that HS conditions degenerate this protective boundary, thus permitting proteolytic enzymes and their cleavage products to transgress this boundary and enter the intestinal wall and systemic circulation. It is in the circulation, where the presence of proteolytic enzymes may cause hemodynamic instability and ultimately organ failure. Across a myriad of experimental shock categories including hemorrhagic, endotoxin, and septic shock, enteral infusion of enzyme inhibitors, particularly serine protease inhibitors, significantly improved mortality in both pig and rat models (13, 16).

D. Vasopressor Hyporesponsiveness

Hemodynamic stability is crucial for the maintenance of blood pressure and organ perfusion and is one of the initial systems imperiled by the onset of HS. One significant manifestation of the pathology induced by HS on the vasculature is that of vasopressor resistance, defined as a deficient contractile response by arterioles following the infusion of vasoactive drugs. This results in the inability of vasopressor agents to sufficient support systemic pressure. Among the population of patients for which HS results in mortality, greater than half require the use of vasopressors and experience blood pressure loss despite vasopressor support (7, 12, 29).

While the origin of hypotension accompanies coinciding with circulatory shock can be attributed to vasodilation from systemic inflammation, no consensus exists as to the mechanisms for vasopressor resistance. A number of hypothesis have, however, been posited. It has been confirmed that systemic vasopressin levels are reduced in shock (28) and that upregulated nitric oxide generation promotes vasodilation and cardiac dysfunction (42). In spite of these advancements in clinical knowledge, therapeutic strategies centered on addressing these pathways have been unsuccessful in garnering a reduction in mortality associated with shock-induced vasopressor resistance (2, 30).

One potential mechanism to account for vasopressor hyporesponsiveness hemorrhagic shock may involve altered expression of the vascular α_1 adrenergic receptor, responsible in part for the regulation of arteriolar tone and thus systemic vascular resistance (SVR). Prior research has indicated that HS impacts the membrane density of transmembrane receptors, as in the case of insulin receptor (15). Decreased transmembrane receptor density may be correlated with the presence pancreatic enzymes derived from the small intestine (4). The mucin boundary layer of the intestine becomes permeable under shock (3, 9, 24), causing an efflux of digestive enzymes from the intestinal lumen into the systemic circulation (13). Once

these proteases reach distal organ, their increased concentration and corresponding activity may result in remote organ failure and mortality (autodigestion) (4).

E. Study Objective

The objective of this study was to investigate a new hypothesis for vasopressor resistance in shock, by assessing density changes in α_1 adrenergic receptor expression on smooth muscle in a rat model of hemorrhagic shock. A therapeutic intervention was also implemented to retain vasopressor responsiveness in hemorrhagic shock, using enteral injections of tranexamic acid (TXA). Used intravenously as a plasminogen inhibitor to reduce bleeding (40), TXA is also an effective trypsin inhibitor and has been shown to improve shock mortality when given enterally (13). Thus, the intent of this study were to determine the effect of TXA on vasopressor responsiveness during reperfusion following hemorrhagic shock, and to determine whether α_1 adrenergic receptor expression in the smooth muscle of large arteries, was altered after hemorrhagic shock and preserved in animals treated with TXA

MATERIALS AND METHODS

A. Experimental Protocol

All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California, San Diego and conform to the Guide for the Care and Use of Laboratory Animals by the United States National Institutes of Health (NIH Publication No. 85-23, 1996).

Twenty-four non-fasted male Wistar rats (300-450 g, Harlan Laboratories, Inc., Indianapolis, IN) were randomly assigned to either Control (no shock) (n=6), hemorrhagic shock (HS) (n=6), hemorrhagic shock with enteral tranexamic acid treatment (HS+TXA) (n=6), or hemorrhagic shock with enteral Golytely® (HS + Vehicle) as a hemorrhagic shock control (n=6). All rats were anesthetized (xylazine, 4 mg/kg; ketamine 75 mg/kg i.m.) with supplemental anesthesia administered intravenously as needed (xylazine, 4 mg/kg; ketamine 7.5 mg/kg i.v.). The right femoral vein and artery were cannulated for blood withdrawal and intravenous supplemental anesthesia and continuous monitoring of arterial pressure, respectively. Body temperature was maintained at 37°C via water-heated support and heat blanket.

Control animals were monitored for 4 hours under anesthesia without other interventions. HS and HS+TXA animals were allowed 5 min for hemodynamic stabilization after induction of anesthesia and vascular line placement. All animals were heparinized (1 unit heparin/cc estimated total blood volume, estimated at 6% body weight, intravenously) to allow for blood withdrawal. Hemorrhage was induced by blood withdrawal through the femoral vein (0.5 cc/min) to a target mean pressure of 35 mmHg. Mean arterial blood pressure (MABP)

was maintained between 30 and 40 mmHg for 2 hours, after which time the withdrawn blood was warmed to 37°C and returned to the animals (0.5 cc/min). Animals were monitored for an additional two hours. To test for vascular responsiveness the selective α_1 adrenergic agonist phenylephrine (2 μg/kg) was administered as intravenous bolus (0.1 ml) bolus once before hemorrhage to obtain baseline response and again serially after reperfusion. Total deflection of MABP from baseline after injection as well as time-to-return-to-baseline (within 10%) (DOR) were measured as indices of vascular response to phenylephrine.

In HS+TXA animals 1 hour into the hypovolemic period (127 mM TXA, Cyclokapron, Pfizer) was administered into the lumen of the small intestine via sequential injections longitudinally along its length. GoLytely® (0.14 g /ml 0.9% normal saline (NS)) was used as carrier solution. A total fluid volume of 15 ml was injected into the lumen of the small intestine. An additional 2 ml of TXA in vehicle was injected into the cecum. The dosage of the TXA was based on previously determined effective concentrations (13). A second group of animals (HS + Vehicle) undergoing hemorrhagic shock was instrumented as above and enteral injections of carrier solution only (GoLytely®) were performed as an additional hemorrhagic shock control. Hemodynamics from this group were indistinguishable from those of the HS group, and all subsequent analyses were carried out on the HS animals. HS, HS + TXA, and HS + Vehicle groups were subjected to phenylephrine challenge (2 μg/kg) as described above once before hemorrhage and at 30, 90, and 120 min after the start of the reperfusion period.

Following a two-hour observation period after return of shed blood (Reperfusion), animals were euthanized with B-Euthanasia (120 mg/kg). Death was confirmed by loss of signal on blood pressure monitor followed by bilateral thoracotomy.

B. Tissue Collection

Renal arteries were harvested following laparotomy, gently rinsed to remove residual blood and homogenized in lysis buffer (ThermoScientific). Homogenates were treated with the addition of a protease inhibitor cocktail (1:25, Roche) for Western blot analysis. Arterial homogenates were stored at −80°C for later use. Right femoral arteries were gently excised, washed four times in 10% formalin for 15 min each, and placed directly into 10% formalin and stored at room temperature for immunohistochemical analysis (IHC).

D. Western blot detection of renal artery proteins

Renal artery homogenate samples for each animal (60 µl/sample; 0.5 µg/µl) were separated by SDS-PAGE. Protein yield from the renal arteries was low compared to that normally used for a Western blot. Large volumes of tissue homogenate were therefore used as well as a more sensitive chemiluminescent substrate. For Western blotting, proteins were transferred to a 0.45 µm pore size nitrocellulose membrane (Bio-Rad Laboratories, Inc., Hercules, CA; #162-0097). Following blockade with 5% nonfat dry milk in buffer (Tris-Buffered Saline and Tween 20, TBST), primary antibodies against trypsin (D-1, 1:100, sc-137077 and α_{1D} adrenergic receptor (H-142, 1:300, sc-10721, Santa Cruz Biotechnology, Santa Cruz, CA) were applied. β-Actin (C4, 1:100, sc-47778, Santa Cruz Biotechnology, Santa Cruz, CA) was used as loading control. Corresponding secondary antibodies were applied at 1:5000 dilution. All antibodies were diluted in TBST. Supersignal West Fempto Chemiluminescent substrate (ThermoFisher Scientific, Waltham, MA; #34096) was used for imaging. The molecular weights of the proteases were estimated by use of an electrophoresis marker (Sigma Aldrich, C1992). Gels were digitized and bands were analyzed by densitometry in ImageJ.

E. Immunohistochemistry (IHC) of Femoral Artery

Femoral arteries were resected, formalin fixed, and mounted longitudinally for sectioning. 30 μm sections were made along the length of the artery and placed in distilled water overnight. Sections were rinsed in distilled water to remove residual formalin. Endogenous peroxidase activity was removed with Bloxall (Vector Laboratories, Burlingame, CA, SP-6000). 2.5% normal horse serum (Vector Laboratories, Burlingame, CA, S-2012) was then used as a blocking reagent followed by primary antibody incubation with α_{1D} antibody (1:200, sc-10721, Santa Cruz Biotechnology, Santa Cruz, CA) for 90 min. Sections were incubated for 30 min in ImmPRESS anti-rabbit Ig (Vector Laboratories, Burlingame, CA, MP-7401) and ImmPACT DAB (Vector Laboratories, Burlingame, CA, SK-4105) was used for staining. Sections were mounted using Vectamount (Vector Laboratories, Burlingame, CA, H-5000). Bright-field imaging was carried out at 10X objective magnification. Images were digitized to 8-bit format and measurements were taken of smooth muscle regions in each section. All analysis was performed on ImageJ. Precautions were taken to assure that all IHC steps were carried out under standard conditions to allow quantitative comparison with digital image analysis.

F. Immunocytochemistry of Smooth Muscle Cells Following Rat Plasma Incubation (ICC)

Blood samples from Control (n = 6), HS (n = 6), HS + TXA (n = 6), and HS + Vehicle (n = 6) rats were drawn and blood was centrifuged at 600g for 10 min to achieve plasma separation. Human carotid smooth muscle cells (SMC) (HCtASMC; Cell Applications, San Diego, CA) were cultured in growth medium (311D-250; Cell Applications, San Diego, CA) at 37 °C in an atmosphere of 5% CO_2 and allowed to reach approximately 100% confluence before being exposed to either starvation medium (310-500: Cell Applications, San Diego, CA), or plasma from Control, HS, HS +TXA, or HS + Vehicle animals. SMCs were incubated in the presence

of plasma variant or starvation medium for 3 hours at 37 °C. Cells were starved in serum free media for 12 h prior to experimentation and plasma samples were diluted in a 1:5 ratio in starvation medium immediately preceding incubation. Cells were stained as described above for rat femoral artery sections. Images were acquired at 10X objective magnification, digitized to 8-bit format and measurements were taken as the average greyscale intensity for individual cells. All analysis was performed on ImageJ. Precautions were taken to assure that all ICC steps were carried out under standard conditions to allow quantitative comparison with digital image analysis.

F. Statistical Analysis

Western, IHC, and ICC results are presented as mean ± standard deviation (SD) for bar charts. One-way analysis of variance (ANOVA) was used where appropriate to evaluate the differences between groups with a post-hoc Tukey correction. All analyses were performed using Graphpad (Graphpad Software Inc., La Jolla, CA). Statistical analysis of phenylephrine MABP response duration and absolute MABP change were done using Kruskal-Wallis test with post hoc Dunn's test. Results are presented as mean ± standard deviation (SD). A value of $p < 0.05$ was considered statistically significant for all tests.

RESULTS

A. Blood pressure response to enteral TXA treatment

All groups subjected to HS were maintained at similar systemic blood pressures until reperfusion, at which point the MABP dropped significantly in the HS and HS + Vehicle groups (**Figure 1**). The animal group treated enterally with TXA (HS + TXA) displayed significantly higher MABP compared to both shock groups.

B. Systemic vasopressor response

Vasopressor resistance *in vivo* during hemorrhagic shock in response to phenylephrine challenge was assessed by duration of response (DOR) and maximum transient increase in MABP relative to blood pressure just before the bolus (ΔMABP) (**Figure 2**). DOR and ΔMABP after phenylephrine challenge were recorded at baseline and at 30, 90, and 120 min after the start of reperfusion for HS, HS + TXA, and HS + Vehicle groups. Average ΔMABP at baseline was 59 ± 10 mmHg. Average DOR was 335 ± 131 sec at baseline; there were no significant differences between groups for either ΔMABP or DOR. At 30 minutes after reperfusion there was a significant decrease in DOR for the HS + Vehicle group (107 ± 64, $p<0.01$) and ΔMABP for both HS and HS + Vehicle groups (31 ± 13 mmHg, and 22 ± 5 mmHg, respectively, compared to baseline, $p<0.01$ (**Figures 3 and 4**)). This decrease in DOR and ΔMABP compared to baseline continued at 90 min after reperfusion in the HS and HS + Vehicle groups (DOR: 158 ± 108 sec and 107 ± 64 for the HS and HS + Vehicle groups vs 335 ± 131 sec at baseline, $p< 0.05$, ΔMABP: 28 ± 13 mmHg for the HS and 28 ± 9 mmHg for the HS + Vehicle group vs 59 ± 11 mmHg at baseline, $p< 0.01$). These groups were also significantly different from baseline at 120 min after reperfusion, with ΔMABP of 34 ± 13

mmHg (HS) and 26 ± 14 mmHg (HS + Vehicle) vs 59 ± 11 mmHg at baseline, $p<0.05$, and 118 ± 49 sec (HS) and 116 ± 70 sec (HS + Vehicle) for DOR vs baseline (335 ± 131 sec), $p< 0.01$). At no time point during the study was the HS + TXA group significantly different from baseline for either DOR or ΔMABP.

C. Renal artery trypsin and α_1-adrenergic receptor levels

The serine protease trypsin and α_1 adrenergic receptor were examined by Western blot in the renal artery to determine whether bowel proteases could be detected in large arteries after shock, as well as determine if there were changes in α_1 receptor density in shock and after treatment with enteral TXA. Trypsin was increased significantly in HS compared to Control ($p<0.01$) (**Figure 5**), while α_1 receptor label density was markedly decreased ($p<0.01$) as determined by Western blot (**Figure 6**). Both trypsin and α_1 receptor label levels were maintained near Control levels by enteral treatment with TXA as determined by Western blot analysis. The two distinct bands for the western refer to different isoforms of trypsin. The band can be seen here http://www.scbt.com/datasheet-137077-trypsin-d-1-antibody.html on the manufacturer's site.

D. Femoral artery α_1-adrenergic receptor immunohistochemistry (IHC) analysis

The femoral artery media was also probed for levels of α_1 receptor density using IHC combined with digital image analysis (**Figure 7**). Receptor levels as detected by immunolabeling were significantly decreased in HS (51.5 ± 14.6 DU, n=6 animals/group) compared to Control (78.9 ± 5.2 DU, n=6 animals/group). Enteral treatment with TXA resulted in light absorbance levels similar to those of the Control group (73.9 ± 7.4 DU for HS + TXA, not significant compared to Control, n=6 animals/group). α_1 receptor levels from the HS + Vehicle group were similar to those found in the HS group (54.5 ± 13.3 DU for HS +

Vehicle, not significant, n=6 animals/group). For imaging, the red coloration was extracted using ImageJ and areas were selected across regions of smooth muscle cells and used to determine light absorbance (**Figure 8**).

E. Human Carotid SMC α_1-adrenergic receptor immunocytochemistry (ICC) analysis

SMCs were examined for α_1 receptor density using ICC (**Figure 9**). α_1 receptor density was significantly decreased in HS (57.9 ± 1.1 DU, $p < 0.001$, n=6 animals/group) compared to Control (67.6 ± 5.3 DU, n=6 animals/group). Incubation with plasma from the HS + TXA group resulted in levels similar to Control (71.5 ± 3.2 DU for HS + TXA, not significant, n=6 animals/group). α_1 receptor levels from the HS + Vehicle and HS groups were also not significantly different from each other (59.7 ± 1.7 DU for HS + Vehicle, not significant, n=6 animals/group).

DISCUSSION

Fulminate decompensated hemorrhagic shock (Class IV) (10), characterized by low responsiveness to fluid bolus and vasopressor therapy, carries a poor prognosis for survival, in part because no effective interventions exist. Lack of responsiveness to vasopressors and failure to maintain vascular tone (e.g., systemic vascular resistance (SVR)), commonly described in septic shock, are not well documented for hemorrhagic shock, and although several hypotheses have been proposed, there currently exists no consensus as to mechanism (17). To study this problem we examined the role of the α_1 adrenergic receptor in experimental hemorrhagic shock. The α_1 receptor is the archetypical adrenergic receptor most responsible for maintaining systemic vascular tone (vasoconstriction) and we hypothesized that failure of this receptor, either through reduced density or function, results in decreased vascular tone and subsequent hemodynamic collapse in hemorrhagic shock (31). Furthermore, we hypothesized that enteral treatment with tranexamic acid (TXA), a mild trypsin inhibitor (5), would lead to improved hemodynamics in hemorrhagic shock, in part by maintaining α_1 receptor activity by prevention of "autodigestion" processes.

This study confirms previous reports that enteral inhibition with TXA leads to improved hemodynamics in experimental hemorrhagic shock (13). Using phenylephrine as a probe for α_1 adrenergic receptor responsiveness, we report here that systemic sensitivity to this receptor decreases in experimental hemorrhagic shock with concomitant decrements in systemic pressure maintenance. The decrease in hemodynamic response is manifest both as a maximum decrease in the change in mean systemic blood pressure (ΔMABP) as well as a decrease in the duration of the response (DOR) to the pressor agent phenylephrine. This reduction in hemodynamic responsiveness begins early in the reperfusion period and continues

unabated through the end of the experiment. Western blot analysis and IHC confirm decreased α_1 adrenergic receptor density in hemorrhagic shock; the decrement in density appears to be a mechanism by which α_1 receptor-mediated hyporesponsiveness is mediated. This result is consistent with reports from other investigators on adrenergic function in shock, which appear to be predominantly related to changes in receptor density rather than function (32).

The mechanisms by which vascular α_1 adrenergic receptor density is modulated in shock are unknown, but results from our study demonstrate that circulating factors in the blood may be operant. These circulating mediators include digestive pancreatic proteases (13) and products of proteolysis that may either directly cleave the receptor (14), or indirectly through vasoactive degradation products (3) affect receptor and vascular function (1). Supporting this hypothesis are our current findings that enteral TXA restores receptor levels to baseline levels with commensurate improvements in hemodynamics in experimental hemorrhagic shock as well as decreases vascular tissue trypsin concentrations by protecting the ischemic small bowel from proteolytic degradation (4, 13, 24); the protection is not seen with intravenous protease inhibitor treatment (26).

There are several limitations to this study. Among these is a need for a more detailed description by which enteral TXA inhibition in the bowel maintains α_1 receptor function systemically. Although the finding of increased trypsin in the (renal) artery in hemorrhagic shock and its relative absence in TXA-treated animals implicate the involvement of bowel-derived proteases in mediating α_1 receptor activity, further investigations are necessary to clarify this point. Likewise, although changes in α_1 receptor responsiveness appear to be secondary to changes in density based on Western blot and IHC analysis rather than functional alterations in the receptors per se, the possibility of abnormal α_1 receptor binding in

hemorrhagic shock may also be possible. Finally, it is acknowledged that other receptors, both adrenergic (e.g., β_1, β_2) and otherwise (e.g., vasopressin), are also operant and may be affected by digestive enzyme leak into the circulation in hemorrhagic shock; further studies are necessary to clarify the function of these receptors in this condition and the possible role of enteral TXA in modulating them.

In conclusion, this study demonstrates that hemorrhagic shock decreases α_1 adrenergic receptor responsiveness and density. Decrements in α_1 adrenergic receptor activity and concentrations are mitigated by enteral treatment with TXA, a relatively mild trypsin inhibitor, with resultant decreased protease trypsin levels in vascular tissues and improved hemodynamics and vascular responsiveness. Maintenance of α_1 receptor function may be a key mechanism by which enteral TXA prevents systemic blood pressure reduction and improve survival in experimental hemorrhagic shock.

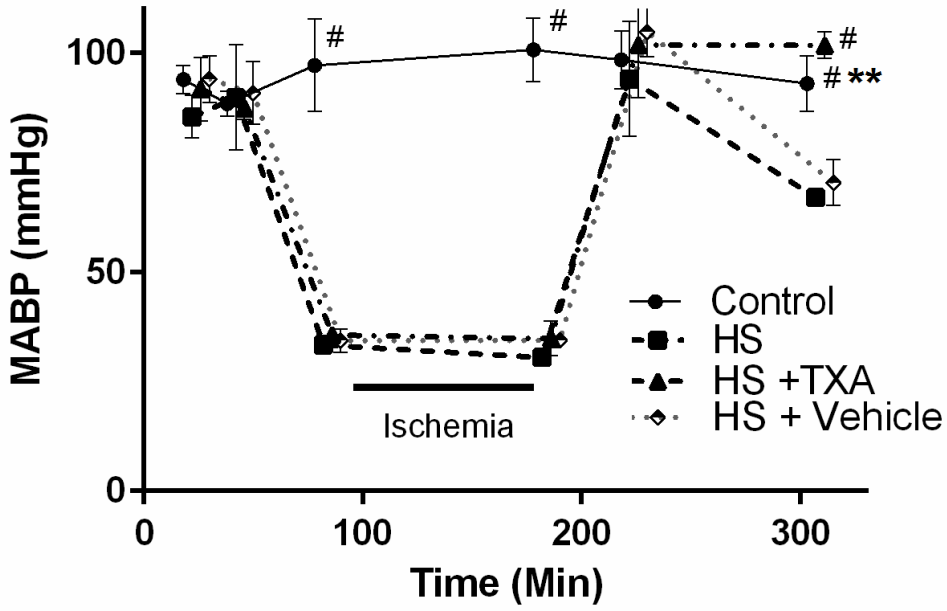

Figure 1: Mean arterial blood pressure (MABP) tracings of Control, Hemorrhagic Shock (HS), Hemorrhagic Shock + enteral tranexamic acid (HS + TXA), and Hemorrhagic Shock + Vehicle (HS + Vehicle) groups. MABP was decreased to a mean of 35 mmHg over several minutes by the serial withdrawal of small aliquots of blood (0.5 ml/min) Reperfusion was carried out in analogous fashion by slow infusion of shed blood (0.5 ml/min). By single factor ANOVA. **$p < 0.01$ Control vs. HS + TXA and #$p < 0.0001$ HS, HS + Vehicle vs. HS + TXA and Control; #$p < 0.0001$ Control vs. HS, HS + Vehicle, HS + TXA during ischemia period as per experimental design. There were no statistical differences between HS and HS + Vehicle groups at any time points. Data points slightly off-set in time for easier readability. Results shown as Mean ± SD.

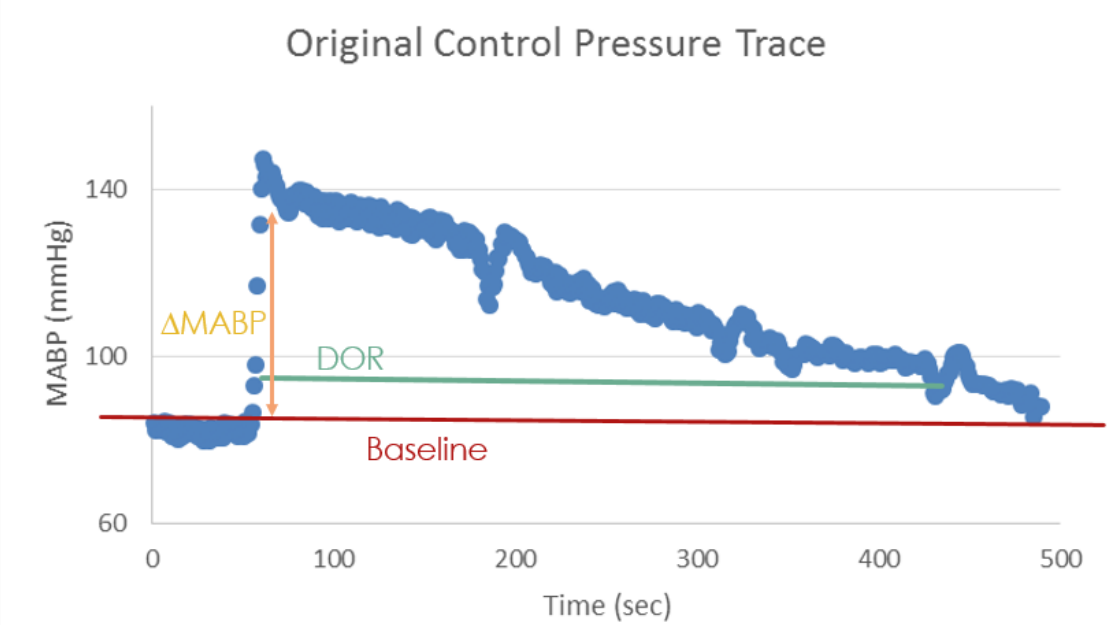

Figure 2: Original blood pressure trace showing the parameters for baseline, ΔMABP and duration of response. ΔMABP is defined as from baseline to the peak of the response while DOR is defined as the start of response to within 3% of the original baseline value.

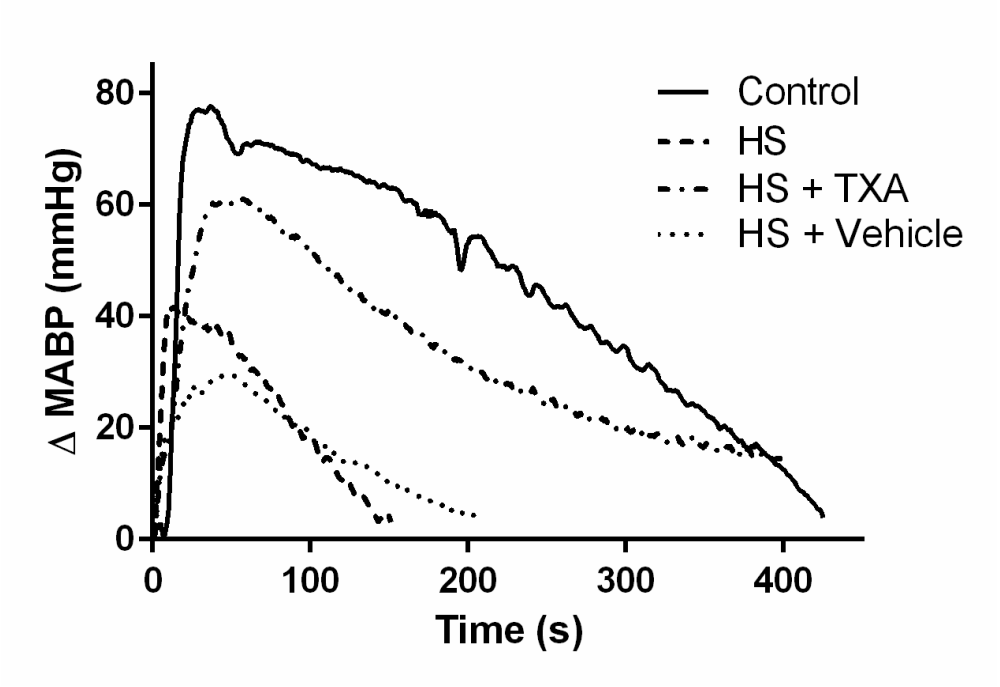

Figure 3: Representative hemodynamic response to phenylephrine challenge (ΔMABP and DOR) at 120 min after start of reperfusion for a single animal in each group.

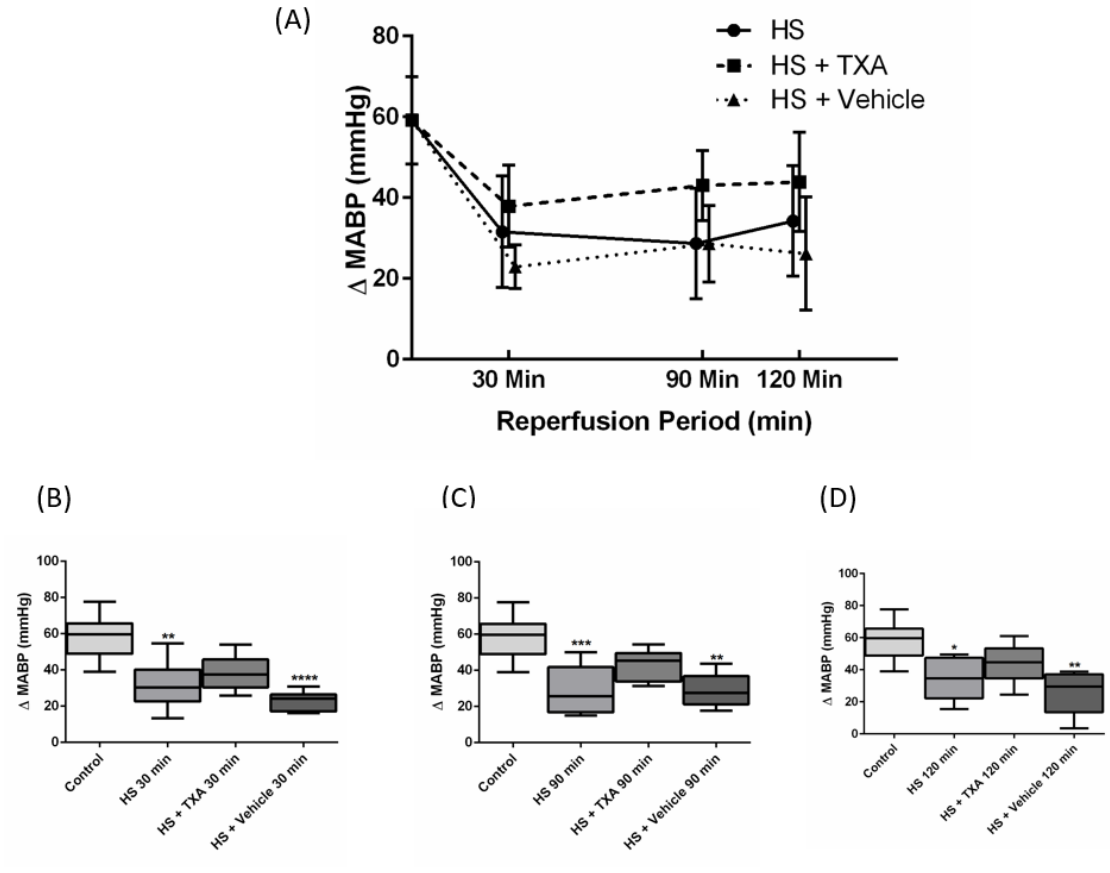

Figure 4: (A) Average peak change in MABP (ΔMABP) per group over time in response to phenylephrine challenge. **(B)** ΔMABP response to phenylephrine challenge 30 min after start of reperfusion, **(C)** ΔMABP response to phenylephrine challenge 90 min after start of reperfusion, **(D)** ΔMABP response to phenylephrine challenge 120 min after start of reperfusion. The first and third quartiles are at the ends of the box, the median is indicated with a vertical line in the interior of the box, and the maximum and minimum are at the ends of the whiskers. *$p < 0.05$ Control vs. HS, **$p < 0.01$ Control vs. HS, HS + Vehicle, ***$p < 0.001$ Control vs. HS, ****$p < 0.0001$ Control vs HS + Vehicle. Results shown as Mean ± SD.

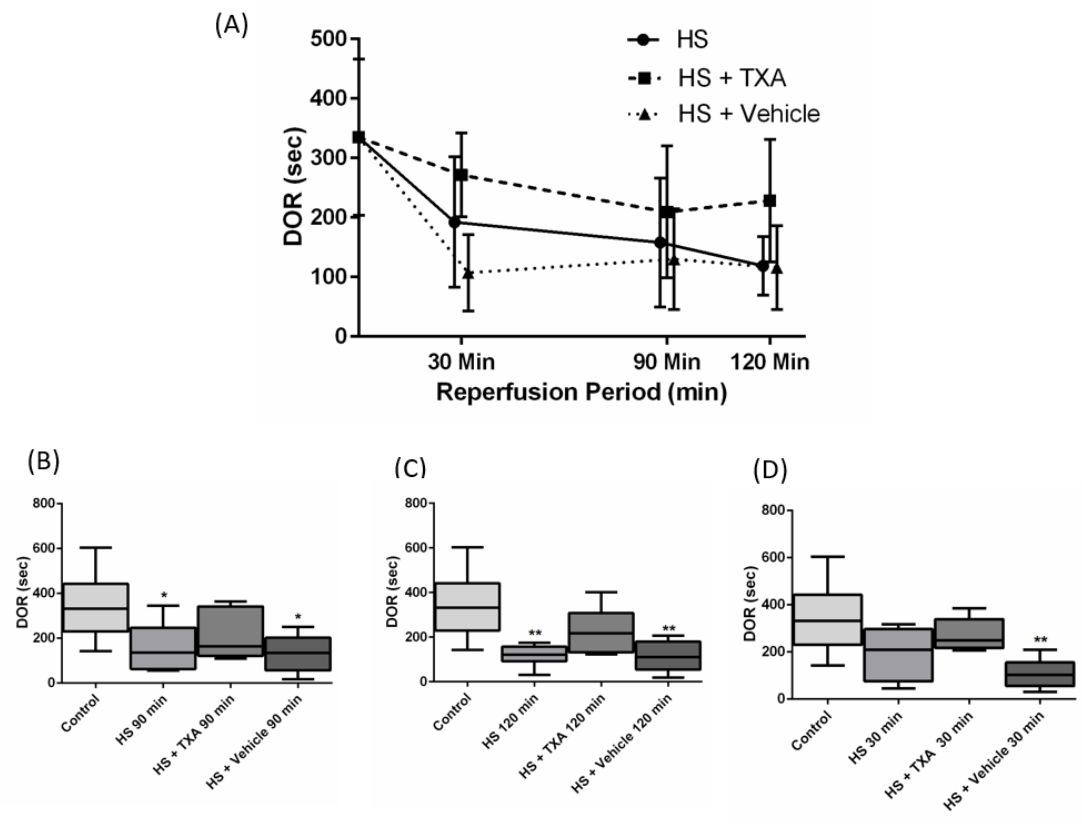

Figure 5: (A) Average response duration to phenylephrine challenge (DOR) per group over time. **(B)** DOR to phenylephrine challenge 30 min after start of reperfusion, **(C)** DOR to phenylephrine challenge 90 min after start of reperfusion, **(B)** DOR to phenylephrine challenge 120 min after start of reperfusion. The first and third quartiles are at the ends of the box, the median is indicated with a vertical line in the interior of the box, and the maximum and minimum are at the ends of the whiskers. *$p < 0.05$ Control vs. HS, HS + Vehicle, **$p < 0.01$ Control vs. HS, HS + Vehicle. Results plotted as Mean ± SD.

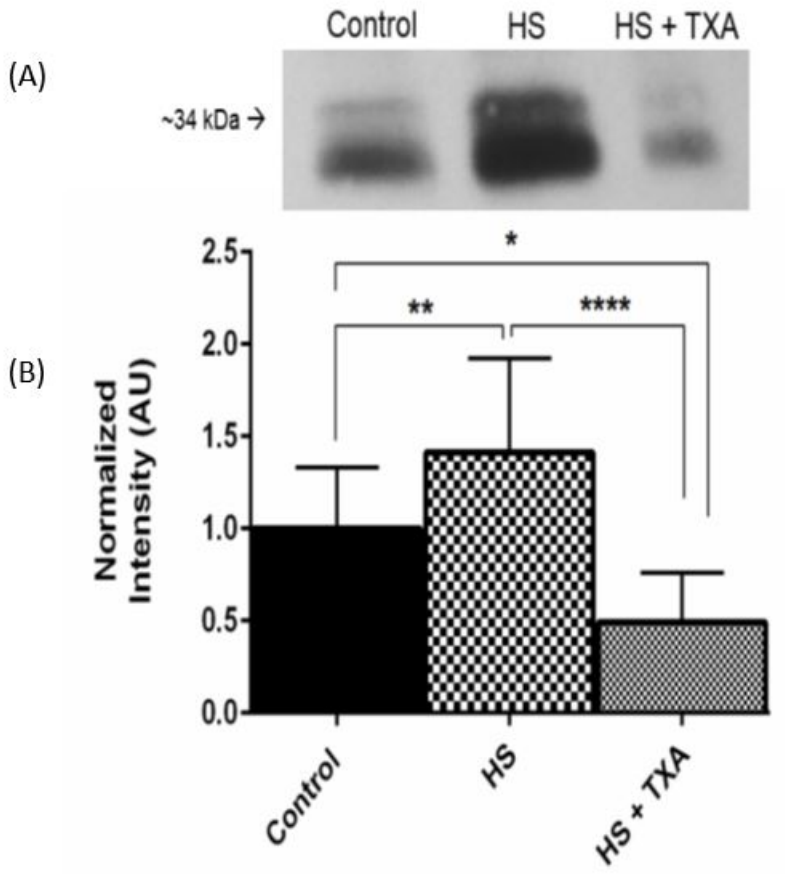

Figure 6: (A) Plasma trypsin density by Western blot with representative plasma trypsin band. The average of the control band densities was used reference. **(B)** Histogram of the average relative band intensities per group (n=6 animals/group). *p < 0.05 Control vs. HS + TXA, **p < 0.01 Control vs. HS and ****p < 0.0001 HS vs. HS + TXA. Results plotted as Mean ± SD.

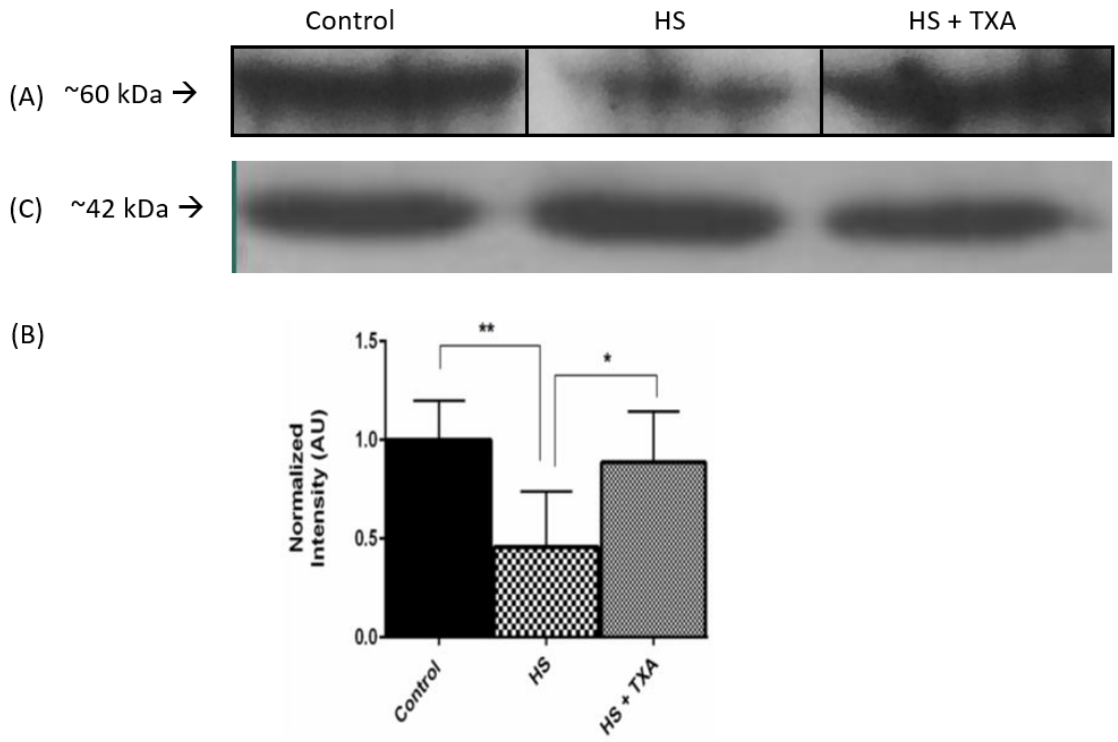

Figure 7: (A) α_{1D} adrenergic receptor band density of renal artery by Western blot with representative α_{1D} renal artery band. The average of the control band densities was used as a reference. **(B)** Histogram of the average relative band intensities per group (n=6 animals/group). **(C)** β-actin is shown as a loading control for the same animals. By single factor ANOVA. *$p < 0.05$, HS vs. HS + TXA; **$p < 0.01$, Control vs. HS. Results plotted as Mean ± SD.

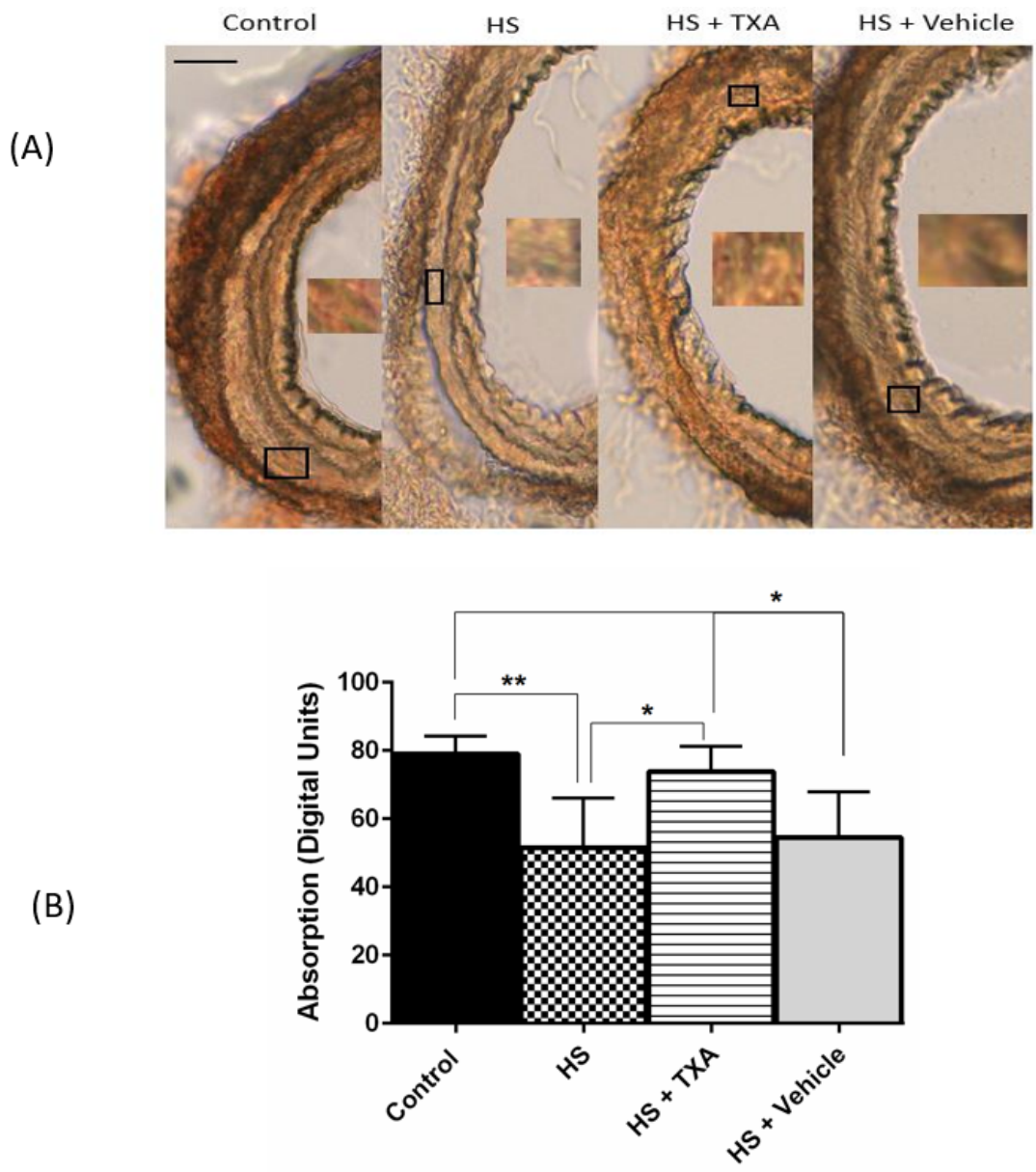

Figure 8: (A) Femoral artery sections labeled for α_{1D}. Boxes indicate smooth muscle regions from which measurements were taken. Inserts are of regions of smooth muscle. **(B)** Histogram of labeling density values in digital units. Scale bar is = 100 microns. **p < 0.01 Control vs. HS, *p<0.05 HS + TXA vs all other groups. Results plotted as Mean ± SD.

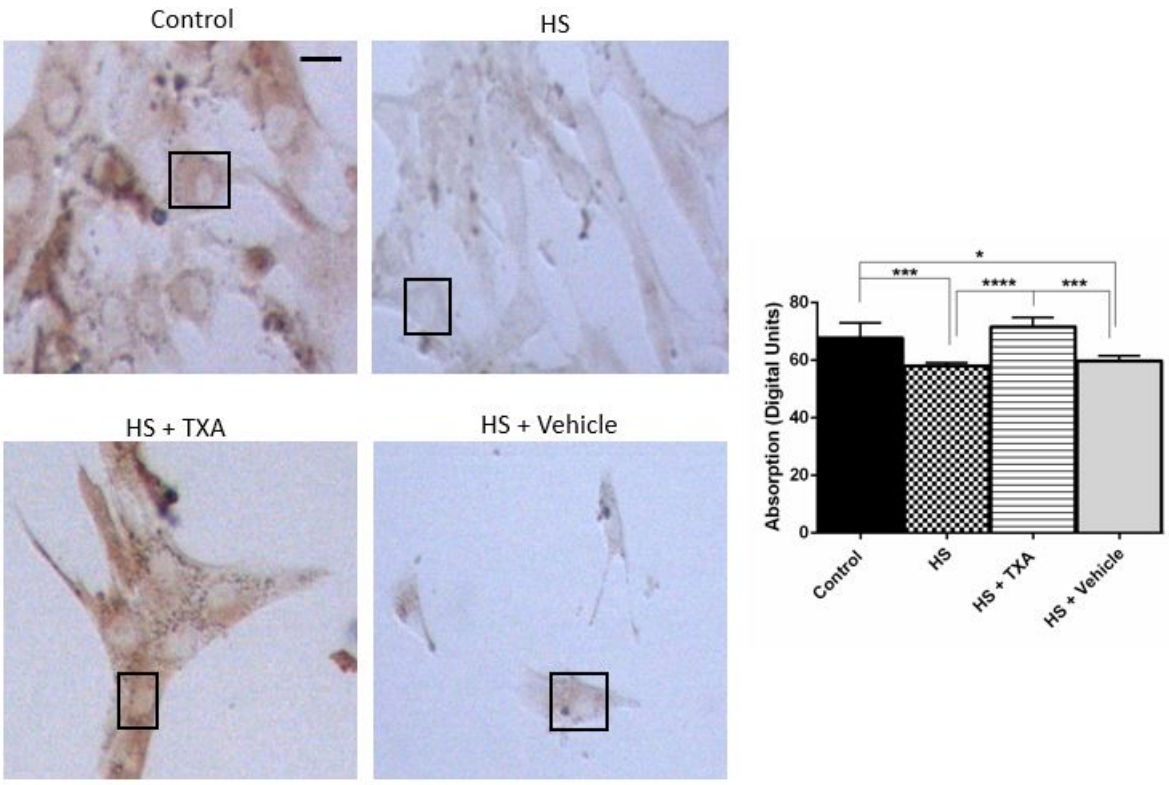

Figure 9: (A) Human carotid SMCs labeled for α1 adrenergic receptor following 3 hour incubation with rat plasma. Histograms display labeling density values in digital units. Scale bar is = 10 microns. *p<0.05 Control vs. HS + Vehicle, ***p < 0.001 Control vs. HS, ***p<0.001 HS + TXA vs. HS + Vehicle, ****p<0.0001 HS + TXA vs. HS. Results plotted as Mean ± SD.

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