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Effect of Ascorbate on Coagulation and Fibrinolytic Factors in the Septic Microvasculature

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A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Pharmacology and Toxicology
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EFFECT OF ASCORBATE ON COAGULATION AND FIBRINOLYTIC FACTORS IN THE SEPTIC MICROVASCULATURE

(Thesis format: Integrated Article)

by

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Graduate Program in Pharmacology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

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Abstract

Sepsis, a systemic inflammatory response to an infection, is a significant cause of morbidity and mortality worldwide. The microcirculation during sepsis fails, in part, due to microthrombosis and the resulting plugging of capillaries, precipitating organ failure. Intravenous injection of ascorbate has been shown to reduce capillary plugging, however the mechanism of this protective effect is unclear. We hypothesized that ascorbate-mediated destabilization of the microthrombi through promoting fibrinolysis could contribute to this protection.

We showed that streptokinase, a pro-fibrinolytic agent, reduced the capillary plugging to a similar degree as ascorbate. This similarity provided the impetus for studying the effect of ascorbate on fibrinolysis. Sepsis increased the urokinase plasminogen activator (u-PA) and tissue plasminogen activator (t-PA) mRNA expression in the skeletal muscle and liver in mice. No effect of ascorbate was observed on u-PA or t-PA expression levels. Sepsis also increased the plasminogen activator inhibitor 1 (PAI-1) mRNA and protein expression and activity in these tissues, but ascorbate did not affect these increases.

The local PAI-1 release by both platelets and endothelial cells may play a critical role in microthrombus formation in capillaries. We observed that PAI-1 released by isolated endothelial cells was not affected by ascorbate. However, thrombin-induced PAI-1 release from platelets was inhibited by ascorbate pH-dependently. We have also discovered that the PAI-1 release from platelets was nitric oxide independent.

It has been shown that PAI-1 has a protective role in sepsis, namely that PAI-1 knockout leads to increased bacterial content, increased neutrophil apoptosis and increased mortality. Therefore, the lack of effect of ascorbate on PAI-1 in the tissue may maintain PAI-1's beneficial role in sepsis. Consistently, we observed that sepsis-induced increases in bacterial count, PAI-1 expression and myeloperoxidase content in various organs were not affected by ascorbate.

Overall, the lack of effect of ascorbate indicates that the protection by ascorbate through reduced capillary plugging is not through a fibrinolytic mechanism. Other mechanisms such as platelet-endothelial cell adhesion and changes in red blood cell deformability in the capillaries should be explored as possible mechanisms of protection by ascorbate.

Keywords

Sepsis, ascorbate, capillary blood flow, plasminogen activator inhibitor-1, fibrinolysis

Co-Authorship Statement

In the investigations entitled:

"Effect of ascorbate on fibrinolytic factors in septic mouse skeletal muscle (Chapter 2)"

Dr. D. Secor assisted with the mouse preparation and intravital microscopy, and conducted the platelet counts in blinded fashion. Ms. F. Li also assisted with the mouse preparation and intravital microscopy, analyzed the capillary blood flow, and conducted the thrombelastography. Drs. P. Gross, C. Ellis, M. Sharpe, and J. Wilson assisted in the experimental design and participated in the manuscript writing.

"Short-term effect of ascorbate on bacterial content, plasminogen activator inhibitor-1, and myeloperoxidase in septic mice (Chapter 3)"

Dr. D. Secor assisted with the mouse preparation and the MPO assay. Drs. C. Ellis, M. Sharpe, and J. Wilson participated in the experimental design and manuscript writing.

"Effect of ascorbate on plasminogen activator inhibitor 1 expression and release from platelets and endothelial cells in an in-vitro model of sepsis (Chapter 4)"

Dr. D. Secor assisted with platelet isolation and the platelet PAI-1 release experiments, and assisted with the endothelial cell harvest and culturing.

In the above investigations, any work not specified above was performed by me. All of the manuscripts were written by me. Dr. K. Tyml assisted in the experimental design, data analysis, writing of the manuscripts and preparation of this thesis.

In Chapters 2-4, I have used the word "we" to acknowledge the co-authorships in these papers. The use of "we" also reflects the versions submitted for publication.

Acknowledgments

I would like to begin by thanking my supervisor, Dr. Karel Tyml, for his support and encouragement throughout the completion of this thesis. His guidance was invaluable and I have learned an immense amount from him about the scientific process, including research skills, experimental design, analysis of data, writing and revising manuscripts. His teachings have allowed me to formulate my own ideas and pursue these new investigations. For these teachings, I will always be indebted to him.

I am grateful to my advisory committee members, Drs. Qingping Feng, Doug Jones, Stan Leung, Sanjay Mehta, Michael Sharpe and Barry Tepperman, for their guidance and advice on my work, as well as reviewing this thesis.

I must thank Ms. Fuyan Li for her teachings and assistance with intravital microscopy. I would like to thank Dr. Dan Secor, a former PhD student in Dr. Tyml's lab, for his technical assistance, intellectual and moral support and friendship over the past 6 years.

I thank the following people for their technical help: Drs. Gedaminas Cepinskas, Sean Gill, Christopher Harle, Dwayne Jackson, John McCormick, Phil Medeiros, Mr. Sean Mclachlan, Kelvin Ng, Andrew Tse, Mrs. Baraa al-Khazraji and Mary Keet.

Table of Contents

List of Tables

List of Figures

List of Appendices

List of Abbreviations

BH4   Tetrahydrobiopterin

CFU   Colony forming unit

CLP   Cecal ligation and perforation

DIC   Disseminated intravascular coagulation

DNA   Deoxyribonucleic acid

DPBS   Dulbecco's phosphate buffered saline

EDL   Extensor digitorum longus

ELISA   Enzyme-linked immunosorbent assay

eNOS   Endothelial nitric oxide synthase

FIP   Fecal injection into peritoneum

GMP   Guanosine monophosphate

IFγ   Interferon gamma

IL-1β   Interleukin 1 beta

IL-6   Interleukin 6

iNOS   Inducible nitric oxide synthase

LPS   Lipopolysaccharide

MPO   Myeloperoxidase

mRNA	Messenger ribonucleic acid
NADPH	β-Nicotinamide adenine dinucleotide phosphate
NFκB	Nuclear factor kappa B
NLR	NOD-like receptor
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
PAGE	Polyacrylamide gel electrophoresis
PAI-1	Plasminogen activator inhibitor-1
PAI-2	Plasminogen activator inhibitor-2
PAP	Plasmin-antiplasmin complex
PAR1	Protease activated receptor 1
PAR4	Protease activated receptor 4
PVDF	Polyvinylidene fluoride
qPCR	Quantitative polymerase chain reaction
RCF	Relative centrifugal force
RLR	RIG-I-like receptor
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
SNAP	S-Nitroso-N-acetylpenicillamine

TEG	Thrombelastography
TLR2	Toll-like receptor 2
TLR4	Toll-like receptor 4
TNFα	Tumor necrosis factor alpha
TNTE	Tris-NaCl-Triton-EDTA
t-PA	Tissue plasminogen activator
TF	Tissue factor
u-PA	Urokinase plasminogen activator
vWF	von Willebrand factor

Chapter 1

INTRODUCTION

This thesis examines the role of the fibrinolytic system on the reduction in capillary perfusion observed during sepsis. To this end, this thesis focuses on the role of plasminogen activator inhibitor 1 (PAI-1), an anti-fibrinolytic factor, in reduced capillary perfusion during sepsis, and examines whether ascorbate (reduced vitamin C) restores capillary perfusion and alters immune response in a PAI-1 dependent manner. This introduction presents background information on: i) sepsis, and its contribution to reduced capillary perfusion, ii) fibrinolysis, including the components of coagulation leading to fibrin formation, and iii) ascorbate, including its beneficial effects in septic patients and animals.

1.1   SEPSIS

1.1.1   Epidemiology and significance

Sepsis is a major cause of mortality worldwide, directly responsible for 1.4% of all deaths (76) and implicated in between 6-10% of all deaths in Canada and the United States of America each year (4, 106). Research on sepsis has not reduced the mortality rate, which was reported to have increased by 0.67% per year between 1995 and 2005 (106). As survival from sepsis is dependent on the availability of intensive care unit services, the mortality rate is most likely higher in developing countries with limited medical facilities (91). The mortality rate of septic patients in Canada, the United States of America and European countries is between 20 and 47% (3, 66, 164).

Sepsis is defined as a systemic inflammatory response to an infection (163). A variety of criteria are used to confirm diagnosis of sepsis, including several scoring systems that assess the severity of particular symptoms. Sepsis is difficult to diagnose as there are a variety of symptoms that may be present in an individual patient. To make a diagnosis of sepsis, a patient must have a suspected or confirmed infection along with several of the following signs and symptoms: fever/hypothermia, tachypnea/respiratory alkalosis, positive fluid balance/edema, general inflammatory reaction, altered white blood cell count, increased levels of biomarkers (C-reactive protein, IL-6, procalcitonin), arterial hypotension, tachycardia, increased cardiac output, low systemic vascular resistance, high venous saturation O_2, altered skin perfusion, decreased urine output, hyperlactatemia (increased base deficit), signs of organ dysfunction, hypoxemia, coagulation abnormalities, altered mental status, hyperglycemia, thrombocytopenia, disseminated intravascular coagulation, altered liver function (hyperbilirubinemia), and intolerance to feeding (altered gastrointestinal motility) (58). In general, increasing numbers of symptoms indicate more severe sepsis, and a higher mortality (164).

In 2011, the only drug approved solely to treat sepsis, Drotrecogin alfa, was withdrawn due to a lack of efficacy (126). As a result, currently the only treatment option available to physicians is supportive in nature, focusing on maintaining blood pressure and respiration while eliminating the bacterial infection, in the hope that the inflammation will resolve on its own (49). Aside from the aforementioned Drotrecogin alfa, treatments targeting the inflammation and coagulation have at times shown promise (1, 108, 149) but were deemed to be either not effective or inconclusive in the treatment of sepsis. Their use is either not recommended or used on a case by case basis as an experimental treatment. Our lack of knowledge of the mechanisms involved in the septic pathophysiology has limited the development of drugs and other treatment strategies.

1.1.2 Pathophysiology of sepsis

The inflammatory response during sepsis is perpetuated by the production of cytokines intended to protect the host from the invading pathogens. Infections caused by gram-

negative bacteria initiate sepsis through the lipopolysaccharide (LPS) present on the bacterial wall. The LPS interacts with toll-like receptor 4 (TLR4), primarily located on leukocytes and a key component of the innate immune system (135). The activation of TLR4 causes the induction of a signaling cascade ultimately resulting in the production of various pro-inflammatory cytokines such as tumor necrosis factor α (TNFα), interleukin-1β (IL-1β) and interleukin-6 (IL-6), all of which are markedly increased in the plasma of septic patients (51). In gram-positive bacterial infections, substances such as lipoteichoic acid are secreted instead of LPS, activating TLR2 with similar production of cytokines (150). Both TLRs signal through activation of NFκB (123). The LPS-induced cytokine storm perpetuates the response by activating other components of the immune system and further recruiting cells such as the neutrophils and macrophages to the site of infection. In addition stimulation of endothelial cells and platelets occur. LPS and/or cytokines increase the levels of pro-aggregation and adhesive proteins, reactive oxygen species (ROS) and nitric oxide (NO) (123, 135). The changes brought about due to the activation of the immune system, endothelial cells and platelets lead to many pathophysiological alterations such as systemic hypotension, reduced capillary perfusion, decreased ability to use the oxygen present, reduced resistance in the systemic vasculature and reduced cell-to-cell signalling in the cells lining the vasculature (14, 32, 58, 77, 161-163). These alterations lead to increasing maldistributions of blood flow and oxygen supply in the capillary bed, causing tissue damage and possible progression to organ dysfunction and failure (58, 140).

1.1.3   Reduced capillary perfusion

As alluded to previously, a reduction in capillary perfusion in various tissues of experimental animals and humans is a hallmark of sepsis (9, 13, 137). Reduced capillary perfusion, defined as an increase in the number of capillaries with stopped flow, is believed to contribute to organ dysfunction and failure due to the reduction in oxygen delivery to the tissue (52, 158). Lower oxygen delivery to the tissues, due to reduced red blood cells traveling through capillary beds lead to local tissue hypoxia and mitochondrial dysfunction. Despite the resolution of septic shock in most patients,

reduced capillary perfusion leading to organ failure was still linked to a third of total patient deaths (131), while mortality increased as capillary perfusion decreased (156). Recent studies suggest that the reduced capillary perfusion is due in part to platelet adhesion and aggregation, and an increase in fibrin in the plugged capillaries (137). However, when platelets were depleted, capillary perfusion did not fully recover (137), indicating that the mechanism of capillary plugging is not exclusively due to platelet adhesion and aggregation.

It is important to note that, in sepsis, reduced capillary perfusion is due to plugging in capillaries themselves rather than plugging in the supplying arterioles or draining venules. It has been observed that blood flow stops in capillaries even when these arterioles and venules are perfused (137, 159). The presence of a precapillary sphincter (i.e., smooth muscle cells surrounding the vessel immediately upstream of the capillary) in skeletal muscle is debated. Studies have found no evidence of a precapillary sphincter (37) or that the sphincter is present but not innervated (45), therefore contraction of a precapillary sphincter as a normal physiological process cannot adequately explain the lack of perfusion in select capillaries of the hind limb skeletal muscle (this process cannot be excluded from some other skeletal muscle beds such as the cremaster muscle (8)). The lack of an influence from a precapillary sphincter points to the presence of a pathological process controlling the reduction in capillary perfusion during sepsis.

There are several possible factors that may contribute to capillary plugging. Leukocytes are large enough that should they adhere to the capillary wall they would fully occlude the capillary vessel. There is merit in this, as leukocyte adhesion has been reported to increase during sepsis or endotoxemia (73, 103). The relative contribution of leukocyte adhesion in the reduction in skeletal muscle capillary perfusion is likely low as very few leukocytes are observed in the septic skeletal muscle, instead being focused in the liver and lungs (104, 144). It is for this reason that it was concluded that leukocytes play little role in the capillary plugging in the septic rat skeletal muscle (125). Previous work in our lab confirmed that capillary plugging in a septic mouse skeletal muscle is not greatly influenced by leukocytes (137, 186). In addition to leukocytes, red blood cells

could potentially contribute to capillary plugging due to their decreased deformability during sepsis (9, 110). The role of red blood cell deformability in capillary plugging has not been directly studied during sepsis. As the major components in microthrombi, platelets and fibrin can be expected to play some role in capillary plugging during sepsis. Indeed, both increased platelet activation and fibrin formation have been observed in septic animals and humans (83, 137, 140). The formation of microthrombi in the capillaries, mediated by platelet sticking to the endothelium and fibrin mesh formation, would likely lead to the occlusion of at least some capillaries, blocking the flow of blood through those particular capillaries. Platelet adhesion has been implicated in capillary plugging (137, 138), however investigating the role fibrin and the mechanism of its breakdown play in capillary plugging will be the focus of the present thesis.

During sepsis, fibrin breakdown decreases with increasing severity (61). This would serve to increase the fibrin content in microthrombi, and combined with platelet trapping in the capillary bed would increase the chance large enough microthrombi could form to fully occlude the capillary flow. Evidence supporting this effect includes our observation that capillaries typically go through a phase of intermittent flow prior to total stoppage, suggesting that the dynamic process of building up and breaking down the microthrombus slowly shifts from equilibrium to a progressively more pro–thrombotic state, ultimately leading to a plugged capillary when microthrombus breakdown is insufficient to maintain flow through the capillary (140).

1.1.4 Oxidant production in sepsis

The major endogenous ROS molecules are the free radicals superoxide, hydroxyl and peroxynitrite. The effects of ROS are localized due to the inability of the radicals to cross the cell membrane. Unlike the free radicals, hydrogen peroxide, a non-radical substance, can freely diffuse across the cell membrane, and therefore spread damage beyond the cell in which the ROS was intitally produced (127). All forms of endogenous ROS are essential in cell signaling and the maintenance of the normal homeostatic state in the body, and must be present in small quantities. Superoxide is converted by

superoxide dismutase, located throughout the body, into hydrogen peroxide. The hydrogen peroxide is inactivated through further conversion into water through the actions of catalase and glutathione peroxidase (34). Superoxide also readily reacts with NO and forms peroxynitrite (127). Under pathological conditions of enhanced NO and superoxide production, peroxynitrite can have detrimental effects on the nearby cells through damage of proteins, lipids and DNA (54).

There are a number of systems that contribute to ROS, however the only enzymes whose primary purpose is to produce superoxide are the NADPH oxidases found, for example, in neutrophils, macrophages and endothelial cells (15, 127). It has been demonstrated that LPS and various inflammatory cytokines stimulate NADPH oxidase, increasing ROS production and thus making an important contribution to the pathophysiological state in sepsis (87, 179, 184).

Septic patients are known to have both greatly increased levels of inflammatory cytokines and reduced levels of the antioxidant ascorbate (28, 46, 63). Indeed, ROS has been shown to have numerous effects during sepsis including inducing the coagulation cascade (62), activating platelets (25, 120) and causing endothelial dysfunction (7). The combination of increased ROS production due to inflammatory cytokine signaling and reduced ascorbate levels likely result in enhanced oxidative stress (27). It has been demonstrated that removal of the NADPH oxidase subunit gp91phox lessened the sepsis induced reduction in capillary perfusion (159) and reduced platelet adhesion (137), while treatment of mice or rats with ascorbate prevented or reversed the observed capillary plugging (5, 159, 160).

1.1.5 Nitric oxide during sepsis

NO has several important functions *in vivo*, including acting as a vasodilator, as a signaling molecule to maintain vascular homeostasis, and as an important component of the immune response. NO has been demonstrated to inhibit platelet activation and aggregation (56, 74) and leukocyte adhesion to the endothelium and platelets (55, 74),

suggesting that local sources of NO may inhibit clot formation at the capillary level. The endogenous source of NO is the three nitric oxide synthases (NOS), endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). The production of NO is facilitated by the NOS enzymes, where L-arginine is converted to L-citrulline in a two step process involving NADPH, H^+ and O_2. NO synthesis occurs only in the presence of the calcium dependent co-factor calmodulin, flavin adenine dinucleotide, flavin mononucleotide, heme and tetrahydrobiopterin (BH_4) (57). The co-factors facilitate the movement of electrons from NADPH to heme, however if any step in this process is interrupted (referred to as uncoupling), such as the consumption of one co-factor, superoxide can be produced and released (42).

During sepsis there is increased NO production evidenced by the rise in nitrite and nitrate levels in the blood plasma of both humans and animals (81, 146). The major source of increased NO is the increase in expression of iNOS through signaling by LPS and inflammatory cytokines such as TNFα (2). It has been demonstrated that the increased NO production causes hypotension through arteriolar hyporeactivity and inappropriate vasodilatation (122). Though iNOS is likely the major source of NO production during sepsis, genetic knockout of this, or either of the other two NOS isoforms (eNOS, nNOS) failed to improve the capillary perfusion, suggesting that the capillary plugging is not related to the arteriolar hyporeactivity or vasodilation resulting from excess NO production (159). In another study, the use of iNOS inhibitors did improve the arteriolar blood pressure (16), suggesting that hypotension is not responsible for the reduced capillary perfusion seen in sepsis. Ascorbate treatment does reduce capillary plugging in septic mice independently of iNOS or nNOS (137). Interestingly, the beneficial effect of ascorbate is eliminated in eNOS$^{-/-}$ mice, suggesting that ascorbate functions through an eNOS dependent pathway. The eNOS isoform is found primarily in the endothelial cells, while other cells such as platelets, erythrocytes and smooth muscle cells also express it (20, 53, 152). The primary role of the NO produced by eNOS is to maintain vascular homeostasis through the regulation of vascular tone and preventing platelet aggregation, apoptosis and smooth muscle cell proliferation (22, 43, 48, 154). During various disease states including sepsis, the supply of BH_4 can become depleted, leading to the uncoupling of the NOS (42). Due to the uncoupling, superoxide is

produced and released by the NOS enzymes, further contributing to the ROS in the vasculature. Additional peroxynitrite production due to impaired eNOS function is believed to cause further damage, particularly in the capillaries, leading to capillary plugging through activation of coagulation (159).

1.1.6   Bacteria and the immune response during sepsis

Though not essential to the development of the inflammatory response, a bacterial infection is most often present in patients presenting with sepsis. Other less common sources of infection are fungi, viruses and parasites. There are a multitude of bacterial infections that can lead to sepsis, with the most common sites of infection being the lungs, abdominal cavity and urinary tract (49). An infection of the abdominal cavity commonly results in peritonitis, an inflammation of the peritoneum lining the peritoneal cavity. Peritonitis can be caused by a ruptured appendix, perforation in the digestive tract or bacteria otherwise entering the peritoneal cavity (100, 124). Many bacteria are typically present in the digestive tract, including both gram-negative and gram-positive species. It has been determined that stool samples from the cecum of mice contain approximately 40 different species of bacteria, with *Escherichia coli*, *Enterococcus* and *Staphylococcus* strains dominating the aerobic bacteria content (147, 171). Peritonitis occurs as a result of the presence of some or all of these bacteria, with the anaerobic bacteria likely playing the greatest role due to the anaerobic nature of the peritoneum (124).

The immune response to a bacterial infection begins with the activation of pattern recognition receptors by pathogen-associated molecular patterns present on bacteria (and other pathogens) and damage-associated molecular patterns released in response to cell damage caused by pathogens (50, 58, 169, 174). There are four main classes of pattern recognition receptors in animals: the TLRs, C-type lectin receptors (CLR), NOD-like receptors (NLR) and RIG-I-like receptors (RLR) (58, 169). In addition, there are a number of secreted pattern recognition receptors, such as C-reactive protein and lectins that are present in the blood and bind to invading pathogens to alert immune cells of the

presence of these pathogens (114). The pattern recognition receptors activate the innate immune system, leading to inflammation and activation of the complement system (174). Inflammation, caused in part by various cytokines, increases blood flow to the infected tissue, leading to increased temperature, swelling and recruitment of leukocytes in an effort to kill the pathogen. Typically, the first leukocytes arriving to a site of bacterial infection are the neutrophils, which phagocytose the invading bacteria, killing them with a respiratory burst involving the NADPH oxidase production of superoxide along with proteins released through degranulation, such as myeloperoxidase (139, 173). Macrophages arrive at the site of infection at the same time as neutrophils and also play a role by removing dead or dying cells, phagocytosing bacteria missed by the neutrophils, and producing inflammatory cytokines (24, 60). The complement system, a cascade of proteins circulating in the blood, is the other component of the innate immune system. The inactive precursor complement proteins are activated by several different pattern recognition receptors, including C-reactive protein and mannose-binding lectin (19, 47). The complement system works by attracting neutrophils and macrophages, along with increasing vascular permeability and opsonizing bacteria (coating the bacteria increases phagocytosis) to weaken and destroy them (19, 128).

In cases of bacterial sepsis, it is believed that the immune response begins normally, with neutrophil recruitment and complement activation. For some reason that has yet to be determined, the inflammatory response becomes aberrant and spreads to the entire body (58, 174). The increase in inflammation leads to the activation of the coagulation system, further production of ROS and a decrease in fibrinolysis (140) (see sections 1.2 and 1.3 for further discussion of coagulation and fibrinolysis). The prolonged inflammatory state directly or indirectly leads to many of the detrimental effects commonly seen in sepsis, including fever, hypotension and organ dysfunction and failure. Though the bacterial infection is the initial trigger, often the inflammatory response actually causes more damage than the infection itself. It is for this reason that antibiotics alone are often insufficient to treat sepsis (112).

1.1.7 Models of sepsis

There are a number of models that have been used previously to study sepsis in a variety of different species. Most studies, largely due to cost, are conducted in mice, though rats, rabbits, pigs, sheep and non-human primates such as baboons have been used in a variety of septic models (40, 168). The models have been developed to attempt to best mimic the pathology observed in septic patients. Most septic models are based on one of two basic pathogenic mechanisms: endotoxemia using a variety of inflammatory cytokines, and bacterial introduction through three main routes: direct injection of bacterial colonies into a host, cecal ligation and puncture (CLP), where the intestinal wall is compromised resulting in peritonitis, and fecal injection into peritoneum (FIP), where peritonitis is caused by administration of feces directly into the peritoneum.

1.2 COAGULATION

1.2.1 Overview

Coagulation is the development of clots from blood. Under physiological conditions, coagulation is essential for the maintenance of vascular homeostasis. Initiation of coagulation begins with platelet activation and adhesion (see section 1.2.4), followed by strengthening of the developing clot with fibrin (see section 1.2.2). Clot development and breakdown (fibrinolysis, see section 1.3) is a dynamic process that is tightly regulated under normal physiological conditions (89). Dysfunction in this regulation, whether through hypercoagulation (excessive coagulation) or hypocoagulation (insufficient coagulation) has been shown to result in various pathological disease states (89, 101, 140).

Coagulation is initiated through two separate pathways: the contact activation pathway and the tissue factor (TF) pathway, both ultimately leading to a common pathway. The contact activation pathway, or intrinsic pathway, is activated when

exposed collagen comes in contact with factor XII activating it to factor XIIa and beginning a cascade of activation first of factor XI to XIa, then factor IX to IXa and activation of factor X to Xa. Under normal conditions, the endothelial cells form a barrier between the blood and collagen. In addition to the contact activation pathway, platelets are directly activated when exposed to collagen and begin to adhere to the exposed site. The TF pathway, or extrinsic pathway (illustrated in Figure 1.1a), is activated when blood comes in contact with TF produced mainly by cells not normally in contact with the blood. This activates factor VII to VIIa, which then directly activates factor X to Xa. Activated factor Xa initiates the common pathway for both coagulation pathways, with the conversion of prothrombin into the active protein thrombin. Thrombin then converts fibrinogen into fibrin, leading to the formation of a fibrin mesh in the developing clot. Thrombin also further activates platelets and endothelial cells (30) and causes a positive feedback activating factor VII to VIIa, therefore increasing the production of thrombin further (89). Under physiological conditions, most TF is produced by vascular cells not in direct contact with the blood, however during pathological conditions, upon stimulation by inflammatory cytokines, endothelial cells and white blood cells have been shown to release TF (35).

It is common in septic patients to observe a wide variety of disorders in the coagulatory system, including disseminated intravascular coagulation (DIC, consumption of coagulation proteins in the microvasculature, ultimately resulting in consumption of coagulatory factors leading to an inability to form clots), while thrombocytopenia (a reduction in platelet count) can occur alone or in conjunction with DIC (84, 140). It has been shown that there is extensive cross-talk between the inflammatory and coagulation proteins, with many inflammatory cytokines activating the coagulation system. LPS itself has been shown to increase the production of TF (148), while cytokines such as TNFα have also been demonstrated to upregulate TF (11). As LPS itself induces the production of TNFα, this would have a positive feedback in generating even more TF and enhancing thrombin production. Inflammation has also been observed to alter coagulation through a reduction in the levels of anti-coagulation proteins protein C (an enzymatic inactivator of factors Va and VIIIa), tissue factor pathway inhibitor (an inhibitor of thrombin as well as factor Xa, and ultimately inhibits the factor VIIa-TF
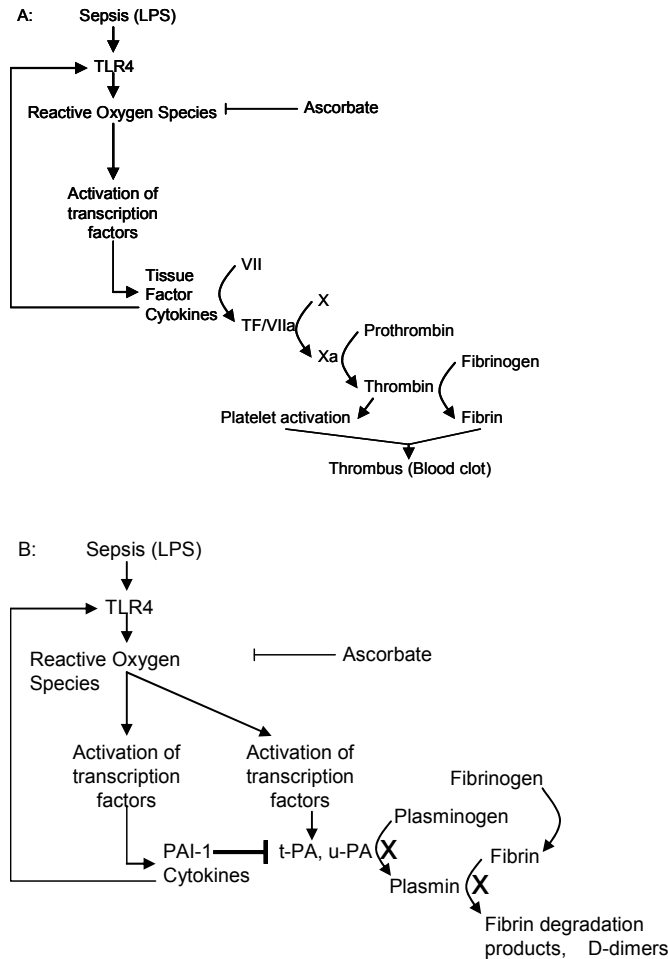
Figure 1.1. Schematic of the coagulation (Panel A) and fibrinolytic (Panel B) pathways during sepsis. Panel A: The coagulation system is initiated in sepsis by the activation of TLR4 by LPS. This generates ROS, TF and cytokines that begin the coagulation cascade. Coagulation occurs when the platelets become activated and fibrin is formed, together composing the thrombus. As ascorbate acts as an antioxidant (see section 1.4), I propose that ascorbate reduces the ROS and thereby inhibiting the activation of the coagulation cascade. Panel B: I propose that activation of TLR4 also initiates production of PAI-1, which then inhibits t-PA and u-PA (see section 1.3). This leads to lower plasmin production and impaired fibrin degradation. Upon ascorbate treatment, PAI-1 expression is reduced restoring plasmin production and fibrin degradation. Activation of factors is indicated by arrows, inhibition is indicated by a T line and reduced production during sepsis is indicated by an X.

complex in conjunction with factor Xa), and antithrombin (an inhibitor of thrombin) (84, 89). In addition to enhancing coagulation, the breakdown of fibrin through the fibrinolytic pathway can also be inhibited by inflammation (see section 1.3) (140).

1.2.2 Fibrin

Fibrin is a fibrous protein formed from its precursor fibrinogen during the blood clotting process. It forms a polymerized mesh giving the blood clot strength and stability. Besides the role in clot formation and support, fibrin is also involved in signal transduction, eliciting cellular responses critical to normal cellular function (44, 178). Fibrin is formed when thrombin cleaves the N-terminus of the fibrinogen α and β subunits. This allows the cleaved ends to polymerize end to end with other fibrin molecules (170). It is the cross-linking of fibrin molecules into a mesh that ensures the structural integrity and three dimensional nature of the clot (172).

During sepsis, fibrin content is markedly increased due to increased TF mediated thrombin generation (84, 89). In addition, fibrin degradation (fibrinolysis, see section 1.3) is reduced during sepsis (140), suggesting that both increased fibrin production and stability contribute to the pathological clot formation during sepsis.

1.2.3 Endothelial cells

The endothelial cells that line the blood vessel walls are essential for vascular function by forming a barrier between the surrounding cells and the vascular lumen as well as facilitating critical components of the coagulation and inflammatory processes. Endothelial cells produce and express several important adhesion proteins including P-selectin and von Willebrand factor (vWF) as well as the anti-fibrinolytic factor PAI-1 (96, 142). Upon activation, the Weibel-Palade bodies that contain the P-selectin and vWF are expressed at the cell surface, facilitating the capture of platelets and coagulation proteins. The secretion of PAI-1 by endothelial cells serves to stabilize the clots through its anti-fibrinolytic activity. It has been demonstrated that endothelial cells can be activated by numerous molecules, proteins and cytokines including LPS, thrombin, and

TNFα (39, 59, 145). Endothelial cells can be damaged during sepsis, resulting in a loss of the normal barrier function, allowing for increased vascular permeability and exposure of the basement membrane and surrounding cells to the vascular lumen (64). The resulting loss of barrier function can result in activation of coagulation and edema (82).

1.2.4   Platelets

Platelets are small anucleated cells derived from the fragmentation of precursor megakaryocytes. They are small in size, their diameter ranging between 1-3 microns. Platelets are essential to maintaining normal vascular homeostasis, primarily through a contribution to the formation of clots. When activated, platelets aggregate and adhere to adhesion molecules expressed on other cells, forming and strengthening the clots. In addition to clot formation, platelets also play an essential role in the repair and regeneration of connective tissues and other damaged tissues surrounding the vasculature (117).   Pathological disorders may result in altered platelet numbers or function. Low platelet count (thrombocytopenia), or reduction in function (thrombasthenia), result in excessive bleeding, while high platelet count (thrombocytosis) leads to pathological clotting (141).

Activation of platelets occurs through exposure to a number of substances, including thrombin, vWF, collagen, adenosine diphosphate, and thromboxane A_2 produced by other platelets (88, 116). Upon activation, platelets change shape becoming more spherical allowing for enhanced adhesion through increased platelet rolling (105). Activated platelets also secrete three types of granules: dense, lambda and alpha. The dense granules release molecules essential for the coagulation response (116). Alpha granules release the pro-coagulation proteins including P-selectin, vWF, fibrinogen, and the anti-fibrinolytic protein PAI-1 along with other proteins (88). The molecules secreted from these granules serve as positive feedback to activate platelets as well as other components of the coagulation and anti-fibrinolytic pathways (88, 116).

Thrombocytopenia is common in septic patients (10, 65), though the exact reasons for the reduction in platelet count remain unresolved. It is known that activation of coagulation does occur during sepsis, including platelet activation. The increased release of thrombin, adenosine diphosphate, and thromboxane A2 (along with other substances) during sepsis activates platelets leading to increased adhesion to the vasculature and increased platelet aggregation (23, 70, 75, 84, 85, 99, 134, 140). In addition, LPS and inflammatory cytokines have been shown to activate platelets (26, 119, 185), resulting in an increase in TF (130, 134). ROS has also been demonstrated to increase platelet aggregation (21, 29, 166), while activated platelets have been shown to release ROS (132, 166).

1.3 FIBRINOLYSIS

1.3.1 Overview

Fibrinolysis is the process by which fibrin is broken down, leading to the dissolution of blood clots. Under normal physiological conditions, fibrinolysis removes clots that are no longer needed, as well as regulating the size of clots so they do not become too large. Fibrinolysis is initiated by the activation of plasmin from its precursor plasminogen (see Figure 1.1B). Plasmin cleaves fibrin into degradation products, dissolving the fibrin mesh and allowing for the dispersal of the clot. Abnormal fibrinolysis, either through inhibition or over activation, has been associated with pathological disease states (67, 102).

Fibrinolysis is inhibited by two separate means, namely inhibiting plasmin production, and directly inhibiting plasmin. Plasmin production is inhibited by the plasminogen activator inhibitors, while α2-antiplasmin is the primary molecule responsible for inactivating plasmin (67, 133). A disease process targeting either pathway could alter fibrinolysis, either through the stabilizing effect of preserving fibrin, or the anti-coagulation effect of uncontrolled fibrin degradation (86, 93, 167).

1.3.2 Plasmin

Plasmin becomes an active enzyme upon cleavage from the inactive zymogen plasminogen. Plasminogen is produced in the liver, circulates in the blood and is incorporated into clots. Two activators of plasminogen, tissue plasminogen activator (t-PA) and urokinase plasminogen activator (u-PA), cleave the molecule into plasmin when plasminogen is bound to a clot or cell surface (90, 107). Active plasmin cleaves fibrin and vWF along with several other pro-coagulation proteins into inactive degradation products, such as D-dimers in the case of fibrin (90, 143). Both t-PA and u-PA are produced by a wide variety of cells, and interact with plasminogen, fibrin and in the case of u-PA, the u-PA receptor, to produce plasmin (90, 94). Plasmin is permanently inactivated by α2-antiplasmin, halting the degradation of fibrin (80). Diseases that cause pathological increases in the expression of t-PA or u-PA lead to hyperfibrinolysis and a lack of clot stability and longevity (36, 118), whereas decreases lead to unchecked clotting (33, 86). Decreased α2-antiplasmin under various disease conditions results in an inability to maintain clots due to enhanced fibrinolysis. Increased α2-antiplasmin has not been reported to occur (18, 71).

During sepsis, both t-PA and u-PA have been reported to have increased expression, however this increase is likely counteracted by increases in the inhibitory plasminogen activator inhibitors (129). Plasmin-antiplasmin complexes are increased during sepsis, supporting the notion that plasmin production is increased during sepsis, however increased plasmin may be offset by increased anti-fibrinolytic activity (86, 93, 102, 157).

1.3.3 Inhibition of plasmin

The production of plasmin is regulated by a dynamic balance between the plasminogen activators (t-PA and u-PA) and their inhibitors PAI-1 and plasminogen activator inhibitor 2 (PAI-2). PAI-1 is the primary inhibitor of the activation of plasmin, acting through the irreversible inhibition of both t-PA and u-PA (31). PAI-1 is produced by a variety of cell

types, however in regards to intravascular clotting, the primary source is endothelial cells (94). Inhibition of t-PA and u-PA eliminates the activation of plasmin therefore stabilizing clots. Active PAI-1, the only form of PAI-1 with a known biological action, is extremely unstable and spontaneously inactivates to a latent form unless stabilized by vitronectin (17). The other known plasminogen activator inhibitor is PAI-2, however this inhibitor is not generally detectable in non-pregnant patients, and has been reported to be no more than five percent of the overall inhibitor content in the body (129). Pathological changes in the production of PAI-1 have been reported, with depressed levels leading to reduced ability to clot, and increased PAI-1 results in an inability to break down fibrin, possibly leading to the formation of fibrosis, which can ultimately lead to organ damage if allowed to occur over the long term (68, 69, 98, 115).

PAI-1 has been demonstrated to be increased during sepsis, while some reports have observed an increase in PAI-2 as well (93, 95, 102, 129, 182). Overall, this leads to an increase in anti-fibrinolysis, where less plasmin is produced. It is unclear whether this increase just counteracts the observed increase in t-PA and u-PA or whether the increased PAI-1 creates an even greater anti-fibrinolytic state (86, 94).

In addition to its fibrinolytic effects, PAI-1 has been demonstrated to play a role in angiogenesis, macrophage migration and neutrophil efferocytosis (17, 79, 121). It is interesting to note that all of these activities are related to wound healing and, as such, PAI-1 clearly plays an essential role in this process. In addition, increasing evidence points to a role of PAI-1 in obesity and cancer, where aberrant expression promotes vascularization and therefore tumor growth (17).

1.4 ASCORBATE

1.4.1 Overview

Ascorbate (a reduced form of vitamin C) is a water soluble antioxidant available extra-
and intra-cellularly in the body. Its primary function is to react with ROS (superoxide,
hydroxyl and peroxynitrite radicals), neutralizing the harm these radicals can cause.
Upon reaction between ascorbate and a free radical, ascorbate loses its reactivity and can
convert to dehydroascorbic acid (175). Ascorbate also plays a critical role in the
recycling of the antioxidant α-tocopherol (vitamin E) (155).

In humans, ascorbate is an essential vitamin obtained from diet, as the ability to
produce ascorbate has been lost. In contrast, most rodents produce their own supply of
ascorbate. During sepsis, blood plasma levels of ascorbate are markedly reduced (e.g.,
from 60 to 10 μM) in both patients and animals regardless of the ability to produce
ascorbate (5, 46). Indeed, there has been a report of a critically ill patient developing
scurvy (vitamin C deficiency) due to depletion of ascorbate in the blood plasma (63). It
is believed that impairment of the mechanisms of ascorbate recycling occurs during
sepsis (12). There is increased consumption of ascorbate in septic patients in order to
neutralize the increased ROS levels (12, 92, 175-177). Decreased plasma ascorbate
levels have been linked with decreased survival and more severe illness in septic patients
(46, 54, 177).

1.4.2 Treatment with ascorbate

Ascorbate, administered with vitamin E has been demonstrated to reduce organ failure,
shorten recovery time, and improve survival in critically ill patients (28, 113). Ascorbate
has also been shown to reduce mortality in burn victims and restore depleted plasma
levels in septic patients when given at high doses of greater than 1g per day (12, 92, 151).
In addition, the antioxidant succinobucol has been shown to reduce TF expression in LPS

stimulated endotoxemia (97), thereby reducing coagulation and suggesting that sepsis derived ROS plays a role in the initiation of coagulation.

Ascorbate given as an intravenous bolus has been demonstrated to enhance capillary perfusion and responsiveness in the arterioles, improve the function of the liver, reverse hypotension and decrease mortality in rodent models of sepsis (5, 137, 159, 160, 180, 181). Ascorbate reduced capillary plugging (137), and platelet activation and aggregation in septic models (138). Capillary plugging has been demonstrated to improve with ascorbate treatment when given with the onset of sepsis (5), as well as six hours into sepsis, at a time when approximately 40% of capillaries were non-perfused (137, 159, 160). The administration of ascorbate restored perfusion to approximately half of the previously non-perfused capillaries (137, 159, 160). The improvement in capillary perfusion in mice with ascorbate treatment is consistent in both a cecal ligation and perforation (CLP) model (180) and a fecal injection into peritoneum (FIP) model (159).

1.4.3   Effects of ascorbate

As ascorbate acts as an antioxidant, it is likely that its protective effects would be at least partly due to its ability to scavenge ROS in septic patients. Indeed, it has been determined that removal of the gp91phox subunit of NADPH oxidase, eliminating the NADPH oxidase ROS contribution, reduces capillary plugging, and that ascorbate improves capillary perfusion to a similar degree (137). In addition, the protective effect of ascorbate is eNOS dependent, likely through eNOS derived NO production, as the addition of endogenous NO or tetrahydrobiopterin (BH_4), an eNOS cofactor, both improved capillary perfusion (159). The use of BH_4 or ascorbate on $eNOS^{-/-}$ mice was ineffective in reversing the capillary plugging (137, 159). As BH_4 is oxidized by ROS, which then uncouples from eNOS, increased ROS during sepsis ultimately leads to the production of superoxide rather than NO, causing cellular damage through the production of peroxynitrite (41, 78, 136). Ascorbate is known to be rapidly and readily taken up by endothelial cells (175, 176). The antioxidant activity of ascorbate likely prevents the

oxidation of BH_4, resulting in a state of normal coupling of eNOS and resulting in restored NO production (177).

NO prevents platelet activation and aggregation, and is one of the mechanisms whereby NO maintains normal vascular homeostasis. One way NO prevents platelet activation is through a reduction in the expression of the adhesion protein P-selectin on both platelets and endothelial cells, though iNOS derived NO actually increases P-selectin expression (6, 56, 183). Reduced P-selectin decreases platelet-endothelial adhesion and thrombus size (38, 56, 153), while ascorbate has been shown to reduce P-selectin expression (138). It must be noted that as PAI-1 is co-localized in the α-granules in platelets, it is possible that a reduction of P-selectin expression may also indicate reduced PAI-1 release, increasing fibrinolysis at the capillary level (88). Interestingly, ascorbate was able to reduce P-selectin surface expression on platelets in a NO independent manner (138), suggesting that α-granule secretion may be controlled by both NO and another mechanism unrelated to its antioxidant properties.

Recently, it has been determined that ascorbate reduces neutrophil extracellular trap formation, resulting in reduced cellular damage from excessive neutrophil activation (109). There is ample evidence that ascorbate affects numerous aspects of the inflammatory and coagulation systems, however the potential protective effects on the fibrinolytic system remain to be determined.

1.5 RATIONALE, HYPOTHESIS AND OBJECTIVES

1.5.1 Rationale

Extensive research on sepsis has been conducted, yet no effective pharmacological treatment targeting the inflammation has been developed. The failure of several experimental treatments at the clinical trial stage reflects the lack of understanding of the pathophysiology of sepsis at the molecular and cellular level. It has been demonstrated

that capillary perfusion is reduced during sepsis, with poorer prognosis with increasing microvascular dysfunction (5, 77, 131). It has been demonstrated that increased platelet adhesion occurs in the skeletal muscle capillaries during sepsis, and that depletion of platelets improves the capillary perfusion (137). In addition, studies have shown increased fibrin deposition and a decrease in fibrinolysis during sepsis (84, 140). However, complete removal of platelets, fibrin or the anti-fibrinolytic factor PAI-1 result in decreased capillary perfusion or markedly reduced survival (72, 111, 137). The poor prognosis through removing these critical components of coagulation highlights the complexity of this process and our lack of understanding of the mechanisms involved. The presence of both platelets and fibrin in capillaries shows that the capillary plugging is caused by a physical blockage due to clot formation (137). To date, the role fibrinolysis plays as it directly relates to capillary plugging has yet to be explored.

It has been many years since ascorbate treatment was shown to have beneficial effects during sepsis, with a link becoming apparent as early as 1990 (165). In the years since, it has been determined that ascorbate improves the capillary plugging (5), survival (159), and most recently decreases platelet adhesion (137, 138). Despite these studies, the mechanisms by which ascorbate facilitates these beneficial effects remains largely undetermined. Ascorbate acts as an antioxidant, and its beneficial effects in reducing capillary plugging is eNOS dependent *in vivo*, likely through the NO produced by eNOS (137). However, it must be noted that ascorbate also has an effect on platelet adhesion that is NO independent (138). This discrepancy suggests at least two separate mechanisms play a role in the protective effect of ascorbate. As platelet depletion does not restore capillary perfusion to the same extent as ascorbate (137), clearly ascorbate has an effect on other systems in sepsis. As fibrin and adherent platelets were both located in the non-perfused septic microvasculature (137), removal of fibrin through increased fibrinolysis may enhance the capillary perfusion in conjunction with reduced platelet adhesion. Exploration of the fibrinolytic state during sepsis will further elucidate the mechanism of ascorbates protective effect as well as support the use of ascorbate as an effective adjuvant therapy for sepsis.

1.5.2   Hypotheses

1) Increased fibrinolysis results in reduced capillary plugging during sepsis.

2) PAI-1 expression in a septic mouse model is increased, leading to decreased fibrinolysis.

3) Ascorbate promotes fibrinolysis in the skeletal muscle through a decrease in PAI-1 expression, restoring capillary perfusion during sepsis.

1.5.3   Objectives

1) Determine the effect of increased fibrinolysis on capillary perfusion in sepsis, and test whether sepsis and ascorbate can alter the levels of pro- and anti-fibrinolytic factor mRNA and protein expressions in an *in vivo* model of sepsis. This objective will be addressed in Chapters 2 and 3.

2) Determine the effect of ascorbate on bacterial levels and the immune response in septic organs. This objective will be addressed in Chapter 3.

3) Develop an *ex vivo/in vitro* model of sepsis to determine the effect of ascorbate on sepsis-induced PAI-1 expression and release from platelets and endothelial cells. This objective will be addressed in Chapter 4.

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Chapter 2

Effect of ascorbate on fibrinolytic factors in septic mouse skeletal muscle

A version of this chapter has been accepted for publication in Blood Coagulation and Fibrinolysis

2.1 INTRODUCTION

Sepsis, the systemic inflammatory response to a local infection, leads to reduced capillary bed perfusion and subsequent organ failure (33). Reduced perfusion of the microvasculature in tissues correlates with organ failure and mortality during sepsis (24). Using intravital microscopy, we have demonstrated that this reduction in microcirculatory flow is due to a significant increase in the number of stopped-flow, or plugged, capillaries (25, 30). The sepsis-induced coagulation of the microcirculation involves platelet activation and adhesion, fibrin deposition, and both the formation and strengthening of micro-thrombi (11). Furthermore, the opposing process of fibrinolysis (breakdown of fibrin) is reduced in blood during sepsis (8). Subsequently, the micro-thrombi within the microvasculature are an important contributor to the blood flow impairment in sepsis. The fibrinolytic process involves the pro-fibrinolytic t-PA and u-PA, and the anti-fibrinolytic PAI-1, which inhibits t-PA and u-PA (29).

We also demonstrated in a mouse model of sepsis that an intravenous injection of the antioxidant ascorbate at 6 h of sepsis (i.e., time when capillary plugging is prevalent) restores capillary blood flow at 7 - 18 h of sepsis (30). Since ascorbate does not affect arterial pressure post-injection (30), a local mechanism within the microvasculature could be responsible for the restoration of capillary blood flow. Numerous local mechanisms could explain this effect (4, 36), including the possibility that ascorbate enhances fibrinolysis in septic capillaries, and thus restores blood flow. To this end, oxidative stress has been shown to increase both mRNA and protein expression of t-PA, u-PA and PAI-1 in endothelial cells (20).

To our knowledge, the effect of ascorbate on the fibrinolytic process in the septic microvasculature has not been examined. The objective of the present study was to determine the effect of sepsis and ascorbate on t-PA, u-PA and PAI-1 mRNA and protein levels in the mouse model of sepsis. We hypothesized here that ascorbate restores blood

flow in septic capillaries by stimulating local fibrinolysis via increasing t-PA and u-PA and/or by inhibiting PAI-1 levels within the septic microvasculature.

2.2 METHODS

2.2.1 Animal preparation

All experimental protocols were approved by the University of Western Ontario Council on Animal Care. Male wild type C57BL/6 mice (weight: 18-25 g, age: 1.5-4 months) were obtained from the Jackson Laboratory (Bar Harbor, ME) and bred in our animal facility. Sepsis was induced by fecal injection into peritoneum (FIP) as detailed previously (25, 30). Briefly, feces was collected from the cecum of a minimum of two donor mice for each experiment, suspended in sterile saline at a concentration of 75 mg of feces/mL and stored overnight at 4°C. The following day, mice were anesthetized with ketamine (80 mg/kg) and xylazine (4 mg/kg) prior to being injected intraperitoneally with 50 mL/kg of the feces solution. For our control sham mice, we substituted sterile saline for the feces solution. All mice were given a subcutaneous injection of 1 mL of sterile saline containing the analgesic buprenorphine (4 µg/mL) 30 minutes prior to FIP.

At 5 - 6 h post-FIP, the mice were anesthetized with ketamine and xylazine and kept anesthetized with supplemental ketamine/xylazine doses for the remainder of the experiment (1.5 h). The extensor digitorum longus (EDL) muscle was surgically exposed and its surface was epi-illuminated and imaged using intravital microscopy as previously detailed by us (25, 30). The EDL muscle was chosen because it is a suitable organ for intravital microscopic examination of blood flow in the capillary bed. Other organs present difficulty with this examination due to the breathing movement of the animal and due to extensive surgery required to expose the capillary bed. It has been demonstrated that the EDL capillary perfusion during sepsis shows the same fundamental features (e.g., blood flow stoppage during sepsis) as the liver, kidney, skin, sublingual surface, heart, lung and brain (1, 24). We have used the EDL as a surrogate organ of the sepsis-induced

capillary derangement in the other organs. Briefly, we used an upright microscope and video-recorded 5 random fields (0.61 x 0.87 mm) per muscle. In each field we drew a test line perpendicular to muscle fibers and counted capillaries perfused with red blood cells and capillaries with stationary red blood cells (i.e., stopped-flow capillaries) crossing this test line. We computed the percentage of stopped-flow capillaries from the total (perfused + stopped) and averaged the percentages from 5 fields. We used the intravital recordings to verify that sepsis impaired capillary blood flow at 5 - 6 h post-FIP and to compute the percentage of stopped-flow capillaries at 7 h (25, 30). At 5 or 6 h, we injected an intravenous bolus (0.1 mL) of streptokinase (Sigma-Aldrich, Oakville, ON, 25000 U/kg), ascorbate (Sigma-Aldrich, 10 mg/kg) (both dissolved in sterile saline), or saline. The ascorbate dose is clinically relevant, as intravenous doses of the order of 1 g/day have been given to critically ill patients (12). To examine the effect of ascorbate on fibrinolytic factors in skeletal muscle, we harvested this tissue at 7 h for mRNA and protein analyses of these factors. We also harvested the liver at 7 h, because the liver could be a major source of fibrinolytic factors (22), which could be released into the bloodstream and thus indirectly affect the microcirculation in the skeletal muscle. Alternatively, we isolated microvascular endothelial cells from the skeletal muscle at 7 h by excising, mincing and digesting the muscle in a collagenase enzyme solution and by subjecting it to immunoseparation using lectin-coated magnetic beads as detailed previously (2, 15). The purity of the endothelial cell isolates were not confirmed due to limited cell availability, however we previously confirmed this method yields high purity through vWF expression (2, 15). To assess the plasma level of the fibrinolytic factors, we punctured the carotid artery to collect a systemic blood sample in sodium citrate. Finally, we also collected blood in heparin for platelet count and thrombelastography.

2.2.2 mRNA expression

To extract mRNA, we used Trizol (Invitrogen, Burlington, ON) according to the manufacturer's directions. We treated the mRNA with DNAse I (Sigma-Aldrich) followed by reverse transcription using Superscript II reverse transcriptase (Invitrogen). We used qPCR to determine the mRNA expression levels of t-PA (primers: forward 5'-

CTGAGGTCACAGTCCAAGCAATGT-3' and reverse 5'-GCTCACGAAGATGATGGTGTAAAGA-3'), u-PA (primers: forward 5'-GCCGCTATCCTACAGAGCAC-3' and reverse 5'-CAAGGGTATTGTTCCCCTCA-3') and PAI-1 (primers: forward 5'-GACAGCACTGTCAGGGTCCATAG-3' and reverse 5'-GCGGTCCTCCTTCACAAAGCTC-3') in the skeletal muscle, liver and endothelial cells. We used β-actin (primers: forward 5'-TCGTGGGCCGCTCTAGGCACCA-3' and reverse 5'-GTTGGCCTTAGGGTTCAGGGGGG-3') as our reference gene. The qPCR was carried out using Quantifast SYBR Green master mix (Qiagen, Mississauga, ON) on a Mini-opticon cycler (Bio-Rad, Mississauga, ON). We used the following cycling protocol: 95°C for 5 min and then 40 cycles of 95°C for 10 s, 60°C for 30 s and 80°C for 30 s. SYBR green fluorescence was acquired at 80°C in each amplification cycle. Subsequently a melt curve was generated, starting at 60°C and taking measurements every 0.2°C until 95°C was reached.

2.2.3 Protein content

Skeletal muscle and liver were homogenized in sodium dodecyl sulfate (SDS) lysis buffer with protease inhibitor cocktail (Sigma-Aldrich). After centrifugation, protein concentrations were determined using a Bradford protein assay. Equal amounts of protein were loaded on a 10% polyacrylamide gel followed by gel electrophoresis and transfer to a PVDF membrane. The membrane was blocked with 3% bovine serum albumin overnight, followed by incubation with primary H-135 PAI-1 antibody and mouse anti-rabbit horseradish peroxidase secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for one hour each. The membrane was then treated with Western Lighting Chemiluminescence reagent (Perkin Elmer, Woodbridge, ON) and exposed to Kodak Biomax MR film (Carestream Health, Rochester, NY). We then stripped the membrane using stripping buffer and reprobed it for β-actin using anti-actin CP01 primary and IgM anti-mouse J1200 secondary antibodies (Calbiochem, La Jolla, CA). To determine the PAI-1 protein level in muscle homogenates by enzyme-linked immunosorbent assay (ELISA), we used muscle from mice where blood was flushed out with saline by means

of abdominal aortic cannulation and cutting of the vena cava. To determine the PAI-1 protein level in plasma, we centrifuged systemic whole blood and used PAI-1 ELISA (for either total (all PAI-1 present in the sample including complexed with t-PA or u-PA) or active (only biologically active PAI-1 in the sample) PAI-1). PAI-1 ELISA kits were purchased from Innovative Research (Novi, MI). Finally, the plasmin anti-plasmin (PAP) complex level in plasma was measured with an ELISA kit from Cusabio Biotech (Newark, NJ).

2.2.4 Fibrin zymography

We used modified fibrin zymograph gels to examine the PAI-1 protein activity in skeletal muscle and liver tissues. Typically, the gel is prepared to include fibrinogen, plasminogen, thrombin and u-PA to induce plasmin-mediated fibrin degradation. The tissue sample is then run on the gel and the capacity of the PAI-1 in the sample to inhibit the u-PA-induced fibrin degradation is assessed (5). In the present study, instead of embedding u-PA in the gel, we mixed a known amount of u-PA with the sample and incubated the mixture for 5 minutes. We then ran this mixture, and u-PA alone, on separate lanes on the gel and assessed PAI-1 activity in the sample by comparing fibrin degradation caused by the mixture against degradation caused by u-PA alone. Again, the PAI-1 activity in the sample was revealed by inhibition of u-PA-induced fibrin degradation.

Briefly, skeletal muscle and liver tissues were homogenized in Tris-NaCl-Triton-EDTA (TNTE) buffer. The homogenate (40 µl of total volume) including 50 µg of protein, 20 µl of non-denaturing loading buffer and 0.001 U of u-PA (all from Sigma-Aldrich) was resolved on a 10% SDS PAGE gel. To confirm the presence of fibrin in the gel (through its digestion), and as a method of standardization, 40 µl of homogenization buffer, 20 µl of non-denaturing loading buffer and 0.001 U of u-PA were mixed together and loaded in an empty lane on the gel. The gel contained 1.2 mg/ml human fibrinogen (from Calbiochem), 0.06 U/ml human plasminogen and 0.25 U/ml human thrombin (both from Sigma-Aldrich). As human and mouse fibrinolytic factors have been demonstrated to

cross-react (5), human factors were used as a substitute for the extremely limited availability of comparable mouse fibrinolytic factors. The SDS in the gel was exchanged with 2.5% Triton X-100 for 1 h. The gel was then incubated in developing buffer (30 mM Tris, 200 mM NaCl, pH 7.4) for 24 h (all from Sigma-Aldrich). The gel was stained with Coomassie blue R-250 (Bio-Rad) staining solution for 20 minutes, then destained and imaged on the Bio-Rad GS-690 imager. Densitometry was completed using the Multi-Analyst 1.1 software package (Bio-Rad).

2.2.5 Platelet count in septic blood

We used heparanized blood samples diluted 200-fold in saline, labeled platelets therein with rhodamine 6G (0.4 µg/mL, Sigma-Aldrich) or calcein-AM (8 µM, Sigma-Aldrich) and counted them in a hemocytometer chamber under a microscope.

2.2.6 Thrombelastography (TEG)

Concurrently with the platelet count, TEG was used to measure coagulability in systemic blood. An aliquot of 360 µL of the heparinized systemic blood was added to a pre-warmed heparinase cuvette of the TEG machine (type 5000, Hemonetics, Braintree, MA) and analyzed according to manufacturer's instructions. In principle, TEG measures the developed mechanical strength of the formed blood clot after the onset of clotting, and it yields parameters reflecting the speed of clotting and the maximal clot strength (13).

2.2.7 Statistical analysis

Data are presented as mean ± SE; n indicates the number of mice per treatment. One skeletal muscle or liver sample was used per mouse. Data were analyzed using one-way ANOVA (repeated measures ANOVA for *ex vivo* model) followed by two-tail test with Bonferroni correction for multiple comparisons, with a level of significance of $p < 0.05$.

2.3 RESULTS

2.3.1 Effect of streptokinase on capillary plugging

Intravital microscopy at 7 h post-FIP showed that sepsis markedly increased the number of stopped-flow capillaries in the skeletal muscle (Figure 2.1). Streptokinase, a putative pro-fibrinolytic agent, reduced this number, suggesting that fibrinolysis plays a role in the restoration of capillary blood flow. Since ascorbate similarly reduced the number of stopped-flow capillaries (Figure 2.1), we pursued the testing of our hypothesis that ascorbate has a pro-fibrinolytic effect in the septic microvasculature. In principle, ascorbate could achieve this effect by elevating the local t-PA and u-PA levels and/or by inhibiting the local PAI-1.

2.3.2 Effect of sepsis on pro-fibrinolytic factors in the skeletal muscle

Sepsis did not statistically alter t-PA mRNA in skeletal muscle homogenate when compared to control; ascorbate did not affect the septic level of t-PA mRNA ($P = 0.15$, Figure 2.2a). Sepsis increased u-PA mRNA expression in muscle but ascorbate failed to alter this increased level (Figure 2.2b). This lack of effect of ascorbate was inconsistent with our hypothesized pro-fibrinolytic effect of ascorbate via increased u-PA level alone. Similar to the muscle homogenate, there was no effect of sepsis and ascorbate on t-PA mRNA in freshly isolated endothelial cells (major source of pro- and anti-fibrinolytic factors) (Figure 2.2a). Sepsis increased u-PA mRNA in the endothelial isolates, but cells from septic mice treated with ascorbate failed to alter this increase (Figure 2.2b).

2.3.3 Effect of sepsis on the anti-fibrinolytic factor PAI-1

In muscle homogenates, sepsis increased PAI-1 mRNA when compared to control; ascorbate inhibited this increase (Figure 2.3a). In endothelial isolates, no effects of sepsis
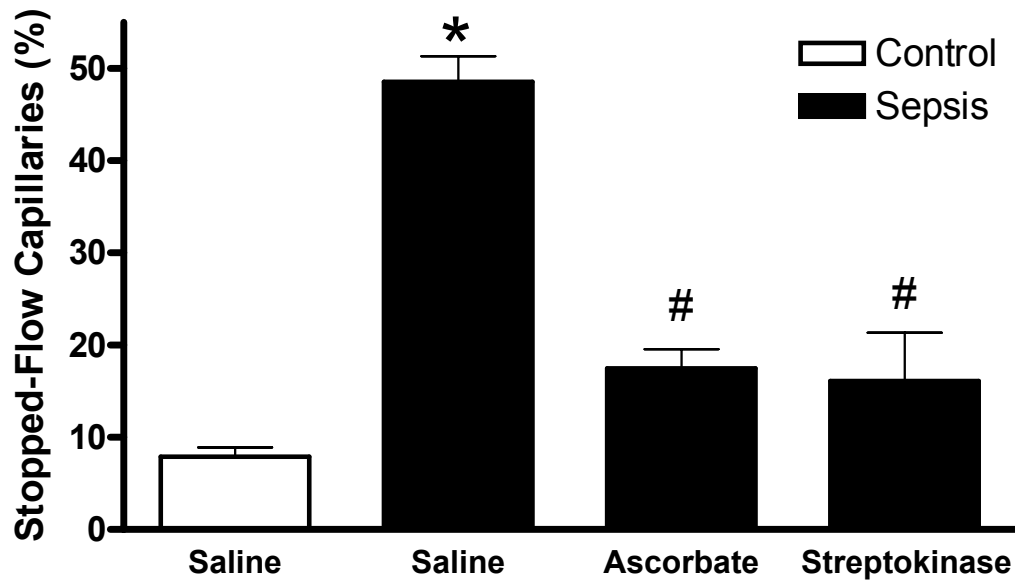
Figure 2.1 Ascorbate and streptokinase reduce the sepsis-induced increase in stopped-flow capillaries. Sepsis was induced by fecal injection into peritoneum (FIP). At 7 h post-FIP, the mouse hindlimb skeletal muscle capillary perfusion was observed using intravital microscopy. There was an increase in stopped-flow capillaries in septic mice. Ascorbate (10 mg/kg) or streptokinase (25000 U/kg), but not saline injected intravenously at 6 h reduced the blood flow stoppage in capillaries. * compared to control, # compared to sepsis+saline group, $P < 0.05$, n = 7 - 9 mice/group.
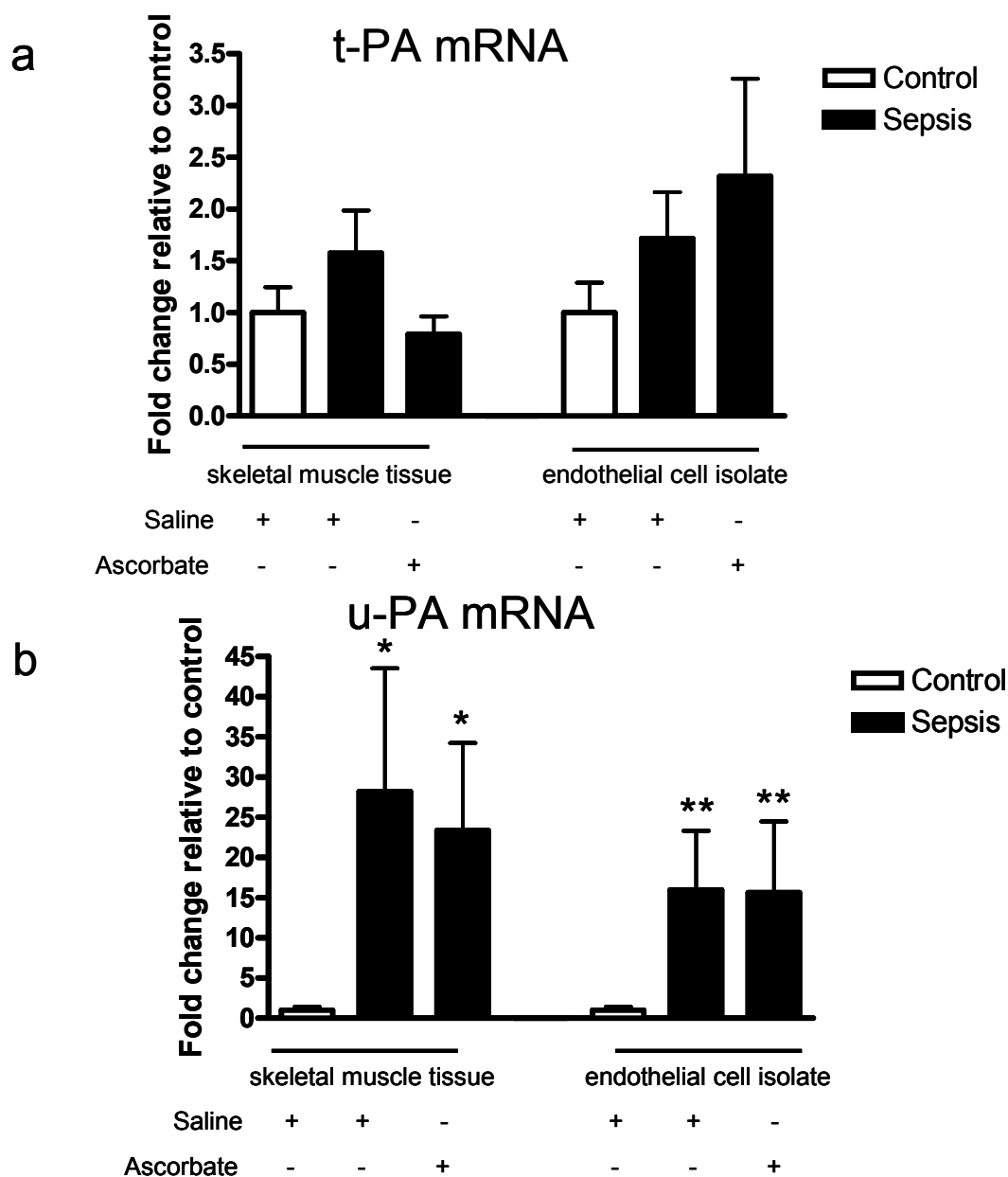
Figure 2.2. Effect of sepsis and ascorbate on t-PA and u-PA mRNA expression in mouse skeletal muscle. Panel a: Sepsis at 7 h did not affect t-PA mRNA in muscle homogenates or in endothelial isolates from this muscle. Panel b: Sepsis increased u-PA mRNA in the muscle and in cell isolates. Ascorbate injected at 6 h did not affect t-PA and u-PA mRNA levels. * P < 0.05 compared to control, ** P < 0.05 compared to cell isolate control, n = 8 - 11 mice/group for t-PA, 6 - 7/group for u-PA , and 6 - 7/group for cell isolates (both panels).
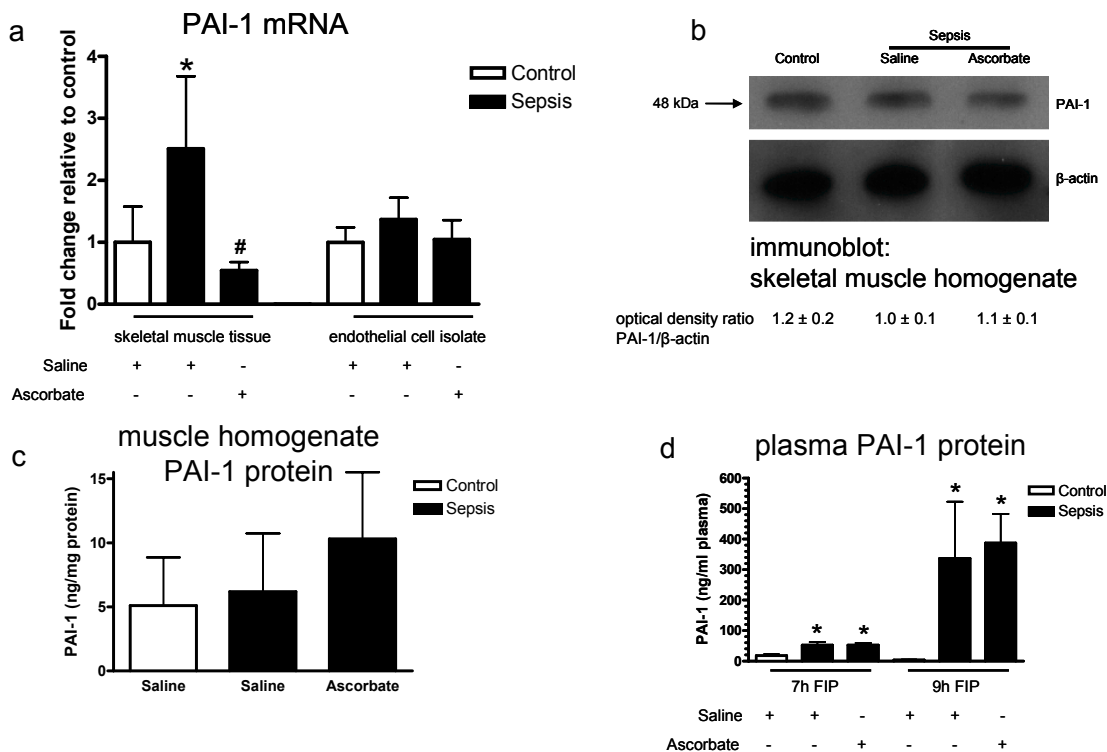
Figure 2.3. Effect of sepsis and ascorbate on PAI-1 mRNA in mouse skeletal muscle, and on PAI-1 protein in muscle and blood plasma. Panel a: Ascorbate injected at 6 h inhibited the sepsis-induced increase in PAI-1 mRNA expression in muscle homogenates at 7 h. Based on western blot analysis (Panel b) or ELISA (Panel c), neither sepsis nor ascorbate affected PAI-1 protein expression in muscle homogenates. Panel d: Sepsis increased PAI-1 protein in systemic blood plasma analyzed by ELISA at 7 and 9 h post-FIP. Ascorbate injected at 6 h did not affect these PAI-1 protein increases. * $P < 0.01$ compared to control, # compared to sepsis+saline group, n = 8 - 9 mice/group for PAI-1 homogenates, 3/group for immunoblots, 4 -5/group for ELISA, and 5 - 7/group for plasma.

or ascorbate were observed (Figure 2.3a). Western blot and ELISA protein analyses of muscle homogenates showed no increase in PAI-1 protein expression in sepsis and no effect of ascorbate on protein levels (Figures 2.3b and 2.3c). Consistently, fibrin zymography of muscle homogenates showed no effect of sepsis and ascorbate on inhibition of u-PA-induced fibrin degradation (Figure 2.4a). However, total blood plasma PAI-1 protein analysis by ELISA revealed a nearly 3-fold increase in PAI-1 in septic mice at 7 h, and a 30-fold increase at 9 h. Ascorbate had no effect on these increases (Figure 2.3d). ELISA analysis of activated PAI-1 in septic mice at 7 h (See Chapter 3, Figure 3.3) showed a significant increase comparable to that of total PAI-1 protein shown in Figure 2.3d.

2.3.4 PAI-1 expression in the liver

Similar to published reports on PAI-1 mRNA in various organs (27, 35), our preliminary data in the septic mouse lung, heart, liver and kidney indicated that the liver is a major source of PAI-1 (See Chapter 3, Figure 3.2). Indeed, sepsis increased PAI-1 mRNA in the liver 6-fold, but only 2.5-fold in the skeletal muscle (Figures 2.3a, 2.6a). Ascorbate did not affect the PAI-1 mRNA expression in the liver (Figure 2.6a). Almost identical to the muscle western blot data (Figure 2.3b), our pilot western blots from liver homogenates showed no effect of sepsis or ascorbate on PAI-1 protein level (Figure 2.5). However, fibrin zymography of liver homogenates showed that sepsis increased the inhibition of u-PA-induced fibrin degradation, while ascorbate failed to alter this effect (Figure 2.4b). Consistent with the dominance of liver in total PAI-1 production during sepsis, the liver homogenates showed a greater inhibition of u-PA-induced fibrin degradation than muscle homogenates (Figure 2.4c).

2.3.5 Pro-fibrinolytic factor expression in the liver

In parallel to the increase in PAI-1 mRNA, t-PA mRNA and u-PA mRNA also increased in the septic liver (Figures 2.6b, 2.6c). In contrast to the muscle, ascorbate inhibited the
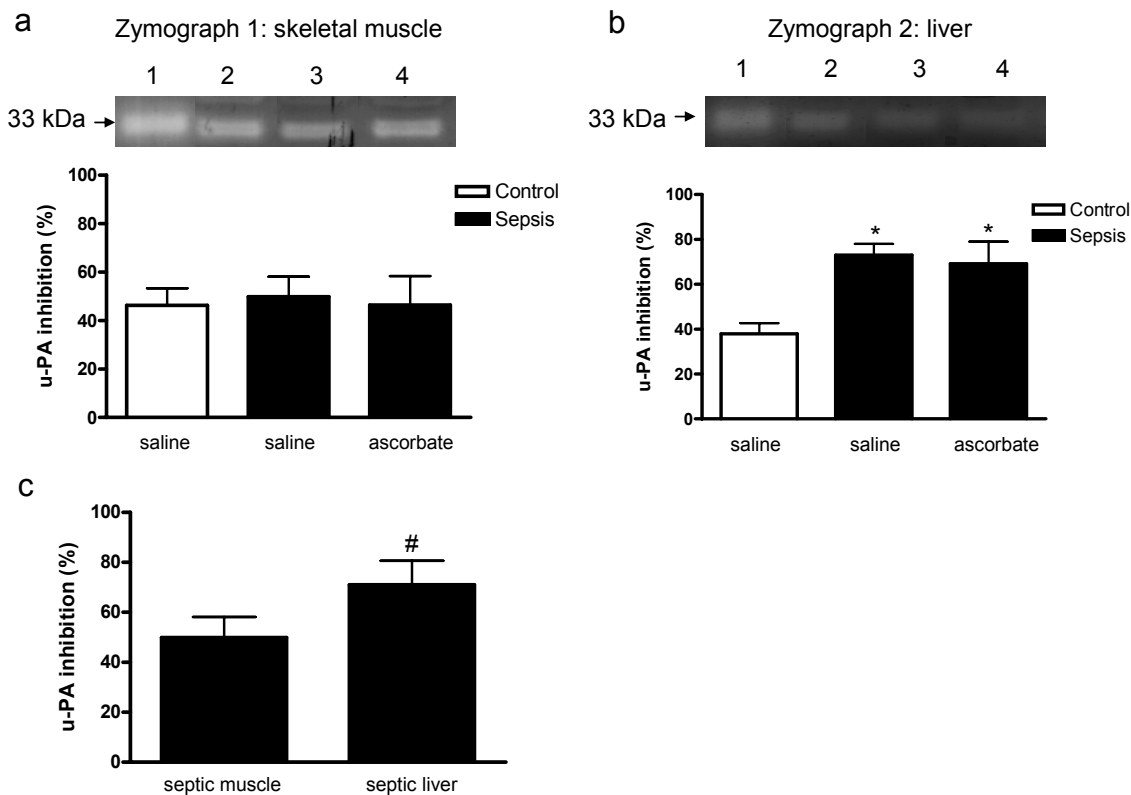
Figure 2.4. Effect of sepsis and ascorbate on PAI-1 protein activity in mouse skeletal muscle and liver analyzed by fibrin zymography. Panel a: Zymograph 1 shows an example of a fibrin zymograph gel used for analysis of PAI-1 activity in skeletal muscle homogenates. In lane 1, exogenous u-PA in loading buffer (40 μl total volume) was run on the gel. This u-PA resulted in plasmin-mediated fibrin degradation in the gel, seen as a bright band at 33 kDa. In lane 2, the control mouse muscle homogenate was mixed with the same amount of exogenous u-PA, and the mixture was run on the gel. When compared with lane 1, the band in lane 2 was dimmer, indicating inhibition of u-PA-induced fibrin degradation (i.e., a measure of inhibition of exogenous u-PA by PAI-1 present in the muscle sample). We used densitometry to set u-PA inhibition to 0 % when the sample band brightness was equal to that in lane 1, and to 100 % when the sample band brightness was equal to that of gel background. In lanes 3 and 4, we mixed u-PA with septic muscle homogenate from mice injected at 6 h with saline or ascorbate, respectively. Below zymograph 1, the 3 bars show a comparable u-PA inhibition among muscles from control, sepsis+saline and sepsis+ascorbate groups. Panel b: Zymograph 2 shows an example of a fibrin zymograph in liver homogenates. With reference to lane 1,

a larger u-PA inhibition was seen in septic liver (lane 3) than in control liver (lane 2). Ascorbate treatment did not alter the sepsis-induced increase in u-PA inhibition. To compare PAI-1 activity between the muscle and liver (sepsis+saline groups), we ran an extra lane with liver in zymograph 1 (lane not shown) and compared this lane and lane 3 with reference to lane 1. Panel c summarizes this comparison, showing a larger u-PA inhibition for septic liver than muscle. In each zymograph, the lanes were not run adjacent to each other. The spaces between lanes were cropped. * $P < 0.05$ compared to control, # $P < 0.05$ compared to septic muscle group, n = 5 - 6 mice/group.
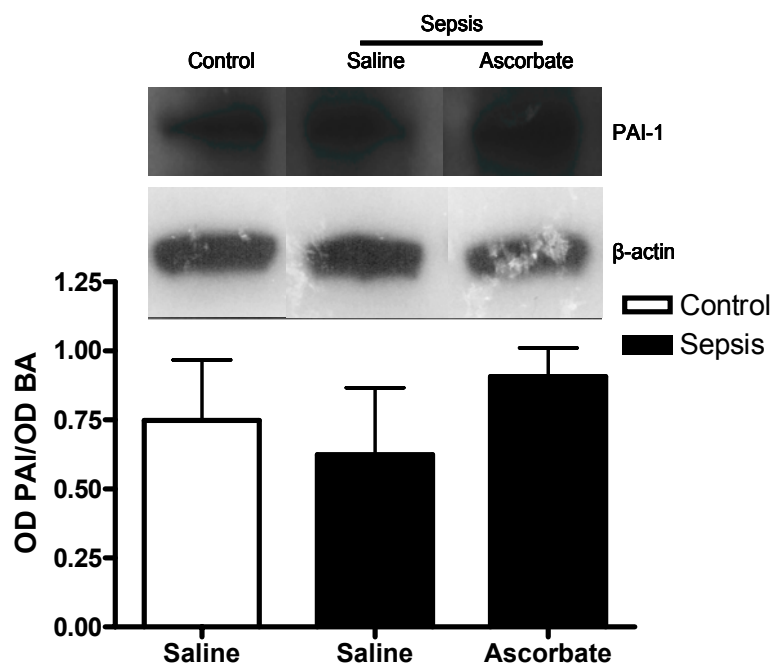
Figure 2.5. Effect of sepsis and ascorbate on the PAI-1 protein expression in the liver. In a pilot experiment, mice were injected with saline or ascorbate at 6 h post-FIP. Neither sepsis nor ascorbate affected PAI-1 protein expression by western blotting in liver homogenates. In the western blot, the lanes were not run side-by-side and therefore the lanes inbetween were cropped. n = 2-3 mice/group.

a

liver PAI-1 mRNA

b

liver t-PA mRNA

c
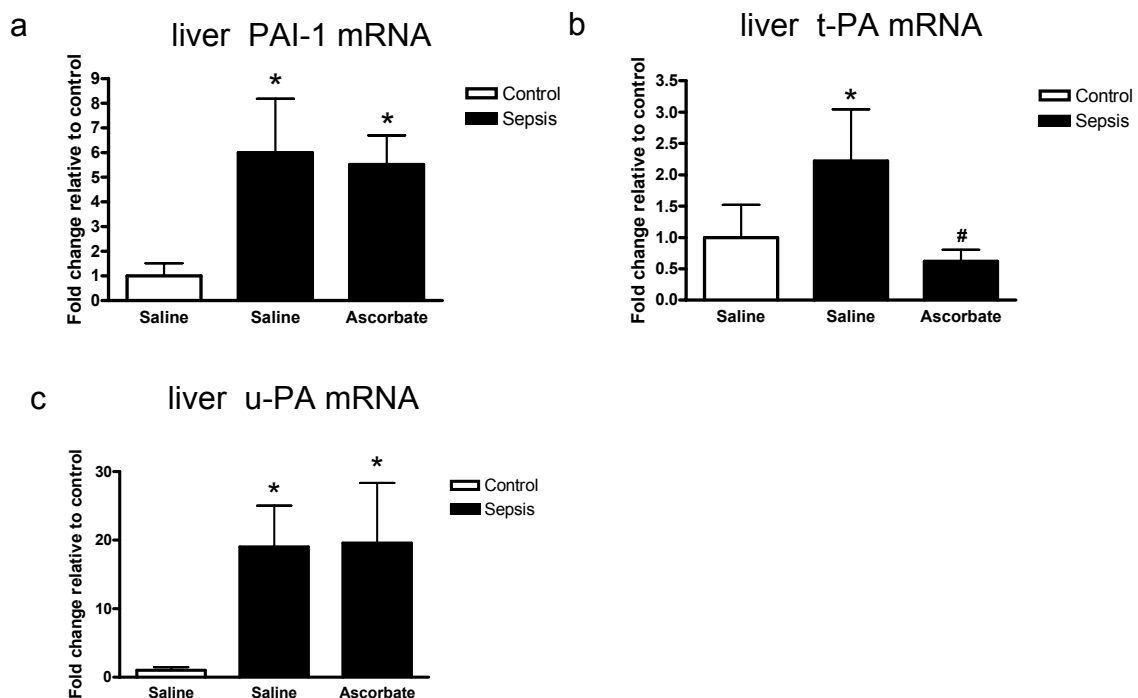
liver u-PA mRNA

Figure 2.6. Effect of sepsis and ascorbate on PAI-1 (Panel a), t-PA (Panel b), and u-PA (Panel c) mRNA expression in mouse liver. Sepsis increased PAI-1, t-PA, and u-PA mRNA expression. Ascorbate inhibited the increase in t-PA expression. * P < 0.05 compared to control, # P < 0.05 compared to sepsis+saline group, n = 6 - 7 mice/group for PAI-1, 7 - 9/group for t-PA, and 7 - 9/group for u-PA.

sepsis-induced increase in t-PA mRNA in the liver (Figure 2.6b). However, the sepsis-induced increase in u-PA mRNA was unaffected by ascorbate in the liver (Figure 2.6c).

2.3.6 Plasmin anti-plasmin expression in the septic blood

To determine whether the parallel increase in pro- and anti-fibrinolytic factors in liver mRNA resulted in an overall pro-fibrinolytic or anti-fibrinolytic effect, we used ELISA to measure the protein level of the PAP complex (an index of fibrinolysis by plasmin) in the systemic blood samples. Sepsis dramatically reduced the PAP level; ascorbate did not affect this response (Figure 2.7).

2.3.7 Alterations in platelet count in the septic blood

Analysis of systemic blood revealed a significant sepsis-induced 34 % drop in platelet count; ascorbate injection increased the platelet count by 23% when compared to the sepsis+saline group (Figure 2.8).

2.3.8 Thrombelastography of septic blood

Finally, we used TEG to measure coagulability in systemic arterial blood. Sepsis reduced the maximal clot strength at 6 h. Ascorbate injected at 5 h did not alter this clot strength (Figure 2.9 and Table 2.1).

PAP
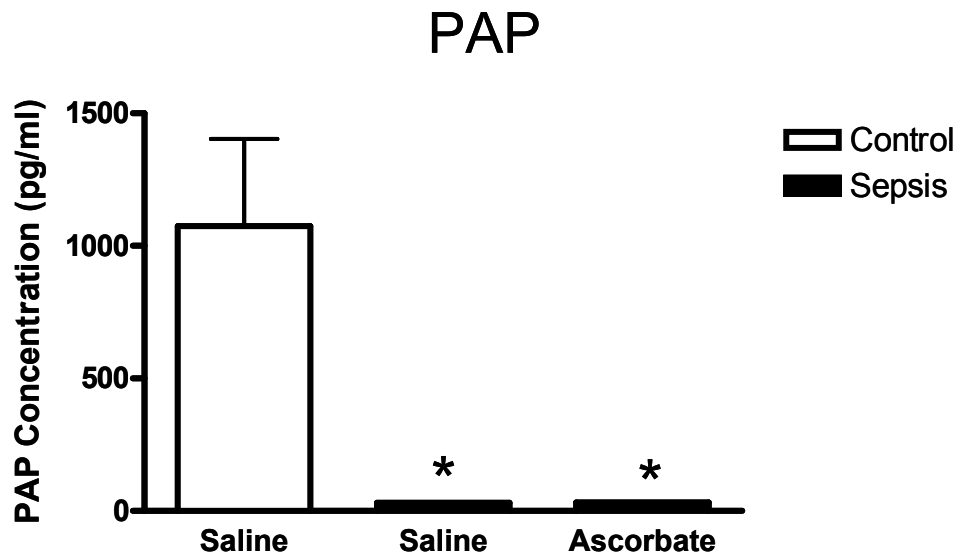
Figure 2.7. Effect of sepsis and ascorbate on plasmin-antiplasmin (PAP) in systemic blood. Sepsis reduced PAP. Ascorbate did not affect this reduction. * P < 0.05 compared to control, # P < 0.05 compared to the sepsis+saline group, n = 6 mice/group.
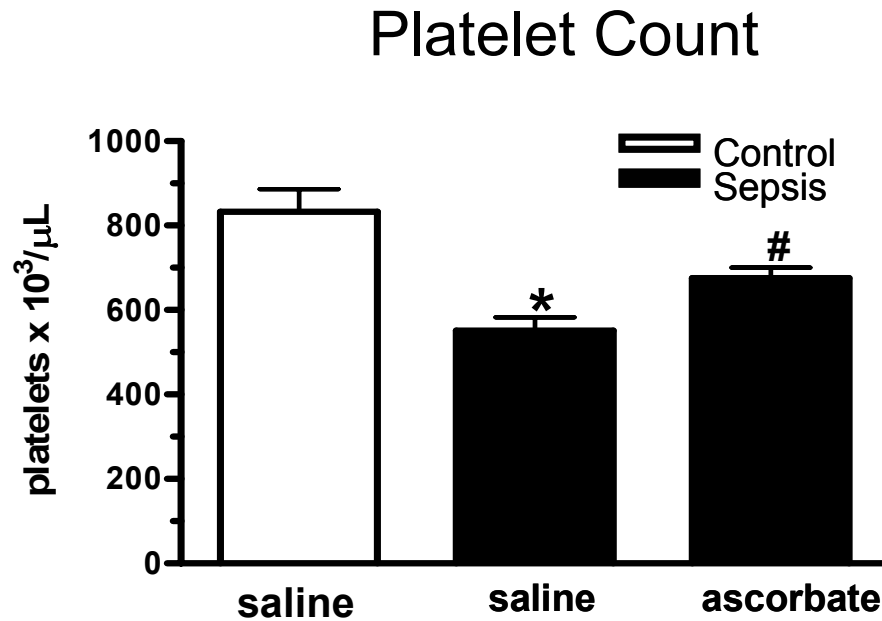
Platelet Count

Figure 2.8. Effect of sepsis and ascorbate on platelet count. Sepsis reduced the platelet count in mice. Ascorbate partially restored the platelet count. * P < 0.05 compared to control, # P < 0.05 compared to the sepsis+saline group, n = 8 mice/group for platelet count.
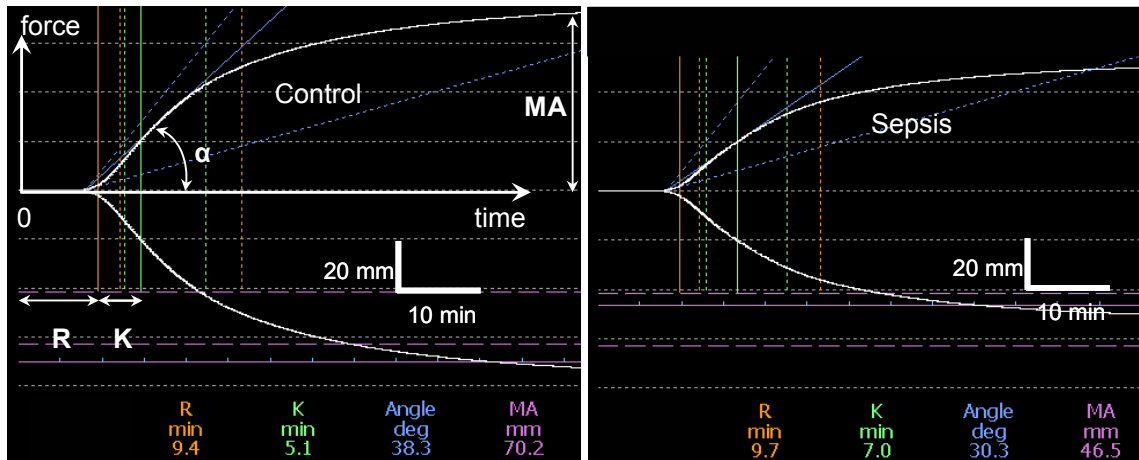
Figure 2.9. Examples of thrombelastography tracings from systemic blood samples obtained from control mice and septic mice at 6 h where saline was injected intravenously at 5 h. R is the reaction time to initial clot formation, K is the kinetic time from the beginning of clot formation until the amplitude reaches 20 mm, α angle reflects the initial rate of force development (i.e., clotting speed) and MA is the maximum amplitude of clot strength.

Table 2.1. Summary of thrombelastography parameters from control, sepsis+saline, and sepsis+ascorbate groups (mean ± SE). The TMRTG and MRTG have been estimated from the TEG tracing.

	R (min)	K (min)	α angle (degrees)	MA (mm)	G (kd/s)	TMRTG (min)	MRTG (100*mm/s)
control (n=5)	13 ± 3	7 ± 1	33 ± 4	66 ± 2	10 ± 1	6 ± 2	6 ± 1
sepsis+saline (n=5)	17 ± 8	13 ± 5	23 ± 7	46 ± 5 *	5 ± 1 *	11 ± 6	5 ± 2
sepsis+ascorbate (n=5)	20 ± 5	11 ± 5	25 ± 6	47 ± 6 *	5 ± 1 *	7 ± 1	5 ± 1

* $P < 0.05$ compared to control. R - reaction time to initial clot formation, K - kinetic time from the beginning of clot formation until the amplitude reaches 20 mm, α angle - initial rate of force development, MA – maximum amplitude, G – shear elastic modulus strength, TMRTG – time to maximum rate of thrombus generation, MRTG - maximum rate of thrombus generation

2.4 DISCUSSION

The present study employed a model of severe sepsis (80 % mortality at 24 h, (30)) associated with a marked drop in platelet count and systemic blood hypo-coagulability. The study examined for the first time the capillary bed perfusion in skeletal muscle in the context of consumption coagulopathy (i.e., DIC), including the role of fibrinolytic factors produced in the muscle and a mechanism of an antioxidant defense against capillary plugging through enhanced fibrinolysis. Analysis of the muscle tissue mRNA indicated that the antioxidant ascorbate did not affect the t-PA and u-PA levels in sepsis but it inhibited the PAI-1 level, resulting in a net pro-fibrinolytic effect in sepsis at the mRNA level. However, analyses of muscle and liver homogenates, and of systemic blood in septic mice, indicated no effect of ascorbate on (i) PAI-1 protein or its enzymatic activity, (ii) PAP protein, and (iii) blood hypo-coagulability assessed by TEG. Together, these results did not support the hypothesis that ascorbate promotes fibrinolysis in sepsis.

2.4.1 Disseminated intravascular coagulation in the capillary bed

DIC, a clinical condition in severe sepsis, has been explained by consumption coagulopathy where platelets and coagulation factors are depleted from systemic blood, resulting in a high risk for bleeding (11). Importantly, decreasing systemic platelet count correlates with increasing mortality rate (17). To our knowledge, DIC has not been examined directly at the level of capillary bed. There is qualitative histological evidence of fibrin deposition in arterioles (11), but such deposition may not lead to stopped blood flow and trapping of coagulation factors in the septic microvasculature. We reported that sepsis stops flow in capillaries but not in the feeding arterioles or draining venules (25). Therefore, the capillary bed may represent a unique micro-environment in the context of DIC.

2.4.2 Methodological considerations

Studies of sepsis are difficult given the complexity of the septic process itself (14) and the experimental shortcomings of animal models of sepsis. A considerable challenge is the animal-to-animal variability when determining the various end-points in sepsis (e.g., PAI-1 mRNA (7)). In the present study we aimed to reduce the variability of the fibrinolytic factors by studying septic mice whose capillary blood flow stoppage was at least 30 % (i.e., one standard deviation below the mean of 48 %, Figure 2.1). In spite of this step, the present study showed a large variability in fibrinolytic factors, including the variability in PAI-1 mRNA and protein expression (i.e., 5 to 7-fold differences between minimal and maximal values in particular groups, precluding reaching statistical significance between groups). This level of variability has been reported for mRNA of fibrinolytic factors (7). The Ct values for the muscle and liver PAI-1 and β-actin were 23.4 ± 0.3, 18.6 ± 0.7, 24.3 ± 0.6 and 18.0 ± 0.9, respectively. The β-actin levels were observed to be similar between sham and septic groups.

2.4.3 Pro-fibrinolytic factors in tissue

Little is known about the pro-fibrinolytic factors in the septic microvasculature. The present data from muscle and liver (Figures 2.2a, 2.6b) agree with reports that sepsis either increases or causes no change in t-PA mRNA and protein levels in mice and rats (16, 21). The presently observed u-PA mRNA increases in muscle and liver homogenates of septic mice also agree with lipopolysaccharide (LPS)-induced increases in u-PA mRNA in various tissues in mice (35) and in sepsis-induced increases in u-PA plasma protein levels in humans (23).

2.4.4 Anti-fibrinolytic factors in tissue

Increased anti-fibrinolytic PAI-1 mRNA and protein expression have been reported for both septic patients (18) and animal models of sepsis (35). Our PAI-1 mRNA/ELISA protein data from plasma agree with these reports, but our PAI-1 protein data based on

western blot/ELISA analysis of tissue homogenates do not. We explain these data by noting that PAI-1 protein could be rapidly externalized and shed into blood stream (19), and/or released into blood from platelets (3), making the sepsis-induced increase in PAI-1 protein preferentially detectable in blood plasma rather than in tissue.

We used the fibrin zymography as an alternative approach to study the effect of sepsis on PAI-1 protein function. This approach does not measure PAI-1 activity directly, but rather it assesses the ability of PAI-1 in the homogenate to inhibit u-PA-induced fibrin degradation in the gel (10). Our zymographs showed that sepsis increased the inhibition of u-PA in the liver but not muscle (Figures 2.4a and 2.4b). This is consistent with the dominant role of liver in PAI-1 production during sepsis. However, in view of the data in Figure 2.4b, it is puzzling why western blot analysis failed to detect a sepsis-induced increase in PAI-1 protein even in this dominant organ. When considering the large change in PAI-1 mRNA detected by qPCR in the liver (Figure 2.6a), it is possible that the western blot in this tissue may not have been sensitive enough to detect the corresponding change at the protein level. Thus, the modest increase seen in PAI-1 mRNA in the muscle (Figure 2.6a) might have resulted in a corresponding protein change undetectable by western blot or zymography (Figure 2.4a). Regarding the discrepancy between the effect of ascorbate on PAI-1 mRNA and protein expression, I discuss the possibility of a delay of the effect of ascorbate on the protein expression in blood plasma in section 5.2.4.

2.4.5 Pro- versus anti-fibrinolytic factors in septic systemic blood

Sepsis increases both u-PA and PAI-1 plasma protein levels (9). In humans injected with tumor necrosis factor (TNFα), the plasma PAP level rises but then it returns towards control (32), indicating a transient increase in fibrinolysis by plasmin in systemic blood. This transient characteristic of PAP could be due to the delayed surge of PAI-1 (31), the inhibitor of u-PA (9). In the present study we found that PAP level was markedly reduced at 7 h of sepsis (Figure 2.7), indicating that the large increase in plasma PAI-1 protein (Figure 2.3d) precipitated inhibition of fibrinolysis in systemic blood at this time.

2.4.6 Effect of ascorbate on fibrinolytic factors in sepsis

We observed that ascorbate did not affect the sepsis-induced u-PA mRNA increase in the muscle or liver. However, ascorbate inhibited the sepsis-induced PAI-1 mRNA increase in the muscle but not liver. These findings are consistent with a pro-fibrinolytic effect of ascorbate in muscle (i.e., maintenance of elevated u-PA and lowering its inhibitor PAI-1). However, analyses of PAI-1 protein/enzymatic activity in muscle and liver homogenates, and of systemic blood in septic mice, indicated no effect of ascorbate. Thus, the protein data do not support the hypothesis that ascorbate promotes fibrinolysis during sepsis.

2.4.7 Hyper-coagulation in the microcirculation versus hypo-coagulation in systemic circulation in sepsis

Severely septic patients with DIC present with a low platelet count (17), hypo-coagulability of systemic blood as assessed by TEG, and high risk for bleeding (28). The present septic mice also showed lowered platelet count and TEG hypo-coagulability (Figures 2.8, 2.9 and Table 2.1). However, these observations disagree with our recent report of hyper-coagulability of blood in capillaries of skeletal muscle in septic mice, where increased platelet adhesion, fibrin deposition and propensity to form micro-thrombi in these capillaries were noted (25). This apparent disagreement could be explained in terms of the concept of DIC where platelets and coagulation factors are trapped in the microcirculation and thereby depleted from systemic blood which then inevitably becomes hypo-coagulable. To our knowledge, however, this possible redistribution between micro- and macrocirculation during sepsis has not been addressed experimentally. There is some indirect evidence for this redistribution. Fisher and coworkers reported that FIP-injected mice demonstrated severe hypo-coagulability of systemic blood (TEG, prothrombin time, activated partial thromboplastin time measures), and that ascorbate injection completely protected against this hypo-coagulability (6).

In the present study, ascorbate reversed the low platelet count in sepsis toward the control level (Figure 2.8). We interpret these data to indicate that the observed beneficial effect of ascorbate (i.e., restoration of capillary blood flow in sepsis) could be at the level

of platelet adhesion rather than fibrinolysis. Recently, we showed that ascorbate reduces platelet aggregation and expression of the adhesion molecule P-selectin in an *ex vivo* model of sepsis (26). Clearly, there may be other mechanisms whereby ascorbate prevents/reverses capillary plugging (26, 34).

In conclusion, we hypothesized that 10 mg/kg ascorbate injection delayed for 6 h after the onset of sepsis has a fibrinolytic effect in the septic microvasculature. Based on the various approaches used to examine PAI-1 protein levels in plasma, and muscle and liver homogenates we failed to demonstrate any effect of the delayed ascorbate dose on these levels.

2.5 REFERENCES

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Chapter 3

Ascorbate does not alter bacterial content, plasminogen activator inhibitor-1, and myeloperoxidase levels in septic mouse organs

3.1 INTRODUCTION

Sepsis, a systemic inflammatory response to an infection, has many detrimental effects on the cardiovascular system. These include hypotension, reduced vascular reactivity and reduced capillary bed perfusion (19, 28, 31, 32). The precise mechanism of reduced capillary perfusion is unknown, but an activated coagulation system including microthrombi formation in capillaries has been suggested (28, 29). Sepsis elevates PAI-1, which may lead to inhibited fibrinolysis and subsequent stabilization of microthrombi and plugging of capillaries (24, 30, 38). However, data from PAI-1 knockout mice suggest elevated PAI-1 during sepsis may be beneficial as survival from sepsis was reduced in these knockout mice (13, 16). These studies indicate the complexity of the effects of sepsis on the coagulation system in various organs.

Sepsis is associated with marked reduction in plasma ascorbate (reduced form of vitamin C), which has been associated with poor outcome in sepsis (27). Importantly, studies in mouse models of sepsis demonstrated that injection of an ascorbate bolus reduces organ injury and mortality in sepsis (8, 32, 34). We have shown that ascorbate injection prevents and reverses capillary plugging in the septic mouse skeletal muscle by decreasing platelet activation and platelet-endothelial cell adhesion (28, 29, 32). Furthermore, antioxidant therapy in septic rats reduced organ dysfunction from oxidative damage caused by neutrophils, consistent with the beneficial effect of ascorbate (2). However, since ascorbate could alter the coagulation system (14, 29), the question arises as to whether it could negatively affect the beneficial role of PAI-1 in sepsis. For example, Jaulmes and colleagues showed that PAI-1 expression depends on NADPH oxidase activity (14), which can be inhibited by ascorbate (33). Given the apparent requirement for PAI-1 in survival during sepsis (16), ascorbate could thus indirectly reduce survival by inhibiting PAI-1. Also, because PAI-1 is required for normal neutrophil efferocytosis (23) and infiltration (18), inhibition of PAI-1 by ascorbate could compromise the neutrophil-mediated immune response in infected organs. Ascorbate has bactericidal properties at very high levels, raising the possibility that the reduced

mortality observed with ascorbate treatment could be due to reduced bacterial loads (12, 37). However, no apparent bactericidal effect of ascorbate in the peritoneum of septic mice has been reported (34).

To investigate the mechanisms of action of ascorbate, our objective was to determine its effect on bacterial content, PAI-1 levels and neutrophil infiltration in septic mice. Based on the fact that ascorbate reduces mortality in sepsis, and that PAI-1 is needed for survival, we hypothesized here that ascorbate does not alter the PAI-1 expression and neutrophil infiltration in organs with presumed high immune response in sepsis (lung, kidney, spleen, liver), preserving the beneficial effect from PAI-1.

3.2 METHODS

3.2.1 Animal model of sepsis

All experiments were performed with approval of the University of Western Ontario Council on Animal Care. Male C57BL/6 mice were bred in our animal facility from mice purchased from Jackson Laboratory (Bar Harbor, ME) or Charles River (Sherbrooke, QC), and were housed in a controlled environment with a 12 h light/dark cycle and access to food and water ad libitum. The mice used were between 2-3 months old and between 20-25 g in weight. Feces was collected from the cecum of donor mice and mixed in sterile saline at a concentration of 75 mg/ml and kept at 4°C. Mice were anesthetized using a ketamine (80 mg/kg) and xylazine (4 mg/kg) mixture given via intraperitoneal injection. Sepsis was induced in our mice by injecting the feces mixture into peritoneum (FIP) as described previously (32). Alternatively, control sham mice were injected with 1 ml sterile saline intraperitoneally. All mice were fluid resuscitated subcutaneously with 1 ml saline containing 2 µg/ml buprenorphine as an analgesic.

3.2.2 Experimental design

We have reported that intravenous injection of ascorbate delayed to 6 h of sepsis (i.e., when capillary plugging is prevalent) restores capillary blood flow in skeletal muscle by 7 h (28). This restoration of flow (or reversal of plugging) could be due to dissolution of microthrombi through enhanced fibrinolysis due to inhibition of PAI-1 by ascorbate. In the present study, we chose to study the effect of this delayed ascorbate injection. At 6 h post-FIP, mice were anesthetized and then injected intravenously with 0.1 ml of sterile saline or ascorbate (10 mg/kg); they remained under anesthesia for 1 h. Upon completion of the 1 hour treatment time (7 h post-FIP), the blood, peritoneal fluid, lung, kidney, spleen, liver, heart and the extensor digitorum longus skeletal muscle were harvested. We chose lung, kidney, spleen and liver because these organs were suspected to be involved in neutrophil recruitment and immune response during sepsis (1, 36). We chose the skeletal muscle because in our previous studies we focused on the microcirculatory response of this organ during sepsis (28, 32); the heart presented another type of muscle to compare with skeletal muscle.

3.2.3 Tissue and blood collection

All chemicals were from Sigma-Aldrich (Oakville, ON) unless otherwise noted. The carotid artery blood was collected 9:1 in acid citrate dextrose solution while peritoneal fluid was diluted with 2 ml sterile saline and collected to determine the bacterial load. The organs located in the peritoneum were washed twice with sterile saline to remove peritoneal fluid from their exterior. Samples were stored in RNAlater for mRNA analysis, frozen at -80°C for protein analyses, or processed for assessing their bacterial content. Samples were diluted 1:5 in sterile saline, homogenized, serially diluted in 10-fold fashion, and plated on Columbia Blood Agar containing 5% sheep blood (MP0351, Oxoid, Nepean, ON) and grown overnight at 37°C. The bacterial colony forming units (CFU) were counted and normalized to the CFU determined in the peritoneal fluid.

3.2.4 PAI-1 mRNA and protein quantification

mRNA was isolated from homogenized tissues using Trizol (Invitrogen) and chloroform as directed by the manufacturer. The isolated mRNA was reverse transcribed using Superscript II (Invitrogen), then quantified using Quantifast SYBR Green (Qiagen, Mississauga, ON) on a Bio-Rad CFX96 thermal cycler (Bio-Rad, Mississauga, ON). The following primers were used to detect PAI-1: (primers: forward 5'-GACAGCACTGTCAGGGTCCATAG-3' and reverse 5'-GCGGTCCTCCTTCACAAAGCTC-3'). β-actin (primers: forward 5'-TCGTGGGCCGCTCTAGGCACCA-3' and reverse 5'-GTTGGCCTTAGGGTTCAGGGGGG-3') was used as a reference gene. The following cycling protocol was used to amplify the target genes: 95°C for 5 min and then 40 cycles of 95°C for 10 s, 60°C for 30 s and 80°C for 30 s.

Blood in citrate was centrifuged at 6000g for 10 minutes and the plasma retained for active PAI-1 protein measurement by ELISA as directed by the manufacturer (MPAIKT, Molecular Innovations, Novi, MI). PAI-1 enzymatic activity was determined by reverse fibrin zymography (5, 17). Briefly, 10% SDS PAGE gels were impregnated with human fibrinogen (1.2 mg/ml, Calbiochem), 0.06 U/ml human plasminogen and 0.25 U/ml human thrombin. Tissues were homogenized in Tris-NaCl-Triton-EDTA buffer, 100 μg of protein was incubated for 5 minutes with 0.001 U exogenous u-PA, mixed with 20 μl of non-reducing loading buffer and run on the SDS PAGE gel. The SDS in the gel was substituted with 2.5% Triton X-100 for 1 h, then developed for 24 h in developing buffer (30 mM Tris, 200 mM NaCl, pH 7.4). Gels were stained with Coomassie blue then destained and densitometric analysis was conducted using Multi-Analyst 1.1 software (Bio-Rad).

3.2.5 Neutrophil infiltration in organs

Neutrophil infiltration in organs was measured using a myeloperoxidase (MPO) assay, which is a conventional method for assessing this infiltration (22). Frozen tissues were homogenized in 20 mM potassium phosphate buffer and centrifuged at 6000g for 20

minutes. The supernatant was then discarded and the pellet was resuspended in 50 mM acetic acid with 0.5% hexadecyltrimethylammonium hydroxide detergent. The samples were then sonicated for 10 s and centrifuged for 5 minutes. The supernatants were mixed with a 1 mM TMB and 0.2 M acetic acid solution, warmed to 37°C, and the reaction initiated with the addition of 3 mM H_2O_2. The samples were left to react for 5-30 minutes (depending on the tissue), then quenched using 1000 U/ml beef catalase and 0.2 M acetic acid. Spectrophotometric analysis was conducted using the Model 680 microplate reader and software version 5.2.1 (Bio-Rad) at 655 nm and compared to an MPO standard.

3.2.6 Statistical analysis

Data are presented as mean ± SE. One sample of each organ was used per mouse. Statistical analysis was conducted using one-way ANOVA and the Bonferroni multiple comparison test. Results were deemed significant with a $P < 0.05$.

3.3 RESULTS

3.3.1 Bacterial content

Samples from sham peritoneal fluid showed zero or very low CFUs (0.002 ± 0.002 CFU/μl (n = 13)), most likely resulting from occasional external contamination during sample collection/handling. Septic mice had peritoneal fluid bacterial counts of 40,300 ± 19,100 CFU/μl (n = 14). We noted about a 7,000 fold range (with respect to the minimum and maximum counts) in these counts despite injecting each mouse with the same amount of feces. When feces were grown separately on agar gels, the bacterial counts were 570 ± 190 CFU/μl of feces suspension (70 fold range with respect to the minimum and maximum counts). Ascorbate injection at 6 h post-FIP did not significantly alter the peritoneal fluid bacterial count. We observed that bacterial counts

were significantly increased in septic mouse blood and all organs compared to sham mice, with the highest mean count observed in the spleen and lowest in the muscle (Figure 3.1). Ascorbate significantly lowered the bacterial count in the blood only.

3.3.2 PAI-1 mRNA expression and enzymatic activity

In control sham mice, the individual mRNA levels in organs were normalized to the mean mRNA level found in each organ. Thus the normalized control levels were 1 ± 0.18 (lung), 1 ± 0.29 (kidney), 1 ± 0.16 (spleen), 1 ± 0.76 (liver), 1 ± 0.16 (heart), and 1 ± 0.14 (muscle). Figure 3.2 shows the fold increase in PAI-1 mRNA expression (relative to the control level) in septic organs from mice treated with saline or ascorbate at 6 h post-FIP. We observed sepsis-induced increases in PAI-1 in the lung, kidney, spleen, liver and skeletal muscle. Ascorbate lowered this increase only in the muscle. Using an active PAI-1 ELISA, we determined that sepsis significantly increased the blood plasma active PAI-1 protein; ascorbate did not affect this increase (Figure 3.3). Our preliminary experiments showed that the ELISA or western blotting approaches were insensitive to detect changes in PAI-1 protein in the organs. For this reason, we used reverse fibrin zymography to indirectly measure PAI-1 enzymatic activity in an organ sample. The PAI-1 activity in the sample mixed together with exogenous u-PA was assessed by the degree of inhibition of u-PA in terms of the degree digestion of the fibrin zymograph (Figure 3.4). Figure 3.5 shows that sepsis increased u-PA inhibition in the lung, heart and liver samples, indicating increased PAI-1 activity in these organs. Sepsis did not affect u-PA inhibition in the kidney, spleen and skeletal muscle. Ascorbate did not affect the sepsis induced changes in u-PA inhibition in any organs (Figure 3.5).

3.3.3 Neutrophil infiltration in organs

Figure 3.6 shows that sepsis increased the MPO levels in the lung and liver, while the level was reduced in the spleen. No change in the MPO level was observed in the heart,
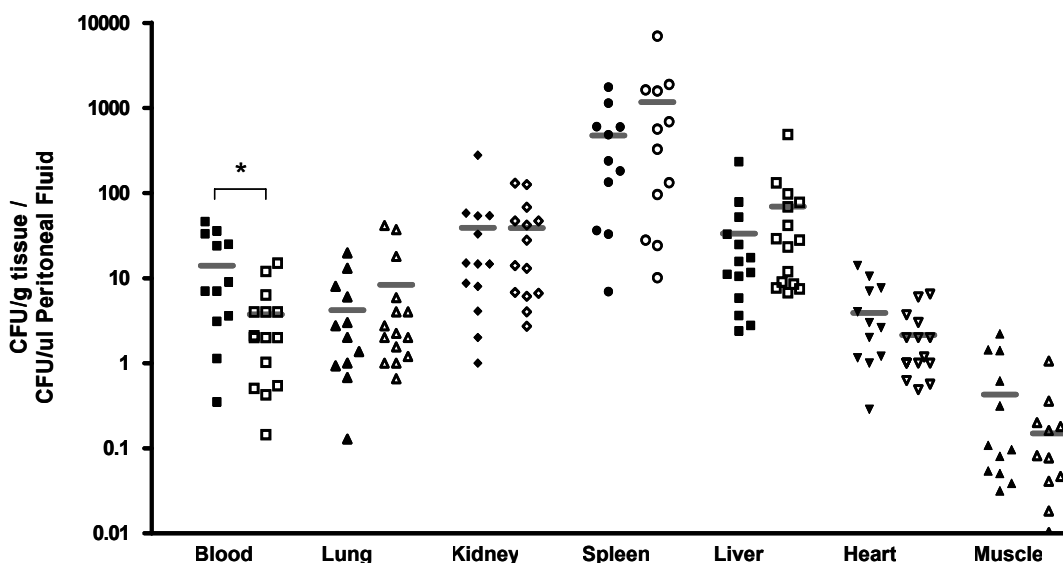
Figure 3.1. Increased bacterial content in septic mouse organs is not altered by ascorbate. Sepsis was induced by feces injection into the peritoneum (FIP). At 6 h mice were injected i.v. with saline or ascorbate. At 7 h post-FIP, the peritoneal fluid and carotid artery blood were collected, the organs harvested and homogenized, and then plated on Columbia blood agar and cultured for 18 h. Bacterial content in fluids and organs in control sham mice was either zero or negligibly small (data not shown). Bacterial content in all septic fluids and organs significantly increased compared to control (P < 0.001). Closed symbols are those of sepsis+saline mice, whereas open symbols are those of sepsis+ascorbate mice. The bacterial content in septic blood was significantly reduced by ascorbate. Several organ bacterial counts were calculated to be 0 and are not included in the figure: two data points in the blood, lung, heart and muscle sepsis + saline groups, one in the kidney and liver sepsis + saline groups and two in the muscle sepsis + ascorbate group. * P < 0.05 compared to sepsis + saline. The P values for comparisons between sepsis + saline and sepsis + ascorbate groups were: 0.32 (lung), 0.99 (kidney), 0.27 (spleen), 0.31 (liver), 0.23 (heart), and 0.21 (muscle). The respective sample sizes for sham, sepsis + saline and sepsis + ascorbate groups were 13, 14, 15 (blood), 13, 14, 15 (lung), 13, 14, 14 (kidney), 10, 11, 11 (spleen), 13, 15, 15 (liver), 13, 14, 15 (heart), 13, 15, 15 (muscle) mice.
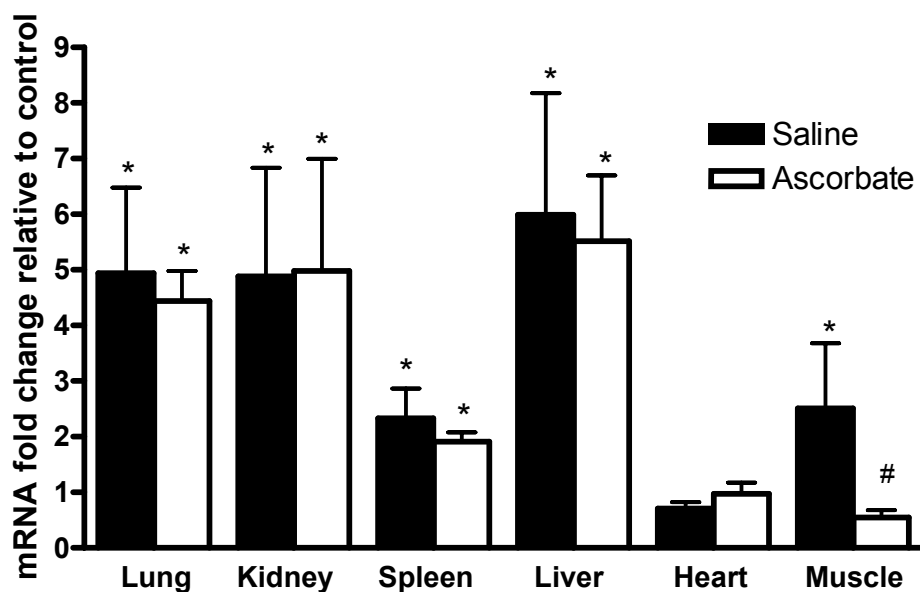
Figure 3.2. Sepsis increases PAI-1 mRNA in organs. Mice were treated as described in Figure 3.1 and RNA was quantified using real time qPCR. The level of mRNA expression in control sham mouse organs was set to 1. Fold increase in PAI-1 mRNA in the septic muscle was inhibited by ascorbate. * $P < 0.05$ compared to control sham level. # $P < 0.05$ compared to sepsis + saline fold change. The respective sample sizes for sham, sepsis + saline and sepsis + ascorbate groups were 5, 5, 6 (lung), 7, 6, 7 (kidney), 8, 7, 8 (spleen), 8, 7, 7 (liver), 6, 5, 7 (heart), 8, 8, 9 (muscle) mice.

Figure 3.3. Ascorbate does not affect increased PAI-1 protein in septic plasma.
Active PAI-1 protein content in 7 h post-FIP septic mice was measured by ELISA. There was no significant difference between sepsis + saline and sepsis + ascorbate. * P < 0.05 compared to control sham. n = 4 mice per treatment.

	exogenous u-PA	Sham	Sepsis +	
			Saline	Ascorbate
% exogenous u-PA inhibition	0	50	66	62
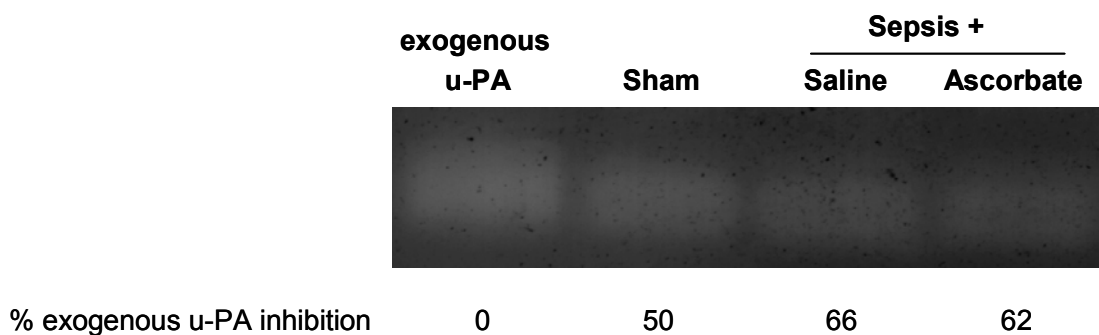
Figure 3.4. An example of reverse fibrin zymography of the mouse heart. In u-PA lane, exogenous u-PA in loading buffer (40 µl total volume) was run on the gel. This resulted in a bright band at 33 kDa, due to plasmin-mediated fibrin degradation in the gel. In the sham lane, control sham mouse heart homogenate was mixed with an equal amount of exogenous u-PA, and the mixture was run on the gel. When compared with the u-PA lane, the sham band was darker, indicating inhibition of u-PA-induced fibrin degradation (i.e., measuring inhibition of exogenous u-PA by the PAI-1 in the sample). Densitometry was employed to set u-PA inhibition to 0 % when the band brightness was equal to that in the u-PA lane, and to 100 % when the brightness was equal to that of the background. In sepsis+saline and sepsis+ascorbate lanes, u-PA was mixed with septic heart homogenates from mice injected with saline or ascorbate, respectively.
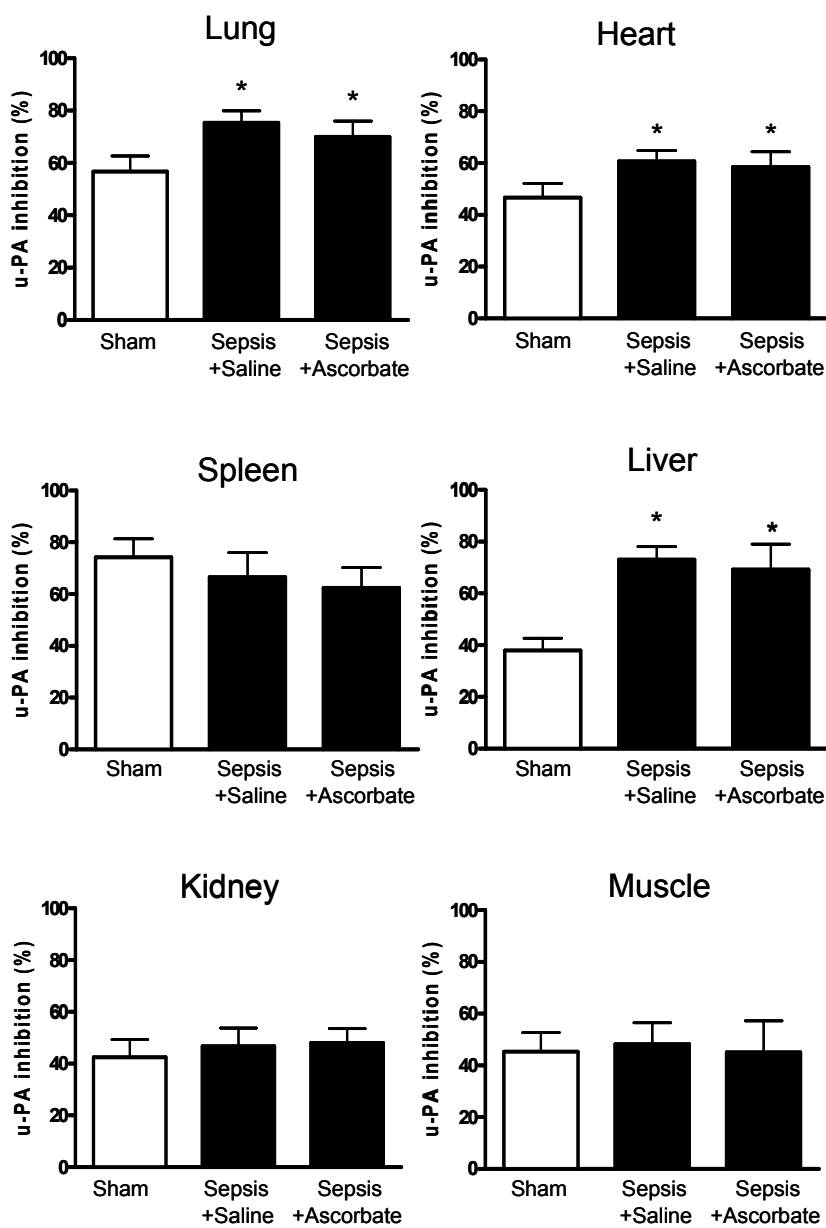
Figure 3.5. Sepsis increases PAI-1 activity in the lung, heart and liver. PAI-1 enzymatic activity in septic mice at 7 h was measured indirectly by reverse fibrin zymography. There were no significant differences between sepsis+saline and sham and between sepsis+saline and sepsis+ascorbate groups in the spleen. * P < 0.05 compared to sham. The respective sample sizes were 5 (lung, muscle), 6 (liver), 7 (heart), 8 (kidney) and 9 (spleen) mice.
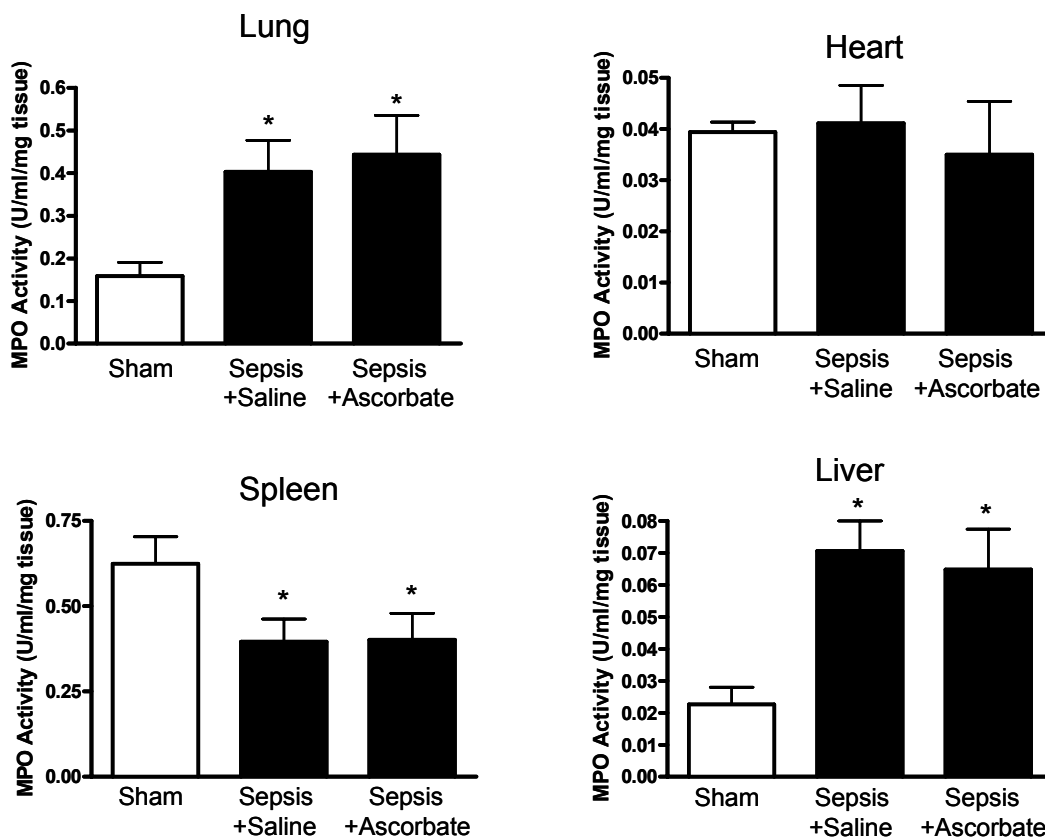
Figure 3.6. Ascorbate does not affect sepsis-induced increase in lung MPO activity, nor decrease in spleen MPO activity. Neutrophil infiltration was determined through an MPO assay in septic organs at 7 h, with saline or ascorbate treatment at 6 h. Ascorbate did not affect MPO activity in any organ compared to sepsis + saline. * $P <$ 0.05 compared to control sham. The respective sample sizes for sham, sepsis + saline and sepsis + ascorbate groups were 10, 9, 9 (lung), 6, 6, 6 (spleen), 6, 6, 6 (liver), 4, 5, 5 (heart) mice.

while the kidney and skeletal muscle samples yielded undetectable MPO levels. Ascorbate did not alter the MPO level in any organ (Figure 3.6).

3.3.4 Relationship between MPO level and PAI-1 activity

Figure 3.7 plots the MPO values of Figure 3.6 against the PAI-1 activity data of Figure 3.5. There was a high linear correlation between these two data sets (slope is significantly different from 0, R^2 value = 0.79). When muscle and kidney data were removed (due to inability to detect MPO), the R^2 value was 0.75; however the slope was not significantly different due to the reduced sample size. It should be noted that MPO and PAI-1 activity measurements were not always collected from the same mice and therefore I had to construct Figure 3.7 using the mean values of MPO and PAI-1 activity.

3.4 DISCUSSION

An intravenous ascorbate dose of 10 mg/kg (equivalent to approximately 700 mg in an average human male) has been shown to improve capillary perfusion in septic mouse skeletal muscle (28, 29, 32). This dose of ascorbate is clinically relevant as humans have been treated with higher doses through parenteral nutrition (20). In the present study, we found that this ascorbate treatment does not affect the bacterial content in the lung, heart, kidney, spleen, liver or skeletal muscle, or PAI-1 mRNA expression/enzymatic activity and neutrophil infiltration in septic mice. These results support our hypothesis that, in parallel to the beneficial effect on capillary bed perfusion, ascorbate does not alter sepsis-induced increases in PAI-1 expression and neutrophil infiltration in the organs with high immune response, preserving the beneficial effect of PAI-1 on the immune function.
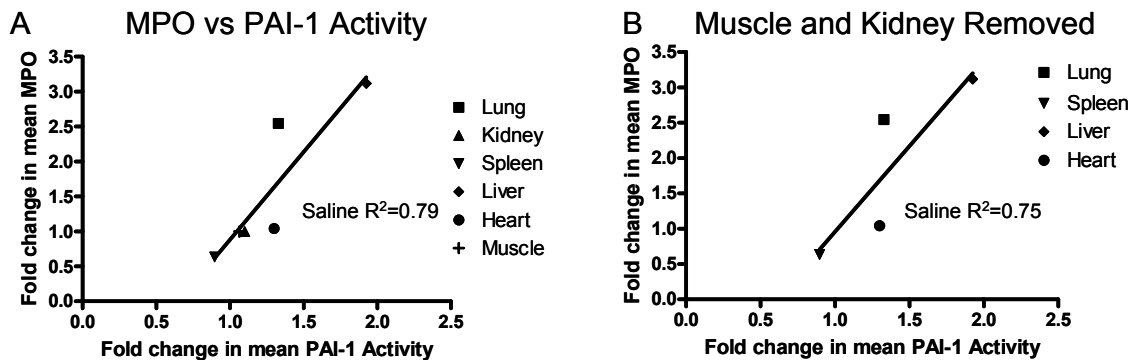
Figure 3.7. MPO content correlates with PAI-1 activity in septic mouse organs.
The mean fold change in MPO from all sepsis+saline mouse organs in Figure 3.6 was
plotted against the mean fold change in PAI-1 activity from Figure 3.5. Panel A: The
slope of the linear regression for sepsis+saline was significantly different from 0. The
fold change in MPO signals for muscle and kidney were set to 1 (MPO levels were
undetectable in these two organs). These results suggest that neutrophil recruitment is
related to the PAI-1 activity. Panel B: When muscle and kidney were removed from the
linear regression due to the inability to detect MPO, the R^2 value was relatively
unchanged, however there were too few points for statistical analysis.

3.4.1 Model of ascorbate administration in sepsis

The low ascorbate levels observed in septic patients (i.e., levels linked to poor outcome (27)), can be reversed by parenteral nutrition containing large doses of ascorbate (1000-3000 mg/day) (20, 27). We and others have shown that a large dose of ascorbate (200 mg/kg) injected into mice at the onset of sepsis protects against the various detrimental effects of sepsis, including high mortality (8, 9, 34, 35). We also showed that a lower dose of ascorbate (10 mg/kg) has similar beneficial effects against sepsis and that these effects are seen even when ascorbate injection is delayed six hours into sepsis (28, 32). The model of delayed injection is clinically relevant for patients presenting with sepsis, and therefore this model was chosen to study the effect of ascorbate in the present investigation.

3.4.2 Methodological considerations

To our knowledge, this study characterized for the first time the effect of FIP on bacterial content in the peritoneal fluid and various organs. This characterization was limited to bacteria which grow on agar plates, including *Escherichia, Staphylococcus* and *Streptococcus* bacterial strains (Certificate of Analysis, Oxoid). Surprisingly, despite ensuring injection of identical amounts of feces solution into mice, there was a large variability in the bacterial content of the peritoneal fluid at 7 h post-FIP (7,000 fold range). Accordingly, the bacterial counts in organs also varied extensively (approximately 100 fold range for each organ, Figure 3.1). This extensive mouse to mouse variability has been observed in other models of sepsis (13, 26). We speculate that the large variability in our FIP model was due to differences in the viability and type of the bacteria in the feces solution (70 fold), variability in the local immune response at the site of injection and in the spread of bacteria from the peritoneum to the blood and organs. Clearly, the observed variability in the initiation of sepsis contributed to the variability seen in the PAI-1 mRNA levels (Figure 3.2). This particular mRNA variability is also consistent with the literature (10, 24). Regarding detection of sepsis/ascorbate-induced changes in PAI-1 protein level in the various organs, for

unknown reasons, our attempts to determine these changes by western blotting and ELISA failed. We speculate that the inability to detect differences in PAI-1 protein levels was due to the high basal levels of PAI-1 found in the non-vascular cells drowning out the relatively small increase in PAI-1 in the vascular cells. Therefore, we used fibrin zymography, where exogenous u-PA inhibition is an indirect measure of PAI-1 activity in organ samples. The fibrin zymography detected sepsis-induced increases in PAI-1 activity in blood plasma, lung, heart and liver (Figure 3.5, 3.8 and 3.9).

3.4.3 Bacterial counts in septic organs

Among the organs studied, the highest levels of bacteria were found in the organs that mount the greatest immune response (kidney, spleen and liver), while relatively few bacteria were found in the skeletal muscle (Figure 3.1). Blood was also heavily infected with bacteria (Figure 3.1). The lack of effect of ascorbate on peritoneal fluid bacterial count is consistent with the reported lack of effect of ascorbate on this count in the septic mouse (34). Previous reports have observed a bactericidal effect of ascorbate (3, 12, 37). We speculate that the discrepancy between the effect of ascorbate on bacterial count in blood (Figure 3.1) and peritoneal fluid could be due to a possible bactericidal concentration of ascorbate in the blood post-intravenous injection..

3.4.4 PAI-1 expression and activity in organs

Sepsis-induced increases in PAI-1 mRNA expression in the lung, kidney and liver (Figure 3.2) are consistent with increases reported by Raeven and colleagues (24). Others have also observed increased PAI-1 mRNA expression in organs with high immune activity during sepsis (25, 26). Ascorbate did not affect the increases in PAI-1 mRNA in the organs with high immune activity, but it inhibited the increase in PAI-1 mRNA in the skeletal muscle (Figure 3.2). It is possible that the effect of ascorbate on PAI-1 mRNA expression differs depending on the immune activity in each organ. To our

	exogenous u-PA	Sham 1	Sepsis 1	Sham 2	Sepsis 2	Sham 3	Sepsis 3
% exogenous u-PA inhibition	0	30	63	-15	64	-40	21
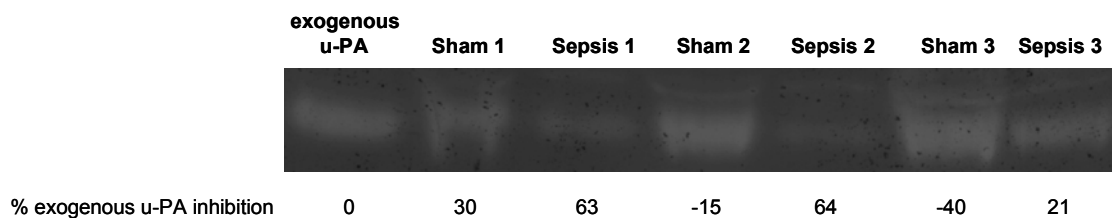
Figure 3.8. An example of reverse fibrin zymography of the mouse blood plasma.
Similar to the reverse fibrin zymography in Figure 3.4, exogenous u-PA was added to
blood plasma from 3 control sham mice (Sham 1, 2 and 3) and 3 sepsis + saline mice
(Sepsis 1, 2 and 3). Sham 1 and Sepsis 1, 2 and 3 lanes were darker than the exogenous
u-PA lane, indicating inhibition of exogenous u-PA by PAI-1 in these samples. Sham 2
and 3 had brighter bands than the exogenous u-PA band, indicating enhanced fibrin
degradation than by exogenous u-PA alone, most likely due to endogenous u-PA present
in these sham samples. According to the definition of exogenous u-PA inhibition in
Figure 3.4, negative values of u-PA inhibition were computed. Side by side comparison
between septic and sham mice clearly shows greater inhibition of exogenous u-PA in the
septic samples.
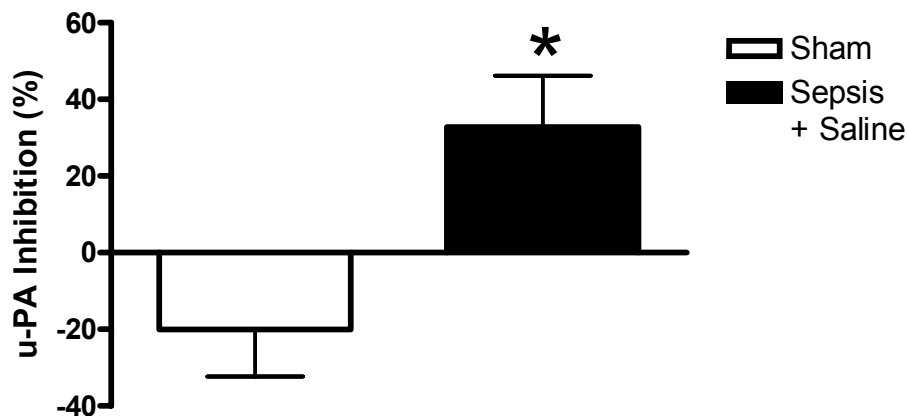
Figure 3.9. Sepsis significantly increases u-PA inhibition in the blood plasma. These data summarize the mouse blood plasma reverse fibrin zymography data exemplified in Figure 3.8. These data agree with data from Figure 3.3, where an Active PAI-1 ELISA showed a significant increase in PAI-1 protein expression in the blood plasma of septic mice. A negative u-PA inhibition indicates a pro-fibrinolytic state, and a positive u-PA inhibition indicates an anti-fibrinolytic state in the blood plasma. These data then validate the use of reverse fibrin zymography to detect PAI-1 in samples. * $P < 0.05$ compared to sham. n = 6 mice per group.

knowledge, the effect of ascorbate or other antioxidants on PAI-1 mRNA/protein expression in septic organs has not been reported.

Our data showing sepsis-induced increases in active PAI-1 protein in blood plasma (Figure 3.3) are consistent with previous reports (31). Ascorbate did not affect this increase. Our preliminary unpublished data at 9 h post-FIP (ascorbate injected at 6 h) also showed a lack of effect of ascorbate on sepsis-induced increase in PAI-1 protein, indicating that even at 3 h post-injection, ascorbate does not affect PAI-1 protein expression. Regarding PAI-1 enzymatic activity in organs measured by zymography, ascorbate did not alter the levels observed during sepsis (Figure 3.5). Out of the six organs examined, the sepsis-induced increased PAI-1 mRNA levels yielded increased activities only in the lung and liver. The lack of increased enzymatic activity, despite increased mRNA level in the kidney and skeletal muscle, suggests several possibilities including decreased mRNA stability resulting in no PAI-1 protein production in certain organs or an organ specific post-translational modification of PAI-1 protein in sepsis. Interestingly, the PAI-1 activity in the septic heart increased despite unchanged mRNA level in this organ. We speculate that PAI-1 protein in septic blood coursing through the richly vascularized heart affected the PAI-1 activity assessed by zymography in this organ.

3.4.5 Immune response during sepsis.

Increased MPO content has been reported in septic organs including the lung and liver (6, 21). Our data in Figure 3.6 (lung and liver) agree with these reports. The lack of MPO signal in the kidney under sham conditions is consistent with the literature (15). Sepsis reduced the MPO content in the spleen and did not change in the heart (Figure 3.6) consistent with the trends observed by Gebska and colleagues (11). To explain the reduced MPO content in the spleen, we speculate that the large number of neutrophils in sham control mouse spleen (associated with the largest MPO signal, Figure 3.6) was reduced in sepsis due to neutrophil recruitment from the blood into organs such as lung

and liver. Ascorbate had no effect on the sepsis-induced MPO content in any organ (Figure 3.6).

The correlation in Figure 3.7 suggests a dependence of the MPO signal on PAI-1 activity. Previously, the level of neutrophils in blood was shown to depend on the PAI-1 protein content (23). This dependence and the correlation shown in Figure 3.7 suggest that PAI-1 may play a role in the immune response during sepsis.

3.4.6 Significance of the lack of effect of ascorbate.

We reported that delayed ascorbate bolus rapidly reverses (i.e., within 1 h) capillary plugging in sepsis. The present data indicate that this effect is not due to ascorbate promoting fibrinolysis of microthrombi (i.e., by ascorbate inhibiting PAI-1) in capillaries in the skeletal muscle. The reversal of capillary plugging could be due to other mechanisms through which ascorbate exerts its effect rapidly (e.g., ascorbate reduces platelet aggregation and platelet-endothelial adhesion (28, 29)).

Ascorbate did not appear to affect the bacterial counts in the various organs within 1 h post-injection. This observation is consistent with the report that short-term ascorbate treatment failed to change the rate of superoxide production by neutrophils (7) indicating that bacterial killing by neutrophil respiratory burst is not affected by ascorbate. Furthermore, administration of 320 mg/kg ascorbate over 2 h did not affect the intracellular concentration of ascorbate in leukocytes (4). We speculate that the lack of short-term effect of ascorbate on bacterial count is of little consequence, because bacteria in the various organs could be killed by leukocytes infiltrating these organs during sepsis.

The lack of effects of ascorbate at 10 mg/kg in the present study does not preclude any effects of ascorbate given at larger doses or at different time points (e.g., at the onset of sepsis or for longer treatment periods). These effects could be subject to future investigations.

In conclusion, we have demonstrated that ascorbate does not affect bacterial content, PAI-1 or MPO in organs with high immune activity. These results suggest that the beneficial effect of ascorbate on capillary bed perfusion is not accompanied by effects of ascorbate on the fibrinolytic or immune responses in septic organs.

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Chapter 4

Effect of ascorbate on plasminogen activator inhibitor 1 expression and release from platelets and endothelial cells in an *in-vitro* model of sepsis

A version of this chapter has been submitted to Microcirculation.

4.1 INTRODUCTION

Sepsis, a systemic inflammatory response to an infection, leads to numerous physiological abnormalities, including a fibrinolytic abnormality due to elevated plasma levels of the PAI-1 (11, 12, 24). The fibrinolytic abnormality could be critical at the microcirculatory level because elevated PAI-1 levels could inhibit the plasmin-mediated dissolving of microthrombi and thus contribute to DIC. To this end, we have shown that sepsis causes plugging of the capillary bed, involving increased occurrence of both fibrin plaques and platelet adhesion/aggregation in capillaries (22, 28, 29). To our knowledge, the role of the fibrinolytic abnormality in sepsis-induced capillary plugging is not known.

We have reported that, in septic mice, an intravenous injection of ascorbate delayed 6 h after the onset of sepsis unplugs capillaries. Although numerous mechanisms could explain this beneficial effect of ascorbate (22), it is possible that ascorbate enhances the dissolving of capillary microthrombi by inhibiting PAI-1, to account for the observed unplugging of capillaries. Oxidative stress has been implicated in the increase in both mRNA and protein expression of PAI-1 in endothelial cells (8).

Because endothelial cells and platelets (i.e., cells in the milieu of capillary microthrombi) can release PAI-1into the extracellular space (2, 16, 21), the objective of the present study was to use a simple *in vitro* and *ex vivo* models of sepsis to examine whether ascorbate affects PAI-1 expression and release from these two cell types. We hypothesized here that ascorbate reduces the expression and release of PAI-1 in endothelial cells or platelets in sepsis. Because the beneficial effect of ascorbate against capillary plugging has been shown to depend on eNOS-derived NO (22), we also hypothesized that the PAI-1 release is NO-dependent.

4.2 METHODS

4.2.1 Animal preparation

All experiments were conducted under protocols approved by the University of Western Ontario Council on Animal Care. Endothelial cells for *in vitro* experiments were obtained from male wild type C57BL/6 mice (age: 1.5 – 3 months) from Charles River Laboratories (Sherbrooke, Quebec, Canada). Preliminary experiments indicated no gender specific differences in platelet function, therefore platelets for *ex vivo* experiments were obtained from male or female wild type mice of the same strain. Mice were anesthetized with ketamine (80 mg/kg) and xylazine (4 mg/kg) prior to cell harvest or platelet collection. All supplies were purchased from Sigma Aldrich (Oakville, ON, Canada) unless otherwise stated.

4.2.2 In-vitro model of sepsis in cultured endothelial cells

Endothelial cells were harvested from the whole hind limb skeletal muscle as previously described (1, 15). We have chosen this tissue because sepsis-induced capillary plugging and the beneficial effect of ascorbate against this plugging has been observed in the mouse skeletal muscle (22, 26). The muscle was excised, cut into 2 mm diameter pieces and digested in 25 mls of digestion solution containing 21 mg collagenase, 3 mg dispase, 3 mg trypsin and 41 mg bovine serum albumin for 30 minutes. The digest was strained through nylon mesh, centrifuged at 805 rcf for 5 minutes, then the pellet was resuspended in culture medium containing 20% fetal bovine serum, heparin (5 U/ml), endothelial growth supplement (100 µl/ml), antibiotic-antimycotic solution (10µl/ml) and L-glutamine (0.1 µg/ml) in DMEM/F12 HAM. The endothelial cells were grown to confluence and then were purified using a GS-I lectin coated magnetic bead immunoseparation. This method has been demonstrated to yield nearly 100% pure endothelial cells as assessed by vWF (1). We randomly chose cell lines to test for vWF to confirm continued specificity of this method. The cells were cultured and used for experiments up to passage 10. For each experiment, cells were grown to confluence and treated with ascorbate (100 µM) for 1, 3, 6 or 24 hours, concurrently with either

lipopolysaccharide (LPS) (10 μg/ml) or tumor necrosis factor alpha (TNFα) (10 ng/ml). We used 100 μM ascorbate as it has been demonstrated to raise the intracellular ascorbate content to approximately 5 mM, a physiologically relevant concentration *in vivo* (31). The doses of LPS and TNFα were chosen as they were demonstrated to induce platelet-endothelial cell adhesion, suggesting activation of the coagulatory pathway (32). Alternatively, cells were treated with thrombin (0.0375 U/ml) for 3 - 5 minutes at the end of the 24 hour period. We used LPS and TNFα as conventional agents of sepsis as these substances have been shown to initiate endotoxemia *in vivo* (6, 7). The chosen dose of ascorbate results in physiological levels of intracellular ascorbate (31). Thrombin (0.0375 U/ml) has been used to activate platelets to mimic sepsis ex-vivo (23). We have used this concentration of thrombin in endothelial cells as an alternative *in vivo* model of sepsis (23). After completion of experiments, cell supernatants were collected and frozen, and the cells were lysed in Tris-NaCl-Triton-EDTA (TNTE) buffer or Trizol (Invitrogen, Burlington, ON, Canada).

4.2.3 Ex-vivo model of sepsis in isolated platelets

Blood was collected 9:1 in acid citrate dextrose solution and platelets were isolated as detailed previously (23). Briefly, the blood was centrifuged at 179 rcf, the supernatant was retained and centrifuged at 774 rcf to pellet the platelets. The platelet pellet was resuspended in DPBS, divided evenly among the treatment groups and stimulated with thrombin (0.0375 or 0.075 U/ml), LPS (10 μg/ml) or TNFα (10 ng/ml) with or without ascorbate (100, 500 or 1000 μM). To ensure autoactivation of the platlets did not occur, a control unstimulated platelet fraction was always included for each individual experiment. The platelets were incubated at 37°C for 5 or 10 minutes, then centrifuged to pellet the platelets. The supernatant was retained and frozen, and the platelet pellet homogenized in TNTE buffer or Trizol. To determine the effect of NO on platelets, the NO donor S-Nitroso-N-acetylpenicillamine (SNAP) (1 μM) was used in conjunction with thrombin (0.0375 U/ml). This level of SNAP inhibits thrombin-induced aggregation of platelets *ex-vivo* (23).

4.2.4 PAI-1 mRNA expression

mRNA was isolated using the phenol-chloroform extraction method. The isolated mRNA was purified using DNAse I and Reverse Transcribed using Superscript II (Invitrogen). The mRNA was quantified using Quantifast SYBR Green (Qiagen, Mississauga, ON, Canada) as directed on a Bio-Rad CFX96 thermal cycler (Bio-Rad, Mississauga, ON, Canada). The primers used to detect the mRNA are as follows: PAI-1 (forward: 5'-GACAGCACTGTCAGGGTCCATAG-3' and reverse: 5'-GCGGTCCTCCTTCACAAAGCTC-3') and β-actin as a housekeeping gene (forward: 5'-TCGTGGGCCGCTCTAGGCACCA-3'and reverse: 5'-GTTGGCCTTAGGGTTCAGGGGGG-3'). The mRNA was amplified using the following cycling protocol: 95°C for 5 min and then 40 cycles of 95°C for 10 s, 60°C for 30 s and 80°C for 30 s.

4.2.5 PAI-1 protein content and release

Both intracellular PAI-1 protein (i.e., PAI-1 in cell lysates) and PAI-1 release (i.e., PAI-1 in cell supernatants) were quantified using the total PAI-1 ELISA Kit (IMPAIKT-TOT, Innovative Research, Novi, MI) according to manufacturers directions. The ELISA can detect PAI-1 within the 0.05 – 50 ng/ml range. Accordingly, endothelial cell samples were diluted with TNTE buffer prior to being added to the ELISA plates.

4.2.6 Statistical analysis

Data are presented as mean ± SE. A minimum of five different cell lines were used for the endothelial cell experiments. A minimum of four groups of pooled platelets were used for the platelet experiments. Statistical analysis was performed using a repeated measures one-way ANOVA and the Bonferroni multiple comparison test. Results are significant with a $P < 0.05$.

4.3 RESULTS

4.3.1 Endothelial cell PAI-1 mRNA and protein expression

mRNA analysis of endothelial cell PAI-1 expression revealed that LPS and TNFα raised expression after 6 and 24 h of stimulation, but not after 1 and 3 h of stimulation, when compared with control unstimulated cells (Figure 4.1). Ascorbate had no effect on the expression of PAI-1 mRNA at 1- 6 h, but there was a tendency for increased PAI-1 mRNA in TNFα groups at 24 h. Interestingly, the LPS- or TNFα-induced increase in mRNA seen at 6 and 24 h did not translate into increased production of PAI-1 protein at 24 h (Figure 4.2a). Surprisingly, in control cells, ascorbate increased the production of PAI-1 protein. In LPS- or TNFα-treated cells, ascorbate had no effect on PAI-1 protein (Figure 4.2a).

4.3.2 PAI-1 protein release from endothelial cells

For all control, LPS and TNFα groups at 24 h, the level PAI-1 protein release was greater than the level of PAI-1 protein remaining in the cells (Figure 4.2b). Ascorbate did not affect the PAI-1 release in any of the groups (Figure 4.2b). Figure 4.3a shows the effect of acute stimulation of endothelial cells with thrombin.   Thrombin had no effect on intra-endothelial PAI-1 protein in control cells treated with either vehicle or ascorbate for 24 h (Figure 4.3a) nor on PAI-1 protein release from these cells (Figure 4.3b).

4.3.3   PAI-1 protein release from platelets

Figure 4.4 shows that neither LPS nor TNFα affected intracellular and released PAI-1 protein levels when compared to the control unstimulated platelets. However, as anticipated, thrombin at a concentration of 0.0375 U/ml, reduced intracellular PAI-1 and elevated released PAI-1 protein levels from platelets (Figure 4.5). Ascorbate (100 µM) did not affect these levels. However, ascorbate at 500 µM and 1000 µM inhibited the
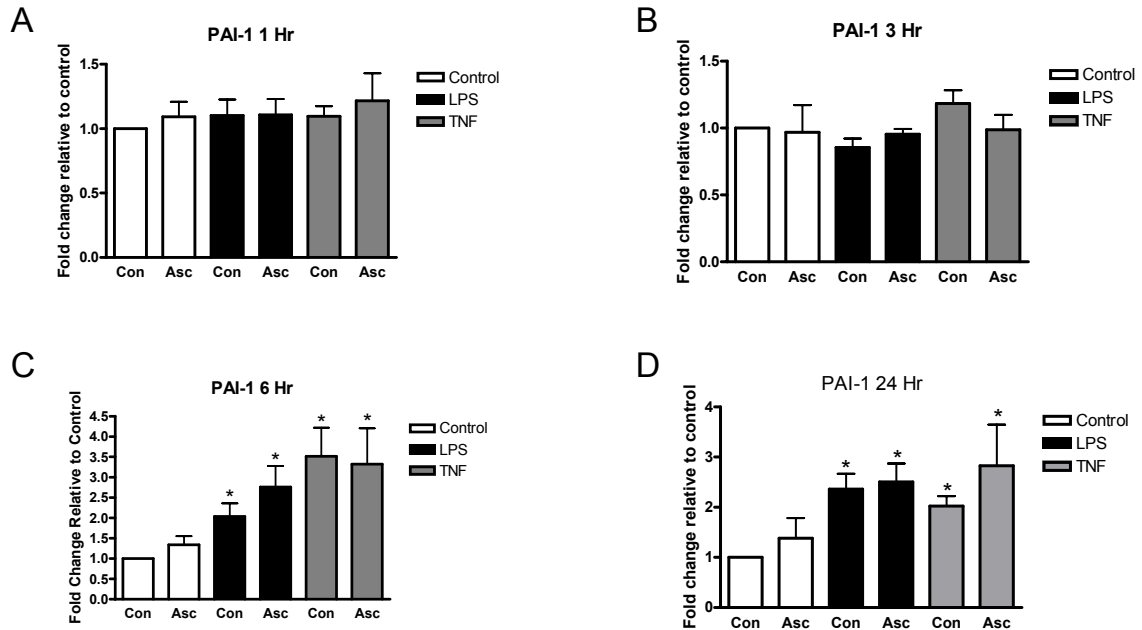
Figure 4.1. Increases in PAI-1 mRNA expression after 6 and 24 hours of endotoxin stimulation were not altered by ascorbate. Isolated endothelial cells were stimulated with either LPS (10 μg/ml) or TNFα (10 ng/ml) for 1, 3, 6 or 24 hours as indicated. Cells were treated with 100 μM ascorbate (Asc) or media (Con) concurrently. Total cell mRNA was extracted, reverse transcribed and PAI-1 mRNA was quantified using qPCR. Neither LPS nor TNFα altered the PAI-1 mRNA expression at 1 hour (A) or 3 hours (B). In contrast, at 6 (C) and 24 hours (D) there was a significant increase in PAI-1 expression with both LPS and TNFα treatment. Ascorbate did not appear to have an effect on this increase in PAI-1 expression. * $P < 0.05$ compared to control. n = 4 – 9 different cell lines per treatment.
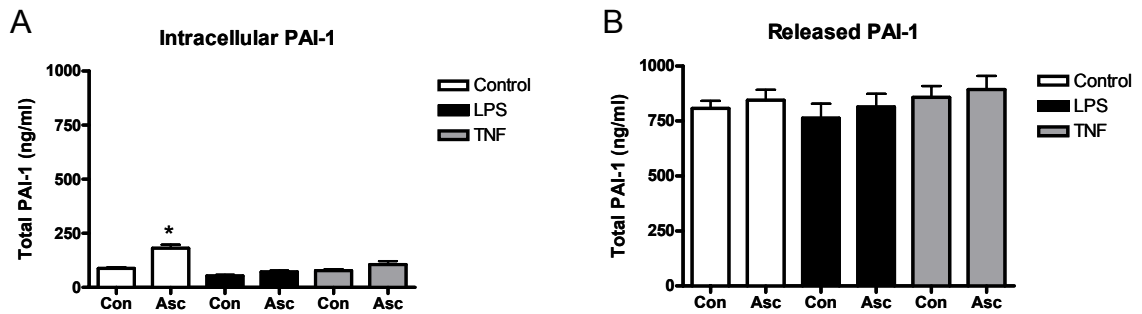
Figure 4.2. Stimulation with LPS or TNFα did not alter the PAI-1 production or release in endothelial cells. Endothelial cells were stimulated with LPS (10 μg/ml) or TNFα (10 ng/ml) for 24 hours, the supernatant was collected and the cells lysed. The PAI-1 content in the cells and released in the supernatant were quantified using an ELISA. Ascorbate (Asc, 100 μM) treatment alone for 24 hours significantly increased the PAI-1 production in the endothelial cells over media alone (Con). Co-treatment with either LPS or TNFα eliminated the observed increase in PAI-1 protein. No stimulation altered the PAI-1 release into the supernatant. * P < 0.05 compared to control. n = 4 cell lines per group.
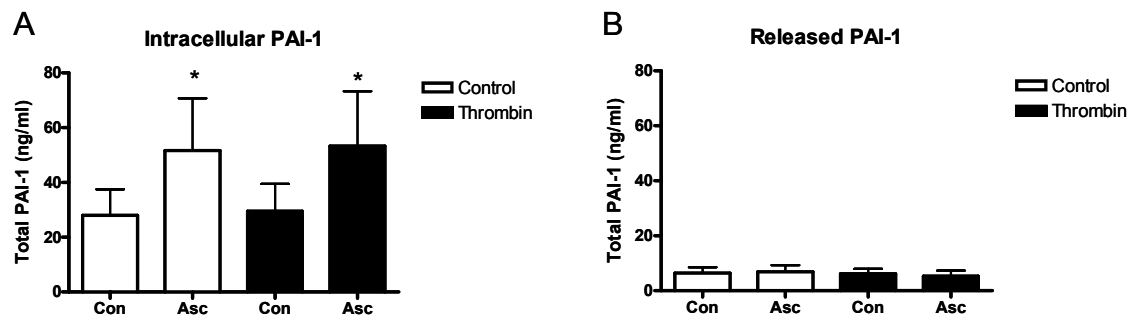
Figure 4.3. Thrombin has no acute effect on the release of PAI-1 protein from endothelial cells. Endothelial cells previously treated with ascorbate (Asc, 100 μM) or fresh media (Con) for 24 hours were stimulated with thrombin (0.0375 U/ml) for five minutes, then the supernatant was collected and the cells lysed. Thrombin had no effect on the release of PAI-1 into the supernatant. Ascorbate treatment significantly increased the PAI-1 production intracellularly. * $P < 0.05$ compared to control. n = 5 cell lines per group.
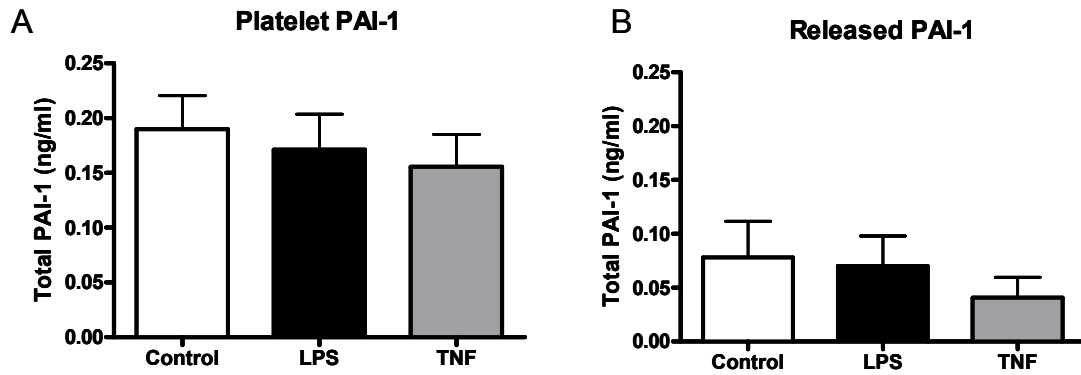
Figure 4.4. PAI-1 release by platelets is not induced by LPS or TNFα. Platelets were isolated from whole mouse blood, stimulated with LPS (10 µg/ml) or TNFα (10 ng/ml) for 10 minutes, then the supernatant and platelet pellets were collected and subjected to ELISA. Neither LPS nor TNFα treatment caused release of PAI-1 from the platelets into the supernatant. n = 6 platelet isolations per group.
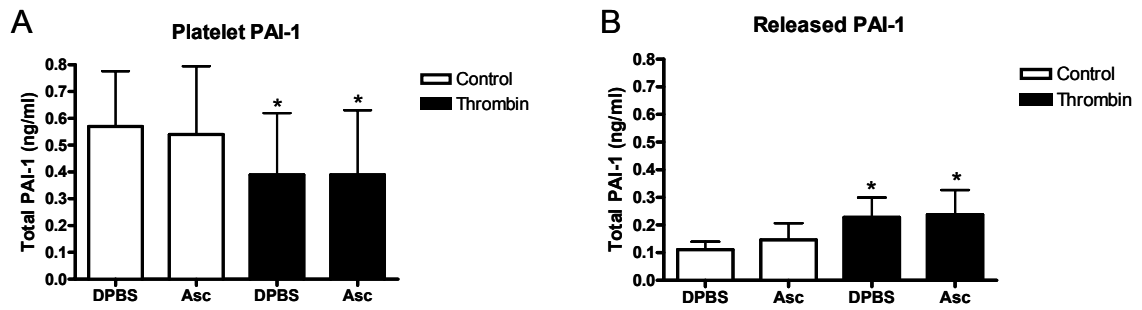
Figure 4.5. Ascorbate (Asc) does not inhibit thrombin induced PAI-1 release from platelets. Isolated platelets were treated with ascorbate (100 µM) or DPBS, followed by stimulation with thrombin (0.0375 U/ml) for 10 minutes, then supernatant and platelet pellets were collected and subjected to ELISA. Thrombin induced a significant release of PAI-1 from the platelets into the supernatant. Ascorbate had no effect on the thrombin induced PAI-1 release. * $P < 0.05$ compared to control. $n = 5$ platelet isolations per group.

thrombin-induced PAI-1 release from platelets (Figure 4.6). As the pH of unbuffered ascorbate is acidic, we tested whether this inhibition is pH-sensitive. Figure 4.7 shows that buffered ascorbate solution (1000 µM, pH adjusted to 7.2) failed to inhibit the thrombin-induced platelet PAI-1 release. When the pH was lowered with hydrochloric acid to the same level as unbuffered ascorbate (pH 5.8 and 3.8), similar reductions in platelet PAI-1 release were observed (Figure 4.8).

4.3.4 Platelet PAI-1 protein release, dependence on NO

Platelet activation by thrombin and the protective effect of ascorbate in the microvasculature have both been reported to be NO-dependent (22, 23). Figure 4.9 shows that the thrombin-induced release of PAI-1 was independent of exogenous NO (i.e., 5 min treatment with SNAP). Since this treatment has been shown to inhibit thrombin-induced platelet aggregation (23), the results in this figure suggest that the platelet PAI-1 release is independent of NO.

4.4 DISCUSSION

In the present study, we have shown that the PAI-1 production and release by the endothelial cells and platelets *in vitro/ex vivo* is not affected by low dose (100 µM) ascorbate treatment. Additionally, we have uncovered a potential pH-mediated mechanism whereby ascorbate may be able to inhibit platelet PAI-1 release. We also explored the NO dependence of the platelet PAI-1 release and determined that platelet PAI-1 release may not be NO-dependent.

We have previously shown that an intravenous bolus of ascorbate can decrease capillary plugging, resulting in improved organ function and improved survival (22, 27, 29). A persistent challenge we have faced is the complexity of the in vivo model of sepsis and the limitations in measuring PAI-1 protein release directly into the blood by both endothelial cells and platelets at the capillary level. The present study uses

A Platelet PAI-1
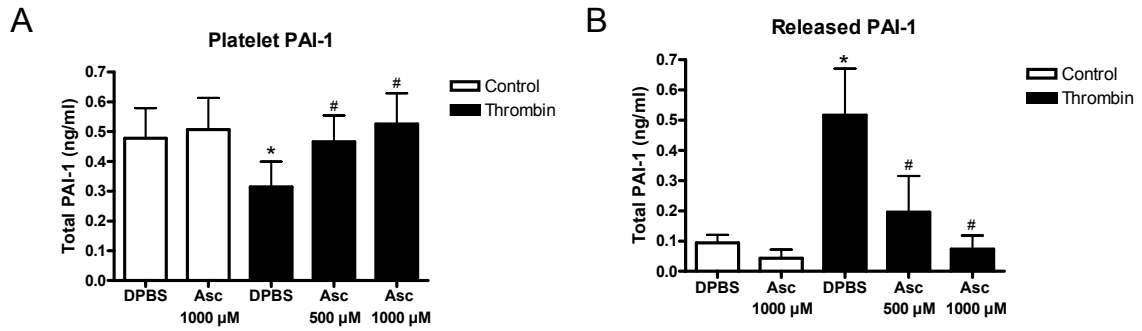
B Released PAI-1

Figure 4.6. High dose ascorbate inhibits the thrombin induced PAI-1 release from platelets. Platelets were treated with unbuffered ascorbate (500 and 1000 μM) or DPBS, followed by stimulation with thrombin (0.0375 U/ml) for 10 minutes. PAI-1 in the supernatant and platelet pellet was quantified using ELISA. Thrombin induced a significant release of PAI-1 from the platelets into the supernatant. Both 500 and 1000 μM doses of ascorbate significantly reduced the release of PAI-1. * $P < 0.05$ compared to control. # $P < 0.05$ compared to thrombin stimulated platelets. n = 6 platelet isolations per group.
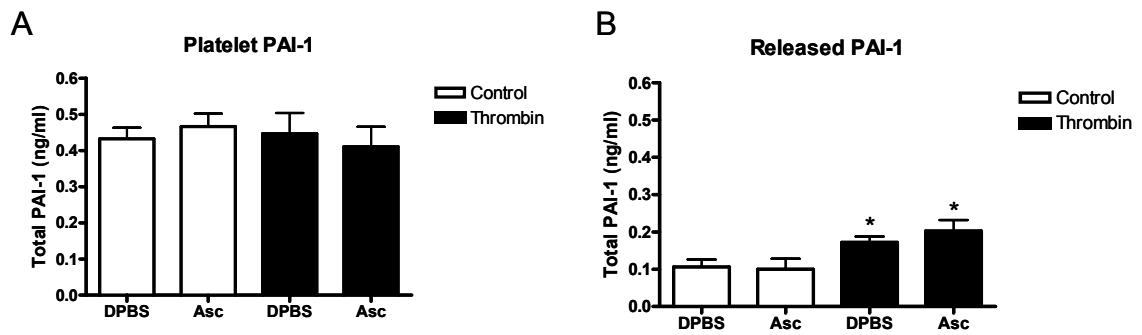
Figure 4.7. pH buffered high dose ascorbate (Asc) does not inhibit the thrombin induced platelet PAI-1 release. Isolated platelets were treated with ascorbate (1000 µM) buffered to pH 7.2 or DPBS, followed by stimulation with thrombin (0.0375 U/ml) for 10 minutes. PAI-1 was then determined in the supernatant and platelet lysate by ELISA. Thrombin induced a significant increase in PAI-1 release by platelets into the supernatant. Ascorbate had no effect on the thrombin induced PAI-1 release. * P < 0.05 compared to control. n = 4 platelet isolations per group.
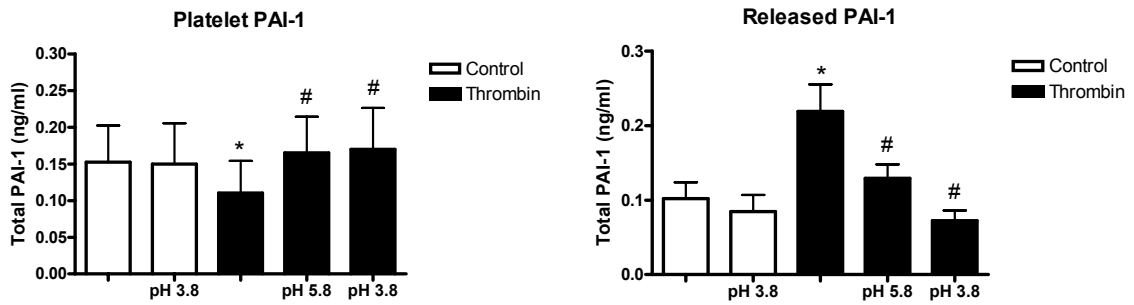
Figure 4.8. Thrombin-induced platelet PAI-1 release is inhibited by low pH.
Isolated platelets were suspended in DPBS at either pH 7.4, 5.8 or 3.8, followed by stimulation with thrombin (0.0375 U/ml) for 10 minutes. PAI-1 protein level was then determined in the supernatant and platelet lysate by ELISA. Thrombin induced a significant increase in PAI-1 release by platelets into the supernatant. Low pH generated a dose-dependent inhibition of the thrombin induced PAI-1 release. * $P < 0.05$ compared to control. # $P < 0.05$ compared to thrombin at pH 7.4. n = 3 platelet isolations per group.
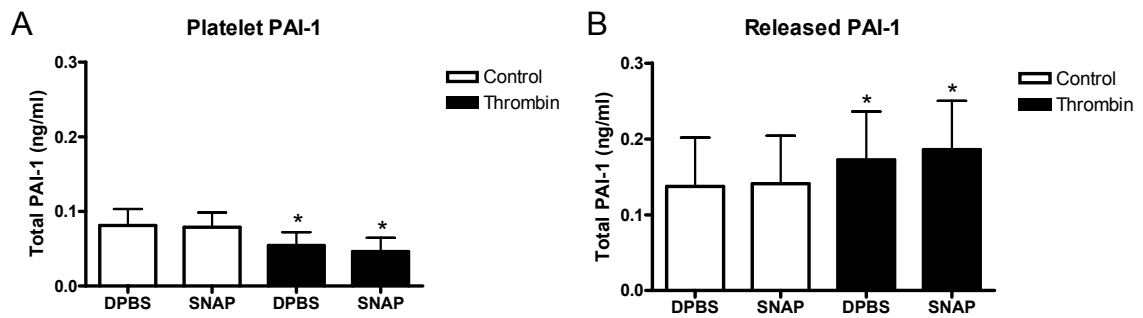
Figure 4.9. Effect of thrombin on PAI-1 release from platelets subjected to exogenous NO. Platelets were isolated and treated with the NO donor SNAP (1 μM) or DPBS, and thrombin (0.0375 U/ml). SNAP did not alter the thrombin-induced platelet PAI-1 release. * P < 0.05 compared to both control and SNAP only treatments. n = 4-5 platelet isolations per group.

simplified *in vitro* and *ex vivo* models to examine the effect of the antioxidant ascorbate on PAI-1 production and release in cells that would be in a very close proximity to a developing microthrombus *in vivo*.

4.4.1 PAI-1 mRNA expression in endothelial cells

Our findings that endothelial cell PAI-1 mRNA is not altered after 1 or 3 h LPS/TNFα treatment are in contrast to the PAI-1 increase reported soon after endotoxin treatment (18). Interestingly, the increase in PAI-1 mRNA observed at 6 and 24 h mirrored that of several previous studies (18, 19). It is possible that inter-species differences between mouse and human cells may be responsible for the delayed response to LPS. Indeed, it has been reported that humans have a markedly increased sensitivity to LPS compared to mice, and that inter-species differences in sensitivity to infections can have profound effects on survival (6, 14). Ascorbate did not appear to have any effect on PAI-1 mRNA expression after 1- 6 h of treatment (Figure 4.1). Ascorbate tended to potentiate the TNFα-induced increase in PAI-1 mRNA (Figure 4.1d).

4.4.2 PAI-1 protein expression and release in endothelial cells

The tendency of increased PAI-1 mRNA observed upon ascorbate treatment in control cells translated into increased PAI-1 protein production (Figure 4.2a). Interestingly, this increase in protein production did not lead to increased release into the extracellular space. Additionally, treatment with LPS or TNFα largely abolished the ascorbate-induced increase in PAI-1 protein expression. It cannot be ruled out that the large amount of PAI-1 released over 24 hours has masked an increase in PAI-1 release late in the treatment period (i.e., if treatment increased release beginning at 23 h, 1 h of increased release may be masked by the first 23 h of release). Consistent with these findings, thrombin also did not initiate increased release of PAI-1 (Figure 4.3). The findings of Figure 4.2 support the notion that endothelial cells make a significant contribution to the total PAI-1 in the vasculature. The lack of increase in PAI-1 protein

levels upon LPS stimulation are in contrast with previous studies in human umbilical vein endothelial cells, where significant increases in PAI-1 were observed (18, 19). Given the demonstrated inter-species variability, it is conceivable that the expression profile of PAI-1 in mouse cells differs from that of human cells.

4.4.3 Effect of ascorbate on PAI-1 protein expression

Regarding the ascorbate-initiated increase in PAI-1 protein production, it is unclear what mechanism is involved in this phenomenon. However, we speculate that ascorbate either increases the stability of PAI-1 enhancing the total content, or it is acting as a stimulant to the initiation of the non-fibrinolytic effects of PAI-1 (e.g., angiogenesis). It would be interesting to determine what effect intravenous ascorbate might have on the vasculature of healthy individuals.

Overall, the long time period required for ascorbate to induce any changes in the endothelial cells (e.g., 24 h may be needed for increased PAI-1 production) argues against ascorbate's acute effects on fibrinolysis. Thus, in the septic microvasculature, restoring capillary blood flow by ascorbate over 1 h period may not occur through PAI-1 inhibition. Interestingly, the increased survival during sepsis seen after ascorbate injection suggests that increased or maintained PAI-1 production during sepsis may not be detrimental to the outcome of sepsis. Despite studies suggesting PAI-1 is essential to survival from sepsis, it is unclear how PAI-1 is beneficial (9).

4.4.4 PAI-1 release from platelets

Consistent with our previous findings of the lack of effect of LPS/TNFα on platelet aggregation (23), in the present study, LPS and TNFα did not alter platelet PAI-1 release. This finding agrees with previous work using platelets isolated from mice using both LPS (20) and TNFα (3), though others have observed spontaneous platelet activation upon LPS stimulation (4). It is important to note that these conflicting studies were conducted with platelets from species other than mice, suggesting inter-species differences may be

involved in the different responses. It is also possible that neither LPS nor TNFα directly stimulate platelets, but rather they may stimulate the production of other signaling molecules (i.e., thrombin) that ultimately activate platelets (10). The present *ex vivo* model may lack the cells necessary for the production of these signaling molecules.

The observed PAI-1 release from platelets upon short-term stimulation with thrombin agrees with the literature (2, 16). Ascorbate at a concentration of 100 µM did not alter the release of PAI-1 from platelets. This is in contrast to our previous report that ascorbate reduces the rate of thrombin-induced aggregation (23). It is possible that we were unable to observe a reduction in the rate of PAI-1 release due to our inability to measure PAI-1 content in real time. We observed a trend towards enhanced PAI-1 release with ascorbate treatment (Figures 4.5 and 4.7). This trend suggests that PAI-1 release may not be directly tied to platelet aggregation.

4.4.5 pH dependent effect of ascorbate on PAI-1 expression

Interestingly, treatment with 500 and 1000 µM ascorbate at an unbuffered pH significantly inhibited platelet PAI-1 release (Figure 4.6). We observed that alteration of pH alone yielded similar inhibition of platelet PAI-1 release (Figure 4.8). This response was abolished when the pH of ascorbate was adjusted to 7.2 (Figure 4.7). Critically ill patients can develop severe acidosis, where the blood pH drops below 6.8 (17), and this level may reach as low as 6.0 in localized regions, particularly during ischemia (5). Although this level is higher than the pH measured in the current study (pH 5.8 at 500 µM and pH 3.8 at 1000 µM), there was a trend of increasing inhibition with increasing dose of ascorbate (Figure 4.6). It is possible that lower levels of unbuffered ascorbate would give a physiological pH, while still inhibiting the release of PAI-1 from platelets. Consistent with our findings of a potential pH dependence of ascorbate, it has been reported that thrombin-induced platelet adhesion is reduced by approximately 25% at pH 7.0 and 50% at pH 6.5 (5). Our preliminary experiments with control sham mice showed that injected ascorbate (10 mg/kg) dropped the blood pH from 7.2 to 7.0 at 5 min post-injection. Thus, our buffered ascorbate solution in the platelet experiments mimicked the

blood's capacity to buffer ascorbate, and indicated that the observed inhibition of thrombin-induced PAI-1 release by ascorbate may not occur under normal physiological conditions *in vivo*. Given the propensity for acidosis during sepsis, we speculate that as the blood pH in septic mice treated with ascorbate may be lower than that in sham mice, it is conceivable that a modest pH dependent reduction in platelet activation and PAI-1 release occurs in our septic mouse *in vivo* model (Chapters 2 and 3).

4.4.6 NO-dependence of PAI-1 release in platelets

Ascorbate has been demonstrated to act in an eNOS dependent manner *in vivo* (13, 22, 23, 25, 26). In the present study, we showed that addition of the NO donor SNAP does not affect the thrombin-induced platelet PAI-1 release. Previously it was demonstrated that SNAP at the same concentration reduces the rate of thrombin-induced aggregation (23). We speculate that the PAI-1 release and the aggregation reflect the different signaling pathways initiated by the thrombin stimulus (16, 30).

In conclusion, we have confirmed the presence and release of PAI-1 protein by both endothelial cells and platelets, two cell types in close proximity to a developing intravascular microthrombus. This local PAI-1 may be critical in the development/stability of the thrombus in sepsis. Ascorbate may reduce the PAI-1 release through a pH-dependent mechanism, thus destabilizing the thrombus and promote restoration of blood flow through plugged capillaries. This potential mechanism warrants further investigation.

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Chapter 5

DISCUSSION

5.1 SUMMARY

Fibrinolysis is a key component in the maintenance of the normal coagulation process, allowing for the dissolution and removal of unneeded clots in the body. During sepsis, capillary blood flow is reduced involving activated coagulation and microthrombi formation in capillaries. Concurrently, fibrinolysis is impaired, stabilizing microthrombi. However, a potential link between reduced blood flow and impaired fibrinolysis has yet to be addressed. Ascorbate has been suggested as a potential treatment for sepsis, as it has been demonstrated to improve capillary blood flow during sepsis. The mechanism of the protective effect remains largely unknown. Given these effects, I hypothesized in Chapter 2 that the capillary plugging observed in sepsis was a result of reduced fibrinolysis, and that ascorbate is protective by increasing fibrinolysis through inhibition of PAI-1. The results of this study are presented in Chapter 2.

As bacteria are found in the blood of septic patients, there is the potential that the spread of bacteria among organs through systemic circulation could be reduced by the impaired capillary blood flow and subsequent increased trapping of bacteria within the organ(s) initially containing the most bacteria. Thus, increased PAI-1 levels during sepsis may actually play a beneficial role by reducing bacterial spread and by promoting the local immune response in this organ(s). For these reasons, I hypothesized in Chapter 3 that ascorbate would not alter the bacterial content, PAI-1 expression or neutrophil recruitment in "immunogenic" organs of mice. Results supporting this hypothesis are presented in Chapter 3.

During sepsis, platelets become activated due to the excessive thrombin expression along with other coagulation proteins. Platelets treated with ascorbate have been demonstrated to have reduced activation and granule secretion. As platelet derived PAI-1 is released through α-granule secretion, I hypothesized in Chapter 4 that ascorbate treatment reduces the platelet PAI-1 expression in response to thrombin stimulation. The results of this study are outlined in Chapter 4.

To support the notion that ascorbate acts in a pro-fibrinolytic manner, I showed in Chapter 2 that streptokinase, a fibrinolytic agent, restored the capillary flow to a similar degree to that of ascorbate. Next, to determine whether fibrinolysis was indeed altered, I studied the mRNA expression of the plasminogen activators t-PA and u-PA, and their inhibitor PAI-1, during sepsis. The mRNA expression of t-PA and u-PA were not altered by ascorbate treatment. Interestingly, the mRNA expression of PAI-1 in the skeletal muscle of septic mice was reduced by ascorbate treatment, suggesting an overall enhancement of fibrinolysis with ascorbate treatment. Unfortunately, the increase in PAI-1 mRNA expression did not translate into increased PAI-1 protein as measured by western blot, ELISA or zymography. Given this discrepancy, I then determined whether there was a difference in mRNA expression between skeletal muscle and liver. Ascorbate did not alter the mRNA expression of u-PA or PAI-1, however t-PA expression was dramatically reduced, suggesting a reduction in fibrinolysis. The increase in PAI-1 expression during sepsis, and the lack of effect by ascorbate, was confirmed by zymography. The plasmin anti-plasmin levels were severely reduced, suggesting a lack of plasmin, likely due to an overall anti-fibrinolytic state. These results failed to support the hypothesis that ascorbate reduces PAI-1, thereby increasing fibrinolysis.

As PAI-1 is not altered by ascorbate, I then wondered if it is beneficial to maintain increased levels of PAI-1 in different organs of the body. First, I confirmed that sepsis significantly increased the bacterial content in all organs, while treatment with ascorbate reduced the bacteria in the blood only. Next, I determined that PAI-1 mRNA is increased in all septic organs studied except the heart. Among these organs, ascorbate reduced the mRNA expression in the skeletal muscle only. Increased PAI-1 protein levels were confirmed in the blood plasma, lung, heart and liver by zymography, while

ascorbate had no effect in any organ. Neutrophil content in the organs was increased in the lung and liver, and decreased in the spleen. Ascorbate did not have any effect on neutrophil content. These results support the hypothesis that ascorbate does not alter (i) the bacterial content, (ii) PAI-1 expression and (iii) neutrophil infiltration in the tissues during sepsis, suggesting that ascorbate has no effect on the immune response during sepsis.

As it is impossible to examine individual cell types in *in vivo* models, I next aimed to determine whether the endothelial cells and platelets, two cell types in direct contact with the blood, have altered PAI-1 expression under septic conditions. I confirmed that long-term exposure to a sepsis-like state increases PAI-1 mRNA expression. In contrast to Chapters 2 and 3, the increase in PAI-1 mRNA did not translate into increased PAI-1 protein expression or release by endothelial cells. Interestingly, I determined that ascorbate alone increases PAI-1 protein expression, but that neither endotoxins nor the coagulation activator thrombin initiated the release of this PAI-1. Next, I showed that thrombin, but not endotoxins, induced PAI-1 release from platelets, and that ascorbate inhibited this release through a pH dependent mechanism. Finally, I showed that the thrombin-induced platelet PAI-1 release is independent of nitric oxide/eNOS. These results partially support my hypothesis that ascorbate inhibits the sepsis-induced increase in PAI-1 production/release, through the discovery of a pH mediated mechanism. The finding that the inhibition of PAI-1 release by ascorbate is NO independent does not support my hypothesis. These findings support the previous *in vivo* studies that ascorbate does not affect PAI-1 expression/release under normal physiological conditions.

In conclusion, the evidence presented in this thesis demonstrates that the sepsis-induced capillary plugging may be perpetuated because of an anti-fibrinolytic environment. The beneficial effect of ascorbate on restoring capillary perfusion likely does not involve alterations in the anti-fibrinolytic state present in the septic microvasculature.

5.2 GENERAL DISCUSSION

5.2.1 Comparison of the models of sepsis

The complexity of the septic process makes it a difficult disease to study. To date, numerous mediators and cell types have been implicated in the pathology of the inflammatory process characteristic of sepsis. A key confounding factor in studying sepsis in humans is the presence of numerous different underlying medical conditions and characteristics present prior to the onset of sepsis in the majority of patients (12). These other diseases (e.g., cancer, diabetes) and factors such as age often alter the septic condition and change the outcome (62). Despite the complexity of sepsis in the clinical setting, animal models have been used to address the various aspects of the septic process (15, 18, 63). The present thesis encompasses three distinct types of models of sepsis, *in vivo*, *in vitro*, and *ex vivo*, to study the fibrinolytic system in the capillaries in relation to sepsis. The relationship between sepsis and fibrinolytic factor content, bacterial load and immune response was explored using an *in vivo* model. The release of PAI-1 from endothelial cells and the mRNA levels of fibrinolytic factors in these cells were studied using an *in vitro* model and the release of PAI-1 from platelets was studied using an *ex vivo* model. Though all models used have limitations, they were used in conjunction to elucidate how ascorbate improves capillary perfusion during sepsis.

5.2.1.1 In vivo models of sepsis

The endotoxemia models most often involve administration of a bolus of lipopolysaccharide (LPS) from the Gram-negative bacteria wall, but alternative models have used initiators such as peptidoglycan, a component of the Gram-positive bacterial wall, and other components elevated during sepsis (15). These components initiate the production of inflammatory cytokines including tumor necrosis factor alpha (TNFα), interferon gamma (IFNγ) and various interleukins (51). The major advantage of the endotoxemia model is the ease in which the experiment can be standardized (11).

Though easily standardized, the use of the endotoxemia model has a major limitation in its lack of clinical relevance, as endotoxins do not accurately mimic bacteria, due to the non-replicating nature of cytokines (11), the inability of LPS to mimic Gram-positive bacteria, (18) and the fact that LPS levels are generally low in septic humans (63).

Bacterial models are considered to be the most physiologically accurate, as the initiation of sepsis is through a bacterial mechanism similar to that of human patients. Direct injection of a known amount of purified bacterial culture, known as a bacteremia model, is the most easily standardized of the three bacterial models (11). Unfortunately, often the bacteria are killed by the immune system prior to replication; therefore the model does not accurately mimic the septic response to an infection (9, 18). Should the bacteria be destroyed, this model largely reverts to an endotoxemia model from the cytokines produced initially, with the drawbacks discussed above.

The remaining two models, CLP and FIP are both based on the same principle, namely they mimic a peritonitis model of sepsis initiated by a bowel perforation. As the bacteria introduced are more numerous both in type and quantity, they sustain cytokine production comparable to that seen in septic patients. Potentially the most biologically accurate model is CLP, where the cecum is ligated to form a bowel obstruction, and is then perforated with a needle. This method allows for a slow release of fecal matter into the peritoneal cavity, and as a result a relatively slow onset to polymicrobial sepsis that fairly accurately mimics that observed in human patients (11). The cytokine release is lower and sustained over a longer period, similar to that observed in septic patients (63). In addition, using different gauge needles, the severity of sepsis can be controlled, allowing for the study of different degrees of perforation and different time points depending on the length of survival for the particular perforation size. The major disadvantages of the CLP model is the high variability in severity of sepsis depending on surgical skill, and the potential for abscess formation around the site of puncture, preventing the fecal matter from escaping from the cecum (15). These two factors contribute to a high degree of variability between one animal and the next, and between the surgical personnel. The necessity to conduct sham operations on control animals further detracts from this model, as any surgery causes cytokine release into the blood

stream, and surgery also has an inherent risk of introducing infection. This leads to the possibility that some control sham animals may also develop sepsis.

The last common model of sepsis is the FIP model, where fecal matter is directly injected into the peritoneal cavity of the mouse. Like the CLP model, the FIP model generates a polymicrobial sepsis similar to that seen in human patients (52). The FIP model relies less on the skill of the experimenter, as it is a simple procedure to inject feces into the peritoneal cavity. In addition, like CLP it is possible to alter the severity by injecting different concentrations of feces. While it is possible for sham mice to become septic due to inadvertent bowel perforation while administering the intraperitoneal dose of sterile saline (to accurately mimic the fluid loading and physical effects on the peritoneum), this is less likely than in CLP as it is much less invasive. Unfortunately, there are drawbacks to the FIP model, including the possibility of abscess formation around the fecal injection and the possibility of injecting the feces subcutaneously instead of into the peritoneal cavity. In addition, the sudden injection of feces is less accurate than the CLP model at mimicking sepsis in humans, as it does not allow for a slow cytokine release in response to a slowly increasing bacterial load (18). It is also possible that the collection of feces from a donor animal and suspension in saline may preferentially select for some types of bacteria over others (in particular, the anaerobic bacteria may not flourish in an aerobic environment), as over 70 different species of bacteria are present in a typical fecal slurry (52).

In the present studies, the FIP model of sepsis was chosen as a compromise to balance enhanced reproducibility with a polymicrobial sepsis model, while maintaining a model closely mimicking human sepsis.

5.2.1.2 Methodological limitations

Research using animal model of sepsis is also difficult due to the methodological limitations inherent in these models. As a result, simplified models are constantly being

developed to attempt to cope with these difficulties. In the present studies, I have employed *in vivo, in vitro* and *ex vivo* models to mimic particular aspects of sepsis.

The *in vivo* model of sepsis is generally regarded as the most physiologically and pathologically relevant. The various different *in vivo* models are detailed above (see section 5.2.1.1). While using the FIP model of sepsis, I encountered one constant confounding issue, the high degree of inter-individual variability between one animal and the next. Despite using exactly the same protocol in inducing sepsis, I still observed a 7,000 fold difference in the number of bacteria in the peritoneal fluid of septic mice (see Chapter 3). This enormous range in infection has likely contributed to the variability observed in the other experimental parameters explored in Chapters 2 and 3. The possibility that a protective effect of ascorbate is masked by the large variability in the FIP model cannot be discounted. I have tried to reduce this possibility by using several different techniques where feasible (i.e., using qPCR, ELISA and zymography to study PAI-1). Another limitation is the inability to isolate the response from individual cell types in the whole organ homogenate. It is entirely possible that alterations in protein expression in the endothelial cells that line the blood vessels were masked by the proteins present in the surrounding tissues collected in the whole tissue sampling. For this reason, I also used an *in vitro* endothelial cell model.

The *in vitro* model employed in the present studies was a purified skeletal muscle microvascular endothelial cell line. Using this model I was able to directly measure mRNA and protein expression in endothelial cells alone. Although this method allows one to zoom at the cellular level, the major drawbacks that I encountered were the difficulty in mimicking sepsis, and the possibility of cell phenotype changing under culturing conditions. I used cytokines known to play a role in the septic pathophysiology, and applied them to an established in vitro model of microvascular endothelial cells. In addition, I have obtained primary endothelial cell cultures from septic mice as a hybrid model that includes the characteristics from the *in vivo* model. The hybrid model allows examination of mRNA expression from a single cell type as in the *in vitro* models (see Chapter 2). Unfortunately due to the small number of cells obtained, this model was not conducive to the determination of protein expression.

To study platelets, I employed an *ex vivo* model. The model involved removing platelets from mice and subjecting them to various chemicals to elicit responses. As platelets are a small component of the body, altered platelet protein expression might be hidden by protein expressed in the tissue cells. Major drawbacks of the platelet *ex vivo* model are that (i) similar to in vitro models, sepsis is difficult to model here, and (ii) platelets by nature tend to activate and aggregate in response to being removed from the body. Similar to the *in vitro* model, I used established methods of mimicking sepsis. I also used great care to prevent platelet activation and used the appropriate controls to determine when autoactivation occurred.

In addition to the limitations of the models, there were other methodological issues. I noted a lack of sensitivity to detect changes in PAI-1 protein using western blotting and ELISA. As I was able to use ELISAs to observe increases in PAI-1 protein levels in blood plasma, it seems that the most likely source of lack of sensitivity was the high basal levels in non-vascular cells masking a modest increase in PAI-1 in the vasculature. Due to the inability to detect PAI-1 protein levels directly, I instead had to rely on an alternative method using reverse fibrin zymography. The zymography employed was modified from published methods of fibrin zymography (7, 27) with one major alteration being the addition of exogenous u-PA. This allowed for the indirect measurement of PAI-1 activity through the inhibition of the exogenous u-PA. As PAI-1 is reported to be responsible for virtually all of the inhibition of u-PA (45), this method has been used as an indicator of PAI-1 activity (and indirectly PAI-1 content, see Chapters 2 and 3). Reverse fibrin zymography on mouse blood plasma mirrored the active PAI-1 protein detected by ELISA, validating this method. As there are two plasminogen activators that are inhibited by PAI-1, u-PA and t-PA, it is possible that the levels of PAI-1 are actually under-reported in the present studies due to some inhibition of endogenous u-PA and t-PA. It is difficult to eliminate this possibility. However, through the use of a known amount of exogenous u-PA, this method gives a measure of the free PAI-1 (total PAI-1 minus PAI-1 reacting with endogenous t-PA and u-PA), and therefore gives a total measure of the anti-fibrinolytic potential of the tissue. Although the various models used had limitations and drawbacks, I have used these models in conjunction with each other to test my hypotheses.

5.2.2 Capillary plugging during sepsis

Sepsis causes many alterations to the vascular system, including hypotension, reduced vascular reactivity and reduced capillary perfusion (2, 6, 56, 61). It has been reported that sepsis can reduce capillary perfusion in the skeletal muscle to the point where less than half of all capillaries have blood flowing through them (57). This impairment of capillary flow has been observed in other animal models of sepsis, in their various septic organs, and in septic patients (4, 5). The exact mechanism of this reduction in perfusion remains elusive, with several possibilities, including a narrowing of the capillary lumen preventing red blood cell flow, stiffening of red blood cells causing them to become lodged in the capillary, the lodging of leukocytes in the vasculature preventing blood flow, closing of precapillary sphincters preventing flow to certain capillaries and the formation of microthrombi that occlude the capillaries (3, 24, 25, 34, 49). Recent evidence lends support to the notion that microthrombi formation contributes partially to this phenomenon, namely that fibrin was observed in the septic capillaries, and that depletion of platelets or inhibition of the coagulation cascade partially restored capillary perfusion (49). This partial restoration suggests that another mechanism is also involved in the capillary plugging other than platelet adhesion and coagulation. That decreased fibrinolysis contributes to this phenomenon is a possibility, given that PAI-1 protein expression is increased during sepsis (33, 55). Increased PAI-1 would only serve to further enhance the stability of the fibrin and, in conjunction with platelet adhesion, increased PAI-1could lead to the formation of stable microthrombi that plug the capillaries.

It has been reported that red blood cells exhibit reduced deformability during sepsis (3). Stiffer red cells in conjunction with platelet adhesion and fibrin deposition may serve to enhance the capillary occlusion leading to the cessation of blood flow. Leukocytes have been observed to adhere to the endothelium during sepsis (24, 34). The contribution leukocyte adhesion has to capillary plugging is unclear as adhesion has almost exclusively been observed in the post-capillary venules only (49). The vascular reactivity has also been observed to decrease during sepsis (35). The loss of vascular reactivity serves to further derange the blood flow to the capillaries, as the arterioles

cannot properly maintain optimal blood flow velocity through contraction and relaxation. However, the plugging of capillaries in sepsis cannot be explained solely in terms of closed venules or arterioles, because plugged capillaries have been observed even when their feeding arterioles and draining venules were patent and perfused with blood (49). A recent study observed a narrowing of the capillary lumen consistent with what might be termed a capillary sphincter (25). It is important to note that this narrowing only occurred sporadically in capillaries in the skin. The implication of this finding on deeper tissue capillaries has not yet been addressed. It must be acknowledged that narrowing of the capillary lumen could also be caused by edema of the surrounding tissue and swelling of the endothelial cells, both common occurrences in sepsis (21, 28), however edema was not observed 24 hours into sepsis when capillary plugging is already present (43). Previous studies examining the capillary plugging revealed that during sepsis, many capillaries are intermittently perfused (i.e., the red blood cells show stop-and-go movement) (2, 57) This could be explained by the possibility that red blood cells encounter a temporary occlusion in the capillary, completely eliminating flow, and then re-start the flow following the release of the occlusion. The observed intermittent flow is in direct contrast to what would be expected through closure of a precapillary sphincter, where the occluding region would be situated immediately prior to the capillary entrance.

The present studies support the notion that during sepsis there is enhanced fibrin stabilization due to sepsis-induced increases in PAI-1 expression. Increased PAI-1 protein levels may be beneficial to survival from sepsis. In my pilot experiments, I was unable to identify fibrin present solely in capillaries of immunohistological sections of the septic skeletal muscle, possibly due to the lack of appropriate antibodies for fibrin. As a result, I could not evaluate the potential for stabilization of fibrin by PAI-1 using immunohistology.

5.2.3 Ascorbate and capillary plugging

An intravenous bolus injection of ascorbate has been demonstrated to decrease both mortality and capillary plugging in septic mice (49, 57, 58) Chapter 2). Ascorbate could

accomplish this beneficial effect partially through reduced platelet activation and aggregation in a P-selectin mediated mechanism (49, 50). As platelet depletion alone cannot account for the reversal of capillary plugging by ascorbate, clearly another mechanism is at play. Ascorbate has been demonstrated to act in an endothelial nitric oxide synthase (eNOS) dependent manner (49), suggesting that it may target any proteins that are regulated by eNOS-derived NO. As vascular smooth muscle cell tone is regulated by eNOS/NO (10), it is possible that ascorbate-mediated increases in eNOS activity may relax the blood vessels. Though capillaries themselves are not covered with smooth muscle cells, it is possible that increased blood flow in the arterioles immediately upstream from the plugged capillaries is able to restore perfusion to these capillaries. Alternatively, relaxation of venous smooth muscle and subsequent dilation would allow blood to properly drain from the capillaries. Ascorbate has been demonstrated to restore the mean arterial pressure in CLP induced septic rats (58). Ascorbate also inhibits the reduced vasoconstriction of arterioles observed in sepsis (35) and restores the arterial response to vasoconstrictors in LPS injected humans (44). These combined arterial alterations may improve the capillary perfusion through increased and better regulated pressure to drive the blood through individual capillaries.

5.2.4 Fibrinolytic effects of ascorbate

Fibrinolysis is known to be depressed during sepsis, contributing to the stability of fibrin in the vasculature. A recent study reported that t-PA mRNA is upregulated with ascorbate treatment during sepsis (16). Several other studies have determined that t-PA is upregulated upon ascorbate administration in other disease processes (26, 59), though others have observed downregulation of t-PA in smokers and diabetics (42, 54). Studies examining the effect of ascorbate on PAI-1 protein are inconclusive, with some evidence for downregulation and other studies finding no effect (1, 26, 42, 54). The present studies, in an attempt to clarify the discrepancies in the literature, aimed to examine the effect of ascorbate in the context of fibrinolysis. The evidence obtained in these studies suggests that ascorbate may not change the sepsis-altered fibrinolytic system. In Chapters 2 and 3, the data indicate that there is no effect of ascorbate on PAI-1 on a

system wide basis. There may be an effect on the mRNA expression of PAI-1 in skeletal muscle, but this finding wasn't consistent with the PAI-1 protein expression. It is entirely possible that ascorbate affects the PAI-1 expression in a delayed manner, however this delayed effect could not explain the reperfusion in capillaries seen already 1 hour after ascorbate injection. Data in Figure 2.3 indicate that this delayed effect is unlikely, as at 3 h post-injection (i.e., 9 h post-FIP), ascorbate had no effect on sepsis-induced PAI-1 protein increase in the blood plasma. In Chapter 4, results *in vitro* suggest that ascorbate treatment for 24 hours increases the production of PAI-1 in endothelial cells. Interestingly, co-treatment with LPS or TNFα abolished this increase. It remains unclear what the implications of increased PAI-1 would be in a healthy individual, though it should be noted that there is no evidence that this increased PAI-1 is released into the extracellular space.

I showed that the platelet PAI-1 protein release is inhibited by ascorbate at low pH. It is worth noting that in a state of acidosis, the combination of reduced platelet PAI-1 release and unchanged PAI-1 release by endothelial cells may result in reduced PAI-1 in the capillary blood. The observed reduction in capillary plugging in sepsis seems unlikely to be exclusively due to a pH-mediated mechanism given the limited evidence that ascorbate reduces blood pH (Chapter 4). Overall, these findings point to another unknown mechanism by which ascorbate acts in a protective manner in the septic microvasculature.

5.2.5 Potential non-fibrinolytic effects of ascorbate

Ascorbate is a well known antioxidant and it is presumable that at least some of its beneficial effect is through a reduction of reactive oxygen species. The fact ascorbate acts in a beneficial effect is somewhat paradoxical, as antioxidants would be expected to diminish the respiratory burst involved in the immune response (46). Theoretically, this could be counteracted by a bactericidal effect of ascorbate at relatively high doses (20, 68). However, in Chapter 3, I have determined that ascorbate did not alter the levels of bacteria in any organ aside from the blood. It is important to note that the animal models

showing a bactericidal effect of ascorbate typically use a chronic dosing of ascorbate. This dosing could explain the apparent discrepancy between these studies and the present one. Nevertheless, it has been demonstrated that six hours after ascorbate injection, the peritoneal bacterial levels were unaltered (67).

Tissue factor, a major initiator of coagulation, has been demonstrated to be activated by ROS. Succinobucol, an antioxidant, reduces this activation (31). It is presumable that the antioxidant action of ascorbate would act in a similar manner, reducing tissue factor, and therefore reducing the initiation of coagulation. As it is commonly accepted that abnormal tissue factor expression is a characteristic of sepsis (32), reduction of tissue factor would reduce coagulation and arrest the further development of microthrombi. In conjunction with any degree of fibrinolysis (whether rescued by ascorbate or not) a reduction in coagulation could lead to a reversal of capillary plugging. Interestingly, depleting tissue factor improves survival during sepsis (40), and therefore a reduction in tissue factor may be another mechanism by which ascorbate reduces capillary plugging.

In addition to changes in the initiation in coagulation, sepsis-induced platelet activation (i.e., P-selectin upregulation, aggregation) has been reported to be inhibited by ascorbate administration (49, 50). This reduction in platelet activation would reduce microthrombi formation and capillary plugging in sepsis (49).

To explain the discrepant results of the reported inhibitory effect ascorbate on platelet activation (50) and my present study on PAI-1 release, it is possible that ascorbate only inhibited some of the thrombin receptors. Protease activated receptors 1 and 4 (PAR1 and PAR4) are cleaved by thrombin and activate the cyclic GMP pathway. It has been demonstrated that platelet PAI-1 release is more sensitive to PAR1 (39), while knockout of PAR4 almost completely abolished platelet aggregation and significantly increased the time required for P-selectin expression (60). Moreover, the use of a PAR1 antagonist did not affect the thrombin stimulated P-selectin release (14). As PARs have been demonstrated to play a role in eNOS derived NO production (23, 64), I speculate that

ascorbate is preferentially altering PAR4 rather than PAR1, reducing platelet P-selectin expression and platelet activation while maintaining normal PAI-1 release.

Another potential non-fibrinolytic effect of ascorbate is to increase red blood cell deformability during sepsis (3). It has been demonstrated that ascorbate is present in red blood cells, and that the levels are identical to that of the blood plasma (29). A reduction in blood ascorbate concentration, as observed during sepsis, would presumably lead to a reduction in red blood cell ascorbate concentration. It is unclear how rapidly the red blood cells lose their ascorbate content. However, at 4°C, red blood cells lost nearly 25% of their ascorbate content after 6 h of exposure to ascorbate-free medium (29). It has not yet been determined what effect the loss of ascorbate would have on red blood cell function. However, it is likely the loss of this antioxidant would reduce red blood cell deformability (and thus promote capillary plugging), because treatment with the antioxidant aminoguanidine inhibited this reduced deformability during sepsis (3).

5.2.6 Ascorbate as a treatment in septic patients

Ascorbate has been demonstrated to be safe even at very high doses. Human cancer patients are routinely given ascorbate at doses approaching, or even exceeding, 1 g/kg body weight with minimal side effects (65). In addition, ascorbate is inexpensive and readily available. Given these characteristics, the only impediment to clinical trials is a lack of understanding as to how ascorbate acts in a protective manner.

As ascorbate levels are markedly reduced during sepsis (48), supplementation with large doses is necessary to restore the blood plasma to normal levels (19, 30, 38). There is no evidence of a detrimental effect of ascorbate at high levels in non-cancer patients (16, 36, 37), therefore given the demonstrated safety of ascorbate, this is not an impediment to its development as a therapy for the treatment of sepsis.

The use of ascorbate administered with vitamin E reduced mortality and multiple organ failure in both septic and non-septic critically ill patients (8, 38). Very recently, the use of ascorbate alone on a small number of severely septic patients (Phase I trial) has been

shown to have beneficial effects on various markers inflammation, the length of hospital stay, and 28-day mortality (17).

A randomized controlled study of critically ill patients concluded that administration of glutamine and vitamins including ascorbate did not improve the mortality after 28 days (22). It is important to note that this study involved enteral administration of ascorbate along with four other vitamins and minerals, rather than a single intravenous bolus of ascorbate as it has been used in the present thesis. It is conceivable that the reduction in capillary plugging observed could reduce survival in the longer term (i.e., after 28 days). Survival studies in mice show that ascorbate injection at the onset of sepsis, or delayed to 6 h of sepsis, significantly decreased mortality (57, 67). I have demonstrated in Chapter 4 that, after ascorbate administration, the bacterial content in the organs is not altered, indicating that ascorbate does not promote the spread of infection, even though increased capillary flow could theoretically reduce bacterial trapping in the organs.

The efficacy of ascorbate as a treatment may be enhanced due to its high bioavailability and ability to accumulate in endothelial cells when given intravenously (66). Ascorbate is rapidly taken up by endothelial cells through vitamin C transporters and accumulates to a steady state concentration of approximately 16 mM (66). Its safety and efficacy is further enhanced by the fact that excess circulating ascorbate is rapidly excreted from the body by the kidneys (13). This protects endothelial cells through the antioxidant capacity of the ascorbate, while allowing for the normal production of ROS in cells involved in the respiratory burst, and preserving the bactericidal role of ROS in the immune system (46).

Despite the relative safety of ascorbate, high doses can result in discomfort due to a number of side effects. Frequently, patients have gastrointestinal disturbances such as nausea, diarrhea and pyrosis and increased urination accompanied by a burning sensation (47). It can also result in red blood cell hemolysis and vitamin B_{12} deficiency (47). In addition, acidification of the urine over a longer time period (months to years) increases the risk of kidney stones (53). The efficacy of certain drugs can be altered by high dose ascorbate, and some pathological conditions, particularly relating to excess transition

metal concentration in the blood (including excess iron due to hemocromatosis, sideroblastic anemia and thalassemia) can lead to a prooxidant state (47). These conditions are relatively rare, but caution would be necessary when using ascorbate as a treatment in these patients.

5.3 Summary, future directions, and overall findings of this research

Overall, the present studies show that sepsis alters the fibrinolytic system, primarily through an increase in PAI-1, and that ascorbate does not appear to alter this increase. In Chapter 2, I showed that ascorbate does not alter the sepsis-induced increase in PAI-1 protein in the skeletal muscle or liver. Ascorbate did partially restore towards control the low platelet count in sepsis. However, there was no effect on any measurements of the coagulability or fibrinolytic potential of the blood. In Chapter 3, I extended my study to confirm that ascorbate did not alter the PAI-1 expression in a variety of different organs, confirmed that ascorbate had no effect on the bacterial levels in tissues aside from the blood, and determined that there is no effect of ascorbate on neutrophil recruitment to the infected organs. Chapter 4 included an exploration of the effect of ascorbate on PAI-1 expression and release by platelets and endothelial cells. I determined that ascorbate may inhibit PAI-1 release by platelets at high concentrations, while ascorbate increased production of PAI-1 in endothelial cells. The effect of ascorbate on platelets was observed to be pH sensitive; however the implications of this finding are unclear, as it is uncertain whether the blood pH would be low enough after ascorbate injection to induce this effect. I demonstrated that PAI-1 mRNA was inhibited by ascorbate in the skeletal muscle in Chapter 2. I have shown that although endothelial cells release PAI-1 protein, the overall contribution to the total blood plasma PAI-1 level might be low (Chapter 2). Overall, the evidence presented points to a lack of effect of ascorbate on sepsis induced PAI-1 expression, suggesting other mechanisms are responsible for the beneficial effects of ascorbate seen in the microcirculation.

Several questions remain unanswered about the effect ascorbate might have on the fibrinolytic system. In Chapter 4, I found that low pH inhibits platelet PAI-1 release. This finding is intriguing, as unbuffered ascorbate will have some effect on the blood pH, particularly near the site of injection. Future studies should explore this further, determining the degree of effect ascorbate has on blood pH, the length of time and threshold pH in order to inhibit platelet PAI-1 release, and how long this phenomenon lasts after the pH increases. As severely septic patients have been known to have severe acidosis with a blood pH below 6.8 (41), the possibility that low blood pH in conjunction with ascorbate might reduce platelet activation and PAI-1 release is intriguing and warrants future study. As a blood pH below 6.8 generally leads to a poor prognosis, it should be investigated whether ascorbate can reduce platelet activation in a slightly acidic environment between 6.8 and 7.0. An important question to be answered is whether human patients can be effectively administered ascorbate in a buffer at pH 7.2 or whether it must be unbuffered to be effective.

Another question to be explored is the implication of ascorbate increasing PAI-1 protein expression in endothelial cells. It is unclear whether ascorbate is increasing the production of PAI-1 or stabilizing the existing PAI-1. It is also unclear why the PAI-1 expression would increase after ascorbate treatment, and whether long-term treatment with ascorbate would boost PAI-1 protein expression in healthy individuals. Future studies should explore the possibility that PAI-1 protein is increased in healthy humans upon ascorbate treatment. If PAI-1 is indeed increased, it would be necessary to determine how this could be beneficial, as this effect would appear to be detrimental at first glance. In addition to the above studies, it is important to continue work with the septic model to determine what other pathways ascorbate may affect. It has yet to be determined whether ascorbate affects red blood cell deformability, as this is another potential mechanism to explain the protective effect in capillaries. Importantly, ascorbate level in red blood cells reflects that of the blood plasma (29). To the best of my knowledge, it has not been determined whether the sepsis-induced decreases in blood plasma ascorbate levels are reflected in the red blood cells.

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Appendices

Appendix 1: Ethics approval for animal use

2011-062::2:
AUP Number: 2011-062
AUP Title: Microvascular dysfunction in sepsis
Yearly Renewal Date: 10/01/2013
The YEARLY RENEWAL to Animal Use Protocol (AUP) 2011-062 has been approved, and will be approved for one year following the above review date.

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office.
   Health certificates will be required.

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.
The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.
Submitted by: Kinchlea, Will D
on behalf of the Animal Use Subcommittee

Curriculum Vitae

Name:	Scott Swarbreck
Post-secondary Education and Degrees:	The University of Western Ontario London, Ontario, Canada 2003 - 2007 B.M.Sc. (Honours) Physiology and Pharmacology
	The University of Western Ontario London, Ontario, Canada 2008 - 2014 Ph.D. in Pharmacology
Honours and Awards:	Ontario Graduate Scholarship in Science and Technology 2010 – 2011
Related Work Experience	Teaching Assistant (Pharmacology 3580Y) The University of Western Ontario 2008 – 2013
	Summer Student (Dr. Karel Tyml's Lab) The University of Western Ontario 2008
	Research Assistant Chemtura Canada 2006 – 2007

Refereed Publications:

Secor, D., **Swarbreck, S**., Ellis, C.G., Sharpe, M.D., and Tyml, K. (2013) Ascorbate reduces mouse platelet aggregation and surface P-selectin expression in an ex vivo model of sepsis. Microcirculation. 20(6): 502-10.

McKinnon RL, Bolon ML, Wang HX, **Swarbreck S,** Kidder GM, Simon AM, Tyml K. (2009). Reduction of electrical coupling between microvascular endothelial cells by NO depends on connexin37. Am J Physiol Heart Circ Physiol. 297(1): H93-H101.

Swarbreck S, Secor D, Li F, Gross PL, Ellis CG, Sharpe MD, Wilson JX, Tyml K. (2014). Effect of ascorbate on fibrinolytic factors in septic mouse skeletal muscle. (In Press), Blood Coagulation and Fibrinolysis BCF-052143.

Swarbreck S, Secor D, Ellis CG, Sharpe MD, Wilson JX, Tyml K. (2014). Short-term effect of ascorbate on bacterial content, plasminogen activator inhibitor-1 and myeloperoxidase in septic mice. (In Press), Journal of Surgical Research JSURGRES-D-13-01713

Manuscripts Submitted for Publication:

Swarbreck S, Secor D, Ellis CG, Sharpe, MD, Wilson JX, Tyml K. (2014). Effect of ascorbate on plasminogen activator inhibitor 1 expression and release from platelets and endothelial cells in an in-vitro model of sepsis. Submitted to Microcirculation.

Siddiqui, M., **Swarbreck, S**., Shao, Q., Peng, T., Laird, D.W., and Tyml, K. (2014). The carboxyl terminal of Cx40 is required for reduced electrical coupling between microvascular endothelial cells in a model of sepsis. Submitted to Microcirculation.