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Mechanism of Ascorbate Protection Against Sepsis-Induced Capillary Blood Flow Impairment

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

MECHANISM OF ASCORBATE PROTECTION AGAINST SEPSIS-INDUCED CAPILLARY BLOOD FLOW IMPAIRMENT

(Thesis format: Integrated Article)

by

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Abstract

Sepsis is a systemic inflammatory response to an infection. The overwhelming inflammation has many deleterious effects, including cessation of capillary blood flow. This cessation may lead to organ failure and subsequent death, but the cause of cessation during sepsis is not fully understood. Ascorbate (reduced vitamin C) has been shown to restore capillary blood flow by an unknown mechanism. I hypothesized that activation of both platelets and the coagulation pathway in sepsis contributes to the cessation of capillary blood flow and that ascorbate protects against cessation by reducing platelet activation.

Using intravital microscopy in the mouse hindlimb skeletal muscle *in vivo*, I observed that sepsis impairs capillary blood flow and increases both adhesion of platelets/platelet aggregates to the capillary wall and deposition of fibrin plaques in the same capillaries. Platelet depletion, blocking of P-selectin (a key adhesion molecule), antithrombin, and eptifibatide (anti-aggregatory agent) all reduced the capillary blood flow impairment and platelet adhesion. Intravenous bolus injection of ascorbate reduced platelet adhesion in capillaries, via the endothelial nitric oxide synthase (eNOS) system.

To study any direct effects of ascorbate on platelet function, I used an *ex vivo* model (isolated mouse platelets) examining platelet aggregation under septic conditions. Here, thrombin, ADP, and thromboxane (agents released into the blood during sepsis), but not lipopolysaccharide (LPS), tumor necrosis factor (TNFα) or septic plasma, increased platelet aggregation and surface P-selectin protein expression. Ascorbate inhibited the increased aggregation and P-selectin expression.

Next, an *in vitro* mouse microvascular endothelial cells model was used to study the effect of ascorbate on platelet-endothelial cell adhesion. LPS and TNFα increased platelet adhesion and P-selectin mRNA expression in endothelial cells. LPS also increased P-selectin-containing endothelial granule secretion. Ascorbate prevented the increased adhesion and granule secretion but did not affect mRNA expression.

Thus, I conclude that impairment of blood flow in the septic microvasculature requires platelets and is reduced by anti-coagulant/anti-aggregatory agents. Ascorbate prevents platelet-endothelial adhesion and platelet aggregation, partly through reducing

P-selectin protein expression at the platelet/endothelial cell surface. Thus ascorbate reduces adhering platelets in septic capillaries leading to restoration of blood flow.

Keywords: Ascorbate, sepsis, capillary blood flow, platelet aggregation/adhesion, P-selectin

Co-Authorship

In the investigation entitled:

Impaired microvascular perfusion in sepsis requires activated coagulation and P-selectin mediated platelet adhesion in capillaries (Chapter 2),

Ms. F. Li assisted in preparation of septic mice including intravenous injections, video recordings, and analysis of blood flow stoppage in capillaries. Drs. M. Sharpe, C. Ellis, P. Gross, and J. Wilson participated in manuscript writing.

Ascorbate reduces mouse platelet aggregation and surface P-selectin expression in an ex vivo model of sepsis (Chapter 3),

Drs. M. Sharpe, C. Ellis, and Mr. S. Swarbreck participated in manuscript writing.

Ascorbate reduces P-selectin mediated platelet-endothelial adhesion in an in vitro model of sepsis via reduced endothelial granule secretion (Chapter 4),

Mr. S. Swarbreck assisted in the preparation of septic mice and mRNA collection and analysis. Ms. S. Seghal measured, in blinded fashion, the platelet-endothelial adhesion to verify my measurements of this adhesion.

In all of the above investigations, all work not specified above was performed by myself. All manuscripts were written principally by myself. Dr. K. Tyml assisted in the experimental design, helpful discussions, and preparation of the final manuscripts and helped in the preparation of this thesis.

In this thesis (Chapters 2-4), I use “we” to retain the original version of the published manuscript. The usage of “we” reflects the co-authorships of the paper.

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List of Abbreviations

5HT	5-hydroxytryptamine (serotonin)
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BH_2	Dihydrobiopterin
BH_4	Tetrahydrobiopterin
cGMP	Cyclic guanosine monophosphate
CLP	Cecal ligation and perforation
DIC	Disseminated intravascular coagulation
DNA	Deoxyribonucleic acid
DNAME	N-Nitro-D-arginine methyl ester
EC	Endothelial cell
EDL	Extensor digitorum longus
eNOS	Endothelial nitric oxide synthase
$FeCl_3$	Ferric chloride
FIP	Feces injection into peritoneum
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GPIIb/IIIa	Glycoprotein IIb/IIIa
HRP	Horse radish peroxidase
ICU	Intensive care unit
IFNγ	Interferon gamma
IL-6	Interleukin 6
iNOS	Inducible nitric oxide synthase

LDL	Low-density lipoprotein
LNAME	N-Nitro-L-arginine methyl ester
LPS	Lipopolysaccharide
Mϕ	Macrophages
MOF	Multiple organ failure
mRNA	Messenger Ribonucleic acid
NADPH	β-Nicotinamide adenine dinucleotide phosphate
NFκB	Nuclear factor kappa B
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
NSF	N-ethylmaleimide sensitive factor
PAI-1	Plasminogen activator inhibitor-1
PBS	Phosphate buffered solution
PE	Phycoerythrin
PECAM	Platelet endothelial cell adhesion molecule-1
PKC	Protein kinase C
PMN	Polymorphonuclear neutrophils
PS	Phosphatidylserine
PSGL1	P-selectin glycoprotein receptor-1
qPCR	Quantitative polymerase chain reaction
RBC	Red blood cell
ROS	Reactive oxygen species
SNAP	S-nitroso-N-acetylpenicillamine
SVCT2	Sodium-dependent vitamin C transporter 2

TNFα	Tumor necrosis factor alpha
TF	Tissue factor
tPA	Tissue plasminogen activator
TRITC	Tetramethylrhodamine isothiocyanate
TXA	Thromboxane A_2
uPA	Urokinase plasminogen activator
US	United States of America
vWF	von Willebrand factor
WBC	White blood cell

Chapter 1

INTRODUCTION

This thesis focuses on the capillary blood flow impairment during sepsis, the coagulation system, a potential key component of this impairment, and the protective effect of ascorbate (a reduced form of vitamin C) against this impairment. The introduction will provide background information on: i) sepsis, including factors that contribute to the capillary blood flow impairment, ii) coagulation, including formation of micro-thrombi, and iii) ascorbate and its possible use as a treatment for sepsis.

1.1 SEPSIS

1.1.1 Clinical significance

Sepsis is the tenth leading cause of mortality in the United States of America (57) and is the leading cause of mortality in non-coronary intensive care units in North America (101), with a mortality rate near 40% (75). Despite extensive research, the mortality rate for sepsis increased between 1995 and 2005 (81). In low-income countries, the mortality rate is likely higher as treatment is dependent on available intensive care unit services. In some countries, there has been no improvement in treating sepsis in the last 10 years (11, 69).

Sepsis is a systemic inflammatory response syndrome due to an infection (15, 27). Patients are considered septic when they have 2 or more of the following features: core

temperature ≥ 38°C or ≤ 36°C, heart rate ≥90 beats/min, respiratory rate > 20 breaths/min or partial pressure of carbon dioxide <32 mmHg or mechanical ventilation, and white blood cell (WBC) counts >12,000/mm^3 or <4,000/mm^3. 'Severe sepsis' is when there is dysfunction in 2 or more organs and 'septic shock' is when hypotension is present (15).

Current treatment focuses on treating the infection and the features (i.e., maintaining blood pressure), while treatments for the inflammatory response are ineffective or only have marginal benefits (27). Despite extensive research and numerous clinical trials, only one pharmacological treatment reached clinical approval for treatment of sepsis, activated protein C, and has since been withdrawn from the market for lack of efficacy (74, 94). The lack of progress in determining effective treatments represents our limited understanding of the mechanisms involved in the pathophysiology of sepsis.

1.1.2 Pathophysiology of sepsis

On the cellular level, the inflammatory response in sepsis is a result of overwhelming production of cytokines by the host to defend against an invading pathogen. In the case of a gram-negative bacterial infection, sepsis is initiated by the endotoxin lipopolysacchride (LPS), a component of gram negative bacteria. LPS results in the release of cytokines such as tumour necrosis factor α (TNFα), interleukin-6 (IL-6), and interferon γ (IFNγ) (2, 97) from macrophages and lymphocytes. This process is mediated through toll-like receptor 4 (90) and proteins of the complement system (125). These cytokines, along with LPS, further stimulate macrophages, neutrophils, platelets, and endothelial cells to express adhesion proteins and chemokines as well as produce reactive oxygen species (ROS) and nitric oxide (NO) (90). The end result of these processes includes many

serious physiological alterations including a multitude of circulatory dysfunctions such as hypotension, decreased systemic vascular resistance, impaired oxygen utilization, maldistribution of blood flow in the microcirculation, impaired capillary blood flow, and decreased cellular communication along the vascular wall (15, 27, 60, 109, 110, 112). These dysfunctions eventually progress towards tissue injury and multiple organ failure (49) (Figure 1.1).

1.1.3 Capillary blood flow impairment

As mentioned, one consequence of systemic inflammation is an impairment of capillary blood flow. This impairment has been observed and defined as a decrease in perfused capillaries and an increase in non-perfused capillaries in rat and mouse skeletal muscle (3, 60, 107, 108). In other words, there is an increase in the percentage of stopped-flow capillaries during sepsis. A similar impairment of capillary blood flow is evident in other organs and in septic patients (10, 14). The microvascular dysfunction leads to poor oxygen transport and tissue hypoxia due to increased oxygen diffusion distances from red blood cells in capillaries to the mitochondria of surrounding cells (30, 40). Tissue hypoxia due to this impairment may account for one third of patients who die from organ failure even when shock is prevented (98, 99). The underlining mechanism of the capillary blood flow impairment is not yet known.

The impairment of flow is heterogeneous in nature meaning that capillaries in a capillary unit supplied by a common arteriole and drained by a common venule include both perfused and stopped-flow capillaries (107). This indicates that the impairment is

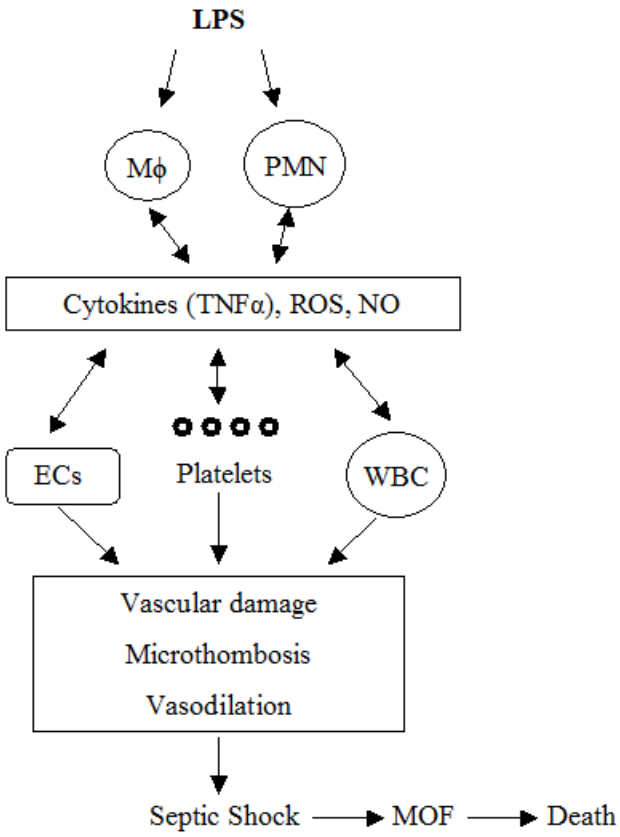

Figure 1.1. A simplified scheme representing the mechanism of LPS-induced sepsis. LPS from gram negative bacteria enters circulation and stimulates macrophages (Mϕ) and neutrophils (PMN) to release cytokines such as TNFα. These cytokines (along with LPS) activate endothelial cells (ECs), platelets, and white blood cells (WBC) to further produce cytokines as well as ROS and NO. These mediators, together with the ECs, platelets, and WBC, cause vascular damage, microthrombosis, and vasodilation potentially leading to septic shock, multiple organ failure (MOF), and/or death.

not due to dysfunction of larger blood vessels. Since the microcirculation in skeletal muscle does not contain precapillary sphincters (31) (i.e., smooth muscle cells encircling the entrance segment of the capillary) there is no obvious physiological process (e.g., constriction and relaxation of sphincters) that could explain this heterogeneous capillary blood flow. Thus, the stoppage of flow in capillaries seen during sepsis is most likely an outcome of pathological processes initiated by sepsis.

There are various microvascular dysfunctions in sepsis that could contribute to the capillary blood flow impairment (Table 1.1, columns 1 and 2, on page 18), including the plugging of the capillary lumen by adhering leukocytes, stiffened erythrocytes and/or micro-thrombi containing platelets and fibrin plaques. Leukocytes are known to have increased adhesion to the endothelium during sepsis (25, 79) and could possibly plug capillaries. However, leukocytes are located primarily in the lung and liver during sepsis (80, 82). Accordingly, it was concluded that leukocytes are not responsible for the impairment in septic rat skeletal muscle (91). Based on this report we predicted that, in our mouse skeletal muscle model of sepsis, leukocytes may have a minor/negligible role in the impairment. Erythrocytes have been shown to have decreased deformity during sepsis (9), and therefore they may contribute to capillary plugging. Platelets become activated during sepsis, leading to their increased aggregation and adhesion to the capillary wall (66). Combined with increase fibrin formation (detailed in section 1.2.2), this would progress to the formation of microthrombi that could block the flow in the capillary. Since little is known about the mechanism of capillary plugging, this thesis will focus on investigating the role of platelets and microthombi in the capillary blood flow impairment

Clinically, a lowered platelet count in systemic blood correlates with increased severity of sepsis (77). One possibility is that platelets are 'trapped' in the capillaries due to the formation of microthrombi. It was observed that the blood flow in a single capillary has intermittent stoppage periods until it ultimately becomes permanently stopped. This could be explained by the process of ongoing formation and breakdown (thrombolysis) of thrombi in capillaries during the initial stage of sepsis. The progressing severity of sepsis eventually tilts the equilibrium towards microthrombi formation leading to the plugging of capillaries (35).

1.1.4 ROS and sepsis

ROS are highly reactive molecules due to an unpaired electron in their outer orbit. The main biological radicals include superoxide, hydroxyl and peroxynitrite radicals, and hydrogen peroxide (non-radical) (41). Superoxide and hydrogen peroxide react with other molecules until they are eventually converted to water by superoxide, catalase, and the glutathione peroxidase enzyme system (28). However, when NO levels are high, superoxide reacts with the NO to form peroxynitrite (96). Peroxynitrite is known to have toxic effects on cells by interacting with lipids, protein, and DNA (41).

The major vascular source of ROS is NADPH oxidase found in neutrophils, macrophages, and endothelial cells (43). The superoxide producing NADPH oxidase is composed of 6 subunits (p22phox, gp91phox, p47phox, p67phox, rac, and p40phox) and translocates to the plasma membrane upon stimulation to form a catalytically active oxidase (54) . LPS and imflammatory cytokines are known to stimulate NADPH oxidase, and thus NADPH oxidase has an important role in the pathophysiology of sepsis (67, 83).

1.1.4.1 ROS in sepsis

Septic patients often show signs of oxidative stress and decreased levels of the antioxidant ascorbate, which correlate with lower levels of survival (22, 38). During sepsis, ROS has been shown to cause endothelial damage and dysfunction (6), increase the expression of adhesion proteins (124), enhance platelet adhesion and activation, and promote coagulation (21, 47, 87). Sepsis was shown to increase ROS production in skeletal muscle in part by NADPH oxidase (123). In particular, LPS+INFγ treatment has been shown to increase superoxide production in endothelial cells (120). It is important to note that increased ROS in the septic capillary will cause the conversion of tetrahydrobiopterin (BH_4) to dihydrobiopterin (BH_2) (59), limiting the available BH_4 needed for proper vascular homeostasis (see section 1.1.5.1).

ROS has been implicated as a potential cause for the sepsis-induced capillary blood flow impairment. Removal of a major source of ROS by gp91phox (a subunit of NADPH oxidase) knockout, reduced sepsis-induced capillary blood flow impairment (107). Furthermore, treatment of septic rodents with the anti-oxidant ascorbate prevented and reversed the capillary blood flow impairment in septic models (3, 107, 108).

1.1.5 NO and sepsis

Although NO is primarily known as a vasodilator, it is a very important signalling molecule for vascular homeostasis with effects including inhibition of platelet adhesion and aggregation (44). Endogenous NO is produced from 3 distinct nitric oxide synthases

(NOS): neuronal NOS (nNOS or NOS1), inducible NOS (iNOS or NOS2), and endothelial NOS (eNOS or NOS3).

NO is synthesized by these enzymes in a 2 step oxidation reaction resulting in the conversion of L-arginine to L-citrulline. This reaction requires oxygen and NADPH as co-subtrates, the calcium-dependent enzyme calmodulin and the co-factors flavin adenine dinucleotide, flavin mononuleotide, heme, and BH_4. During this reaction, electrons are passed from the NADPH to the heme in the oxygenase domain with assistance from the co-factors. This flow of electrons needs to be tightly regulated and if interrupted it will lead to the production and release of superoxide. This is referred to as the 'uncoupled' state of NOS. One such disruption to the flow is when the availability of BH_4 becomes limited (33).

1.1.5.1 NO effects during sepsis

Sepsis is associated with the excessive production of NO as plasma levels of nitrite and nitrate are elevated (5) in septic patients. This elevation occurs due to increased expression of iNOS by LPS and inflammatory mediators such as TNFα, IL-6, and IFNγ (53). The increased NO is responsible for the impaired vascular reactivity and vasodilation leading to hypotension during sepsis (89).

The sepsis-induced capillary blood flow impairment is not affected by genetic deletion of each of the 3 NOS isoforms (eNOS, nNOS, or iNOS)(107). This suggests that the impairment is not due to the overexpression of iNOS or the subsequent changes in vascular reactivity. As mentioned previously, ascorbate treatment rescued the capillary blood flow in septic mice. Interestingly, this reversal was not seen in the $eNOS^{-/-}$ mice

(107). Located in endothelial cells and platelets (93), eNOS generates the majority of vascular NO under normal conditions (33) and contributes to vascular homeostasis by regulating vasomotor tone (88). NO also inhibits vascular smooth muscle cell proliferation (39), and platelet aggregation and adhesion (1). Since, during sepsis, BH_4 may be limited (due to conversion to BH_2 by ROS), eNOS can become uncoupled from BH_4, leading to reduced NO synthesis and thus a reduction of its local effects. Furthermore, the uncoupled eNOS produces superoxide, further contributing to the effects of ROS in the capillary in particular, increasing the formation of peroxynitrite and subsequent damage caused by this highly reactive molecule. Impaired eNOS function, combined with other factors, contributes to the impairment of the capillary blood flow (107).

1.2 COAGULATION

1.2.1 Overview

Coagulation is the process by which thrombi are formed to produce and maintain vascular homeostasis. It contains both a cellular component (platelets; see section 1.2.1) and a protein component (fibrin; see section 1.2.2). Thrombus formation is in constant equilibrium with the breakdown of the clot by fibrinolytic factors (35). Both excessive (hypercoagulation) and insufficient (hypocoagulation) coagulation will lead to disease states (26, 35, 35). Thus any alteration to the equilibrium can lead to a pathological state.

The coagulation cascade has 2 separate pathways that ultimately lead to platelet activation and fibrin formation. The intrinsic or contact pathway begins with exposed

tissue collagen (normally separated from the blood by endothelial cells forming the wall of blood vessels) which will directly activate the platelets and provide a site for the formation of a complex that will eventually convert prothrombin into thrombin. The extrinsic or tissue factor (TF) pathway begins when the protein, tissue factor, comes in contact with the blood. TF is normally exposed on cells that are not normally in contact with the blood such as fibroblast and smooth muscle cells. However endothelial cells and WBCs can express TF on their surface following stimulation by inflammatory mediators (63). Tissue factor will bind and activate factor VII to form the tissue factor-factor VIIa complex. This complex will activate factor X (to factor Xa) that subsequently cleaves prothrombin into its active form thrombin (95). Thrombin then cleaves fibrinogen to fibrin as well as activating platelets and endothelial cells(24). Thrombin is one of the strongest platelet activators *in vivo* (63).

Septic patients commonly manifest coagulation disorders such as thrombocytopenia (reduced platelet counts) and disseminated intravascular coagulation (DIC) (63). This is due to cross-talk between the inflammatory and coagulation pathways. For example, TNFα can upregulate TF (12). Furthermore, direct stimulation by LPS can also increase TF on endothelial cells (19). Besides affecting thrombin generation by TF, inflammation can alter coagulation by reducing the function of the anticoagulation system. This system includes protein C (degrades coagulation factors Va and VIIIa), antithrombin (inhibits thrombin), and tissue factor pathway inhibitor (inhibits TF-factor VIIa complex) (63). Finally, inflammation can disrupt the coagulation system by impairing the fibrinolytic system (34).

1.2.2 Platelets

Platelets are fully differentiated anuclear cells formed from megakaryocytes in the bone marrow. They are approximately 1 – 3 microns in diameter and have a lifespan of 7 days. They circulate in the blood and contribute to vascular homeostasis. When activated, they form and strengthen thrombi at the site of vascular damage. Pathological disorders can occur when platelet function is altered. High counts or overactive platelets will lead to enhance clotting disorders (e.g., stroke), whereas low counts or inactive platelets will lead to bleeding complications (50).

Platelets are activated by a variety of agents such as thrombin, adenosine diphosphate (ADP), thromboxane A_2 (TXA), collagen, and serotonin (5HT) (32, 68). Three major events accompany platelet activation: shape change, granule secretion, and cell surface integrin activation. Platelets change to a more spherical shape with pseudopods on its surface. This new shape favours platelet rolling thus increasing adhesion (58). Platelets secrete 2 types of granules: alpha and dense. Dense granules contain small molecules (ADP, adenosine triphosphate (ATP), serotonin and calcium) whereas alpha granules contain pro-coagulatory proteins (P-selectin, fibrinogen, thrombospondin, von Willebrand factor and others). The release of molecules from these granules acts as a positive feedback for further platelet activation (32, 68). Activation of cell surface integrins such as glycoprotein IIb/IIIa (GPIIb/IIIa) contributes to platelet adhesion and aggregation (85).

Reduced platelet counts (thrombocytopenia) is a common feature during sepsis (64). However, the cause of this reduction is unclear. Due to cross-talk between the inflammatory and coagulation pathways, in particular the increased levels of thrombin,

ADP, TXA, and 5HT (20, 51, 63, 71), platelets can become activated during sepsis leading to increased aggregation and adhesion to the vascular wall (64, 65, 102). Platelets can be activated by both endotoxin (LPS) and inflammatory cytokines (126, 127). Activated platelets can in turn increase the expression of tissue factor on monocytes (104). Furthermore, ROS will potentiate platelet aggregation (46, 56, 100, 113) and activated platelets will release ROS (another positive feedback mechanism) (8, 113).

1.2.3 Fibrin

Fibrin is an insoluble fibrous protein that forms 3-dimensional matrix structures that participate in the formation of a clot. Besides physically supporting the clot, fibrin triggers signalling events that mediate cellular functions (117). Fibrin matrices form when thrombin cleaves the insoluble fibrinogen into fibrin monomers that polymerise and branch to form the fibrin network. The architecture of the fibrin network determines the structural integrity of the clot (114).

The breakdown of fibrin (fibrinolysis) is also very important for homeostasis. Fibrin is lysed by the protease plasmin, which is formed by cleavage of the circulating plasminogen. There are 2 plasminogen activators that facilitate this process: tissue plasminogen activator (tPA), and urokinase plaminogen activator (uPA). Both of these enzymes are inhibited by plasminogen activator inhibitor-1 (PAI-1) (42).

Fibrin deposition is a common consequence of sepsis. Inflammatory mediators activate mononuclear cells and endothelial cells to express tissue factor (63). Moreover, septic patients have elevated PAI-1 levels (103). Although, levels of tPA are also

increased, the net effect is antifibrinolytic (103). These 2 points suggest that sepsis produces the state of fibrin clot formation and stabilization.

1.2.4 Endothelial cells

Endothelial cells line the blood vessel walls, providing a barrier between the vascular lumen and the surrounding cells. Besides the barrier function of the cells, they also participate in both the inflammation and coagulation process. Endothelial cells express various adhesion proteins which participate in the inflammatory response in the microvasculature (111). Specifically, endothelial cells contain pro-coagulatory factors such as von Willebrand factor (vWF) and P-selectin in Weibel-Palade bodies that are exposed to the surface when the cells become 'activated'. Endothelial cells can be activated by LPS, inflammatory mediators such as TNFα, and thrombin (37, 52, 73). Also, it is well established that there is endothelial damage and dysfunction during sepsis resulting in a loss of barrier function including microvascular leak and exposure of the subendothelial matrix (or basement membrane) (62).

1.2.5 Platelet adhesion, aggregation, and thrombi formation

On the molecular level, there are many proteins that facilitate the adhesion of platelets to the endothelium, to fibrin, and to other platelets to form micro-thrombi. These include P-selectin (CD62P), platelet endothelial cell adhesion molecule-1 (PECAM), vWF, and glycoprotein IIb/IIIa (GPIIb/IIIa or integrin $\alpha_{IIb}\beta_3$) (85).

P-selectin (CD62P) is a 140 kDa glycoprotein that is found in the alpha granules in platelets and Weibel-Palade bodies in ECs. Upon stimulation of either cell type, P-

selectin is exposed on the surface of the cell where it can interact with its counter receptor P-selectin glycoprotein receptor-1 (PSGL1)(13). PSGL1 is found on both platelets (36) and ECs (23). Thus, the P-selectin-PSGL1 interaction could occur between platelet-endothelium, endothelium-platelet, and platelet-platelet. Once bound, the interaction between the proteins leads to platelet rolling, adhesion, and aggregation. Furthermore, the binding can signal other effects such as platelet activation (13, 17).

Adhesion of platelets to the endothelium by PECAM, although possible, does not occur under physiological conditions and is not the primary function of this protein (118).

vWF participates in platelet adhesion and aggregation by acting as a binding partner, linking proteins together to cause adhesion. Like P-selectin, vWF is stored in alpha granules and Weibel-Palade bodies and is released upon stimulation. vWF will interact with GPIIb/IIIa as well as the platelet integrin GPIb, thus linking 2 platelets together and initiating platelet aggregation. Furthermore, vWF will bind collagen (a part of the sub endothelial matrix). Therefore vWF will link platelets to the sub-endothelial matrix, causing platelet adhesion to the vessel wall (72).

GPIIb/IIIa binds to vWF, fibrinogen or fibrin, fibronectin, and vitronectin. Like many integrins, GPIIb/IIIa can exist in a low active or high active state depending on its conformation (85, 92). Found constitutively expressed on the surface of platelets, GPIIb/IIIa will change conformation to a high active state when activation of the platelet occurs (68, 92). When in this state, GPIIb/IIIa binds to vWF (causing platelet aggregation and adhesion), fibrinogen or fibrin (linking the platelet to the clot), and to fibronectin and vitronectin (i.e., proteins found in the sub-endothelial matrix) to participate in platelet adhesion.

All of these processes come together during sepsis, which increases (i) coagulation, leading to increased platelet activation, (ii) endothelial cell activation and (iii) fibrin formation (which increases P-selectin expression, vWF release, and GPIIb/IIIa activation). The resulting binding interactions between platelets, ECs, the subendothelial matrix, and the fibrin network lead to the formation of microthrombi.

1.3 ASCORBATE

1.3.1 Ascorbate treatment

Ascorbate (reduced vitamin C) is the most abundant water soluble antioxidant. It will react with most ROS including superoxide and peroxynitrite. After reacting, the ascorbate radical has a low reactivity due to resonance stabilization of the unpaired electron and readily dismutates to ascorbate and dehydroascorbic acid (18). Ascorbate can also recycle other antioxidants, such as α-tocopherol (vitamin E) and glutathione from the radical species (78).

Circulating ascorbate levels are significantly lowered in septic patients and animal models of sepsis (3, 38). In these models, this is likely due to impairment of cellular mechanisms of ascorbate production during sepsis (55). In critically ill patients, this depletion is due to increased requirement for ascorbate (7, 70) caused by increased ROS levels (115, 116). Reduced ascorbate levels correlate with increased severity and mortality of septic patients (16, 38, 41).

Clinically, ascorbate, in combination with vitamin E, has been shown to reduce mortality in critically ill patients in a randomized, double-blind, placebo controlled trial

(22). Furthermore, ascorbate and vitamin E reduced multiple organ failure and shortened the ICU stay in patients post trauma or major surgery (84). Finally, an extremely high dose of ascorbate decreased mortality of severely burned patients (105).

In experimental animal models of sepsis, ascorbate bolus given intravenously has been shown to improve arteriolar responsiveness, arterial blood pressure, liver function, capillary blood flow, and survival (3, 107, 108, 121, 122). In particular, ascorbate bolus prevented the sepsis-induced capillary blood flow impairment when administered at the onset of sepsis (3). Further, when administered late into sepsis (i.e. when the capillary blood flow impairment was already present), ascorbate improved capillary blood flow (107, 108). Finally, ascorbate has been shown to improve survival in a mouse CLP (cecal ligation and perforation) model when given early (121) and in a mouse FIP (feces injection into peritoneum) model when given late (107). Table 1.1 (columns 3 and 4) indicates the protective effects of ascorbate and their poteintial role in the capillary blood flow impairment during sepsis.

1.3.2 Mechanism of protection by ascorbate

We have recently discovered that the protective effect of ascorbate in sepsis is eNOS-dependent. Furthermore, application of exogenous NO, or the eNOS cofactor BH_4 directly on the impaired capillaries also reversed the impairment (107). Application of BH_4 was ineffective in $eNOS^{-/-}$ mice indicating that its protective effect is also eNOS-dependent (107). As stated previously, BH_4 is known to be oxidized by ROS and, when in this state, is no longer a cofactor for eNOS and thus will 'uncouple' from eNOS (59, 61). In this uncoupled state, eNOS produces superoxide instead of NO, effectively

reducing NO production to the local environment (61) and further increasing ROS due to the formation of peroxynitrite. Therefore, we propose that ascorbate is readily absorbed into the endothelial cells (115, 116), where it prevents and reverses the oxidization of BH_4, effectively 're-coupling' to eNOS and thus restoring the local NO production. This local NO then provides beneficial effects alongside any other effects of ascorbate (Figure 1.2).

NO is known to inhibit platelet aggregation and adhesion thus contributing to the maintenance of blood flow in the microvasculature. Primarily known to prevent platelet activation through a cGMP pathway, NO has also been shown prevent this activation by other pathways. Specifically, NO has been shown to reduce P-selectin expression on ECs (4) and on platelets (44) thus reducing platelet-endothelium adhesion (44, 45, 76, 86). Thus ascorbate, through NO, may modulate P-selectin function. Since NO has anti-platelet properties, and platelets are believed to involve in septic capillary plugging, the restored local NO production will lead to a reduction in the capillary blood flow impairment.

Table 1.1. Possible underlying processes/events involved in the sepsis-induced capillary blood flow impairment, and possible protective effect of ascorbate treatment (intravenous bolus injection).

Pathophysiological process/ event during sepsis	Involvement of process/event in capillary blood flow impairment	Effect of ascorbate treatment on process/event	Protection by ascorbate ?
Decreased blood pressure (3)	Unlikely	Prevents decrease (3)	None
Increased drainage pressure (49)	Unlikely	No effect	None
Increased WBC adhesion (80)	Likely*	Reduced *in vitro* (25)	None
Increased RBC stiffening, adhesion (10,29)	Likely	Unknown	Possible
Increased platelet adhesion (67)	Likely	Unknown	Possible
Increased microthrombi formation (64)	Very likely	Unknown	Possible
Increased EC swelling (47)	Possible	Possibly reduced	Possible
Increased edema (63)	Possible	Unknown	None

WBC – white blood cell, RBC – red blood cell, EC – endothelial cell, *unlikely in skeletal muscle

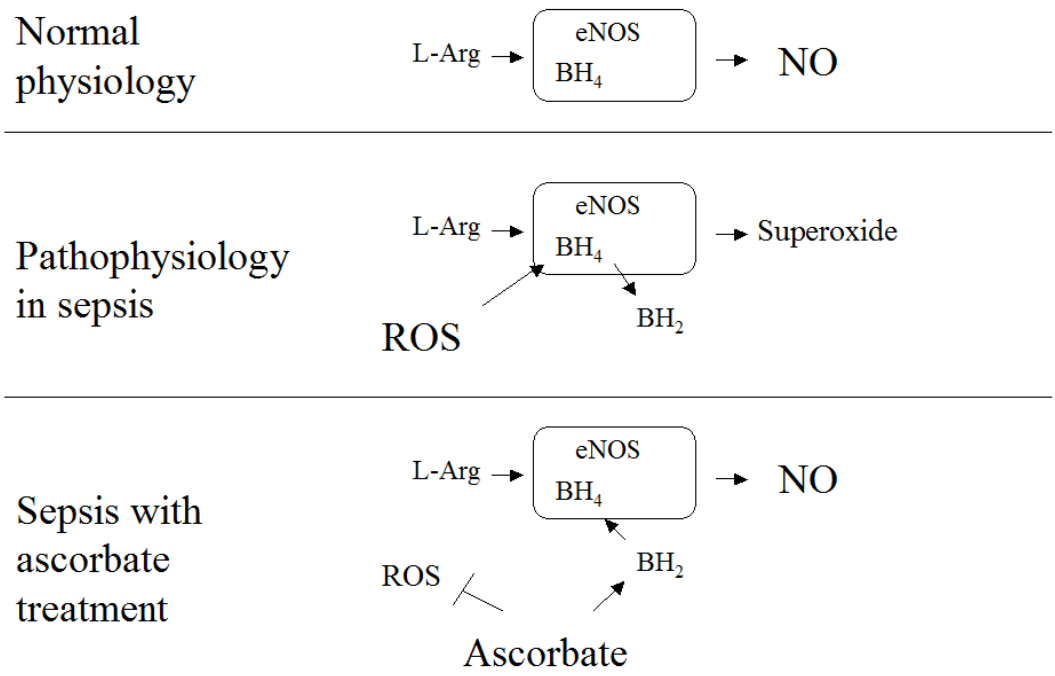

Figure 1.2. Mechanism of restored nitric oxide (NO) production by ascorbate during sepsis. Under normal physiological conditions, local NO is produced from L-arginine (L-arg) through the enzyme endothelial nitric oxide synthase (eNOS) with the aide of the cofactor tetrahydrobiopterin (BH_4). During sepsis, increased levels of reactive oxygen species (ROS), causes the conversion of BH_4 to dihydrobiopterin (BH_2). BH_4 becomes limited, effectively 'uncoupling' from eNOS, which produces superoxide instead of NO. Treatment with ascorbate will decrease the ROS as well as convert BH_2 back to BH_4 restoring eNOS function and NO production.

1.4 RATIONALE, HYPOTHESIS, and OBJECTIVES

1.4.1 Rationale

Despite extensive research, there is no pharmacological therapy for the treatment of sepsis. This represents a lack of understanding of the complex cellular and molecular mechanisms that govern the disease. The sepsis-induced capillary blood flow impairment has been associated with poor prognosis of sepsis and is a possible mechanism for the multiple organ failure seen in severe sepsis (99). However, the precise mechanisms of this impairment remain unclear. The impairment is a physical hindrance of blood flow in the capillary and one of the most likely candidates to cause this hindrance is the formation of micro-thrombi. To this extent, the roles of platelets and fibrin with regards to the capillary blood flow impairment have not been investigated.

Over 10 years ago, Armour and coworkers (3) first demonstrated that treatment with ascorbate had beneficial effects on capillary blood flow during sepsis. Since then, there have been numerous studies confirming the beneficial effect of ascorbate in sepsis (3, 106-108, 119, 121, 122). The mechanism of ascorbate's effect on the capillary blood flow impairment is unresolved. It is known that the effect of ascorbate is eNOS dependent, presumably due to the restoration of local NO. NO plays an important role in the homeostasis of coagulation, and thus any contribution to the capillary blood flow impairment by the coagulation system (i.e., microthrombi formation), will be altered by the increased local NO. Further information regarding this will (i) further the

understanding of the molecular interactions occurring during sepsis, and (ii) encourage the use of ascorbate as a therapy for septic patients.

1.4.2 Hypotheses

1) Increased platelet adhesion, aggregation and microthrombi formation during sepsis lead to plugging of capillaries, resulting in capillary blood flow impairment.

2) Ascorbate lowers platelet aggregation and adhesion by decreasing P-selectin expression, thus improving capillary blood flow during sepsis.

1.4.3 Objectives

1) Determine the effect of sepsis on capillary blood flow and on platelet adhesion in capillaries of mouse skeletal muscle, and examine the roles of the coagulation system, ROS and NO in sepsis-induced impairment of capillary blood flow and platelet adhesion. This objective will be addressed in Chapter 2.

2) Use an *ex vivo* model to examine i) the effect of sepsis on platelet aggregation and P-selectin expression and ii) the protection by ascorbate against sepsis-induced aggregation and P-selectin expression. This objective will be addressed in Chapter 3.

3) Use an *in vitro* model of microvascular endothelial cells to examine i) the effect of sepsis on platelet-endothelial cell adhesion and P-selectin expression in endothelial cells and ii) the protection by ascorbate against sepsis-induced platelet-endothelial adhesion and P-selectin expression in endothelial cells. This objective will be addressed in Chapter 4.

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Chapter 2

Impaired microvascular perfusion in sepsis requires activated coagulation and P-selectin mediated platelet adhesion in capillaries

A version of this chapter has been published previously

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2.1 INTRODUCTION

Severe sepsis is a systemic inflammatory response in which impaired microvascular perfusion precipitates organ failure and death (31). Impaired perfusion is seen as increased number of stopped-flow capillaries and decreased number of perfused capillaries (13). The impairment increases the diffusion distance for oxygen to parenchymal cells, leading to tissue hypoxia and organ failure (3, 9). Septic impairment of capillary blood flow has been visualized in animal organs by intravital microscopy (6, 13) and in human tissues by orthogonal polarization spectral imaging and sidestream dark-field imaging (8, 26). Despite the prevalence of capillary flow impairment and its importance in determining the clinical outcome of sepsis (26), the mechanisms of this impairment are unknown (19).

Intravascular coagulation in sepsis (2, 7) could be a contributory mechanism. Inflammatory and coagulation pathways cross-activate (15), including initiation of coagulation and activation of platelets by lipopolysaccharide (LPS) and inflammatory cytokines (15, 16), as well as augmentation of inflammation by activated platelets (1, 11, 17, 23, 32). Therefore, sepsis-induced platelet adhesion to capillary endothelium, platelet aggregates and/or subsequently formed microthrombi could plug capillaries. To our knowledge, there is no report addressing this possible mechanism. The overall aim of the present study was to use high-resolution intravital microscopy to examine in "real time" platelet adhesion and microthrombi formation in septic capillaries. The study had 2 specific objectives. First, we used treatments known to affect platelet and coagulation functions to establish the role of these functions in blood flow stoppage in septic capillaries. Second, we

manipulated the production of reactive oxygen species (ROS) and nitric oxide (NO) in the microvasculature to determine if the role of platelets in septic blood flow stoppage is oxidant- and NO-dependent. We discovered that platelet adhesion co-localized with fibrin deposition in septic capillaries contributes critically to blood flow stoppage.

2.2 METHODS

2.2.1 Animal preparation

We used a fluid resuscitated model of polymicrobial sepsis in mice as described previously (28, 29). Briefly, male wild type, $eNOS^{-/-}$, $iNOS^{-/-}$ and $gp91phox^{-/-}$ mice (C57BL/6, 18 - 27 g body weight, Jackson Laboratory, Bar Harbor, ME) were injected with feces into the peritoneum (FIP; 50 ml/kg of mixture of saline and feces at 75 mg/ml). For controls, we used naive or sham mice (saline-injected intraperitoneally, 50 ml/kg). Immediately after FIP, mice were injected subcutaneously with 1 ml saline containing the analgesic buprenorphine (4 μg/ml). Mice were studied by intravital microscopy at 6-7 h post-FIP because capillary blood flow impairment is established at this time (29). The experimental protocol was approved by the University of Western Ontario Council on Animal Care.

2.2.2 Intravital microscopy

Mice were anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (4 mg/kg) at 5.5 h post-FIP (or post-saline injection in shams) and the extensor digitorum longus (EDL) muscle was prepared for intravital microscopy as a bioassay for capillary blood

flow impairment (29). To assess impairment, we used an upright microscope (Leitz) and video-recorded 5 random fields (0.61 x 0.87 mm) per muscle, counted capillaries with moving red blood cells (CD_{PER}) and stationary red blood cells (CD_{STAT}) in each field, normalized counts per mm of a test line drawn across the muscle fibers, averaged CD_{PER} and CD_{STAT} from 5 fields (29), and computed the percentage of stopped-flow capillaries as 100 % x $CD_{STAT}/(CD_{PER} + CD_{STAT})$.

Platelets and fibrin were observed with a high resolution inverted microscope (Olympus IX81, 20x objective, N.A.= 0.75) yielding 0.35 x 0.47 mm field of view. To visualize platelets, fluorescent dye rhodamine 6G (0.08 mg/ml saline; Sigma, St. Louis, MO) was filtered (0.22 μm) and then injected in a 0.05 ml bolus into the penile vein 15 min before visualization. We used fluorescent epi-illumination with TRITC filter and digital recording to determine adherent platelets in 3 random fields per muscle. Each field was recorded during two 10 s periods spaced 1 min apart. We verified that objects fluorescing under TRITC illumination were labeled platelets by viewing with a high-resolution 40x objective (N.A.= 0.90) under both TRITC and bright light illumination. Platelets were counted as adherent if they were stationary during both recording periods (counts were confirmed by a "blind" observer). In each field, we used bright light illumination to measure the total length of visible capillaries projected to the surface plane of the muscle. Platelet adherence was normalized to 1 mm capillary length. Since rhodamine 6G also labels leukocytes (identified by their large size under bright light illumination), we similarly measured the occurrence of adherent leukocytes in control and septic capillaries.

To visualize fibrin, a 0.1 ml saline bolus containing 200 μg/ml of Alexa 488-labeled anti-fibrin antibody (Fibrin II beta clone: T2G1, Accurate Chemical, Westbury, NY) was injected intravenously 15 min prior to visualization (25). This antibody does not bind fibrinogen. To assess fibrin deposition in capillaries, we recorded 3 - 6 random fields for 15 s, measured the total length of capillaries containing fibrin, and expressed it as the percentage of total capillary length.

In several experiments we determined platelet adhesion specifically in capillaries with formed fibrin. Here, we first injected the anti-fibrin antibody and then, 20-30 min later, rhodamine 6G.

2.2.3 Biochemical and blood platelet count analyses

Following intravital microscopy, 0.2 ml blood was collected by carotid artery puncture and analyzed for lactate (iStat analyzer and CG4+ cartridges, Abbott Laboratories, Mississauga, ON). Blood was also analyzed for platelet count by diluting 200-fold in saline, platelets were labelled by rhodamine 6G (0.4 μg/ml) and were counted in a hemocytometer chamber using an Olympus microscope.

2.2.4 Experimental design: (a) Role of platelet and coagulation functions in blood flow stoppage

We studied septic mice with lactate above 1 mmol/l at 6-7 h post-FIP. To determine if platelets are required for blood flow stoppage in septic capillaries, mice were injected intravenously with platelet-depleting antibody (AIAD31440, purchased from Accurate Chemical) at 0.5 h post-FIP (5 μl/mouse), P-selectin blocking antibody (2 mg/kg in 0.1

ml of saline, time 0; clone RB40.34 purchased from BD Biosciences) or control immunoglobulin (2 mg/kg; purchased from Accurate Chemical). P-selectin is a key adhesion protein mediating platelet-endothelial interaction (20). To examine the role of activated coagulation, mice were injected intravenously with antithrombin (250 U/kg, 0.5 h), a glycoprotein IIb/IIIa inhibitor, eptifibatide (180 μg/kg, 6 h), or saline. Thrombin and glycoprotein IIb/IIIa are required for fibrin and thrombus formation (18). Finally, to assess the thrombogenic potential of the septic capillary bed at 6 h, we flooded the EDL muscle with a $FeCl_3$ solution (50 mmol/l in saline, 0.1 ml volume, 5 min) and then examined capillary blood flow. Topical application of $FeCl_3$ is a conventional approach to assess thrombosis in microvessels (33).

2.2.5 Experimental design: (b) Roles ROS and NO in platelet adhesion in the septic capillary bed

To examine the role of ROS, mice were injected intravenously with the antioxidant ascorbate (10 mg/kg; freshly dissolved, 0 or 6 h) or saline. Alternatively, we used mice with genetically deleted gp91phox , a subunit of NADPH oxidase (major source of ROS in septic microvasculature (34)). To assess the role of iNOS-derived NO, we used $iNOS^{-/-}$ mice. To examine the role of local NO available near capillaries, we flooded the EDL muscle with the NOS cofactor tetrahydrobiopterin (BH_4; 0.1 μmol/l, in repeated 0.1 ml bolus applications over 1 h), the NO donor S-nitroso-N-acetylpenicillamine (SNAP; 5 μmol/l in one 0.1 ml bolus), decomposed SNAP, or saline. The glass coverslip normally covering the muscle surface was slightly lifted to permit introduction of the bolus between the muscle surface and coverslip. To determine if the effects of ascorbate and

BH_4 treatments were eNOS-dependent, ascorbate/BH_4 treatments were done in $eNOS^{-/-}$ mice.

2.2.6 Statistics

Data are presented as mean ± SE; n indicates the number of mice (one muscle/mouse). Data were analyzed by Student t-test or ANOVA followed by Bonferroni multiple comparisons test. Significance was assigned as $p < 0.05$.

2.3 RESULTS

2.3.1 Role of platelet and coagulation functions in blood flow stoppage

Sepsis markedly increased blood flow stoppage in capillaries in mice (Figure 2.1; control data in naive and sham-injected mice did not differ, and were therefore pooled here). No stoppage was seen in arterioles or venules. Sepsis also markedly increased platelet adherence and fibrin deposition in capillaries (Figure 2.1). We observed single-adhering platelets and aggregates up to 5 platelets. Platelets preferentially adhered in stopped-flow capillaries (4.5 ± 1.0, n = 7) rather than in perfused capillaries (0.2 ± 0.1 platelets/mm, n = 7). The number of adherent leukocytes in capillaries was negligible (0.11 ± 0.04 leukocytes/mm in septic mice; not significantly different from zero in control mice, n = 5). Formed fibrin occurred only in stopped-flow capillaries; none was seen in arterioles/venules. We also determined platelet adhesion specifically in capillaries with formed fibrin. Platelet adhesion in these specific septic capillaries was more extensive (3.6 ± 0.8 platelets/mm, n = 4) than adhesion in any septic capillaries (1.6/mm, Figure

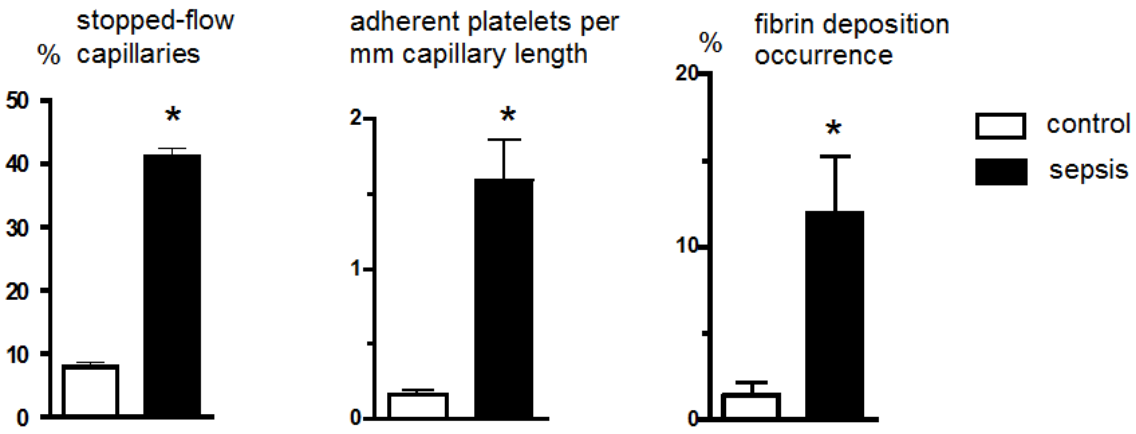

Figure 2.1. Sepsis increases blood flow stoppage, platelet adhesion and fibrin deposition in capillaries in mouse skeletal muscle. Sepsis was induced by feces injection into peritoneum (FIP). Flow stoppage was determined in 18 control and 16 septic mice at 6-7 h, adhesion in 9 control and 7 and septic mice at 7 h, and fibrin deposition in 2 control and 4 and septic mice at 7 h post-FIP. * Difference from control, $p < 0.05$.

2.1), confirming that septic platelet adhesion and fibrin deposition occurred in the same capillaries.

Platelet-depleting antibody dramatically lowered serum platelet counts in control (from 850 ± 50 to 40 ± 20) and septic mice (610 ± 40 to 60 ± 20 x 10^9/l) (n = 8-13). In septic mice, the antibody decreased the abundance of stopped-flow capillaries (41 ± 2 to 28 ± 2 %, $p < 0.05$, n = 16, 12, respectively), implicating platelets in septic impairment of capillary flow.

P-selectin blockade, antithrombin and eptifibatide significantly reduced platelet adhesion and blood flow stoppage in septic capillaries (Figure 2.2), indicating that P-selectin and coagulation activation were required for this adhesion/stoppage. Injection of control immunoglobulin did not affect septic adhesion/stoppage (1.32 ± 0.10 platelets/mm; 41 ± 3 % stopped-flow capillaries, n = 5). Finally, $FeCl_3$ was topically applied on the EDL surface. Topical application of $FeCl_3$ on microvessels is a conventional approach to assess the thrombogenetic potential in these microvessels (33). Fifty micromolar of $FeCl_3$ in a 0.1 ml volume applied for 5 minutes stopped flow more extensively in septic than control capillaries (Figure 2.3), suggesting that sepsis increased the propensity of microthrombi formation in capillaries.

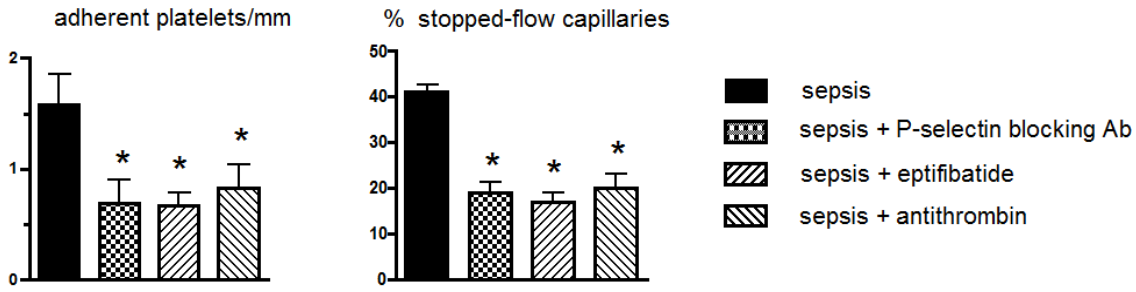

Figure 2.2. **Effect of P-selectin blocking antibody, eptifibatide, and antithrombin on platelet adhesion and blood flow stoppage.** Injections of P-selectin blocking antibody at 0.5 h, eptifibatide at 6 h (glycoprotein IIb/IIIa inhibitor), and antithrombin at 0.5 h inhibited platelet adhesion and blood flow stoppage in capillaries at 6-7 h post-FIP. * Difference from sepsis, $p < 0.05$, $n = 5$-9 for adhesion groups, and 5-16 for stopped-flow groups.

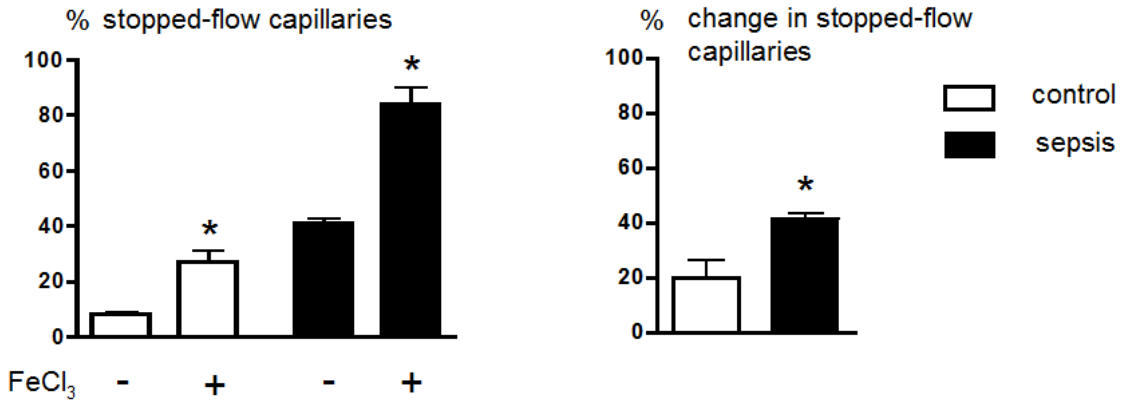

Figure 2.3. Sepsis increases thrombosis in capillaries. The thrombosis potential was tested by flooding the muscle with $FeCl_3$ at 6 h post-FIP. *Left panel:* * Difference between post-$FeCl_3$ and pre-$FeCl_3$ percentage of stopped-flow capillaries, $p < 0.05$, n = 5-18. *Right:* Change in stopped-flow percentage = (post-$FeCl_3$ percentage) - (pre-$FeCl_3$ percentage). * Difference from control, $p < 0.05$, n = 5 per group.

2.3.2 Role of ROS and NO

Prophylactic injection of ascorbate, or gp91phox deletion, prevented platelet adhesion and flow stoppage in septic capillaries (Figure 2.4). Moreover, delayed injection of ascorbate reversed septic adhesion/stoppage (Figure 2.5). These latter effects were eNOS-dependent, since they were absent in $eNOS^{-/-}$ mice (Figure 2.5). BH_4 and NO donor SNAP at 6-7 h significantly reduced septic platelet adhesion and flow stoppage (Figure 2.6, top). Flooding of the muscle surface with decomposed SNAP or saline did not affect septic flow stoppage (44 ± 2 and 47 ± 2 % stopped-flow capillaries, n = 7, 3, respectively, versus 41 ± 2 %, n = 16 in non-flooded septic muscles). The reversal effects of BH_4 were eNOS-dependent, since they were absent in $eNOS^{-/-}$ mice (Figure 2.6). Consistently, the effects of SNAP did not depend on eNOS (Figure 2.6). eNOS knockout did not affect blood flow stoppage and platelet adhesion in septic capillaries (Figure 2.5), but iNOS knockout inhibited platelet adhesion (Figure 2.6, bottom). Flow stoppage in control $gp91phox^{-/-}$, $eNOS^{-/-}$ and $iNOS^{-/-}$ mice (12 ± 2, 12 ± 5, and 5 ± 1 % stopped –flow capillaries, $p< 0.05$, n = 3, 5, 4, respectively) was not statistically different from that of control wild type mice (8 ± 1 %, n = 18) (Figure 2.1).

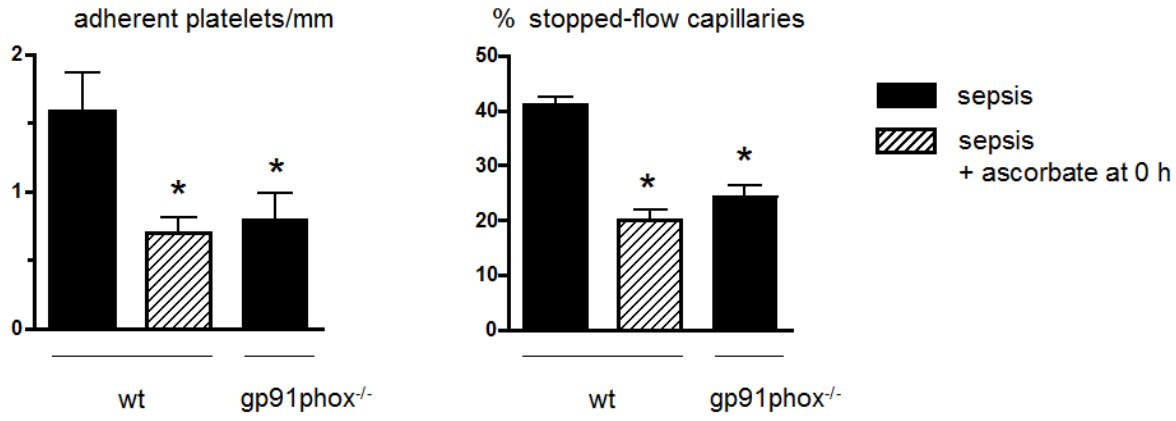

Figure 2.4. Effect of ascorbate and gp91phox knockout on platelet adhesion and blood flow stoppage. Ascorbate injection at 0 h and gp91phox knockout prevent platelet adhesion and blood flow stoppage in capillaries at 6-7 h post-FIP. * Difference from sepsis wild type (wt) group, $p < 0.05$, $n = 6$-7 for adhesion groups, and 5-16 for stopped-flow groups.

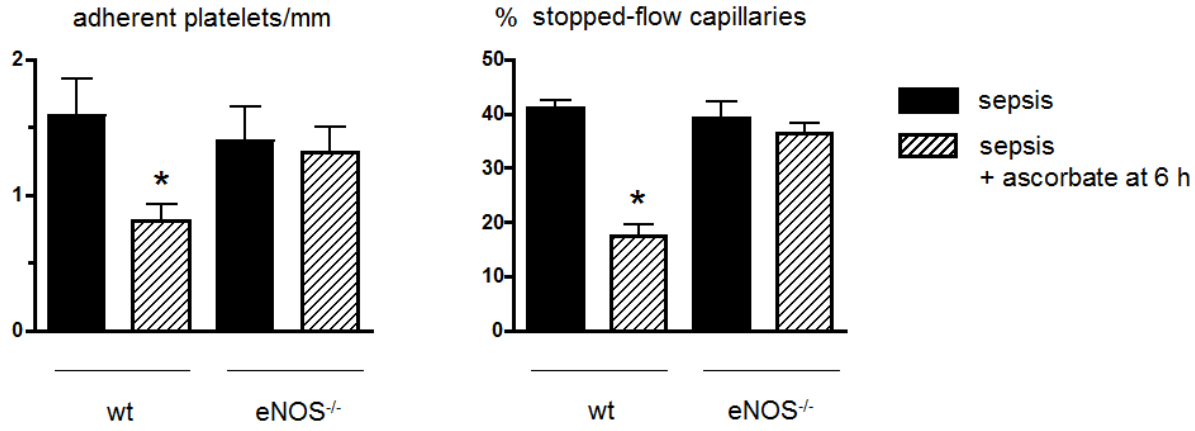

Figure 2.5. Effect of ascorbate on platelet adhesion and blood flow stoppage in wild type and eNOS knockout mice. Ascorbate injection at 6 h reverses platelet adhesion and blood flow stoppage in capillaries at 7 h post-FIP in wild type but not eNOS$^{-/-}$ mice. * Difference from sepsis group, $p < 0.05$, n = 6-17 for adhesion groups, and 8-16 for stopped-flow groups.

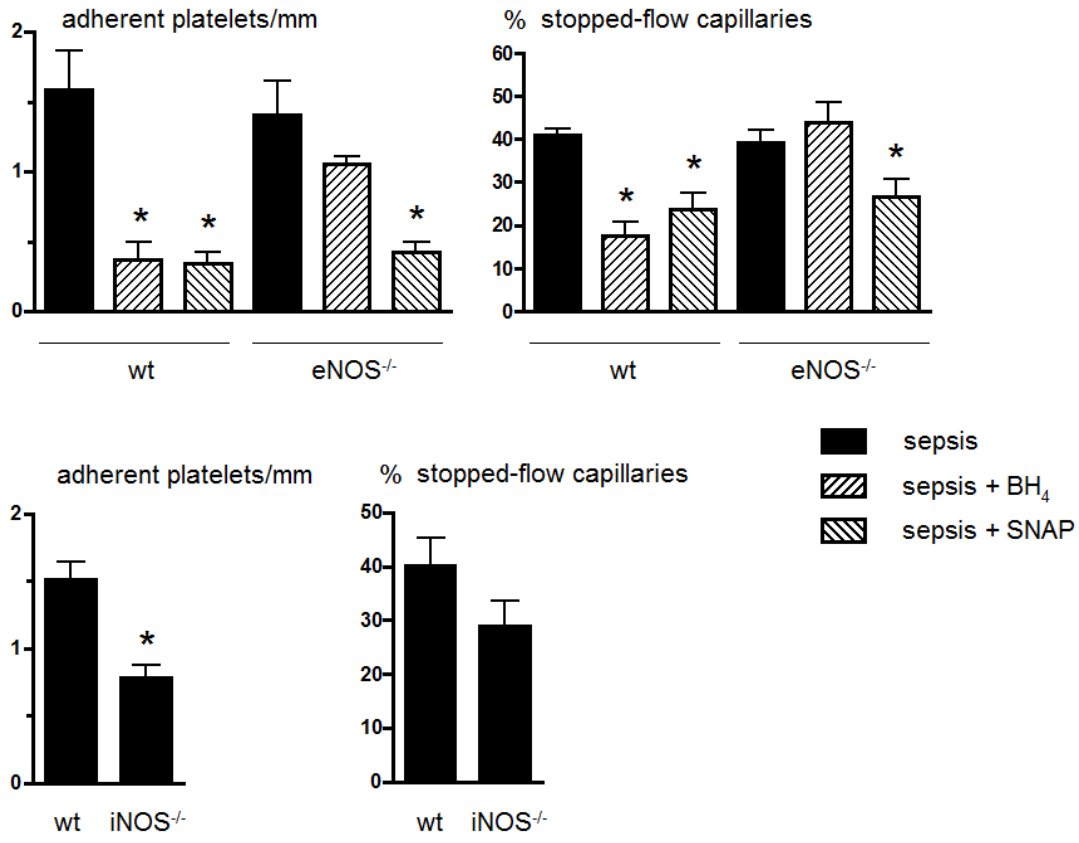

Figure 2.6. **Effects of tetrahydrobiopterin (BH_4) and the NO donor SNAP on platelet adhesion and blood flow stoppage in capillaries of wild type and eNOS$^{-/-}$ mice (top), and effect of iNOS knockout on adhesion/stoppage (bottom).** At 6 h, 0.1 ml boluses of BH_4 (repeated over 1 h period) flooded the muscle surface to determine platelet adhesion and flow stoppage at 7 h post-FIP. Alternatively, a single 0.1 ml bolus of SNAP flooded the surface, and platelet adhesion and stoppage were determined 15 min later (when the temporary SNAP-induced vasodilation had ended). BH_4 reversed platelet adhesion and flow stoppage in septic capillaries of wild type but not eNOS$^{-/-}$ mice. SNAP reversed platelet adhesion and flow stoppage in both types of mice. iNOS knockout inhibited septic platelet adhesion at 6 h post-FIP. * Difference from appropriate sepsis group, $p < 0.05$, n = 5-10 for adhesion groups, and 5-16 for stopped-flow groups in top row, and 6 and 10 for adhesion, and 6 and 10 for stopped-flow groups at bottom, respectively.

2.4 DISCUSSION

The present study addressed the mechanism of blood flow stoppage in septic capillaries. We report for the first time that (i) sepsis markedly increases platelet adhesion, fibrin deposition and propensity of thrombosis in capillaries, (ii) capillary flow stoppage requires platelets, P-selectin, and coagulation activation, and (iii) capillary platelet adhesion can be prevented or reversed by gp91phox and iNOS deficiencies, ascorbate, and local BH_4 and exogenous NO.

2.4.1 Role of platelet and coagulation functions in blood flow stoppage

In the present study, platelets adhered in septic capillaries and venules consistent with the literature (30). However, unlike in capillaries, adhering platelets in venules were not associated with fibrin deposition or stopped blood flow, indicating that rheological findings in larger microvessels cannot be extended to capillaries. In view of negligible leukocyte adhesion observed in capillaries, we sought leukocyte-independent mechanism(s) of capillary plugging in sepsis.

Possible mechanisms could be platelet activation, aggregation, and subsequent critical narrowing/plugging of the capillary lumen by adhering platelets (17, 32), or plugging by adhering platelets and formed microthrombi within the capillary. LPS and inflammatory cytokines increase expression of P-selectin and von Willebrand factor on endothelial cells to initiate platelet adhesion (4, 16). LPS and cytokines also increase tissue factor expression at the cell membrane of monocytes and endothelial cells (15), initiating coagulation. The present data are consistent with the mechanism of platelet

adhesion and microthrombi formation. Sepsis increased platelet adhesion in capillaries, whereas platelet depletion and blockade of P-selectin significantly reduced septic blood flow stoppage (Figure 2.2). Sepsis-induced platelet adhesion and fibrin deposition were seen to co-localize in stopped-flow capillaries, while anticoagulants decreased septic platelet adhesion and flow stoppage in capillaries (Figures 2.1, 2.2).

In critically ill patients, a 30 % drop in platelet count independently predicts death (21). In the present study, a comparable 28 % drop occurred in septic mice. Since the life span of platelets is longer than 6 h (22), we hypothesize that most of this platelet consumption is due to platelet adhesion in the microcirculation. To estimate how platelet adhesion in capillaries predicts septic blood flow stoppage, we used the present data to plot the mean values of stopped-flow capillaries (%) versus values of platelet adhesion/mm (Figure 2.7). The plot has a significant linear correlation, indicating that platelet adhesion in capillaries predicts ~ 90 % of capillary blood flow stoppage. If decreased platelet count were due to platelet adhesion in capillaries, then the 28 % drop implicates a substantial plugging of the capillary bed (e.g., 40 % plugging in skeletal muscle).

2.4.2 Role of ROS and NO in septic capillaries

We showed that sepsis increases ROS production in mouse skeletal muscle (35) and that septic capillary blood flow impairment depends on NADPH oxidase (29). ROS promote expression of P-selectin at the surface of platelets and endothelial cells, and enhance platelet adhesion to the endothelium and coagulation (12, 15, 24). Our data are consistent with ROS-mediated blood flow stoppage due to enhanced platelet adhesion in capillaries. The

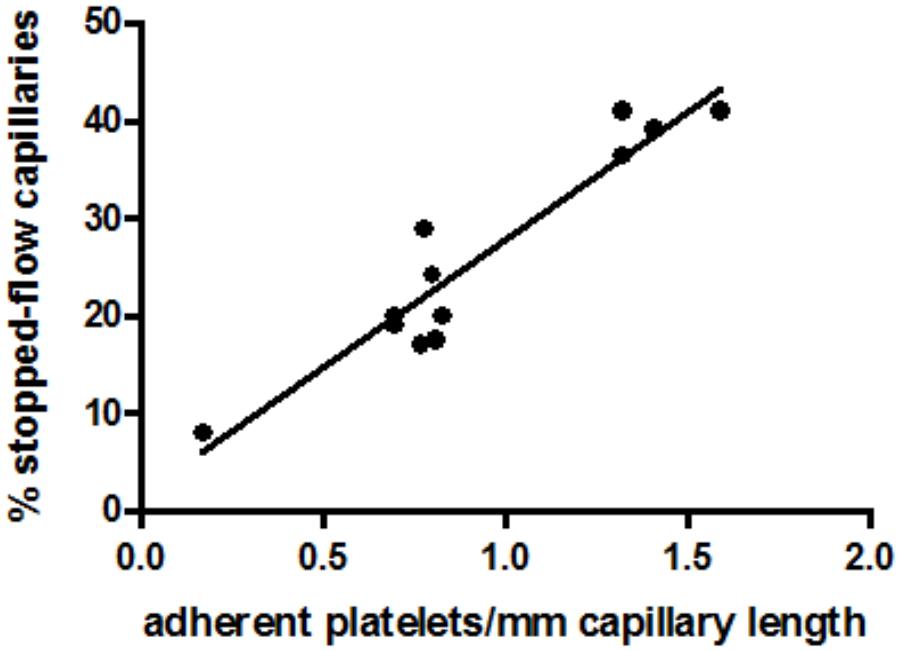

Figure 2.7. Platelet adhesion in septic capillaries predicts capillary blood flow impairment. The average values of percent stopped-flow capillaries in experiments of Figures 2.1, 2.2, 2.4, 2.5 and 2.6 (bottom) were plotted against the corresponding average values of adherent platelets/mm. In these experiments, no systematic difference existed between the treatment/concentration of agents in surface and deep capillaries (e.g., agents were injected intravenously, rather than introduced by flooding of the muscle surface). Computed line of linear regression has $r^2 = 0.89$ and slope different from 0, $p < 0.05$.

antioxidant ascorbate and gp91phox knockout prevented/reversed septic platelet adhesion and flow stoppage (Figures 2.4, 2.5) (an effect consistent with ascorbate's ability to significantly improve septic mouse survival at 24 h) (29, 35).

Recently we proposed that increased ROS level in sepsis oxidizes the eNOS cofactor BH_4, uncouples it from eNOS in platelets and endothelial cells and thus stops NO production in these cells (29). Because low physiological levels of NO are anti-aggregatory and anti-adhesive (24), uncoupled eNOS promotes platelet adhesion/aggregation and flow cessation in capillaries. The present data are consistent with this proposed mechanism. BH_4 or NO applied locally reversed platelet adhesion and flow stoppage in septic capillaries (Figure 2.6). Further, the beneficial effects of ascorbate and BH_4 were eNOS-dependent (Figures 2.5, 2.6).

Knockout of iNOS reduced septic platelet adhesion (Figure 2.6, bottom), suggesting that iNOS-derived NO is pro-adhesive. At first glance, this result contradicts the beneficial effect of local NO (Figure 2.6, top). To reconcile these observations, we note that iNOS enzymatic activity is negligible in the mouse skeletal muscle at 6 h of sepsis (29) and hypothesize that (i) iNOS activity is higher in other septic tissues and (ii) NO overproduction here could promote platelet adhesion in the skeletal muscle. Excess NO could react with superoxide, form peroxynitrite (24), and lead to activation/priming of blood-borne platelets to adhere in tissues. Increased iNOS expression results in decreased eNOS activity and expression (27) further reducing the local production of NO. The apparent opposite effects of NO (Figure 2.6) underscore the complex role NOS/NO may play during sepsis (5, 14).

2.4.3 Methodological limitations

The use of the platelet-depleting antibody to study the role of platelets in septic blood flow stoppage was problematic. Both control and septic mice injected with the antibody were noticeably sicker than their non-injected counterparts (mice hunched in the cage, had erected fur, and did not respond to tactile stimuli). Further, the antibody significantly increased (40 %) capillary flow stoppage in control mice. Consistent with reported increased mortality in mice by platelet-depleting antibody (10), the present deleterious effects of the antibody on animal health might have obscured the full beneficial effect of platelet depletion against septic capillary flow stoppage in skeletal muscle.

Finally, the present value of adherent platelets/mm in sepsis may underestimate the actual platelet adherence, since plugged septic capillaries may not permit plasma flow and detection of platelets with a fluorescent dye. Capillary obstructions could also limit the full impact of agents injected at 6 h on platelet adhesion and blood flow stoppage studied at 7 h.

In conclusion, we demonstrated that polymicrobial sepsis increases platelet adhesion and fibrin deposition in skeletal muscle capillaries and that septic impairment of capillary blood flow requires platelet adhesion, P-selectin and activated coagulation. Since platelet adhesion and capillary flow impairment can be inhibited by the antioxidant ascorbate and exogenous NO, administration of ascorbate and/or NO donors to attenuate platelet accumulation in septic capillaries is an important consideration for future development of novel adjuvant therapies for sepsis.

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Chapter 3

Ascorbate reduces mouse platelet aggregation and surface P-selectin expression in an *ex vivo* model of sepsis

A version of this chapter has been published previously

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3.1 INTRODUCTION

Sepsis is a systemic inflammatory response due to an infection. Sepsis results in circulatory dysfunction that can lead to multi-organ failure and death (6, 60). An exaggerated coagulation response contributes to the severity of the disease in the form of microvascular thrombosis, leading to disseminated intravascular coagulation (DIC) and organ dysfunction (28). Furthermore, it has been shown that a reduced platelet count in blood (i.e., an event contributing to DIC) correlates with higher mortality (27, 37). Adhering platelets and coagulation (fibrin deposition) in capillaries may lead to cessation of blood flow in these microvessels, an event frequently observed during sepsis (1, 26, 56, 57, Chapter 2). Cessation of flow will result in increased diffusional distance for oxygen to reach cell mitochondria, leading to tissue hypoxia and organ failure (14, 17).

It is known that septic plasma potentiates platelet activation (22). The resulting platelet aggregation and platelet-endothelial adhesion may be critical in capillary flow impairment in sepsis. Platelets can be activated by a large number of agonists including thrombin, adenosine diphosphate (ADP), thromboxane, serotonin, and collagen (15, 29). Thromboxane, serotonin, and ADP are subsequently released from activated platelets to further aggregation (42). Recently, reactive oxygen species (ROS) have been implicated to modulate platelet function (25). The levels of platelet-activating agonists and the level of ROS are increased during sepsis (12, 20, 32, 35, 36, 39, 55). The excess of ROS during sepsis will oxidize tetrahydrobiopterin which is a cofactor for endothelial nitric oxide synthase (eNOS). When tetrahydrobiopterin is oxidized, eNOS produces superoxide instead of nitric oxide (NO). Superoxide will interact with NO to form peroxynitrite, further reducing the bioavailability of NO. NO is an important factor in the homeostasis

of platelets and is a known inhibitor of platelet activation/adhesion (41). The antioxidant ascorbate (reduced vitamin C) has been shown to prevent and reverse the oxidation of tetrahydrobiopterin thus restoring the NO production via eNOS (23, 38).

Clinically, there is no effective pharmacological treatment for sepsis. Current management strategies aim to increase oxygen delivery to counteract the rising levels of lactate (13). One possible treatment that has been under investigation is the use of ascorbate (33). An intravenous bolus of ascorbate has been shown to increase survival of septic mice as well as to prevent and reverse the capillary blood flow impairment (1). Ascorbate also inhibits the sepsis-induced increase in adhesion of single platelets and their aggregates in capillaries (Chapter 2). Although several mechanisms have been proposed to explain the beneficial effect of ascorbate in the septic microvasculature (55), the precise mechanism of this effect is unclear. Ascorbate could act directly, because oxidative stress of the platelet plays an important role in the platelet activation (54), and also indirectly because it could restore NO production by the aforementioned eNOS pathway and thus inhibit platelet adhesion/aggregation (55, 56, Chapter 2).

We have recently reported that the treatment of septic mice with a blocking antibody against P-selectin reduces adhesion of single platelets/aggregates in capillaries (Chapter 2). P-selectin is a key platelet-endothelium and platelet-platelet adhesion molecule (5). In our study, it was not possible to determine whether the platelet and/or endothelial P-selectin are involved in platelet adhesion to capillaries. To address this issue, the present study employed a simpler *ex vivo* model of isolated platelets to examine (i) the effect of sepsis on the platelet surface P-selectin expression, and (ii) the effect of ascorbate on this expression. We have also reported that the inhibitory effect of ascorbate

against adhesion of platelet aggregates in septic capillaries was eNOS-dependent (Chapter 2). However, we could not differentiate whether the endothelial and/or platelet eNOS were involved. In the present study, we used the *ex vivo* model to examine the platelet eNOS involvement. Overall, we hypothesized that the surface P-selectin expression and aggregability of activated platelets are reduced by ascorbate in an eNOS-dependent manner.

3.2 METHODS

3.2.1 Reagents used

Lipopolysaccharide (LPS), tumor necrosis factor alpha (TNFα), ADP, N-Nitro-L-arginine methyl ester (LNAME), N-Nitro-D-arginine methyl ester (DNAME), S-Nitroso-N-acetyl-DL-penicillamine (SNAP), and ascorbate were purchased from Sigma-Aldrich (St. Louis, MO). U46619, a thromboxane A2 receptor agonist, was purchased from Enzo Life Sciences (Farmingdale, NY). Serotonin was purchased from Alfa Aesar (Ward Mill, MA). The phycoerythrin (PE) conjugated P-selectin antibody (clone: Wug.E9) and PE conjugated IgG control were purchased from Emfret Analytics (Eibelstadt, Germany). A fluorescein isothiocyanate (FITC) conjugated CD41 antibody was purchased from BD Pharmingen (Mississauga, ON). Eptifibatide was from Shering-Plough (Kenilworth, NJ).

3.2.2 Experimental design

To investigate the effect of sepsis on platelet aggregation and P-selectin surface expression *ex vivo*, we used plasma from septic mice and the conventional septic

mediators LPS and TNFα. We also used platelet-activating agents known to be released into bloodstream during sepsis, namely thrombin, ADP, serotonin and U46619 (thromboxane A2 analog) (12, 20, 35, 37, 39). The involvement of NOS in the responses to the activating agents was examined by using the non-selective inhibitor LNAME.

3.2.3 Platelet collection and isolation, and plasma collection.

Experimental protocols were approved by the University of Western Ontario Council on Animal Care. Wild type C57BL/6 mice (weight: 18-25 g, age: 1.5 - 4 months) were obtained from Charles River (Sherbrooke, QE) and bred in our animal facility. We collected platelets from control male mice anaesthetized with ketamine and xylazine (Chapter 2). Blood was collected via carotid artery puncture into an acid citrate dextrose solution in a 6:1 blood to solution ratio. Platelets were isolated as described previously (53). Briefly, platelet rich plasma was collected by centrifuging the whole blood at 120 g for 8 min. The plasma was then re-spun to remove any collected leukocytes. Finally, the platelets were pelleted at 735 g for 10 min and resuspended in calcium free Tyrode's buffer. Immediately before testing, normal Tyrode's buffer was added (1:5 ratio) to the platelet solution.

We also used the blood collection procedure to obtain plasma from septic mice. Mice were made septic by fecal injection into peritoneum as previously described (56, Chapter 2). Blood was collected 4 h post fecal injection.

3.2.4 Platelet aggregation

Platelet aggregation was measured using a custom built Born aggregometer (7). Briefly,

isolated platelets were placed in a specialized cuvette and treated with thrombin at 0.075 U/ml (i.e., the EC_{50} value from dose-response curve, Figure 3.1) or 0.0375 U/ml, ADP (1 μM), U46619 (320 nM), serotonin (10 μM), LPS (10 μg/ml), or TNFα (10 ng/ml) at effective concentrations published in the literature (3, 19, 30, 48, 58, 62). Some experimental groups of platelets were treated simultaneously with ascorbate (100 μM), LNAME (5 mM), DNAME (non-functional LNAME analogue, 5 mM), SNAP (1 μM), inactive SNAP (10 μM, exposed to room air for at least 24 h), eptifibatide (clinically used anti-aggregatory agent, 1.8 μg/ml), and/or septic plasma (1:1 ratio with normal Tyrode's buffer solution with platelets). The platelets were continuously stirred and the light transmittance was recorded. Aggregation was determined as the change in light transmittance in the aggregometer from the initial light transmittance reading prior to adding any platelet-activating agent.

3.2.5 Flow cytometry

Isolated platelets were treated with thrombin (0.0075 - 7.5 U/ml), LPS (10 μg/ml), or TNFα (10 ng/ml) for 5 min at room temperature. Some experimental groups of platelets were treated simultaneously with ascorbate (100 μM), LNAME (5 mM), and/or DNAME (5 mM). Platelets were then incubated with a PE conjugated P-selectin antibody (clone: Wug.E9) and a FITC conjugated CD41 antibody (platelet specific marker) for 15 min prior to stopping the experiment by addition of phosphate buffered solution. As a negative control, a PE conjugated IgG was used. The labeled platelets were then analyzed with a flow cytometer (Coulter EPICS XL-MCL, Beckman Coulter, Brea, CA, USA). The percent of total events indicated the number of events that were both P-selectin and

CD41 positive over the total number of detectable signals within the assigned region of the scatter plot (forward scatter 70 – 400, side scatter 6 – 60).

3.2.6 Statistical analysis

Data are presented as mean ± SE; n indicates the number of separate platelet isolations. Blood from one mouse was used for each isolation. Data were analyzed by student t test or ANOVA followed by Bonferroni multiple comparisons test. Significance was assigned as $p<0.05$.

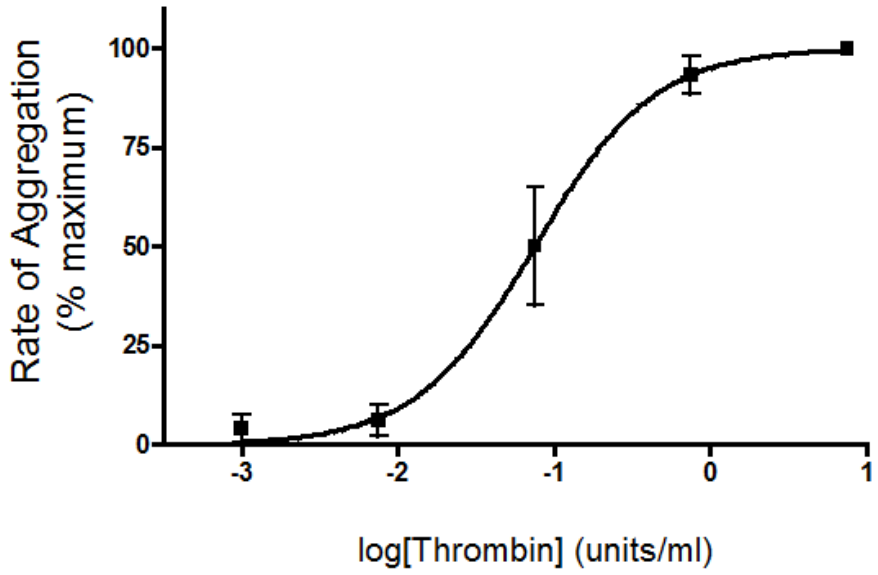


Figure 3.1. Dose response to thrombin on mouse platelet aggregation ex vivo. Isolated platelets were treated with thrombin (0.001, 0.0075, 0.075, 0.75, 7.5 units/ml) and then placed in an aggregometer. Aggregation was defined as the change in light transmittance normalized to transmittance at time 0 for each experiment. The initial rate of aggregation is shown as the percentage of the maximum rate (i.e., caused by thrombin) per experiment. EC_{50} value is 0.075 units/ml. n = 6 per group.

3.3 RESULTS

3.3.1 LPS and TNFα do not initiate platelet aggregation nor increase P-selectin surface expression

LPS or TNFα did not affect platelet aggregation (Figure 3.2). This figure shows a strong effect of thrombin (0.075 U/ml) used as positive control. The standard error bars represent the day-to-day variability of the platelet aggregation. LPS did not potentiate the aggregation caused by thrombin at a sub-threshold concentration of 0.0075 U/ml (i.e., initial rates of aggregation were negligible for thrombin at 0.25 ± 0.16 and for thrombin+LPS at 0.21 ± 0.15 % maximum; n = 5 per group). Furthermore, collected septic plasma applied for 5 min did not cause aggregation and also it did not potentiate platelet aggregation caused by thrombin (initial rate of aggregation for septic plasma was 0.01 ± 0.01 and for septic plasma+thrombin (0.0075 U/ml) it was 0.01 ± 0.01 % maximum; n = 8 per group). Septic plasma applied for 1 h did not cause aggregation and did not potentiate the effect of thrombin at 0.0075 U/ml (initial rate of aggregation for septic plasma was 0.01 ± 0.01 and for septic plasma+thrombin it was 0.01 ± 0.01 % maximum; n = 6 per group).

Using flow cytometry, we measured the P-selectin surface expression on the platelets after treatment with LPS and TNFα. Neither LPS nor TNFα increased the P-selectin expression (Figure 3.3). Again, thrombin (0.075 U/ml) was used as positive control.

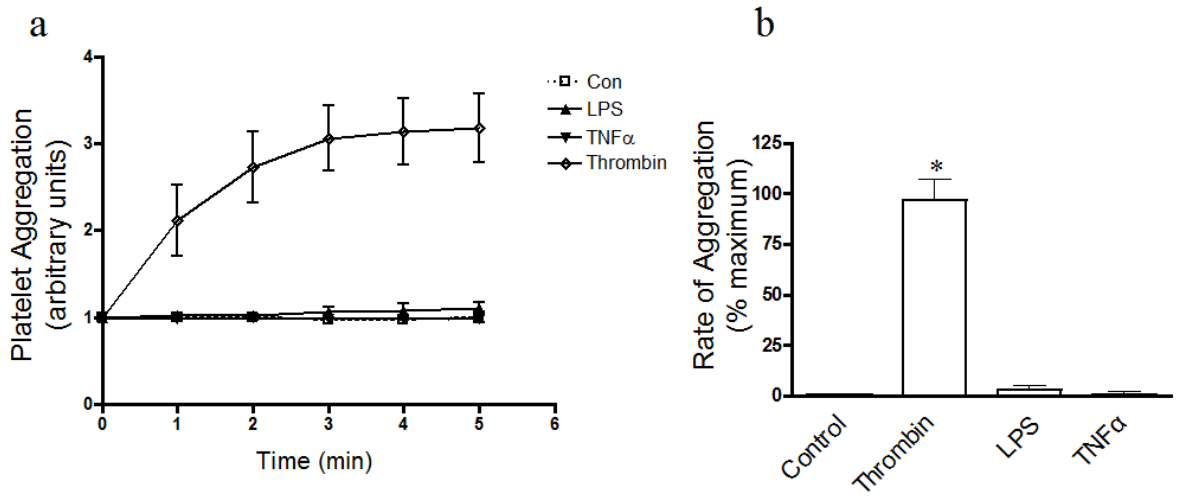


Figure 3.2. LPS and TNFα have no effect on mouse platelet aggregation *ex vivo*. Panel a: Isolated platelets were treated with LPS (10 μg/ml), TNFα (10 ng/ml), or thrombin (0.075 units/ml) and were placed in an aggregometer. Aggregation was defined as the change in light transmittance normalized to transmittance at time 0 for each experiment. Platelet aggregation was measured over 5 minutes. Panel b: The initial rate of aggregation is shown as the percentage of the maximum rate (i.e., caused by thrombin) per experiment. * $P < 0.05$ compared to control, $n = 5$ per group.

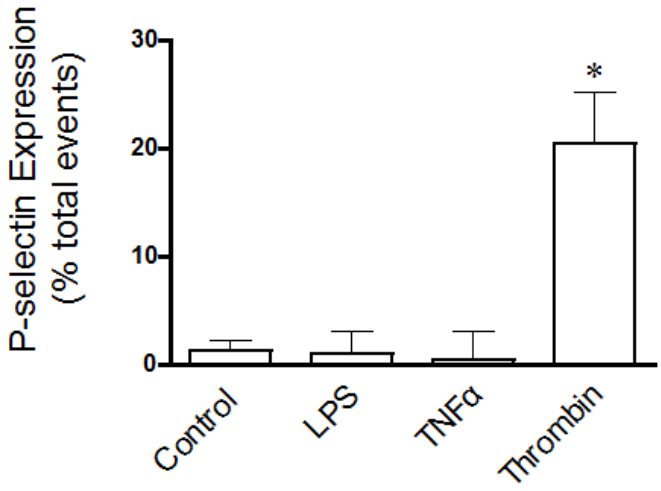


Figure 3.3. Effect of LPS, TNFα, and thrombin on P-selectin surface expression on isolated platelets. Isolated platelets treated with LPS (10 μg/ml), TNFα (10 ng/ml), or thrombin (0.075 units/ml) for 20 min were examined for surface P-selectin expression by flow cytometry. LPS or TNFα had no effect. Thrombin significantly increased P-selectin surface expression. P-selectin surface expression is represented by percent of events that were both positive for P-selectin and CD41 (platelet marker). * $P < 0.05$ compared to control, n = 5 per group.

3.3.2 Ascorbate reduces platelet aggregation to thrombin, ADP, and U46619

Thrombin, ADP, and U46619 (thromboxane A2 analog) caused platelet aggregation as expected (Figures 3.4 a-f). Serotonin did not initiate aggregation in our model (initial rate of aggregation was 2.09 ± 1.22 versus 1.54 ± 0.86 % maximum in untreated controls, n = 5 and 8, respectively). Ascorbate inhibited the initial rate of aggregation due to thrombin, ADP, and U46619 by 44, 20.4, and 51.5 %, respectively (Figure 3.4). In comparison with ascorbate, eptifibatide similarly inhibited the thrombin-induced aggregation (initial rate of aggregation was 40.2 ± 4.50 % maximum, n = 6). The inhibition by ascorbate depended on the concentration of thrombin employed to aggregate platelets. When the lower concentration was used (i.e., 0.0375 U/ml), ascorbate inhibited aggregation by 74 % (initial rate of aggregation was 25.8 ± 9.3 % compared to thrombin alone, n = 5). Ascorbate alone had no effect on platelet aggregation (initial rate of aggregation was 2.38 ± 1.50 versus 1.54 ± 0.86 % maximum in untreated controls; n = 4 and 8, respectively).

3.3.3 Ascorbate reduces the sensitivity of P-selectin surface expression to thrombin

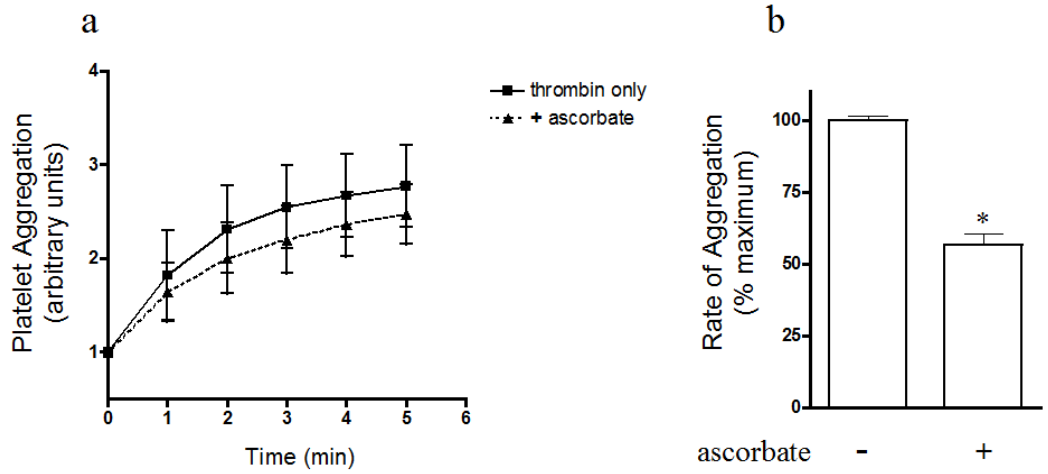
Since it is difficult to determine the concentration of thrombin found within the septic microvasculature in vivo and then test the effect of ascorbate at this concentration, we examined the effect of ascorbate across multiple concentrations of thrombin. Figure 3.5 represents a dose-response of P-selectin surface expression to thrombin and to thrombin in the presence of ascorbate. Ascorbate caused a significant increase in the EC_{50} value from 0.26 ± 0.03 to 0.40 ± 0.04 units/ml (Figure 3.5 insert), indicating that it reduced the

sensitivity of platelets to thrombin. Platelets treated only with ascorbate had P-selectin surface levels similar to controls (ascorbate = 1.16 ± 0.86, control = 0.79 ± 0.65 % of total events detected by flow cytometer, n = 8 per group).

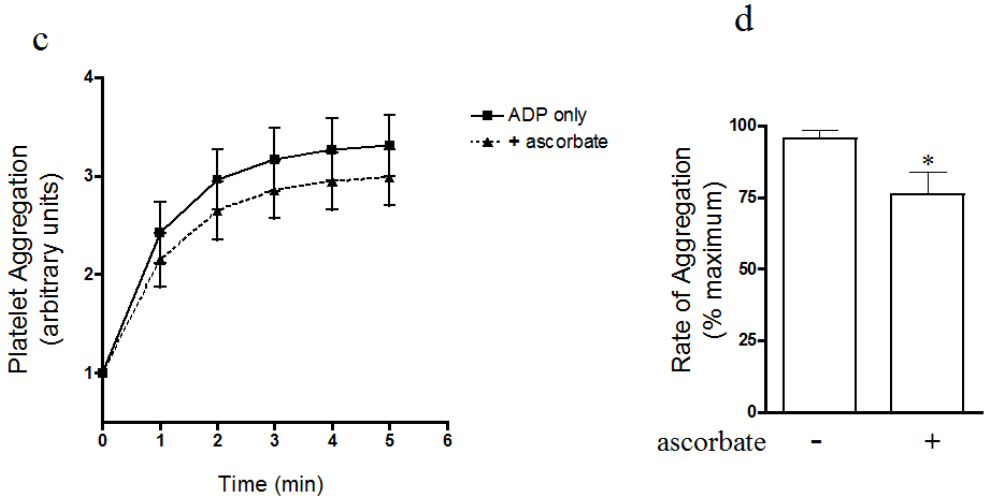
3.3.4 Ascorbate reduces thrombin-induced platelet aggregation independent of NOS

Figure 3.6 shows that the non-specific NOS inhibitor LNAME did not alter the effect of ascorbate on platelet aggregation due to thrombin. This indicates that the effect of ascorbate was not mediated by eNOS in our ex vivo model. Importantly, platelets in this model had their ability to respond to exogenous NO, because SNAP significantly inhibited thrombin-induced aggregation. In the thrombin+SNAP group (n= 5) the initial rate of aggregation was 66.2 ± 6.6 % of the control group (thrombin+inactive SNAP), (n = 4, $p< 0.05$). L-NAME had no effect on thrombin-induced aggregation in the absence of ascorbate (0.075 U thrombin: 2.67 ± 0.15, thrombin + LNAME: 2.65 ± 0.10 units after 5 min of stimulation, n = 3 per group). L-NAME did not alter the effect of ascorbate on thrombin-induced P-selectin expression (Figure 3.7). EC_{50} for thrombin+ascorbate+LNAME was 0.341 ± 0.025 and EC_{50} for thrombin+ascorbate+DNAME was 0.339 ± 0.021 units/ml. L-NAME had no effect on thrombin-induced P-selectin surface expression in the absence of ascorbator (0.075 U thrombin: 64.8 ± 5.2, thrombin + LNAME: 57.4 ± 8.0 % of total events detected by flow cytometer, n = 4 per group).

Thrombin



ADP



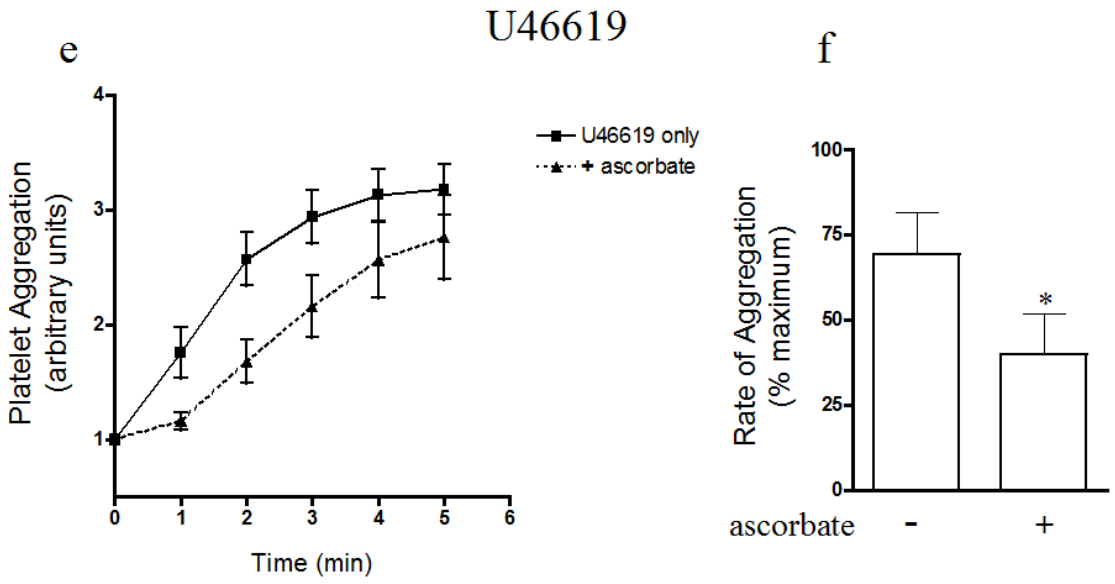


Figure 3.4. Ascorbate reduces thrombin-, ADP-, and U46619-induced platelet aggregation. Isolated platelets were treated with thrombin (0.075 units/ml, Panels a and b), ADP (1 μM, Panels c and d) or U46619 (320 nM, Panels e and f) with or without ascorbate (100 μM) and then placed in an aggregometer. * $P < 0.05$ compared to thrombin alone group, n = 5 (Panel b), compared to ADP alone group, n = 6 (Panel d), and compared to U46619 alone group, n = 5 (Panel f).

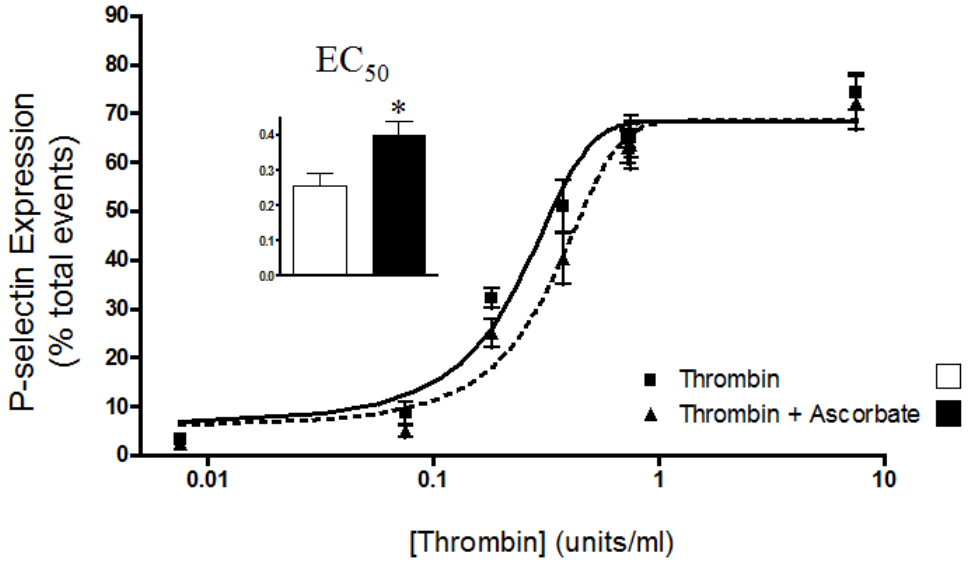


Figure 3.5. Dose response to thrombin and to thrombin plus ascorbate on P-selectin surface expression on isolated platelets. Isolated platelets were treated for 20 min with thrombin (0.0075, 0.075, 0.181, 0.375, 0.7, 0.75, 7.5 units/ml), concurrently with or without ascorbate (100 μM), and then they were examined for surface P-selectin expression by flow cytometry. P-selectin surface expression was represented by the percent of events that were both positive for P-selectin and CD41 (platelet marker). Ascorbate significantly increased the EC_{50} value from 0.26 to 0.40 units/ml (inset). * $P < 0.05$ compared to thrombin alone, n = 5 per group.

Thrombin

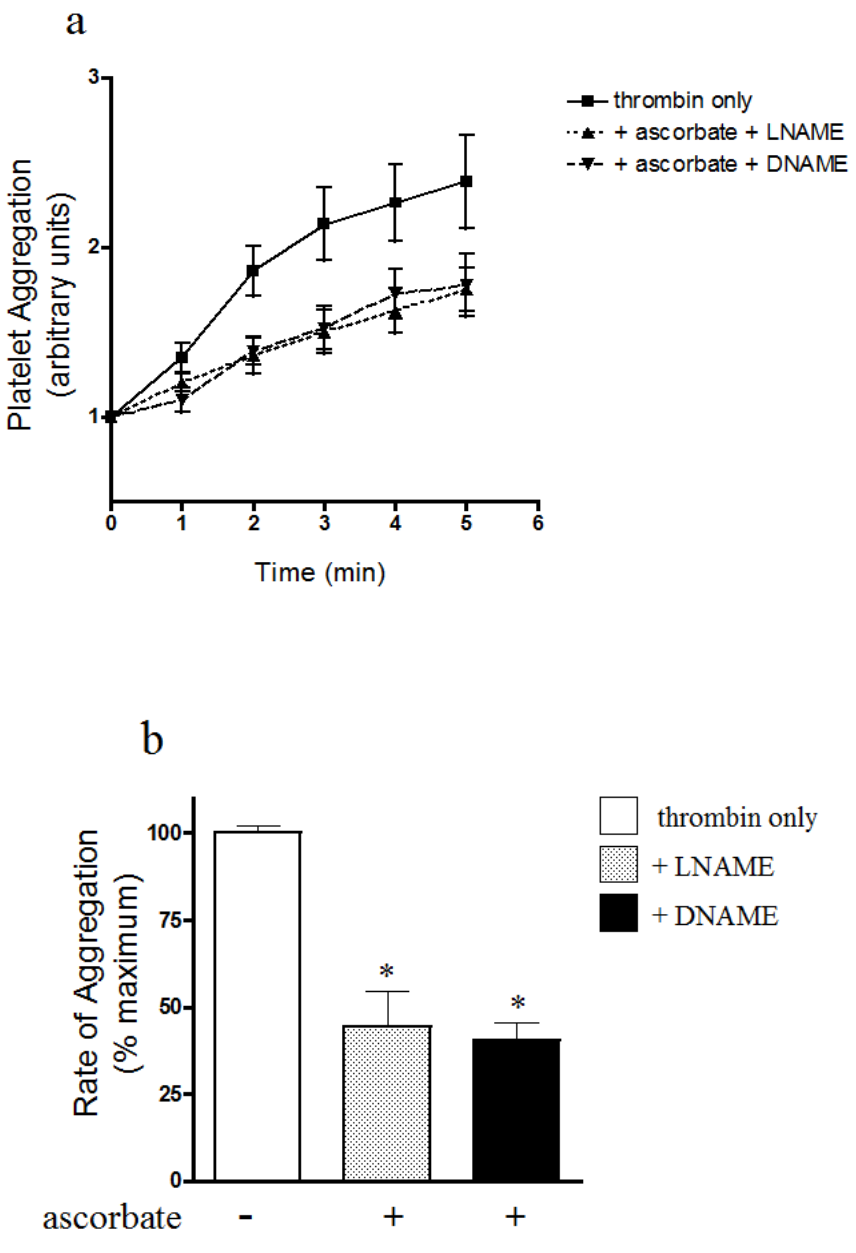

a
Platelet Aggregation (arbitrary units)
Time (min)
thrombin only
+ ascorbate + LNAME
+ ascorbate + DNAME
b
Rate of Aggregation (% maximum)
thrombin only
+ LNAME
+ DNAME
ascorbate
-
+
+

Figure 3.6. Effect of ascorbate on thrombin-induced platelet aggregation is independent of platelet NOS. Panel a: Aggregation of isolated platelets treated with thrombin (0.075 units/ml) alone or concurrently with ascorbate (100 μM) plus LNAME (5 mM) or ascorbate plus DNAME (5 mM)**.** Panel b: The initial rate of aggregation for thrombin, thrombin+ascorbate+LNAME and thrombin+ascorbate+DNAME, shown as the percentage of the maximum rate per experiment. There was no difference between the thrombin+ascorbate+LNAME and thrombin+ascorbate+DNAME groups. * $P < 0.05$ compared thrombin alone group, n = 4 per group.

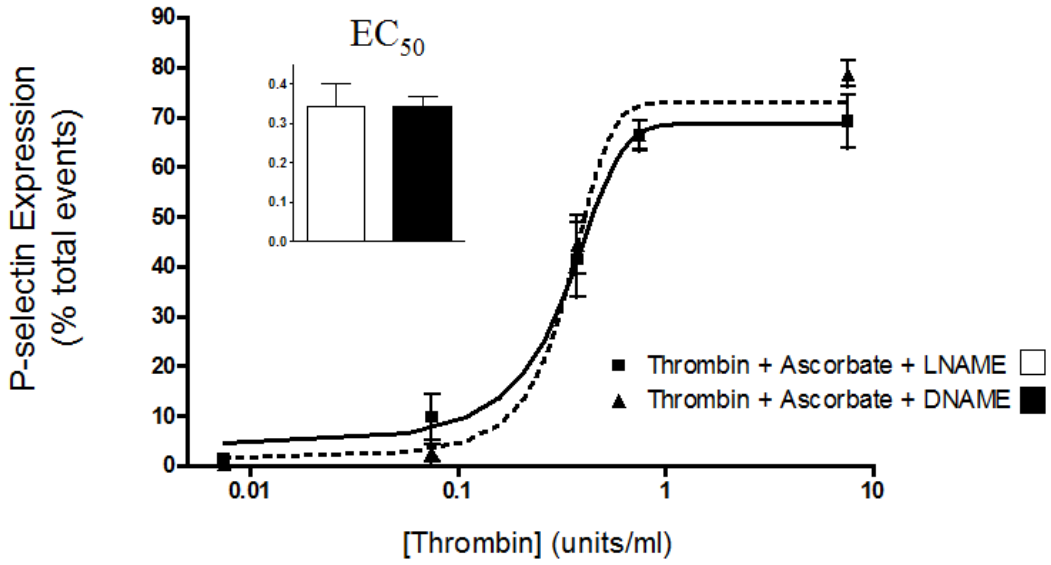


Figure 3.7. Effect of ascorbate on thrombin-induced P-selectin surface expression on isolated platelets is independent of platelet NOS. Isolated platelets were treated for 20 min with thrombin, ascorbate, LNAME, and/or DNAME. P-selectin surface expression was then measured by flow cytometry. There was no difference in EC_{50} between the thrombin+ascorbate+LNAME and thrombin+ascorbate+DNAME groups (inset), n= 4 per group.

3.4 DISCUSSION

In the present *ex-vivo* study we found that ascorbate reduced platelet aggregation and P-selectin surface expression on platelets activated with thrombin, ADP and U46619. This effect of ascorbate was NOS independent. In contrast to these agents, LPS, TNFα and plasma from septic mice did not alter platelet aggregation nor P-selectin surface expression.

Our findings that LPS does not alter platelet aggregation and expression of P-selectin are consistent with earlier reports (10, 47, 63). However, recent studies have shown otherwise, namely that LPS enhances thrombin-induced platelet aggregation and increases P-selectin expression (11, 52, 64). The reason for these discrepancies is unclear. We suspect that inter-species differences may be important here because our data agree with the study of Rumbaut and co-workers (47) who also used platelets from mice. Consistent with previous reports (8, 40), treatment with TNFα also did not initiate aggregation or increased P-selectin expression. Regarding the lack of effect of plasma from septic mice, it should be noted that the present protocol for plasma collection from septic mice included an inevitable delay before its application to the platelet-Tyrode suspension (about a 30 min delay). During this delay the concentration or effectiveness of these platelet-activating agents might have been minimized (e.g. half-life of thrombin in plasma is 56 sec (46)), thus explaining the lack of effect of septic plasma on platelet aggregation or P-selectin surface expression. To our knowledge, there are no reports addressing the direct effect of septic plasma on this aggregation/expression under similar *ex-vivo* experimental conditions.

There are numerous reports that sepsis increases the plasma levels of thrombin, ADP, and thromboxane (12, 20, 32, 35, 36, 39, 55). The present data showing that thrombin, ADP, and thromboxane promote platelet aggregation are consistent with the literature (15, 29). Furthermore, thrombin-induced P-selectin expression on platelets has been reported in humans and dogs where the EC_{50} value was approximately 0.05 units/ml (21, 44). Our higher EC_{50} of 0.26 units/ml is most likely due to the differences between species and experimental protocol used.

The novel finding of this chapter is that ascorbate reduces platelet aggregation and P-selectin surface expression. Administration of anti-oxidants ebselen, resveratrol and trolox have also similar effects (31, 54). Pignatelli and coworkers (45) reported that vitamin C reduces platelet CD40 expression. However, vitamin C had no effect on platelet aggregation and P-selectin expression in this report. This discrepancy may be due to the use of human platelets instead of mouse platelets.

We observed that the inhibitory effect of ascorbate was larger against thrombin-induced than against ADP-induced platelet aggregation (Figure 3.4). This could be explained by 2 mechanisms. First, platelets produce ROS when exposed to various agonists and these ROS augment the aggregation response (18, 24, 49, 61). The various agonists will produce different amounts of ROS (2, 4, 25). Therefore, the aggregation response to a particular agonist may be more sensitive to antioxidant treatment than the response to another agonist. Second, stimulations with various agonists follow different pathways to activate platelets. The receptors for thrombin and thromboxane are G_q coupled, leading to the activation of protein kinase C (PKC) whereas the receptor for ADP is G_i coupled (29). Both antioxidants vitamin E and gallic acid inhibit PKC activity

in stimulated platelets (9, 16) although vitamin E may have effects that are independent of its antioxidant ability. Furthermore, PKC activation in platelets increases the expression of the ascorbate transporter SVCT2, and therefore increases ascorbate levels in the platelets (50). This would explain the greater inhibition by ascorbate in thrombin- and U46619- stimulated platelets as compared with ADP-stimulated.

Since P-selectin plays a role in both aggregation and platelet-endothelial adhesion, reducing P-selectin expression/activity would decrease the formation of microthrombi, leading to reduced capillary plugging. In the previous chapter, we found that blocking P-selectin reduces the capillary blood flow impairment (Chapter 2). The ability of ascorbate to reduce the surface expression of P-selectin on platelets may be one of the mechanisms by which ascorbate reduces the plugging of capillaries.

Our data suggest that, despite the ability of platelets to respond to exogenous NO, the effect of ascorbate on thrombin-induced platelet aggregation and P-selectin surface expression is independent of platelet-derived NO. Others have reported that platelet-derived NO may only have a minor role in aggregation and P-selectin surface expression and have indicated that non-platelet sources of NO within the microvasculature may still have an important inhibitory role in platelet function (43, 51, 59).

The effect of ascorbate *in vivo* has been shown to be eNOS-dependent through a mechanism that restores eNOS function during sepsis (23, 55, 56, Chapter 2). The restored eNOS produces NO that could reduce platelet aggregation in 2 ways. First, NO reduces platelet activation by the classical cyclic GMP pathway (41). Second, NO reduces platelet adhesion by reducing P-selectin surface expression via a reduction in granule secretion. This second pathway includes the nitrosylation of N-ethylmaleimide

sensitive factor (NSF) which is necessary for proper granule docking to the plasma membrane (34). Nitrosylated NSF has a diminished function leading to reduced granular exocytosis and reduced P-selectin surface expression. Our data show that ascorbate works independently of platelet-derived NOS and imply that ascorbate could reduce platelet adhesion/aggregation in the absence of restored eNOS function within the microvasculature. Thus, in the context of plugging of septic capillaries, the inhibition of platelet aggregation directly by ascorbate may not be enough to fully restore capillary blood flow. We propose that, in addition to the reduced adhesion/aggregation caused directly by ascorbate, NO-derived from non-platelet sources (e.g., endothelial eNOS) may also be required for the full *in vivo* effect of ascorbate.

Although the inhibitory effect of ascorbate seemed modest (Figures 3.4 and 3.5), this finding might have reflected the particular conditions of our model. The conditions were pro-aggregatory with thrombin at 0.075 U/ml, since the anti-aggregatory agent eptifibatide inhibited aggregation to a similar modest degree as ascorbate (44% inhibition). Using a lower concentration of thrombin at 0.0375 U/ml, ascorbate inhibited aggregation more robustly (74% inhibition). Furthermore, the present conditions did not exclude the effect of endogenous ascorbate remaining in the platelets after isolation. This remaining ascorbate could have masked the true difference between the control untreated and ascorbate-treated aggregation of platelets.

In conclusion, we used an *ex vivo* model of sepsis in mice to demonstrate that ascorbate (i) reduces platelet aggregation stimulated by thrombin, ADP, and U46619, and (ii) lowers platelet surface P-selectin expression induced by thrombin, in NOS-independent manner. The ability of ascorbate to inhibit platelet P-selectin expression and

platelet aggregation could be an important mechanism by which ascorbate inhibits capillary plugging. Further studies are needed to determine if this inhibition of platelet aggregation/plugging reduces the organ dysfunction occurring during sepsis.

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Chapter 4

Ascorbate reduces P-selectin mediated platelet-endothelial adhesion in an *in vitro* model of sepsis via reduced endothelial granule secretion

A version of this chapter has been submitted to *Microcirculation*

4.1 INTRODUCTION

Sepsis is the tenth leading cause of mortality in the US (19) with a mortality rate near 40% (25). Circulatory dysfunctions including cessation of blood flow in septic capillaries can lead to multiple organ failure (6, 38). Contributing to the capillary blood flow impairment is increased platelet adhesion and fibrin deposition in the capillary suggesting the formation of microthrombi (Chapter 2). This could explain the reduced platelet counts seen in septic patients which correlate with increased mortality (27). The cessation of capillary blood flow leads to increased diffusional distance for oxygen to reach cell mitochondria, tissue hypoxia and organ failure (10, 14).

Enhanced coagulation contributes to the severity of sepsis resulting in microvascular thrombosis and disseminated intravascular coagulation (DIC, (20)). During sepsis, increased platelet-endothelial adhesion may be critical in capillary flow impairment (Chapter 2). P-selectin is a key platelet-endothelium adhesion molecule (4) as inhibition of P-selectin results in decreased platelet adhesion in the septic capillary. Inhibition also prevents the cessation of capillary blood flow (Chapter 2). P-selectin is stored in Weibel-Palade bodies in endothelial cells and, upon stimulation, it is expressed at the surface of the cell where it can facilitate platelet adhesion through binding its counter receptor P-selectin glycoprotein ligand-1 (PSGL1) (23). Reactive oxygen species have also been implicated in the capillary blood flow impairment as sepsis increases the level of ROS (21, 36). ROS have been shown to modulate platelet function by enhancing platelet activation (18) and indirectly, by altering endothelial function (3). Furthermore, ROS can reduce local nitric oxide production by oxidizing the eNOS cofactor BH_4. When

BH_4 is oxidized, eNOS production of nitric oxide (NO) is reduced. NO is an important factor in the homeostasis of the microcirculation and is a known inhibitor of platelet activation/adhesion (30). Furthermore, NO can prevent expression of P-selectin (2), and it can inhibit P-selectin surface expression by reducing the exocytosis of the Weibel-Palade bodies in endothelial cells (24). The antioxidant ascorbate (reduced vitamin C) prevents and reverses the oxidation of BH_4 thus restoring local NO production via eNOS (16, 29).

Clinically, there is no effective pharmacological treatment for the systemic inflammation component of sepsis. Ascorbate has been under investigation as a possible treatment (22). An intravenous bolus of ascorbate has been shown to prevent and reverse the cessation of blood flow in septic capillaries as well as increase survival of septic mice (1, 37). Sepsis-induced increase in adhesion of single platelets and their aggregates in capillaries are also inhibited by ascorbate treatment (Chapter 2). Although several mechanisms have been proposed to explain the beneficial effect of ascorbate in the septic microvasculature (36), the precise mechanism of this effect is unclear.

Our recent study showed that ascorbate could reduce platelet adhesion during sepsis as well as improve capillary blood flow (Chapter 2). However, it was not possible to determine if ascorbate was acting directly on the platelet adhesion or that the reduced platelet adhesion was a consequence of other effects of ascorbate. To address this issue, the present study employed a simple *in vitro* platelet-endothelial cell adhesion assay. This *in vitro* model could determine (i) the effect of sepsis on the endothelial P-selectin expression, and (ii) the effect of ascorbate on this expression. We hypothesized that sepsis-induced platelet-endothelial adhesion and endothelial P-selectin genomic expression and surface expression are reduced by ascorbate.

4.2 METHODS

4.2.1 Reagents used

Lipopolysacchride (LPS), tumor necrosis factor alpha (TNFα), calcein-AM, protease inhibitor cocktail, Superscript II, and ascorbate were purchased from Sigma-Aldrich (St. Louis, MO). The phycoerythrin (PE) conjugated P-selectin antibody (clone: Wug.E9) and PE conjugated IgG control were purchased from Emfret Analytics (Eibelstadt, Germany). A P-selectin blocking antibody (clone: RB40.34) and a rat IgG1 lambda isotype control were purchased from BD Pharminogen (Mississauga, ON). TRIZOL was purchased from Invitrogen (Burlington, ON). A horse radish peroxidase (HRP) tagged anti-rat IgG antibody was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). A GAPDH antibody and a HRP anti-rabbit IgG antibody were purchased from Cell Signalling Technology (Danvers, MA). Quantifast SYBR Green master mix was purchased from Qiagen (Mississauga, ON).

4.2.2 Endothelial cell harvest and culture

All experimental protocols were approved by the University of Western Ontario Council on Animal Care. Skeletal muscle microvascular endothelial cells were harvested from wild type (C57BL/6) mice and isolated as performed previously in our lab (5, 41). Briefly, skeletal muscle from the hind limb of mice was collected and digested. The digest was plated and grown to confluency. Endothelial cells were isolated by lectin

coated magnetic beads. Cells were cultured to confluence and cell type was confirmed by immunocytochemistry, checking for the endothelial marker von Willebrand factor. To model sepsis, endothelial cells were treated with LPS (10 μg/ml) or TNFα (10 ng/ml) for 1h. Ascorbate (100 μM) was applied to cells as a pre-treatment 4 h prior to LPS or TNFα treatment. In some experiments, a P-selectin blocking antibody (clone: RB40.34, 1:1000) or a control IgG was added cells concurrently with LPS or TNFα.

4.2.3 Platelet collection and isolation

Blood (600 μl) was collected from anaesthetized naive male mice via carotid artery puncture into a vial containing 100 μl of acid citrate dextrose solution. Platelets were isolated by the method describe by Singer *et al* (32). Briefly, platelet rich plasma was collected by centrifuging the whole blood at 120 g for 8 min. The plasma was then re-spun to remove any collected leukocytes. Finally the platelets were pelleted at 735 g for 10 min and resuspended in phosphate buffer solution (PBS).

4.2.4 In vitro adhesion model to mimic the platelet-endothelial cell interaction

To visualize platelets with fluorescence microscopy, isolated platelets were incubated with 8 μM calcein-AM for 10 minutes. The platelets were pelleted at 735 g for 10 min to remove excess calcein-AM and resuspended in fresh PBS. Following endothelial cell treatment, ~3 x 10^6 platelets were added to the a confluent monolayer of 0.25 x 10^6 endothelial cells and co-incubated under static conditions for 1h, and then washed. The remaining platelets adhering to the endothelial cells were visualized with a Zeiss

fluorescence microscope using a 20 x magnification objective. Labelled platelets were then counted in the entire area of the microscopic field of view (0.43 mm x 0.32 mm). Each treatment group was done in triplicate and 5 arbitrary microscopic fields of view (chosen blindly) were used per replicate. Counts of attached platelets were repeated by a separate individual blinded to the conditions.

4.2.5 Septic mouse model

Male mice were anaesthetized with an intraperitoneal (i.p.) injection of a mixture of ketamine (80 mg/kg) and xylazine (4 mg/kg). Sepsis was induced by feces injected into peritoneum (FIP) method previously described by our lab (37). Briefly, mice were given an i.p. injection of 50 ml/kg volume of saline containing 75 mg/ml of feces. Control (sham) mice received sterile saline i.p. injection 50 ml/kg. Both FIP and sham mice were given 1 ml of saline containing 4μg/ml buprenophine sub-cutaneously, providing fluid resuscitation and analgesics.

4.2.6 P-selectin and PSGL1 mRNA expression in cultured endothelial cells and skeletal muscle homogenates

P-selectin and PSGL1 mRNA were measured by real time qPCR. After treatment, mRNA from the endothelial cells was collected by TRIZOL according to the manufacturer's instructions. Hindlimb skeletal muscle was collected from sham and septic mice at 7 h post-FIP. Prior to this, septic mice were injected intravenously (penile vein) with 1 ml of sterile saline or with 1 ml saline containing ascorbate (10 mg/kg) at 6 h (i.e., when capillary plugging is prevalent (37)). Similarly, sham mice were injected with saline at 6

h. The muscle was homogenized and mRNA was collected by TRIZOL. mRNA was then reversed transcribe to cDNA by Superscript II. We used qPCR to determine the mRNA expression levels of P-selectin (primers: forward 5'-GTCCACGGAGAGTTTGGTGT-3' and reverse 5'-AAGTGGTGTTCGGACCAAAG-3') and PSGL1 (primers: forward 5'-CTTCCTTGTGCTGCTGACCAT-3' and reverse 5'-TCAGGGTCCTCAAAATCGTCATC-3') and 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 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.

4.2.7 Von Willebrand factor (vWF) ELISA

The purpose of this procedure was to assess indirectly P-selectin protein expression at the endothelial cell surface. Originally, we carried out pilot experiments using immunocytochemistry and flow cytometry to detect P-selectin. Unfortunately, for unknown reasons these procedures failed to detect P-selectin under any experimental conditions. For this reason we have used the vWF ELISA as a surrogate measure for P-selectin surface protein detection. P-selectin is contained together with vWF in Weibel-Palade granules under the surface of endothelial cells. Exocytosis of these granules delivers to the surface both vWF and P-selectin (23).

Endothelial cells grown to confluence were treated with dialyzed serum medium with or without ascorbate (100 μM) for 4 h. The medium was replaced by fresh medium without ascorbate and the cells were treated with LPS (10 μg/ml) or TNFα (10 ng/ml) for 1 h. The cell culture media after the endothelial cell treatments was collected and measured for von Willebrand factor using a commercially available ELISA kit (Cusabio, Wuhan, China).

4.2.8 Western blots

Hindlimb extensor digitorum longus skeletal muscle was collected and homogenized in SDS lysis buffer with protease inhibitor cocktail. The collected protein was run on an acrylamide gel and transferred to a PVDF membrane. The membrane was probed with a P-selectin antibody (Clone: Wug.9, at 1:1000) and a HRP tagged anti-rat IgG secondary antibody (1:5000). To control for protein loading, the membrane was also probed with a GAPDH antibody (Clone: 14C10, 1:1000) followed by a HRP anti-rabbit IgG secondary antibody (1:2000).

4.2.9 Statistical analysis

Data are represented as mean ± standard error and n represents the number of mice or separate cell culture experiments where cells were harvested from at least 3 mice. Data were analyzed by one-way ANOVA followed by Bonferroni comparison. P values less than 0.05 were considered significant.

4.3 RESULTS

4.3.1 Ascorbate prevents increased platelet-endothelial cell adhesion in an in vitro model of sepsis

Both LPS and TNFα increased platelet-endothelial cell adhesion under static conditions (Figures 4.1, 4.2). LPS or TNFα-induced increase in adhesion was prevented by blocking P-selectin (Figure 4.1). The control IgG had no effect (LPS-induced platelet adhesion with control IgG and TNFα-induced platelet adhesion with control IgG were 2.4 ± 0.7 and 2.5 ± 0.9 fold, respectively, over vehicle treated control cells with control IgG, n = 3 per group). Pretreatment of endothelial cells with ascorbate prevented both the LPS-induced and TNFα-induced increases in platelet-endothelial cell adhesion (Figure 4.2). Ascorbate has no effect in vehicle treated cells.

4.3.2 LPS and TNFα increase P-selectin mRNA expression but not PSGL1 mRNA

LPS and TNFα increased P-selectin mRNA 3 and 4 fold, respectively, in endothelial cells. However, pre-treatment of the endothelial cells with ascorbate did not affect this increase (Figure 4.3). Ascorbate has no effect in vehicle treated cells. LPS or TNFα treatments did not alter PSGL1 mRNA (Figure 4.4).

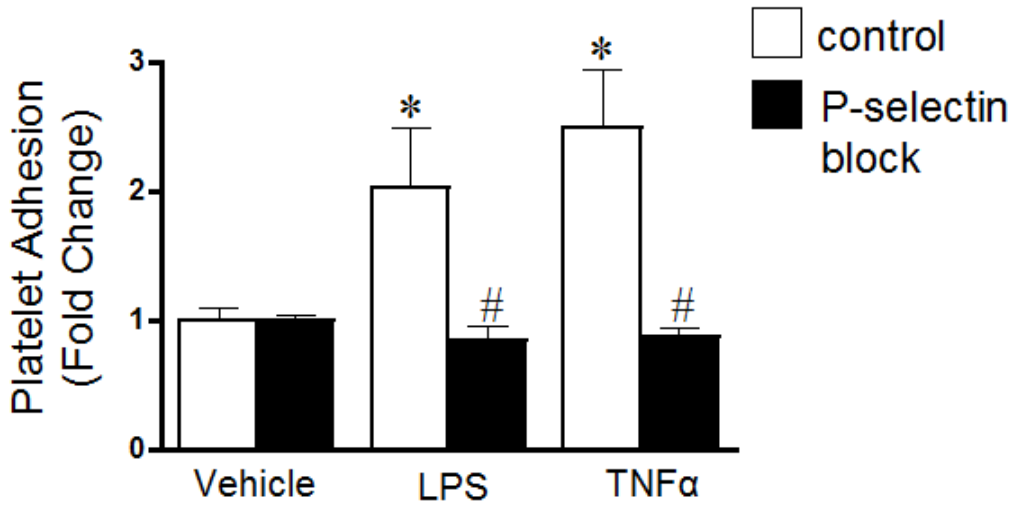

Figure 4.1. Effect of P-selectin block on LPS or TNFα treated endothelial cell-platelet adhesion. Endothelial cells treated with LPS or TNFα for 1 h were co-incubated with untreated platelets for 1 h, washed, and adherent platelets were counted by fluorescence microscopy. A separate group of cells was co-treated with ascorbate a P-selectin blocking antibody during the TNFα treatment. LPS or TNFα treatment significantly increased the endothelial cell-platelet adhesion. P-selectin block prevented this increased adhesion. (*significantly different from control untreated group, #significantly different from control group with the same treatment, $p < 0.05$, $n = 5$).

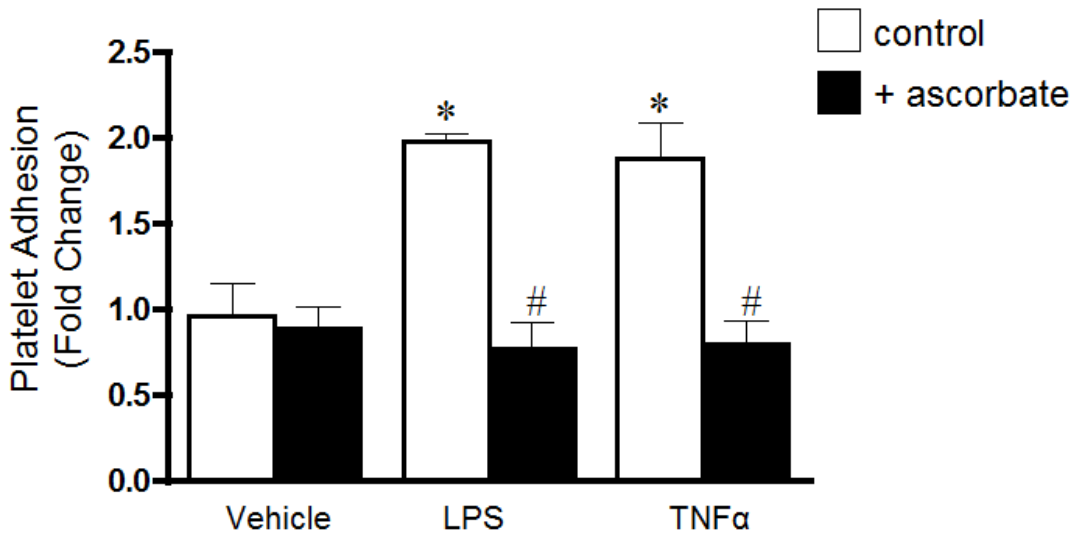

Figure 4.2. Effect of ascorbate on LPS or TNFα treated endothelial cell-platelet adhesion. Endothelial cells treated with LPS or TNFα for 1 h were co-incubated with untreated platelets for 1 h, washed, and adherent platelets were counted by fluorescence microscopy. A separate group of cells was pretreated for 4 h with ascorbate prior to LPS/TNFα treatment. LPS or TNFα treatments significantly increased the endothelial cell-platelet adhesion. Ascorbate prevented this increased adhesion. (*significantly different from control untreated group, #significantly different from control group with the same treatment, $p < 0.05$, $n = 5$ -8).

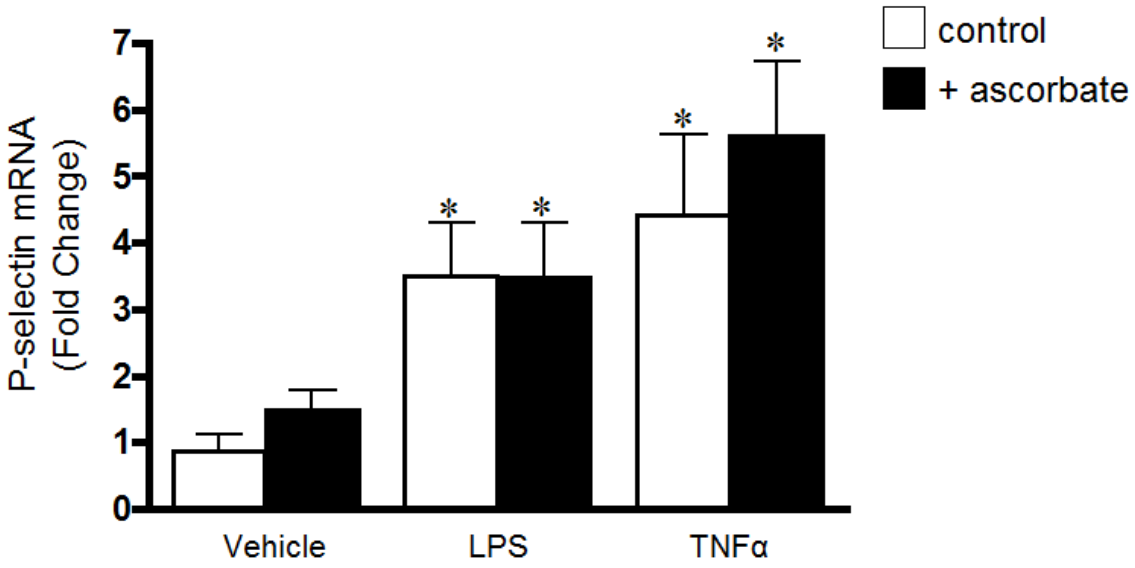

Figure 4.3. Effect of ascorbate on P-selectin mRNA expression in LPS or TNFα treated endothelial cells. Endothelial cells treated with LPS or TNFα for 1 h were assayed for P-selectin mRNA expression by real time qPCR. A separate group of cells was pretreated for 4 h with ascorbate prior to LPS/TNFα treatment. LPS or TNFα treatments significantly increased P-selectin mRNA. Ascorbate did not affect this increased expression. (*significantly different from control untreated group, $p < 0.05$, n = 6 - 7).

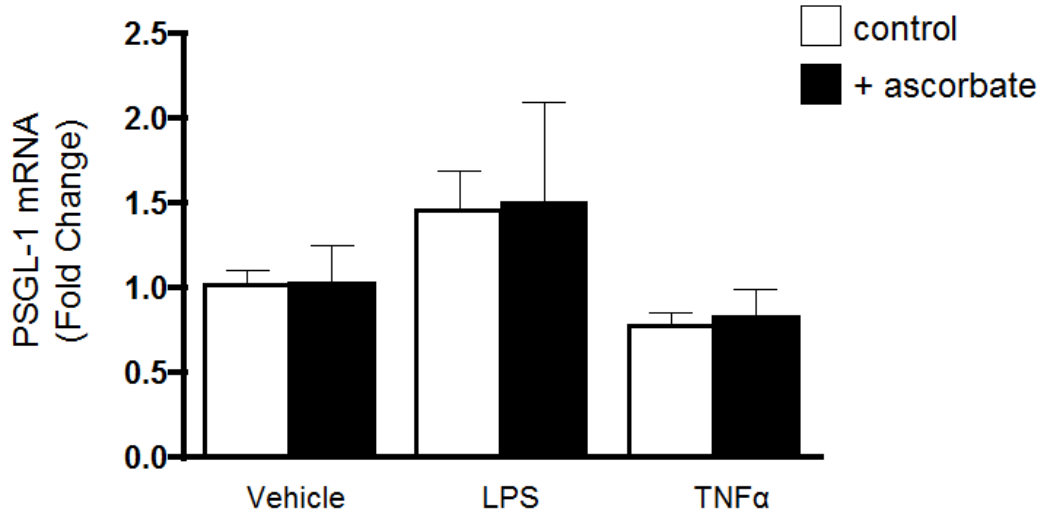

Figure 4.4. Effect of ascorbate on P-selectin glycoprotein ligand-1 (PSGL1) mRNA expression in LPS or TNFα treated endothelial cells. Endothelial cells treated with LPS or TNFα for 1 h were assayed for PSGL1 mRNA expression by real time qPCR. A separate group of cells was pretreated for 4 h with ascorbate prior to LPS/TNFα treatment. LPS and TNFα did not affect PSGL1 mRNA. (Means are not significantly different, n = 6 - 7).

4.3.3 Ascorbate reduces vWF release in LPS treated endothelial cells

LPS but not TNFα treatment increased vWF granular secretion from endothelial cells (Figure 4.5). Ascorbate pre-treatment prevented the LPS-induced vWF release. Ascorbate showed no effect in vehicle treated cells. Fold change over the vehicle treated control cells was used due to large variability in the day-to-day experimental data (range of vWF protein amount in vehicle treated control cells: 0.2 – 5.5 ng/ml).

4.3.4 Sepsis increases expression of P-selectin mRNA but not protein in muscle homogenates

Muscle homogenates from septic mice at 7 h has a 7 fold higher P-selectin mRNA compared to sham mice (Figure 4.6). However, Figure 7 shows that there was no difference in P-selectin protein content between the septic and sham mice homogenates. Ascorbate injected at 6 h did not affect either the mRNA or protein when compared to the saline treated control group (Figures 4.6, 4.7).

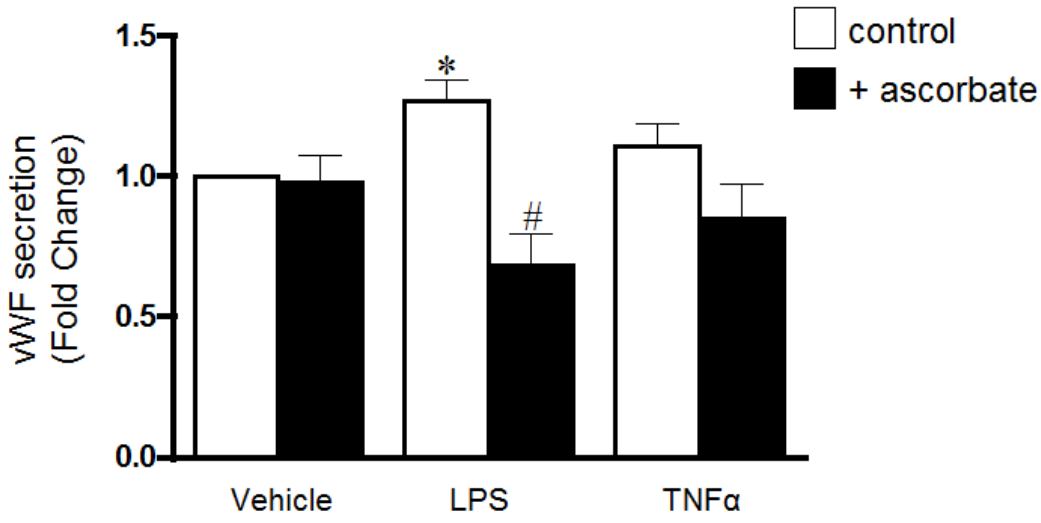

Figure 4.5. Effect of ascorbate on endothelial cell granule secretion. Media from endothelial cells treated with LPS or TNFα for 1 h was collected and assayed for von Willebrand factor (vWF) expression by ELISA. A separate group of cells was pretreated for 4 h with ascorbate prior to LPS/TNFα treatment. LPS but not TNFα increased vWF release from endothelial cells. Ascorbate prevented this increased vWF release. (*significantly different from control untreated group, $p < 0.05$, $n = 5$).

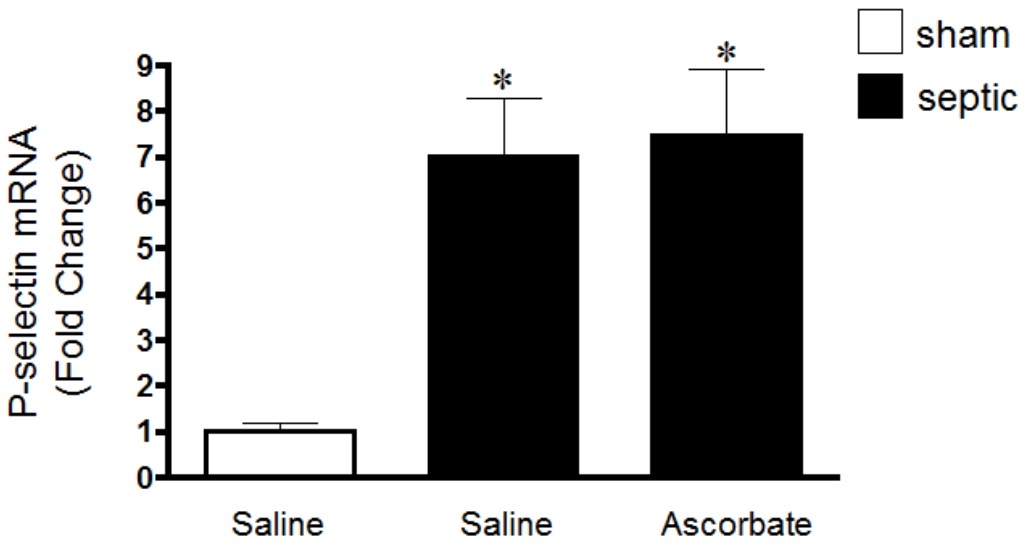

Figure 4.6. Effect of ascorbate on P-selectin mRNA expression in septic mice. At 6 h after sham injection or feces injection into peritoneum (FIP, model of sepsis), mice were given an i.v. bolus of saline or ascorbate. At 7 h post-FIP, hindlimb muscle was collected, homogenized, and analyzed for P-selectin mRNA by real time qPCR. Sepsis increased P-selectin mRNA expression. Ascorbate did not affect this increase. (* significantly different from sham, $p < 0.05$, $n = 9$-13).

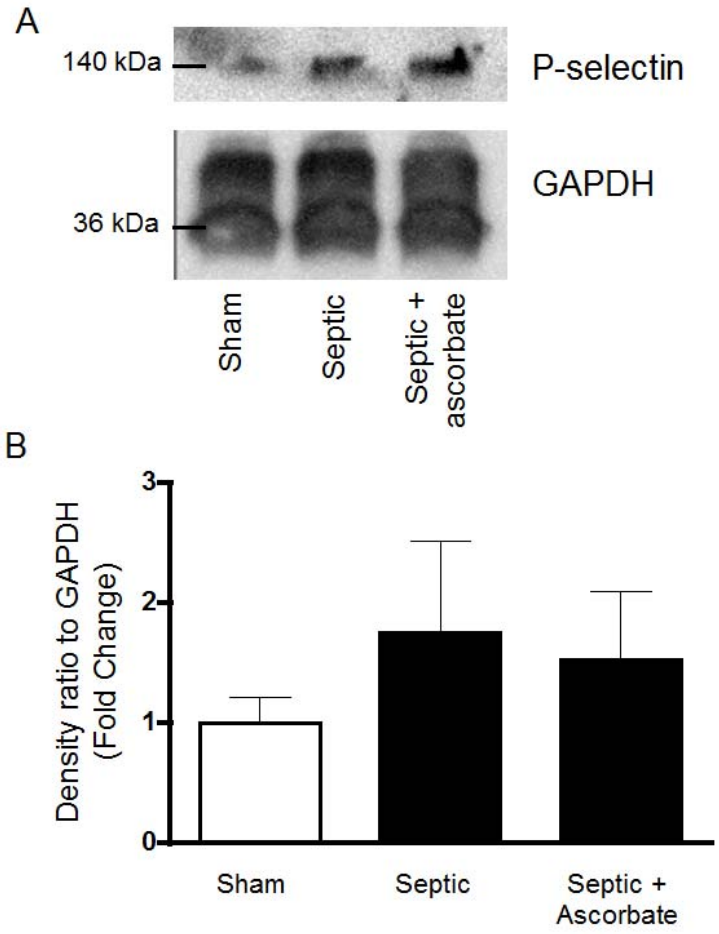

Figure 4.7. Effect of ascorbate on P-selectin protein expression in septic mice. At 6 h after sham or FIP injection, mice were given an i.v. bolus of saline or ascorbate. At 7 h post-FIP, hindlimb muscle was collected, homogenized, and analyzed for P-selectin protein by western blot. A) Shows a representative blot. B) densitometry ratio of the P-selectin:GAPDH signal. Data is displayed as the fold change when compared to the sham mice. (Means are not significantly different, n = 6).

4.4 DISCUSSION

In the present study we found that ascorbate reduced platelet adhesion to endothelial cells stimulated by LPS or TNFα. Ascorbate reduced vWF granular secretion from endothelial cells activated by LPS. On the other hand, ascorbate did not affect P-selectin mRNA expression in LPS or TNFα-stimulated endothelial cells and in skeletal muscle homogenates from septic mice.

Sepsis has been observed to induce platelet adhesion to the capillary wall *in vivo* (Chapter 2). Our present *in vitro* model successfully mimicked this observation. Both LPS and TNFα have previously been shown to increase platelet-endothelial cell adhesion (17, 42). In our model, this increased adhesion was P-selectin dependent as blockage of P-selectin via an antibody prevented stimulated adhesion. We interpret these observations to indicate that the P-selectin played a direct role in the observed adhesion. However, since P-selectin-PSGL1 binding can produce further signalling that also leads to adhesion involving other adhesion proteins (4), the role of P-selectin in the observed adhesion could also be indirect.

We previously reported that ascorbate reduces platelet adhesion in septic capillaries (Chapter 2). However, to our knowledge, ascorbate has not been tested in an *in vitro* assay to probe for the mechanism of this reduction. Previous reports have shown that other antioxidants namely vitamin E and quercetin, can reduce platelet-endothelial adhesion *in vitro* (11, 34). Szuwart and co-workers (34) found that the inhibitory effect of vitamin E on platelet adhesion was seen in platelet rich plasma and not in isolated platelets. Since only the endothelial cells received the treatments in the present *in vitro* model, activation of only these cells was responsible for the changes in the platelet-

endothelial adhesion. Therefore we propose that the LPS or TNFα stimulus causes an increase in P-selectin expression in the endothelial cells leading to the increased adhesion.

LPS or TNFα increased P-selectin mRNA expression in our endothelial model which is consistent with other reports (13, 33). However, treatment of the endothelial cells with ascorbate did not affect this expression. Our results disagree with a previous report that the antioxidant nebivolol inhibits P-selectin mRNA expression on human macrovascular endothelial cells stimulated with oxidative low density lipoprotein (12). The difference could be explained by the use of a different stimuli, species, or cell type. Inflammatory mediators do not affect PSGL1 mRNA expression (9), which agrees with our present data showing no effect of TNFα or LPS on PSGL1 mRNA in our *in vitro* model.

Granular secretion measured by vWF release was used as a surrogate biomarker for P-selectin surface expression. LPS but not TNFα increased vWF release from endothelial cells (Figure 4.5). This, too, is consistent with the current literature. LPS has been shown to increase vWF release (28) as well as increase P-selectin surface expression (7) whereas TNFα failed to affect vWF secretion (31) and P-selectin surface expression (7). This suggests that LPS and TNFα increase platelet-endothelial adhesion by different mechanisms. Here we show for the first time that ascorbate can inhibit vWF release from murine microvascular endothelial cells and thus reduce P-selectin surface expression. Wang and coworkers (39) showed similar results with the antioxidant genipin on thrombin stimulated cells, NOS dependently. Likewise, other studies have shown that

antioxidants reduce P-selectin surface expression caused by thrombin (35) or hypoxia/re-oxygenation (15) in human umbilical vein endothelial cells.

In our model, ascorbate could prevent LPS-stimulated granular secretion by restoring eNOS function. Proper Weibel-Palade body exocytosis requires N-ethylmaleimide-sensitive factor (NSF) regulating membrane fusion (26). When nitrosylated by NO, NSF has reduced function and thus lowers granule secretion (26). Therefore, ascorbate could restore local NO production, nitrosylate NSF, and subsequently reduce Weibel-Palade body exocytosis including P-selectin surface expression. The effect of ascorbate on TNFα induced platelet-endothelial adhesion is unclear. TNFα did not increase vWF release and thus did not affect P-selectin surface expression on endothelial cells. However, TNFα-induced platelet adhesion was prevented by blocking endothelial P-selectin.

Although P-selectin mRNA was increased in septic murine muscle tissue, there was no change in the protein expression when assessed by immunoblot (Figure 4.7). The apparent discrepancy between mRNA and protein data could be explained by the fact that the mRNA from the muscle homogenates comes exclusively from the endothelial cells whereas the protein comes from both the endothelial cells and the platelets. Thus the platelet protein content could mask any changes in the endothelial cell protein that may occur during sepsis. Since ascorbate did not affect P-selectin expression on either the mRNA or protein level, we must conclude that the protective effect of ascorbate (i.e., reduction in adhesion) is independent of changes in the overall P-selectin levels.

4.4.1 Methodological limitations

In the present study, treatment of cultured endothelial cells with P-selectin blocking antibody (but not with control IgG) prevented platelet adhesion to these cells. Together with observed P-selectin mRNA expression in these cells, the effect of blocking strongly indicated that these cells expressed P-selectin protein. However, our inability to detect P-selectin surface protein in these cells by immunocytochemistry and flow cytometry and the total P-selectin protein by immunoblotting pointed to a limitation of the cell culture model (Figure 4.8). In the platelet adhesion assay, we counted about 5 - 20 adhering platelets in each microscopic field of view including about 180 endothelial cells. This implies that only a low percentage of endothelial cells presented P-selectin at their surface to "capture" platelets for adhesion, after excess of platelets was subjected to co-incubation with the confluent endothelial monolayer. To explain this low percentage, we speculate that the culturing conditions reduced the number of cells expressing P-selectin protein and/or the level of this expression per cell. This level could have been too low to be detected by immunocytochemistry or immunoblot. The skeletal muscle vascular bed could also have a low P-selectin expression. This low expression has been reported for liver sinusoids (8, 40). Regarding detection of P-selectin surface expression by flow cytometry, this limitation would be further worsened by the procedural steps necessary for cell preparation. Detachment of cells from dishes, suspension and fixing of cells could all negatively affect the presence of this protein at the cell surface.

In conclusion, we used an *in vitro* model of sepsis in mice to demonstrate that ascorbate (i) reduces platelet-endothelial adhesion stimulated by LPS or TNFα and (ii) lowers LPS-induced vWF granular release from endothelial cells. The ability of ascorbate to inhibit P-selectin surface expression could be an important mechanism by which ascorbate inhibits capillary plugging.

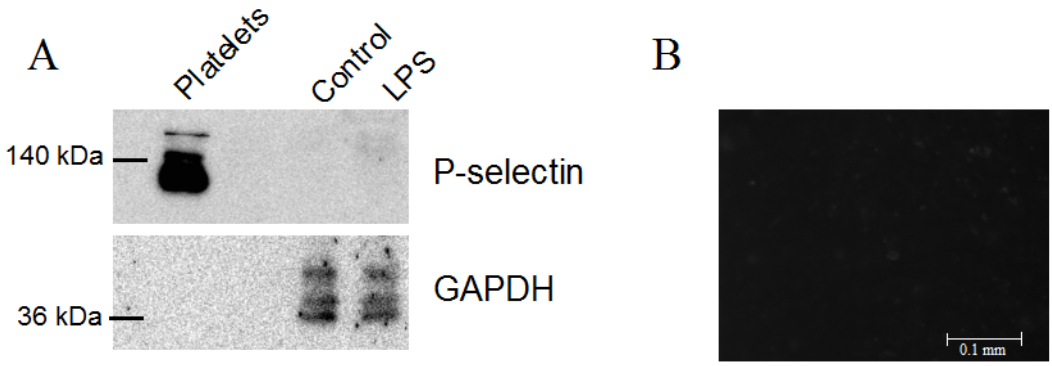

Figure 4.8. P-selectin protein expression in the endothelial cell model. A) Endothelial cells treated with LPS for 6 h were assayed for P-selectin protein expression by Western blot. Shown is a representative blot of 6 endothelial cell lines originating from 6 mice. P-selectin was not detected in the endothelial cells. Platelet lysate was used as a positive control. B) Endothelial cells treated with LPS for 1 h were assayed for surface P-selectin protein expression by immunocytochemistry. A FITC conjugated anti-P-selectin antibody was co-incubated with the cells (Clone: Wug.9 at 1:100 for 1 h). No staining was observed. Treatment of isolated platelets with the antibody was used a positive control.

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Chapter 5

SUMMARY, DISCUSSION, and FUTURE DIRECTIONS

5.1 THESIS SUMMARY

One of the key characteristics of sepsis is the cessation of capillary blood flow that leads to tissue hypoxia and possibly multiple organ dysfunction. The mechanism behind the cessation of flow is currently unclear. A possible mechanism is physical hindrance of the flow due to the formation of microthrombi in the septic capillary. The coagulation system has been known to be involved the pathophysiology of sepsis; a reduced platelet count in blood is a particularly telling index of the severity of sepsis. Thus, I hypothesized that increased platelet adhesion, aggregation and microthrombi formation during sepsis leads to plugging of capillaries resulting in cessation of blood flow. This hypothesis was addressed in Chapter 2.

Treatment with ascorbate prevented and rescued the capillary blood flow impairment and increased survival in our animal model of sepsis. However, the means by which ascorbate provides the beneficial effects are unknown. The effect of ascorbate is eNOS dependent, therefore locally produced NO could be involved in the protective effect of ascorbate. NO is a known platelet inhibitor and thus may modulate the formation of microthrombi in the septic capillary. I hypothesized that ascorbate lowers platelet aggregation and adhesion by decreasing P-selectin expression, thus improving capillary blood flow during sepsis. This has been addressed in Chapters 3 and 4.

In Chapter 2, I show that removal of platelets from the circulation significantly reduced the capillary blood flow impairment in septic mice. Next, I observed that platelet adhesion to the capillary wall and fibrin deposition in capillaries are increased during sepsis. Septic capillaries also showed an increased thrombogenic potential. Finally, administration of the general anticoagulant antithrombin, the platelet anti-aggregatory agent eptifibatide, and an antibody that blocks P-selectin (platelet and endothelial adhesion molecule) reduced the capillary blood flow impairment in septic mice. These data support our hypothesis that increased microthrombi formation leads to the capillary blood flow impairment.

Platelet adhesion in septic capillaries was decreased by an intravenous bolus of ascorbate administered after before the onset of sepsis or later when the cessation of capillary blood flow was already established. Also, platelet adhesion was reduced in gp91phox knockout mice suggesting that ascorbate works by its antioxidant properties. This effect, however, was not seen in eNOS knockout mice. Thus, similar to the capillary blood flow impairment, reduction in platelet adhesion by ascorbate during sepsis is eNOS dependent. Furthermore, I showed that topical application of BH_4 and the NO-donor SNAP reduced platelet adhesion in wild type septic mouse capillaries but only SNAP reduced adhesion in $eNOS^{-/-}$ mice. Therefore, I proposed that ascorbate works by restoring eNOS function through increasing BH_4 availability (via preventing or reversing oxidative alterations to BH_4), leading to local NO production that reduces platelet adhesion.

Although ascorbate reduces platelet adhesion *in vivo*, this decrease in platelet adhesion could be a result of the other beneficial effects of ascorbate (i.e., increase blood

pressure) restoring capillary blood flow. Thus, I wanted to take a closer look at platelet adhesion and aggregation independent of flow and other *in vivo* parameters. Platelet aggregation was first examined in Chapter 3. Here, I showed that LPS, TNFα, or septic plasma do not initiate platelet aggregation nor platelet P-selectin surface expression (a marker of platelet activation). Thus, I developed our *ex vivo* model, using thrombin, ADP, and thromboxane as the stimulating agents, because the concentration of these molecules in blood is known to be increased during sepsis. I showed that ascorbate reduced platelet aggregation initiated by these factors. Furthermore, ascorbate reduced the sensitivity of platelet P-selectin surface expression induced by thrombin by effectively increasing the EC_{50} of the dose-response curve from 0.26 to 0.40 units/ml. Ascorbate reduction of thrombin-induced aggregation or P-selectin surface expression was independent of platelet NOS activity as LNAME (non specific NOS inhibitor) treatment had no effect. We concluded that ascorbate directly inhibits platelet aggregation by reducing P-selectin surface expression. However, this reduction of platelet aggregation alone may be insufficient to improve capillary blood flow during sepsis.

Finally, I examined platelet-endothelial adhesion in Chapter 4. Primarily, I focused on the effect of ascorbate on this adhesion. To this end, I developed an *in vitro* system to model the septic platelet-endothelium interactions. Here, I show that LPS or TNFα increased platelet-endothelial adhesion similar to the increased adhesion in the septic capillary. Moreover, I demonstrated that ascorbate treatment prevented the increase in adhesion caused by LPS or TNFα, indicating that ascorbate can directly affect platelet-endothelial adhesion. To further investigate how ascorbate prevents this adhesion, we focused on the role of endothelial cell P-selectin expression. LPS or TNFα increased P-

selectin mRNA expression, however ascorbate treatment had no effect on this increase expression. By using vWF release as a marker for granule secretion from endothelial cells, I potentially showed that LPS increased granule secretion and thus P-selectin surface expression. Ascorbate treatment prevented the LPS-induced increase in vWF release suggesting a potential mechanism by which ascorbate reduces platelet adhesion caused by LPS. Finally, I showed that septic muscle tissue has increased P-selectin mRNA expression but that ascorbate treatment does not affect this increase.

In conclusion, the sepsis-induced capillary impairment is, in part, due to the formation of platelet-fibrin microthrombi. Ascorbate reduces the impairment by decreasing platelet adhesion and aggregation via a reduction in P-selectin surface expression (Figure 5.1).

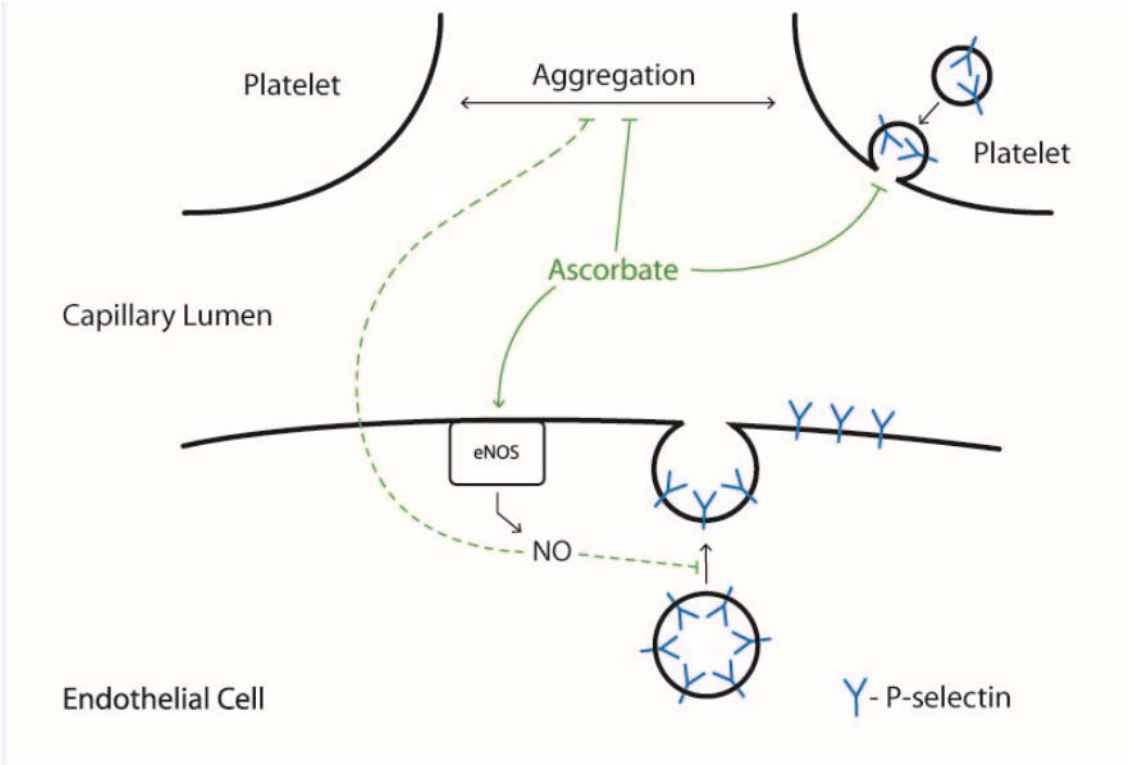

Figure 5.1. The effect of ascorbate on platelet-endothelial function in septic capillaries. Ascorbate can directly reduce platelet aggregation and P-selectin surface expression (i.e., platelet activation). Furthermore, ascorbate could restore local NO production via eNOS. The local NO may further reduce platelet aggregation and endothelial P-selectin surface expression. The summation of these effects leads to reduced platelet adhesion and aggregation in the septic capillary leading to restoration of blood flow.

5.2 GENERAL DISCUSSION

5.2.1 Comparing the in vivo, ex vivo, and in vitro models of sepsis

Sepsis is a complex inflammatory disease that involves numerous cell types and mediators. In addition, sepsis can be confounded by pre-existing diseases, making it very difficult to study (29). The studies presented in this thesis use *in vivo*, *ex vivo*, and *in vitro* models to investigate the effect of sepsis on capillary platelet-endothelial and platelet-platelet interactions. These models are used to gain insights on how ascorbate treatment benefits capillary flow during sepsis. Capillary impairment in the mouse extensor digitorum longus (EDL) muscle, platelet-platelet interactions of isolated platelets, and platelet interactions with mouse microvascular endothelial cells were used as *in vivo*, *ex vivo*, and *in vitro* models of sepsis, respectively. These models have both advantages and limitations.

5.2.1.1 Mouse models of sepsis

There are several animal models used to study the mechanisms governing the pathophysiology of sepsis. The goal of these models is to best mimic the pathogenesis observed in septic patients. The most commonly used models are (i) direct administration of endotoxin or viable bacteria into the animal and (ii) causing peritonitis by disrupting the barrier of the gastrointestinal tract by cecal ligation and puncture (CLP) or direct administration of fecal material into the peritoneum (FIP).

Endotoxemic models usually involve bolus injections of lipopolysaccharide (LPS), a component of the wall of Gram-negative bacteria. This model is used primarily

for its ease in standardizing the experimental conditions (22). The major criticism of this model is its lack of clinical relevance, as the LPS challenge does not have the focus of infection seen in clinical sepsis, because LPS levels are usually low in human patients (7). Furthermore, the LPS model does not account for the septic cases that involve Gram-positive bacteria (41).

The bacteremia model of sepsis involves the injection of cultures of viable bacteria (16) and thus may be more clinically relevant than the endotoxemic model. However, the injected bacteria are usually not able to replicate within the host because they are destroyed by the immune system (13). This results in an endotoxemic model rather than an infectious model causing the model to suffer from the same criticisms as the LPS model. Both of these models lead to many of the clinical characteristics of sepsis (hypotension, multiple organ dysfunction) but tend to have more acute and intense increases in inflammatory mediators than seen in septic patients (22).

Both the FIP and CLP models of sepsis are better associated with clinical sepsis. As well as having a peritoneal focus of infection and displaying the characteristic symptoms of clinical sepsis, both models produce a continuous and sustained production of cytokines similar to human patients (21, 22, 59). For the present series of investigations, the FIP model was used over the CLP because it is a more standardized model. The resulting infection due to CLP can vary greatly depending on the amount of fecal material that leaks into the peritoneum. Thus the precision in replicating the surgical procedure is essential for accurate data. The FIP model eliminates this factor by standardizing the amount of feces injected into the peritoneum. Also, the sham control animals for the FIP model receive an intraperitoneal injection of saline as opposed to a

sham surgery in the CLP model, which itself leads to the release of inflammatory cytokines and thus confound the outcome of bacterial infection. When comparing the FIP and CLP models, the major drawback for the FIP model compared to the CLP model is that it is less clinically relevant as clinical septic patients have varied degrees of infections.

5.2.1.2 Methodological limitations to the models

In Chapter 2, mouse EDL muscle was observed by intravital microscopy. Although skeletal muscle is not a critical organ, it is used to model capillary beds because the capillaries can be easily visualized microscopically when compared to other organs that move (e.g., lungs, heart). Moreover, the EDL muscle was chosen because it can be surgically exposed without disturbance to the muscle and because it has evenly distributed parallel capillaries. Also, it is possible to visualize platelets in these capillaries in real time. I used the epi-illuminated EDL muscle preparation where scattered light is reflected from the muscle surface to the microscope objective, thus limiting the optical resolution at a higher magnification. Therefore, platelets (about 1 μm diameter) needed to be labelled with a fluorescence dye to be visualized under fluorescent illumination. The labelling makes it difficult to distinguish between single platelets and small aggregates of 4 or less platelets. Furthermore, labelling of the platelets requires administering the dye just before the visualization (e.g., 6 h into sepsis when cessation of flow in capillaries has already occurred). Thus, any platelets situated in capillaries that are completely plugged (and therefore, have no flow) would not be labelled by the dye. This causes an underestimate in the reported platelet adhesion in septic capillaries.

Because ascorbate may have many effects *in vivo*, the reduction in platelet adhesion in the capillaries after ascorbate administration could be a result of these other effects (e.g., increased blood pressure to restore flow) as oppose to the direct effect ascorbate may have on platelet-endothelial interactions. To this end, we employed *ex vivo* and *in vitro* models to eliminate the complexities of the *in vivo* model. I used isolated platelets in the *ex vivo* model to measure platelet function under septic conditions. One disadvantage of this model was that LPS or TNFα treatments, which represent the initiating and inflammatory cytokine stages of sepsis, respectively, did not produce any platelet activation. Therefore, to mimic the septic condition, the stimulating agents thrombin, ADP, and thromboxane A_2 were used instead. Another limitation to the *ex vivo* model is that the conditions in the aggregation assay are pro-aggregatory (i.e., platelets aggregate spontaneously when left in the cuvette for longer than 1 - 2 h), thus diminishing the potential full inhibitory effect of ascorbate I have investigated.

The final model used is an *in vitro* model using mouse microvascular endothelial cells to investigate platelet-endothelial interactions. Although there was an increase in platelet adhesion after treatment with LPS or TNFα, the overall number of adhering platelets was very low in comparison to the total number of platelets used in the assay (i.e., ~ 0.1 % platelets adhered out of the total number of platelets applied to the endothelial monolayer). Pilot experiments with higher concentrations of LPS, TNFα, or thrombin failed to increase the platelet adhesion greater than that observed. It is proposed that culturing the endothelial cells reduces in their ability to interact with platelets. This is further evident when examining P-selectin expression in these cells. P-selectin protein was not detected in the cells using western blot, immunocytochemistry, and flow

cytometry techniques. However, P-selectin mRNA was present and blocking of P-selectin reduced platelet-endothelial adhesion (Figures 4.3, 4.1) indicating the presence of P-selectin in the cells. If P-selectin was expressed in very low amounts or only present in a small number of the cells, then this would account for the relative low adhesion seen in this model.

5.2.2 Mechanism of capillary blood flow impairment during sepsis

The impairment of capillary blood flow during sepsis was first discovered and defined in skeletal muscle almost 20 years ago (36). Since then it has been seen in other organs as well as in septic patients (4, 6). Essentially, the impairment is cessation of blood flow in approximately 50% of septic capillaries (61). The underlying pathophysiological processes that prevent the flow of blood in these septic capillaries are yet to be fully elucidated. Table 1.1 summarizes some of the possible processes that may contribute to the cessation of flow.

The blood flow in capillaries can be visualized by the intravital microscopy in terms of the movement of RBCs situated in these capillaries. Any process that prevents RBCs from this movement will contribute to the impairment seen in sepsis. This includes sepsis-induced changes in blood pressure or changes in the ability of the RBC to deform to progress through the vessel. Changes in the perfusion pressure across the capillary bed include both a decrease in arteriolar pressure at the capillary entrance as well as an increase in venular pressure (drainage pressure) at the capillary exit (2, 5, 28). Although a large enough decrease in arterial blood pressure could prevent flow in a capillary, Boczkowski *et al* (5) showed that decreases in mean arterial pressure due to haemorrhage

did not cause a dysfunction in capillary blood flow seen in LPS-treated rats. Venular pressure is also increased in sepsis (28), and could potentially inhibit flow through a capillary. However, I have observed directly with intravital microscopy a septic capillary bed supplied by a single arteriole and drained by a single venule. Here about 50 % of capillaries had stopped flow, while both the arteriole and the venule remained perfused with blood (Chapter 2). Thus, capillary plugging does not require stoppage of blood in the arteriole or the venule.

Even though blood pressure changes in the capillaries are unlikely to result in capillary blood flow impairment, they could combine with other processes in the septic microvasculature (e.g., increased wall permeability) to contribute to capillary flow stoppage. Edema and vascular leak are key features of sepsis that promote swelling of the tissue surrounding the capillaries (37). If the swelling becomes severe enough, the resulting decrease in the capillary diameter could potentially inhibit the passage of RBCs in the capillary. A similar situation could occur due to the swelling of endothelial cells, another event that occurs during sepsis (26). Blood flow could also be stopped by blocking the passage of RBCs by physical means such as adhering non-deformable RBCs, adhering WBCs, or microthrombi. RBCs have been shown to become adhesive to LPS treated endothelial cells (19) and stiffen in during sepsis (3). Leukocyte adhesion is markedly increased during sepsis (12, 45, 55). However, this event is usually measured in post-capillary venules and therefore the contribution of leukocytes to the capillary plugging is unclear. Due to the targeting of leukocytes to specific organs during sepsis (47), the role in the cessation of capillary blood flow by WBCs is organ-dependent. Roller *et al* (55) observed increase leukocyte trapping in lung capillaries in a mouse CLP

model, and Croner *et al* (12) showed increased leukocyte adhesion in liver sinusoids in a rat CLP model. On the other hand, in our skeletal muscle model I did not notice any significant increase in leukocytes in septic capillaries (Chapter 2). I showed an increase in platelet adhesion and fibrin disposition in septic capillaries in our mouse FIP model (Figure 2.1). These 2 factors combined suggest the presence of microthrombi in the capillaries that could prevent blood flow. I further showed that removal of platelets or inhibition of thrombi formation reduces the number of stopped-flow capillaries in septic mice (Chapter 2).

Overall, the stoppage of blood flow that defines the capillary blood flow impairment is most likely caused by a combination of these factors (for example, a single platelet adhering to the endothelium may not itself block flow but might reduce the capillary diameter enough that a stiffened RBC cannot fit through). The interventions that I used in Chapter 2 to reduce microthrombi formation did not reduce the capillary blood flow impairment back to sham levels. This indicates that, in addition to microthrombi formation, other mechanism(s) are contributing to the observed impairment.

5.2.3 Capillary plugging in septic skeletal muscle

As mentioned in section 5.2.2, there are 3 main cell types that can be involved in impeding capillary blood flow: leukocytes, RBCs, and platelets clumped in microthrombi. Since leukocyte involvement in the skeletal muscle in minimal, our skeletal muscle model allowed addressing the impact of only the 2 cell types (platelets and RBCs) on the capillary plugging.

5.2.3.1 Microthrombi formation

The exact size, shape, and composition of a microthrombus necessary to plug a capillary are not known. Single adhering platelets are not large enough to prevent the blood flow alone. I have observed that platelet adhesion and fibrin deposition is increased in the septic capillary and that they both usually occur in the same capillary. However due to limitations in our methodology I cannot accurately determine (i) the size (number of platelets) per aggregate, (ii) the architecture of fibrin depositions, and (iii) the relative contribution of platelets and fibrin to the microthrombi. I observed that septic conditions promote both platelet aggregation and platelet-endothelial adhesion (Chapters 3 and 4). This adhesion contributes to the microthrombi formation in the septic capillary. However, the role of fibrin in the plugging of septic capillary has not been studied.

Although fibrin has other roles, its primary function is to support clot formation and provide integrity to the thrombi (66, 68). In the septic capillaries I observed the fibrin depositions as long strands as opposed to clumps (Figure 5.2). From these observations I cannot rule out that, after fibrin is formed (by cleavage of fibrinogen), whether it remains in the plasma or it lines the vessels without participating in clot formation (thus not contributing to the plugging of the capillaries). Fibrin deposition has also been observed in capillaries of endotoxemic rat liver and kidney but not in the lung (60), further indicating that the mechanism of capillary plugging is organ specific. This study used immunohistochemistry techniques, which like our study only indicates that fibrin is present. The specific role and necessity of fibrin in the capillary plugging in sepsis will need to be further investigated to completely understand its role in capillary impairment.

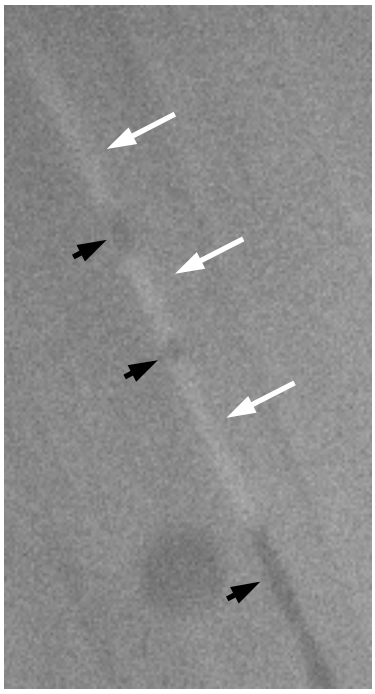

Figure 5.2. Image of fibrin plaques in septic capillaries. Fibrin plaques (white arrows) were visualized with fluorescence illumination using Alexa 488-labelled anti-fibrin antibody, injected i.v. 10 min prior visualization (antibody does not bind to fibrinogen). Plaques were seen only in capillaries with stationary red blood cells (black arrows).

5.2.3.2 RBC adhesion

In a sepsis-like state, RBCs are activated, causing red blood cell adhesion *in vitro* (18, 19). Adhering RBCs may contribute to the microthrombus formation and could potentially plug capillaries. Under septic conditions, RBCs are known to express phosphatidylserine (PS) on their surface (33). In view of the emerging evidence of endothelial cells expressing a PS binding receptor (57), this suggests a possible mechanism for RBC-EC adhesion that may contribute to the capillary plugging. Furthermore, PS will bind to thrombospondin, which will also bind to the vitronectin receptor on endothelial cells suggesting another role for PS expression on RBCs to participate in RBC-endothelium adhesion (24). I have observed RBCs in plugged capillaries; however, thus far, I was unable to determine whether they contribute to capillary stoppage or they are simply just caught behind the blockage. I conducted pilot studies in our lab using an *in vitro* approach (RBCs flowed over ECs in a parallel plate system). In these pilots, I did not visualize any obvious increase in RBC adhesion under septic conditions (LPS or TNFα treatment of ECs). Due to methodological difficulties and lack of any meaningful observations, further studies here were not pursued.

5.2.4 Ascorbate and capillary blood flow impairment during sepsis

It has been shown that an intravenous bolus of ascorbate prevents and reverses the sepsis-induced capillary blood flow impairment (2, 61, 62, Chapter 2). Although we have made progress in understanding the capillary blood flow impairment on the tissue level (i.e., oxidative stress and eNOS dysfunction contribute to the impairment) and in understanding how ascorbate inhibits impairment at this level (i.e., it reduces oxidative

stress and restores eNOS function), the potential mechanisms of how ascorbate achieves this inhibition at the cellular/molecular level are still poorly understood. In Figures 2.4 and 2.5, I show that ascorbate reduces platelet adhesion in the septic capillary, which suggests that ascorbate reduces the capillary plugging by preventing microthrombi formation. Overall, it is very likely that ascorbate reduces the capillary blood flow impairment by more than one mechanism.

Ascorbate may reduce capillary plugging by affecting platelets, RBCs, and leukocytes. Data in this thesis indicate that ascorbate decreases platelet adhesion and aggregation under septic conditions through a reduction in P-selectin surface expression. Ascorbate may also reduce RBC adhesion by decreasing PS exposure on RBCs. Ascorbate reduces PS exposure in RBCs in an apoptotic model (40). Reduced PS exposure prevents RBC adhesion by lowering its exposure to its counter receptors (described in section 5.2.3). Finally, ascorbate may reduce capillary plugging by lowering leukocyte adhesion. Ascorbic acid can reduce *in vitro* leukocyte adhesion to endothelial cells (15). Also, vitamin C can reduce *in vivo* leukocyte adhesion in diabetic and oxidized LDL models of oxidative stress (32, 38). Although the effect of ascorbate on leukocyte adhesion would only have a minor beneficial effect on capillary blood flow in skeletal muscle, this effect could be very important in other organs.

Ascorbate reduces the permeability of LPS-stimulated endothelial monolayers (25) and therefore it may reduce endothelial swelling during sepsis. There is evidence that antioxidants decrease endothelial swelling in an ischemia/reperfusion model (64). Also, antioxidants have been shown to decrease edema in lung and heart tissues in animal models of sepsis (34, 58, 69). Furthermore, the antioxidant tirilazad can reduced RBC

stiffness in an animal stress model (8). Thus, ascorbate may increase capillary diameter and RBC deformability to prevent the RBCs from getting stuck in the capillaries.

Ascorbate may influence the capillary blood flow during sepsis indirectly, since it prevents the drop in mean arterial blood pressure seen in CLP rats (62). Ascorbate improves arteriolar function by inhibiting the sepsis-reduced conducted vasoconstriction in mouse arterioles (46) and by reversing arterial hyporesponsiveness to vasoconstrictors in human patients injected with LPS (53).

5.2.5 The protective effect of ascorbate and eNOS

Our lab has shown that the protective effect of ascorbate on the sepsis-induced impairment of capillary blood flow is eNOS dependent (61). Figure 2.5 shows that ascorbate's inhibitory effect on platelet adhesion in septic capillaries is also eNOS dependent. However, using the *ex vivo* approach, the reduction in platelet aggregation by ascorbate was NOS independent suggesting that ascorbate reduces aggregation directly. This reduction in aggregation must be insufficient to improve capillary blood flow in septic capillaries without eNOS function (Chapter 3).

The NO derived from eNOS maintains vascular homeostatisis by regulating vasomotor tone (51), inhibiting platelet aggregation (1), inhibiting leukocyte-endothelium adhesion (35), and inhibiting apoptosis (9). NO acts directly on guanylyl cyclase to produce cGMP that activates downstream target protein kinase G. This pathway mediates vascular smooth muscle relaxation and platelet inhibition. NO may also modulate protein function by nitrosylation of thiol residues in the amino acid sequence. One example is the nitrosylation of N-ethylmaleimide-sensitive factor (NSF), a protein critical for proper

granule docking with the plasma membrane. Nitrosylated NSF has reduced function resulting in reduced granule secretion (43).

In vivo, ascorbate prevents and reverses the oxidation of BH_4 and thus restores proper eNOS function (Figure 1.2). The local NO can then participate in the unplugging of capillaries by reducing platelet adhesion and aggregation. In the *ex vivo* model, platelet aggregation was reduced by ascorbate, independent of platelet derived NO. I proposed that endothelial derived NO may further inhibit both platelet aggregation and P-selectin surface expression (Chapter 3). Thus restoration of endothelial eNOS function may be required for ascorbate's full anti-platelet effects *in vivo*. Data in Chapter 4 suggest that ascorbate reduces platelet-endothelial adhesion by at least 2 mechanisms. One of these mechanisms could be reduced surface P-selectin protein expression on endothelial cells due to nitrosylation of NSF. It would be interesting to know whether the effect of ascorbate on LPS or TNFα-induced platelet-endothelial cell adhesion *in vitro* is NOS dependent since LPS and TNFα increase adhesion by different mechanisms (and NO could play a different role in each case).

5.2.6 Ascorbate as a treatment for sepsis

The data in the thesis suggest a major role of the coagulation system in the pathophysiology of sepsis. Specifically, Figure 2.2 shows that inhibition of coagulation by antithrombin or eptifibatide prevents or reverses the capillary blood flow impairment respectively. This indicates that targeting the coagulation pathway would be of interest for treating septic patients. However, clinical trials with anticoagulants have not been successful in improving survival (14).

Ascorbate in combination with vitamin E was shown to decrease mortality in critically ill patients (11) as well as reduce multiple organ failure in critically ill surgical patients (49). Although the above studies have indicated a beneficial affect of ascorbate, the administration of ascorbate alone has not yet being used as a therapy for septic patients.

A recent trial involving critically ill patients by Heyland *et al* (27) showed no improvement in 28-day mortality with antioxidant treatment. However, the treatment in this study was a mixture of antioxidants given orally as oppose to just ascorbate given intravenously as presented in this thesis. It is possible that reduction of platelet adhesion and restoration of capillary blood flow could have negative consequences on survival. Platelet adhesion to neutrophils is necessary for the formation of neutrophil extracellular traps that have a role in bacteria trapping (10). Restored blood flow may promote spreading of the infection. Thus, a negative effect of ascorbate treatment could be prolonging the infection.

Ascorbate may be more beneficial than other antioxidants because it is readily absorbed into microvascular endothelial cells and platelets through high affinity vitamin C transporters (67). This leads to the accumulation of high levels of intracellular ascorbate (up to 16 mM in endothelial cells and greater than 1mM in platelets (44, 67). Furthermore, excess ascorbate is quickly excreted (over 4 - 5 h) through the kidneys (50). This is beneficial because ROS are required as a defense mechanism against infections (including bactericidal effects and the up-regulation on inflammatory cytokines). Removal of ROS may be the reason that other antioxidants have not always offered protective results in septic patients (17). Moreover, mice with a major source of ROS

genetically removed (gp91phox knockouts, a subunit of NADPH oxidase) have reduced 24 h survival in a FIP model of sepsis when compared with wild type mice (unpublished observations from our lab). Thus, complete suppression and/or continuous antioxidant therapy in septic patients could be detrimental. Ascorbate being quickly absorbed or excreted would reduce the effect of this possible complication.

Septic patients have reduced plasma ascorbate levels (23). High doses of ascorbate are needed to restore plasma ascorbate levels in critically ill patients back to control (39, 49, 56). There is no evidence to suggest toxic effects of high dose ascorbate on organ function. In healthy volunteers, intravenous injections of 7.5 g, 33 mg/kg, or 2 g of ascorbate induced no pro-oxidant changes in plasma markers of oxidative stress (48), no abnormalities in blood cell counts, or liver or kidney function (42), and did not alter superoxide production by neutrophils (20), respectively.

5.2.7 Overall implications of this research

Sepsis leads to the cessation of capillary blood flow which results in tissue hypoxia and potentially multiple organ failure (36, 61). The cellular mechanisms that cause this impairment have yet to be fully determined. Furthermore, treatment of septic animals with ascorbate has shown to prevent and reverse the cessation in capillary blood flow (2, 61, 62). Again, the mechanisms underlying ascorbate's beneficial effects are not completely clear.

The research in this thesis is the first to demonstrate the participation of the coagulation system in the capillary blood flow impairment during sepsis. In particular, we show increased platelet adhesion and fibrin deposition in septic capillaries suggesting the

presence of microthrombi in the capillaries. The increase in platelet adhesion is reduced by ascorbate eNOS-dependently similar to the improvement of capillary blood flow by ascorbate. This demonstrates another mechanism by which ascorbate could improve the outcome in sepsis.

Ascorbate reduces platelet aggregation and platelet-endothelial adhesion in *ex vivo* and *in vitro* models of sepsis, respectively; by a reduction in the cell surface P-selectin protein expression. The reduced aggregation and adhesion by ascorbate leads to a decrease in the formation of microthrombi in the capillaries. Removal of microthrombi leads to the rescue of capillary blood flow, attenuating tissue hypoxia. This thesis demonstrates a mechanism of ascorbate providing a beneficial effect during sepsis and thus supports investigations of the use of ascorbate as an adjuvant treatment for septic patients.

5.3 FUTURE DIRECTIONS

5.3.1 Effect of eNOS inhibition on platelet adhesion, in vitro

As mentioned previously, the protective effects of ascorbate against the impairment of capillary blood flow and increase platelet adhesion to the capillary wall during sepsis is eNOS dependent. In Chapter 4, I show that ascorbate can reduce LPS-induce platelet-endothelial adhesion by reducing P-selectin surface expression as assayed by vWF granule expression. I propose that ascorbate acts to restore eNOS function similar to our *in vivo* model. The NO produced by restored eNOS may nitrosylate NSF, causing a reduction in granule secretion, and thus reduce surface P-selectin protein expression (43).

Furthermore, ascorbate is expected to reduce TNFα-induced platelet-endothelial adhesion by a different mechanism since TNFα does not cause an increase in granule secretion. Investigating the effect of NOS inhibition in the *in vitro* model of LPS-induced platelet-endothelial adhesion should clarify the mechanism of ascorbate's inhibitory effect.

5.3.2 Mechanism of ascorbate inhibition of TNFα-induced platelet adhesion, in vitro

TNFα caused an increase in platelet-endothelial adhesion in our *in vitro* model. However, this increase was not due to increased P-selectin protein expression on the endothelial cell surface. Blockade of P-selectin prevented this increase in adhesion indicating the increased adhesion still involved P-selectin. Ascorbate reduced the platelet adhesion through a mechanism that did not include reduction in granule secretion. It has been suggested that ascorbate could affect the protein kinase C (PKC) pathway in platelets (Chapter 3), but this pathway has not been examined in endothelial cells. TNFα is known to increase the activity of the transcription factor nuclear factor κB (NFκB) through a PKC pathway (54). NFκB up-regulates inflammatory cytokines and numerous genes that regulate cell adhesion including P-selectin (52), E-selectin (65), vascular cell adhesion molecule (VCAM)-1 (30), and intercellular adhesion molecule (ICAM)-1 (63). Moreover, PKC has been shown to modulate the function of platelet-endothelial cell adhesion molecule (PECAM)-1 (31), which has the potential to facilitate the adhesion of platelets to the endothelial cells in our model. The role of PKC in TNFα-induced platelet adhesion in our *in vitro* model could be examined by pharmacological inhibition of PKC

by calphostin C. PKC activity assays in platelets and endothelial cells could be used to determine if ascorbate alters PKC function.

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Appendices

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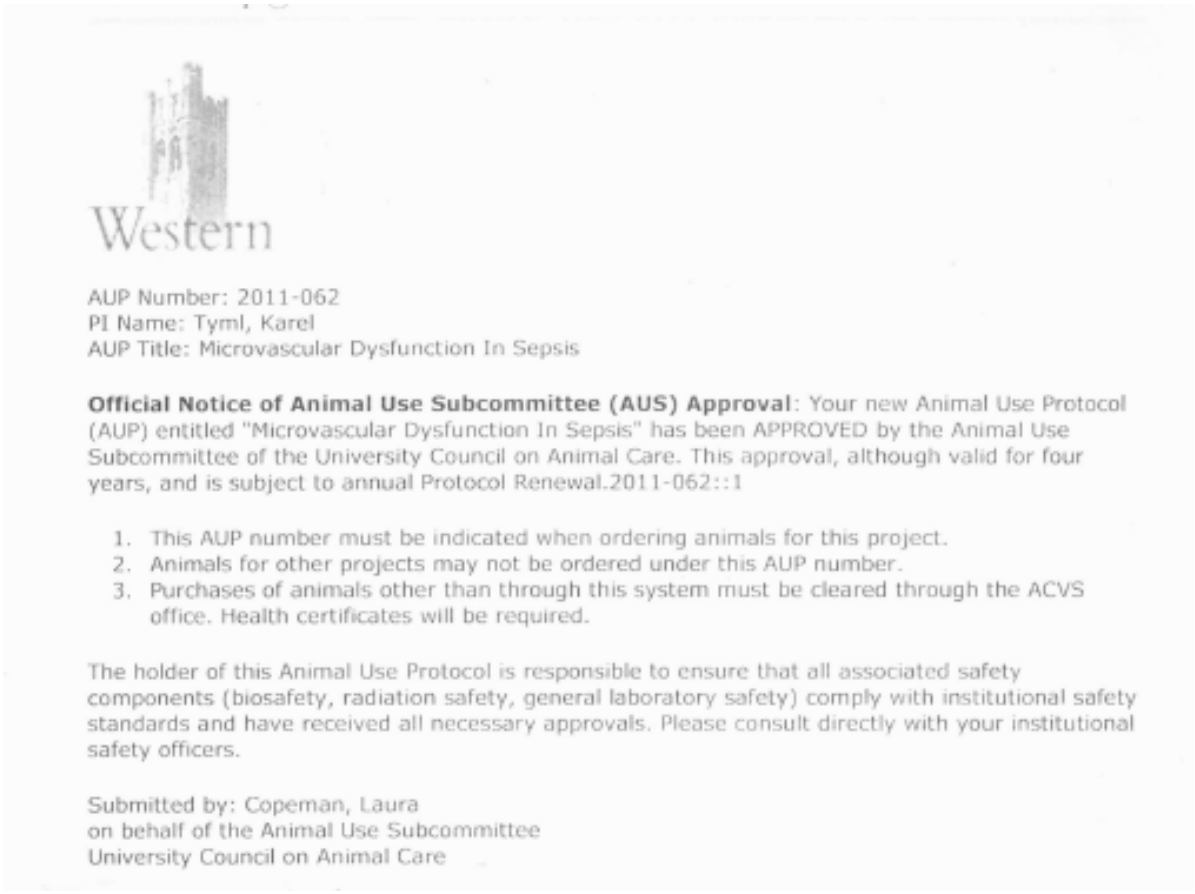
Appendix 2: Ethics approval for animal use



Western

AUP Number: 2011-062
PI Name: Tyml, Karel
AUP Title: Microvascular Dysfunction In Sepsis

Official Notice of Animal Use Subcommittee (AUS) Approval: Your new Animal Use Protocol (AUP) entitled "Microvascular Dysfunction In Sepsis" has been APPROVED by the Animal Use Subcommittee of the University Council on Animal Care. This approval, although valid for four years, and is subject to annual Protocol Renewal.2011-062::1

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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.

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Submitted by: Copeman, Laura
on behalf of the Animal Use Subcommittee
University Council on Animal Care

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REFERREED PUBLICATIONS

Secor D, Swarbreck S, Ellis CG, Sharpe MD, Tyml K. Ascorbate reduces mouse platelet aggregation and surface P-selectin expression in an ex vivo model of sepsis. *Microcirculation*, 2013.

Secor D, Li F, Ellis CG, Sharpe MD, Gross PL, Wilson JX and Tyml K. Impaired microvascular perfusion in sepsis requires activated coagulation and P-selectin-mediated platelet adhesion in capillaries. *Intensive Care Med.* 36: 11: 1928-1934, 2010.

MANUSCRIPTS SUBMITTED FOR PUBLICATION

Secor D, Swarbreck S, Ellis CG, Sharpe MD, Tyml K. Ascorbate reduces P-selectin mediated platelet-endothelial adhesion in an in vitro model of sepsis via reduced endothelial granule secretion. Submitted to *Microcirculation*, 2013.

Swarbreck S, **Secor D**, Li F, Ellis CG, Sharpe MD, Gross PL, Wilson JX and Tyml K Effect of ascorbate on fibrinolytic factors in septic mouse skeletal muscle: disparity between microcirculatory and systemic blood assessment. Submitted to *Microcirculation*, 2013.

PUBLISHED ABSTRACTS

Secor D, Li F, Ellis CG, Sharpe MD, Wilson JX and Tyml K. Septic impairment of capillary blood flow requires activated coagulation pathway and is reversed by ascorbate through eNOS-dependent dislodging of platelets in capillaries. FASEB J 23:593.6