Clinical and experimental studies on treatment of acute mesenteric ischemia
Schoots, I.G.

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Treatment of acute mesenteric ischemia

Clinical and experimental studies

Ivo G Schoots
Clinical and experimental studies on treatment of acute mesenteric ischemia
The studies described in this thesis were performed at the departments of Surgical laboratory (Professor T.M. van Gulik), and Vascular Medicine (Professor M.M. van Levi), Academic Medical Center, University of Amsterdam, Meibergdreef 9, 1105 AZ, Amsterdam, the Netherlands, and at the department of Molecular and Vascular Medicine (Professor W.C. Aird), Beth Israel Deaconess Medical Center, Harvard Medical School, 330 Brookline Avenue, 02215 Boston, Massachusetts, United States of America.

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Prof. Dr. M.M. Levi

Overige leden: Prof. Dr. W.C. Aird
Prof. Dr. H.A. Büller
Prof. Dr. H.R. Büller
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Faculteit der Geneeskunde
“Don’t race against others, only race against yourself”
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Outline of thesis

“The results of diagnosis and management of mesenteric ischemia have improved significantly over the past 100 years but remain poor. The best part of the history of mesenteric ischemia remains to be written.”

Scott J. Boley
Acute mesenteric ischemia represents a major clinical problem, not only because it is associated with a poor prognosis with mortality rates up to 93%, but also because this vascular disorder may progressively increase in time. As the average life expectancy increases and subsequently the number of elderly in our hospitals grows, the vasculopathy of acute or chronic mesenteric ischemia will rise. Unfortunately, despite the progress in our understanding of the pathophysiology, diagnosis and treatment of acute mesenteric ischemia, mortality rates in clinical series in the last 15 years remain as high as they did a century ago. The aim of this thesis is to provide better insight into the complex syndrome of intestinal ischemia and reperfusion. Acute mesenteric ischemia will be addressed from a clinical and an experimental point of view.

In the first part of this thesis clinical articles review acute mesenteric ischemia and surgical as well as new treatment strategies.

In chapter 1 we introduce the clinical syndrome of acute mesenteric ischemia and summarize the different surgical treatment modalities. At present-day, surgical therapy is still the far most favorable treatment modality as it includes the assessment of intestinal viability, determination or conformation of the underlying cause, revascularization, and resection of the nonviable intestine. However, relatively new treatment modalities may serve as an adjunct to surgical intervention in some cases.

In chapter 2 we discuss the difficulties of analyzing the diversity of the clinical syndrome of acute mesenteric ischemia. We attempted to subdivide this condition according to disease etiology. The etiology of acute mesenteric ischemia remains often undefined in reported studies, as the correct diagnosis can usually only be confirmed at autopsy. Furthermore, the relative infrequency of acute mesenteric ischemia and the varied clinical presentation constitute an obstacle to analyze each etiological subset individually and to undertake randomized or case–control trials. Most of the previous retrospective studies assessed, calculated a mortality rate based on data compiled from all etiological subsets taken together. This has the drawback of obscuring differences in clinical presentation and characteristics, diagnostic investigation, disease progression, mortality and response to therapeutic modalities that are specific to disease etiology. Systematic evaluation of research results, even if only observational data and small case series are available, is necessary to move forward, certainly in view of improved imaging and current thrombolytic strategies. The aim of this systematic analysis of the literature on acute mesenteric ischemia was to investigate the relationships between disease etiology (arterial embolism, arterial thrombosis, venous thrombosis and non-occlusive mesenteric ischemia), mode of treatment and mortality.

In chapter 3 we evaluate thrombolytic therapy for acute superior mesenteric artery occlusion as an alternative or adjunctive treatment modality to surgical therapy in order to provide current knowledge for timely and informed decisions regarding treatment of acute mesenteric ischemia. Therefore, we performed a systematic analysis of the available literature from 1966 to 2003 regarding thrombolytic therapy for superior mesenteric artery thromboembolism.

In the second part of the thesis we discuss the eponym Riolan’s anastomosis (chapter 4). Riolan’s anastomosis or arc is eponymously used to indicate the arterial anastomosis between the superior and inferior mesenteric arteries. Vascular as well as gastro-intestinal
surgens are well-acquainted with this collateral mesenteric pathway for retrograde perfusion of the superior mesenteric artery when the origin of the latter is occluded. The eponym suggests that Jean Riolan (1580-1657), a famous 17th century French anatomist, was the first to describe this arterial anastomosis. Riolan was a strong defender of traditional Galenic doctrine in medicine and therefore, proved a vigorous opponent of the new concept of the circulation of the blood as exposed by William Harvey (1578-1657). This makes it unlikely that Riolan would have conceived an arterial collateral pathway in the mesocolon, a notion confirmed by examining his anatomy book published in 1649.

In the third part of this thesis experimental studies of acute mesenteric ischemia in rats are described, regarding different mechanisms that may play a role in the induction of ischemia and reperfusion injury.

In chapter 5 we briefly review these different mechanisms as addressed in the following chapters relating to intestinal ischemia. First we focused on the coagulation cascade that may lead to the formation and deposition of fibrin, consequently occluding the microvasculature which is crucial in the oxygenation of the intestinal epithelium. We discuss the anticoagulant mechanisms and fibrinolysis system that may be insufficient to inhibit microvascular occlusion or to restore blood flow in the occluded microvasculature, respectively. Furthermore, we describe the potential crosstalk of coagulation and inflammation in the ischemia and reperfusion syndrome. In addition to these different mechanisms, we address the role of drug-intervention in ischemia and reperfusion syndrome, with the aim of restoring the imbalance within these mechanisms and attenuating the ischemia and reperfusion injury.

In chapter 6 we investigate intravascular coagulation and thrombotic obstruction in the splanchnic vasculature after intestinal ischemia and reperfusion in relation to epithelial integrity and function. Ischemia and reperfusion-induced endothelial cell injury results in a procoagulant and fibrinolysis-suppressing environment giving rise to intravascular fibrin deposition which may further compromise the (micro)circulation of for example the intestine and promote necrosis in distal tissue. Moderate and more severe intestinal ischemia was induced in rats by superior mesenteric artery occlusion. Intestinal injury was assessed by histological analysis, biochemical markers and functional studies. During reperfusion, portal and systemic blood samples were collected to analyze activation of coagulation and fibrinolysis.

In chapter 7 we review situations of ischemia and reperfusion in which activated protein C might be effective. The efficacy of activated protein C in sepsis may rely on the fact that it can modulate both coagulation and inflammation. Therefore, a potential beneficial effect of activated protein C may be present in disease states that are also characterized by simultaneous activation of these systems. Ischemia and reperfusion injury of various organs may represent such a state. Published articles on experimental and clinical studies of activation of both coagulation and inflammation in various disease states (including intestinal ischemia) were analyzed.

In chapter 8 we examine whether administration of activated protein C or antithrombin reduce local splanchnic derangement of coagulation and inflammation and attenuate intestinal dysfunction and injury following intestinal ischemia/reperfusion. Mechanisms that have been incriminated to play a role in the procoagulant response following ischemia and reperfusion are the upregulation of tissue factor in combination
with dysfunctional anticoagulant pathways, along with suppression of fibrinolysis mainly due to increased levels of the inhibitor of fibrinolysis: plasminogen activator inhibitor-1. Regulatory anticoagulant pathways, in particular the antithrombin system and the protein C system, appear to be ineffective in inhibiting thrombin generation following ischemia and reperfusion. Physiological anticoagulants such as antithrombin and activated protein C, in addition to reducing thrombin generation, may exert anti-inflammatory properties including modulation of cytokine expression, regulation of cell migration and promotion of apoptosis. Restoration of these defective, physiological anticoagulant mechanisms form a logical approach to the treatment of local or remote post-ischemic reperfusion injury. Rats were subjected to superior mesenteric artery occlusion and a randomized intravenous administration of vehicle, heparin, antithrombin, or activated protein C was performed during ischemia, briefly before reperfusion. Coagulation and fibrinolysis parameters obtained from portal blood, were correlated with mucosal fibrin deposition (determined by anti-rat fibrin antibody staining), intestinal function (glucose/water clearance) and intestinal injury (histological evaluation by Park/Chiu score).

In **chapter 9** we analyze the effect of enhanced fibrinolysis in the same rat model of intestinal ischemia and reperfusion that results in local activation of coagulation, suppression of endogenous fibrinolysis and mucosal fibrin deposition. Intestinal ischemia and reperfusion causes local inhibition of endogenous fibrinolysis in combination with activation of coagulation. This may lead to thrombotic obstructions that compromise microcirculation and promote intestinal injury. This led to the hypothesis that “recanalization” of the thrombotic microvasculature by fibrinolysis may attenuate the sequelae of intestinal post-ischemic, reperfusion injury. Inhibited fibrinolysis following intestinal ischemia and reperfusion is related to increased plasminogen activator inhibitor-1. Therefore, fibrinolysis was enhanced by intravenous administration of recombinant tissue plasminogen activator (rt-PA) or by inhibition of PAI-1 by administration of monoclonal antibody MA-33H1F7. Again, coagulation and fibrinolysis parameters obtained from portal blood, were correlated to mucosal fibrin deposition, intestinal function and intestinal injury.

Intestinal ischemia and reperfusion may lead to profuse secretion of water and electrolytes. The underlying mechanisms have been related to increased hydrostatic pressure, to denudation of intestinal villi and recently, to adenosine-mediated enhancement of chloride secretion. Considering the complex interplay between vascular, subepithelial and epithelial factors in *in vivo* models of ischemia and reperfusion, and taking into account that compared to *in vivo* studies, monolayers of intestinal cell-lines show a notably different response to hypoxia, it is of interest to study the effects of hypoxia and reoxygenation in *in vitro* small intestinal preparations. In **chapter 10** we report the determination of baseline electrophysiological parameters, glucose absorption, glutamine absorption, cAMP-mediated secretion induced by forskolin, Ca\(^{2+}/PKC\)-mediated secretion induced by carbachol or histamine, and epithelial barrier function using disodium-fluorescein and horseradish peroxidase as permeability probes, during varying periods of hypoxia and reoxygenation in rat ileum mounted in Ussing chambers.

In the fourth part of this thesis we analyze the role of reactive oxygen species in intracellular mechanisms of the endothelial cell in response to vascular endothelial growth factor (VEGF). An important goal in vascular biology is to understand the molecular
mechanisms underlying the modulation of endothelial cell phenotypes in health and disease. Reactive oxygen species (ROS) have traditionally been viewed as cytotoxic molecules, predominantly generated in pathological conditions such as ischemia and reperfusion syndromes, but they are now recognized to play a critical role in signal transduction and transcriptional regulation within the vascular tree. VEGF is important for the growth of new blood vessels (vasculogenesis and angiogenesis) and the maintenance of vascular integrity.

In chapter 11 we investigate the hypothesis that NADPH oxidase-derived ROS serve to modulate selective VEGF-dependent signaling pathways, transcriptional profiles and biological functions in endothelial cells. VEGF signaling in the endothelium involves a number of different pathways, including PI3-kinase/Akt, MAPK, and PKC. It has recently been shown that VEGF-induced proliferation, migration, and downstream expression of some but not all genes in endothelial cells are dependent upon a Rac1-regulated NADPH oxidase-derived ROS, suggesting that VEGF signaling in the endothelium is tightly coupled to NADPH oxidase activity.
Part I

Clinical studies
A surgical approach to acute mesenteric ischemia

Ivo G. Schoots\textsuperscript{1,2}, Marcel Levi\textsuperscript{2} and Thomas M. van Gulik\textsuperscript{1}

Departments of \textsuperscript{1} Surgery and Surgical Laboratory, and \textsuperscript{2} Vascular and Internal Medicine, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands

\textit{Surgical intervention in acute mesenteric ischemia – the golden standard}

Adapted from \textit{J Vasc Interv Radiol.} 2004 (in press)
Abstract

Bowel ischemia may arise from a number of causes affecting the arterial and venous compartments of the mesenteric circulation. The rapid onset of acute mesenteric ischemia and the potential rapidity with which bowel infarction may occur explain the lethality of this disease. This review addresses the causes and consequences of arterial obstruction, focusing on the acute phenomenon of mesenteric ischemia. Therapeutic strategies are dependent on reversible or irreversible ischemic damage on bowel integrity. Surgery is far the most favorable treatment, as it includes the assessment of intestinal viability, determination or conformation of the underlying cause, revascularization, and resection of the nonviable intestine. A variety of operative procedures are described to restore blood flow and secure the viability of the remaining bowel. In this context, a number of new treatment modalities are reported, which have been introduced in the last decades of the previous century. These treatment modalities may benefit a certain population of patients with acute mesenteric artery occlusion and may improve outcome of this lethal disease.
Chapter 1

Introduction

Whereas great advances in diagnosis and treatment have been obtained over the past decades, there is still no reason to express optimism concerning acute mesenteric ischemia. Continued poor outcome of most of the patients with acute mesenteric ischemia makes this disease a dreaded condition with mortality rates up to 90% \(^1\). Since the first report of mesenteric ischemia described almost two hundred years ago \(^2\), successful diagnosis and treatment has always been a great challenge for any physician to salvage the intestine and subsequently the life of the patient.

Tiedman \(^3\) and Virchow \(^4\), in 1843 and 1847 respectively, described acute mesenteric ischemia as a clinical entity, but it was Elliott \(^5\) in 1895 who reported the first patient to recover from resection of necrotic and gangrenous bowel. Schnitzler \(^6\) in 1901 described our present-day understanding of chronic mesenteric ischemia; a patient with years of postprandial pain who at autopsy had infarction of the small intestines from a thrombus superimposed on atherosclerosis of the superior mesenteric artery. In the early 1900s, more articles and reviews appeared in the literature which identified accurately the spectrum of mesenteric ischemia: occlusions of the mesenteric arteries or veins by means of embolism, thrombosis or atherosclerosis, with chronic or acute appearance were described \(^7,8\). However, it lasts until 1943 that Thorek documented precisely bowel necrosis in the absence of arterial or venous obstruction, which may represent the first report of a patient with nonocclusive mesenteric ischemia \(^9\). During this period Rendich and Harrington \(^10\) suggested that radiographic examination might be helpful in the diagnosis of mesenteric ischemia. In the 1950s Shaw and Rutledge \(^11\) but also Klass \(^12\) reported a patient in whom embolectomy of the superior mesenteric artery without resection of bowel was followed by survival. The first successful revascularization procedure - a thromboendarterectomy - for superior mesenteric artery thrombosis with survival was described by Shaw and Maynard \(^13\) in 1958, and remains unequaled present-day. Until present day, the revascularization procedures have been expanded including aortomesenteric and iliomesenteric bypasses and balloon dilations of stenotic and occluded arteries, however, the prognosis after mesenteric arterial thrombosis appears to be worse than that after mesenteric arterial embolism. The involvement of at least two of the major splanchnic arteries may be relevant in the poor prognosis of this etiological subset of mesenteric ischemia \(^14\).

In 1967, Aakhus and Brabrand \(^15\) first suggested the value of angiography in diagnosing acute superior mesenteric artery insufficiency. Early diagnosis of acute mesenteric ischemia was difficult before intestinal infarction had occurred, because physical findings, radiographic examination and laboratory tests were, and still are, non-specific. Subsequently, in 1973, Boley and coworkers \(^16\) proposed an aggressive radiographic and surgical approach to acute mesenteric ischemia, with the liberal use of angiography in patients at risk and the infusion of papaverine through the angiographic catheter as part of the treatment of both occlusive and nonocclusive mesenteric arterial insufficiency. Jamieson and coworkers \(^17\) described the pioneering thrombolysis of acute superior mesenteric artery embolism in 1979, which was soon followed by the successful percutaneous transluminal angioplasty of stenoses of both celiac and superior mesenteric artery in 1980 \(^18\). In addition to this catheter-directed balloon dilatation, the first stent was placed in the superior mesenteric artery in 1996 \(^19\), followed by combining these latter therapies in 1997 \(^20\).
Anatomy

The blood supply of the gastrointestinal tract consists of three main aortic branches: the celiac trunk, the superior mesenteric artery and the inferior mesenteric artery (Figure 1). The blood flow to the small intestine is predominantly supplied by the superior mesenteric artery. The superior mesenteric artery supplies the transverse and ascending duodenum, the jejunum and ileum, and the large bowel to the splenic flexure, however, great variability in the vascular anatomy has been described. From the superior mesenteric artery four major branches arises; the inferior pancreaticoduodenal artery, the middle colic artery, the right colic artery, and the ileocolic artery. These branches give rise to numerous jenunal and ileal branches and vascular circuits, and ultimately supply the straight end arteries of the terminal arcade. These arteries enter directly the layers of the intestinal wall.

The three main aortic branches are connected with each other, by means of arcades, through anastomoses and collateral pathways. The pancreaticoduodenal arcade anastomoses the celiac axis and the superior mesenteric artery by the superior and the
inferior pancreaticoduodenal branches. These branches originate from the common hepatic artery and superior mesenteric artery, respectively. The meandering mesenteric artery or the arc of Riolan connects the superior and the inferior mesenteric artery by the left branch of the middle colic artery and the ascending branch of the left colic artery. The marginal artery of Drummond, which also connects the superior and the inferior mesenteric artery, runs along the large intestine and may play a less important role in superior mesenteric artery occlusion.

Despite these collateral pathways the middle of the jejunum renders most vulnerable for developing intestinal ischemia from superior mesenteric artery occlusion, as this part of the small intestine is far away from collateral blood supply from celiac axis or inferior mesenteric artery.

**Etiology and pathogenesis**

Acute mesenteric ischemia can be grossly classified into ischemia of thrombotic or non-thrombotic origin (Figure 2). Non-occlusive mesenteric ischemia, the dominant non-thrombotic cause of acute mesenteric ischemia, results from low-flow states (e.g. cardiogenic shock, sepsis, hypovolemia), and is usually superimposed on already stenotic (e.g. atherosclerotic) mesenteric arteries. Thrombotic conditions include mesenteric arterial embolism, arterial thrombosis and venous thrombosis. This thesis focuses on superior mesenteric artery occlusion as the cause of acute mesenteric ischemia.

Approximately 70% of acute mesenteric vasculopathy is caused by arterial occlusion, i.e. 30-50% by arterial embolism and 15-30% by thrombosis. The superior mesenteric artery is susceptible to embolic occlusion because of its large diameter and its arising from the aorta at a narrow angle. Emboli usually originate from the left side of
the heart, less frequently from ulcerated atherosclerotic plaques or thrombosed aortic aneurysm\textsuperscript{26}. Cardiac arrhythmias, especially atrial fibrillation, are the main cause of thrombus dislodgement and embolization, in up to 80\% of patients\textsuperscript{26,27}.

Acute thrombosis of the superior mesenteric artery is generally localized at a stenosis at the origin of the superior mesenteric artery (\textit{Figure 3}), which is often a result of chronic atherosclerosis\textsuperscript{28}. These patients typically have extensive and diffuse atherosclerotic disease, with prior coronary, cerebrovascular, or peripheral arterial insufficiency.

\textbf{Clinical presentation}

Most patients with acute superior mesenteric artery occlusion present with severe abdominal pain, which persists for more than 2 to 3 hours and is classically more impressive than the physical findings. Acute mesenteric ischemia may be caused by an embolus if acute abdominal pain is followed by rapid and forceful bowel evacuation, whereas a thrombus may be suggested if a history of chronic abdominal pain for weeks or months is followed by an acute abdominal insult\textsuperscript{29}. Furthermore, the stools may contain occult blood in up to 75\% of the patients. Diarrhea, nausea, and vomiting are common, but overall the clinical symptoms and signs are relatively non-specific for acute mesenteric ischemia and can be seen in a wide variety of gastrointestinal disorders. Patients older than 50 years with congestive heart failure, cardiac arrhythmias, recent myocardial infarction, arterial stenosis, hypovolemia, hypotension, or sepsis, have been identified as patients at risk in retrospective studies\textsuperscript{30} and should be evaluated. Immediate evaluation of a possible superior mesenteric artery occlusion is mandatory when prompt

\textbf{Figure 3:} Localization of thrombosis and emboli in the vascular tree of the superior mesenteric artery.
acute abdominal pain occurs after a recent myocardial infarction, cardioconversion or cardiac catheterization. Cardiac surgery, aorta reconstruction and blood dialysis may also result in intestinal ischemia, whereas previous embolic or thrombotic events should raise clinical suspicion for superior mesenteric artery occlusion.

Thromboembolism of the superior mesenteric artery does not always result in acute abdominal pain. Patients with atherosclerotic narrowing of the superior mesenteric artery trunk often stimulate the formation of collateral mesenteric circulation. Thrombotic obstruction of a pre-existing stenosis of the superior mesenteric artery may often sustain asymptomatic.

**Diagnosis**

The diagnosis of acute superior mesenteric artery occlusion in its early stages is important to improve survival. Serum markers to establish or exclude the diagnosis of superior mesenteric artery occlusion in its early phase are lacking. Elevations in the levels of serum markers most suggestive of intestinal ischemia (for example leucocytosis, metabolic acidosis, hyperamylasemia, elevated levels of lactate dehydrogenase, alkaline phosphatase, or intestinal fatty acid-binding protein) usually occur only after transmural bowel infarction has developed. A recent study, however, demonstrated potential use of D-dimers in detecting acute mesenteric ischemia; further studies are advocated to evaluate the sensitivity and specificity of this serum marker for detecting intestinal ischemia in its early phase.

**Figure 4:** (A) CT findings of a patient with atrial fibrillation and abdominal pain shows a large embolus lodged in the origin of the superior mesenteric artery (arrow), which has been surgically proven. (B) Transverse arterial phase CT image of a patient with atrial fibrillation and thrombus at echocardiography who presented with abdominal pain. Note relative lack of enhancement of the descending colon (arrowhead) when compared with the ascending colon (arrow). Ischemic segments of both small and large bowel were found at surgery.

Findings on plain X-ray films of the abdomen associated with acute mesenteric ischemia, such as pneumatosis, portal venous gas, or thumbprinting usually are nonspecific and occur late in the course of the disease and correlate with a high mortality rate. Abdominal plain X-ray films are useful in exclusion of other identifiable causes of abdominal pain, for example perforated gastro-duodenal ulcer, in a patient who is suspected of having acute mesenteric ischemia.
A surgical approach to acute mesenteric ischemia

Duplex sonography, valuable in detection of peripheral vascular occlusion, is of no value in detecting emboli beyond the proximal superior mesenteric artery or in diagnosing non-occlusive mesenteric ischemia. Despite the high specificity of almost 100%, duplex sonography shows a sensitivity of only 70-89% for identification of proximal occlusions or severe stenoses of the mesenteric vessels. The paralytic ileus associated with mesenteric ischemia and the gaseous component of the abdominal distention make duplex sonography technically very difficult and unreliable. Furthermore, due to abundant collateral vessel formation a patient with superior mesenteric artery occlusion can be asymptomatic, which indicates that identification of significant arterial occlusion alone does not establish the diagnosis of intestinal ischemia.

Figure 5: (A) Selective angiography of the superior mesenteric artery without arterial obstruction or narrowing. (B) Selective mesenteric angiography of the superior mesenteric artery of a patient with surgically proven arterial embolism causing mesenteric ischemia.

Computed tomography (CT) in acute intestinal ischemia has recently been reviewed. Analysis of acute and exclusively arterio-occlusive transmural bowel infarction demonstrated that the necrotic small intestine may show dilated and fluid-filled or gas-and fluid-filled loops with a "paper thin wall" (Figure 4). Furthermore, CT has the ability to show a thrombus or embolus within the origin of the superior mesenteric artery or celiac trunk. The CT has emerged as the primary imaging test for acute abdominal disorders, supported by the rapid image acquisition time (less than 1 minute) and the comprehensive nature of an abdominal CT, which may diagnose other frequent mimics of acute mesenteric ischemia, including appendicitis, diverticulitis or acute abdominal aortic disorders. Despite the accurate demonstration of changes in ischemic intestine and the help in determining the primary cause and coexistent complications of acute mesenteric ischemia, most findings on CT associated with acute mesenteric ischemia are nonspecific and late in disease progression. It is to be suspected that the new multi detection-row CT-angiography becomes important in abdominal emergencies, because of its ability to
detect the causes of intestinal ischemia. However, future studies are advocated to demonstrate the additional value of this technique in diagnosing acute mesenteric thromboembolism in its early phase.

**Figure 6**: Intestinal ischemia that mandates resection

Magnetic Resonance (MR) imaging provides a noninvasive alternative for the initial evaluation of patients with suspected acute mesenteric ischemia. Gadolinium-enhanced MR angiography provides excellent morphologic information of the proximal mesenteric vasculature. However, impediments to more widespread use in the acute setting of superior mesenteric artery occlusion include a limited availability of MR imaging scanners, and the complexity, length and costs of MR imaging examinations. At present day, the additional value of MR imaging of acute superior mesenteric artery occlusions is lacking.

Selective mesenteric angiography with up to 100% sensitivity and specificity is considered to be the golden standard for the diagnosis of arterial occlusions of the small intestine. Abrupt cutoff of the angiographic image of the superior mesenteric artery with the absence of collateral circulation is diagnostic of an acute thromboembolic occlusion. Only angiography or explorative laparotomy (open or laparoscopic) enables early diagnosis which may convince the use of angiography in detecting acute mesenteric ischemia in suspected patients. Selective mesenteric angiography, which is described by Bakal et al. in detail, provides an entrée for postoperative angiographic follow-up studies and inclines to initiate immediate treatment strategies, such as endovascular angioplasty or stent placement, catheter-directed vasodilator or thrombolytic therapy.

**Therapy**

**Surgical therapy**

The basis of treatment of patients with acute mesenteric ischemia traditionally emphasizes early diagnosis, resection of nonviable bowel, targeted surgical or non-surgical restoration of blood flow to the ischemic intestine, second-look procedures and supportive intensive care. Only surgery includes the assessment of intestinal viability, determination or confirmation of the underlying cause, revascularization, and resection of the nonviable...
A surgical approach to acute mesenteric ischemia

Embolectomy requires longitudinal or transverse arteriotomy after exposure of the superior mesenteric artery (Figure 8a). A 3-4 F Fogarty catheter is passed proximally or distally to extract clot and establish flow (Figure 8b and 8c). Distal thrombectomy may establish flow in smaller vessels (Figure 9). If recirculation is not established, bypass may bring solution after closing arteriotomy primarily or with a vein patch. Chronic mesenteric occlusive disease commonly becomes symptomatic when the celiac axis and the superior mesenteric artery inflow are significantly narrowed or suddenly occluded.

Endarterectomy can be carried out via the origin of the superior mesenteric artery, celiac artery or through the aorta (Figure 10). The origins of the arteries may be approached either via an anterior abdominal wall incision, followed by medial visceral rotation, or using a thoraco-abdominal approach from the eleventh rib, which gives retroperitoneal exposure. Multivessel occlusive disease associated with the clinical picture of chronic ischemia mandates revascularization of the celiac axis and the superior mesenteric artery.

For bypass grafting not only the superior mesenteric artery, but also the aorta and the iliac vessels should be exposed. A short antegrade reconstruction (Figure 11a and 11b) can provide better hemodynamic results than a long retrograde bypass (Figure 11c). But under certain circumstances, a retrograde bypass may be the procedure of choice, as for the most cases of acute mesenteric thromboses. For the retrograde superior mesenteric
**Figure 8a:** Exposure of the superior mesenteric artery in the root of the mesentery as it crosses over the junction of the third and fourth portions of the duodenum.

**Figure 8b:** A proximal thromboembolectomy, performed with a No. 4 balloon catheter.

**Figure 8c:** The propagated clot is extruded with a No. 3 catheter.

artery revascularization the infrarenal aorta is used for inflow. The retrograde blood flow into the superior mesenteric artery may be a (theoretical) disadvantage. A major drawback, however, is kinking of the graft. After the viscera are returned to their normal locations, a saphenous vein or unsupported conduit may kink, resulting in immediate failure of the reconstruction. By using a long externally supported ePTFE graft, which makes a gentle loop as the viscera are returned to their normal conditions, kinking of the prosthesis is prevented. Similar patency rates are obtained compared to those for antegrade bypass.
When bowel resection may be necessary, it is advisable to refrain from the implantation of a prosthetic graft in patients with acute mesenteric ischemia. A single-vessel (i.e. superior mesenteric artery) is an acceptable option in this setting (*Figure 12*).

Once blood flow is restored, assessment of bowel viability should be performed, and non-viable bowel resected. The decision, whether viable bowel after resection should be primarily anastomosed or brought out as stomas, is based on the stability of the patient and the health of the bowel to be anastomosed.
A surgical approach to acute mesenteric ischemia

Figure 11a: Antegrade bypass to the celiac and superior mesenteric artery beds for proximal celiac and superior mesenteric disease.

Figure 11b: Grafting of the celiac orifice and superior mesenteric artery. Bifurcated prosthesis or straight grafts can be used as alternative antegrade celiac and superior mesenteric artery bypass.

Figure 11c: Retrograde grafting of the superior mesenteric artery.

Figure 12. Single-vessel implantation of the superior mesenteric artery directly into the aorta is advisory when bowel resection is mandatory, as prothetic material may increase the risk of infection.


Non-surgical therapy
Patients with acute mesenteric ischemia frequently present with established bowel infarction, which eliminates endovascular management as an emergency laparotomy because bowel resection is indicated. However, when transmural infarction and subsequent peritonitis has not occurred, endovascular treatment strategies may be an alternative or adjunctive to surgical therapy. The non-surgical treatment modalities for superior mesenteric occlusion are percutaneous transluminal angioplasty, endovascular stent placement, catheter-directed vasodilation or thrombolytic therapy. At present, limited studies are available in the literature and these procedures continue to be controversial, however, results are promising. Undoubtedly, as the average life expectancy increases and subsequently the number of elderly in our hospitals grows, the need for endovascular thrombolytic therapy, angioplasty, and stenting in either acute or chronic mesenteric ischemia will increase, especially when surgical therapy in some elderly is neither indicated nor safe.
Conclusion

The rapid onset of acute mesenteric ischemia and the potential rapidity with which bowel infarction may occur explain the lethality of this disease. The relative infrequency of acute mesenteric ischemia, the variable pathogenesis and the broad spectrum of ischemic injury of the small and large intestines make it almost impossible to study this disease and its diagnostic and therapeutic strategies in clinical randomized or case-controlled trials. Surgery is far the most favorable treatment, as it includes the assessment of intestinal viability, determination or confirmation of the underlying cause, revascularization, and resection of the nonviable intestine. However, in the last decades of the previous century, a number of new treatment modalities have been introduced, which may benefit a certain population of patients with acute mesenteric artery occlusion and may improve outcome of this lethal disease. In this thesis thrombolytic therapy of acute superior mesenteric artery occlusion as an adjunct to surgical therapy will be discussed. Furthermore, the ability to reduce ischemia and reperfusion injury by using drug therapies in experimental settings of acute mesenteric ischemia will be analyzed.
References


Systematic review of survival after acute mesenteric ischaemia according to disease aetiology

Ivo G. Schoots, Geert I. Koffeman, Dink A. Legemate, Marcel Levi and Thomas M. van Gulik

Departments of Surgery and Surgical Laboratory, and Vascular and Internal Medicine, Academic Medical Centre, University of Amsterdam, Amsterdam, the Netherlands

A little optimism – still a dreaded condition

Abstract

Background: Differentiation of acute mesenteric ischaemia on the basis of aetiology is of great importance because of variation in disease progression, response to treatment and outcome. The aim of this study was to analyse the published data on survival following acute mesenteric ischaemia over the past four decades in relation to disease aetiology and mode of treatment.

Method: A systematic review of the available literature from 1966 to 2002 was performed. Results: Quantitative analysis of data derived from 45 observational studies containing 3692 patients with acute mesenteric ischaemia showed that the prognosis after acute mesenteric venous thrombosis is better than that following acute arterial mesenteric ischaemia; the prognosis after mesenteric arterial embolism is better than that after arterial thrombosis or non-occlusive ischaemia; the mortality rate following surgical treatment of arterial embolism and venous thrombosis (54.1 and 32.1 per cent respectively) is less than that after surgery for arterial thrombosis and non-occlusive ischaemia (77.4 and 72.7 per cent respectively); and the overall survival after acute mesenteric ischaemia has improved over the past four decades.

Conclusion: There are large differences in prognosis after acute mesenteric ischaemia depending on aetiology. Surgical treatment of arterial embolism has improved outcome whereas the mortality rate following surgery for arterial thrombosis and non-occlusive ischaemia remains poor.
Introduction

Despite considerable advances in medical diagnosis and treatment over the past four decades, mesenteric vascular occlusion still has a poor prognosis with an in-hospital mortality rate of 59–93 per cent. The pessimistic view offered by Cokkinis more than 75 years ago, namely that '..the diagnosis is impossible, the prognosis hopeless and the treatment useless.', seems to remain valid up to the present day. Whether an aggressive approach to acute mesenteric ischaemia, consisting of early diagnosis, restoration of arterial perfusion to the ischaemic intestine, resection of the necrotic intestine, second-look laparotomy and supportive intensive care, has improved survival during the past decades is unclear from the literature.

Several factors underlie this uncertainty. The aetiology of acute mesenteric ischaemia is often undefined in reported studies, as the correct diagnosis can usually only be confirmed at autopsy. This information is often not available. Furthermore, the relative infrequency of acute mesenteric ischaemia (1–2 per 1000 hospital admissions) and the varied clinical presentation constitute an almost insurmountable obstacle to undertaking randomized or case–control trials.

Mesenteric ischaemia can be classified grossly into ischaemia of thrombotic or non-thrombotic origin. Non-occlusive mesenteric ischaemia, the dominant non-thrombotic cause of acute mesenteric ischaemia, results from low-flow states (for example cardiogenic shock, sepsis, hypovolaemia) whereas thrombotic conditions include arterial embolism, arterial thrombosis and mesenteric venous thrombosis.

Systematic evaluation of research results, even if only observational data and small case series are available, is necessary to move forward, certainly in view of the impact of improved imaging and current thrombolytic strategies. The aim of this systematic analysis of the literature on acute mesenteric ischaemia was to investigate the relationships between disease aetiology (arterial embolism, arterial thrombosis, venous thrombosis and non-occlusive mesenteric ischaemia), mode of treatment and mortality.

Patients and Methods

Inclusion and exclusion criteria

Studies were eligible for inclusion if patients with acute mesenteric ischaemia were divided into aetiological subsets (arterial embolism, arterial thrombosis, venous thrombosis and non-occlusive mesenteric ischaemia) and reported in conjunction with in-hospital mortality rates. Studies that reported data concerning only one aetiological subset were excluded, because they focused on aspects other than aetiology and mortality, such as patient and clinical characteristics, risk factors and diagnostic methods. Patients with acute mesenteric ischaemia secondary to arteritis, mechanical obstruction, adhesion or aortic aneurysm repair, or caused by occlusion of the inferior mesenteric artery (ischaemic colitis), were excluded. Studies dealing with chronic mesenteric ischaemia were also excluded. All studies included had to provide information on the methods used to ascertain the diagnosis (angiography, laparotomy, histopathology or autopsy).

Search strategy

Two authors independently performed a formal computer-assisted search of the medical databases Medline (January 1966 to January 2002, search updated to August 2002), Cochrane Database of Systematic Reviews, Cochrane Clinical Trial Register and Embase (January 1988 to January 2002). Keywords and medical subject heading (MeSH) terms used were ‘mesenteric vascular occlusion’, ‘mesentery’ and ‘ischemia’, limited to ‘human’ studies; clinical studies written in English, Spanish, German, French and Italian were identified. A manual cross-reference search of the eligible papers
was performed to identify additional relevant articles. Data quoted as unpublished or data from abstracts were not used.

**Data collection**
Two authors independently assessed the selected studies and extracted data on study design (retrospective, prospective), population, aetiology and outcome measures, and judged whether the publication met the stated inclusion criteria. Retrospective data were defined as those extracted from patient charts or routine data sources. Prospective data were defined as specific information the collection of which started before disease diagnosis in specified patients. Disagreements concerning inclusion of studies and data extraction were resolved by group discussion. The checklist proposed by the Meta-analysis Of Observational Studies in Epidemiology (MOOSE) group was used as a guideline for performing this quantitative analysis. Observational studies were defined as reports that used data from existing databases, cross-sectional studies, case series, case-control studies, or studies with a historical control or a cohort design.

**Aetiological subsets**
Patient data from each study were divided into the following aetiological subsets: superior mesenteric artery embolism, superior mesenteric artery thrombosis, mesenteric venous thrombosis and non-occlusive mesenteric ischaemia. When no distinction in aetiological origin was made between superior mesenteric artery embolism and artery thrombosis, patients were included in the group ‘artery embolism and artery thrombosis’.

**Mortality**
Mortality was defined as in-hospital death, and was categorized according to the defined aetiological subsets and analysed from 1966 to 2002. The median years of the inclusion period of each included study were used as independent variables in the regression analysis.

**Treatment strategy**
To discriminate between the outcomes of various treatment strategies, data on treatment modalities (when available) were divided into treatment subsets: ‘supportive care’, ‘exploratory laparotomy’, ‘resection’, ‘revascularization’ and ‘revascularization with resection’. Patients were included in the group ‘resection, revascularization or both’ when type of surgical intervention was not clearly stated. Supportive care was defined as conservative treatment without diagnostic or surgical intervention. Supportive care was used in patients who refused surgery, in patients who did not qualify for operation, or in those whose condition improved during diagnostic follow-up. A diagnostic exploratory laparotomy only was performed when total necrosis of the small and large bowel was found, in which case resection would be incompatible with life. Patients who were included in the subset ‘resection’ underwent resection of varying lengths of small bowel with or without large bowel resection during primary laparotomy or subsequent relaparotomy. The subset ‘revascularization’ included patients who underwent embolectomy, thrombectomy, patch angioplasty, endarterectomy or aortoiliac-mesenteric bypass with or without autologous material. The authors were not able to specify the subdivision of each individual surgical procedure owing to lack of data. Patients who were included in the subset ‘revascularization and resection’ underwent revascularization of the splanchnic circulation and subsequent resection of part of the large and/or small bowel, during the same operation or during subsequent relaparotomy.

**Prognostic studies**
Prognostic studies which contained all patients with acute mesenteric ischaemia, including those treated with supportive care or diagnosed at autopsy, were distinguished from prognostic studies in which patients treated with supportive care were not included. This subdivision was made on the assumption that inclusion of patients treated with supportive care only, who were most often moribund or not eligible for interventional treatment, might influence survival negatively.
Chapter 2

Statistical analysis

The primary outcome measure was the in-hospital mortality rate according to aetiological subsets in the individual studies. A relative risk for mortality was calculated. The statistical heterogeneity of the included studies was assessed with the \( \chi^2 \) test with \( k - 1 \) degrees of freedom. Estimates of mortality risk in the aetiological subsets were expressed as pooled relative risks using either the fixed-effects model according to Mantel and Haenszel\(^8\) or the random-effects model according to DerSimonian and Laird\(^9\), depending on the degree of heterogeneity of the included studies. When significant heterogeneity was found, the random-effects method was used to calculate the pooled relative risk. \( P \) values were calculated with the \( \chi^2 \) test; \( P < 0.050 \) was considered statistically significant. Data analysis was performed using Review Manager 4.1 © software (Cochrane Collaboration, Oxford, UK).

Results

Excluded studies

The initial search yielded 933 articles of which 863 did not meet the inclusion criteria. The majority of the excluded papers covered a variety of topics, including diagnostic modalities and treatment strategies. Other excluded articles comprised review articles, articles on chronic mesenteric ischaemia or papers lacking data on aetiology. Retrieval of the 70 candidate papers led to the exclusion of a further 25 because of insufficient data on aetiology\(^10\), unclear primary endpoints\(^22\), 23-27 publication of the same dataset in two languages\(^28\), or because data on acute and chronic mesenteric ischaemia or bowel strangulation\(^29\) was published.

Included studies

The 45 studies\(^35\)-\(^79\) included in the analysis are listed chronologically in Table 1. Only observational studies were identified, of which one was prospective and 44 were retrospective case series, published between 1967 and 2002. The total number of patients was 3692 with a female : male ratio of 1.06.

Clinical characteristics

The median (range) ages of patients in the different aetiological subsets were comparable: 69 (60-75) years for those with arterial embolism (\( n = 280 \)), 71 (59-78) years for patients with arterial thrombosis (\( n = 264 \)), 70 (43-74) years for patients with venous thrombosis (\( n = 108 \)) and 69 (57-76) years for patients with non-occlusive mesenteric ischaemia (\( n = 152 \)). In the subsets of arterial embolism, arterial thrombosis and non-occlusive mesenteric ischaemia, there were more females than males, but the female : male ratios in these subsets were not significantly different (\( \chi^2 \) test): 1.23 for arterial embolism (\( n = 268 \)), 1.46 for arterial thrombosis (\( n = 244 \)), 0.78 for venous thrombosis (\( n = 41 \)) and 1.17 for non-occlusive mesenteric ischaemia (\( n = 117 \)).

Mortality

Mortality was expressed as in-hospital death in all studies. The overall mortality rates of patients within the aetiological subsets in the two groups of prognostic studies, either with or without patients treated with supportive care, were mean (range) 73.9 (33.3-94.6) and 63.7 (24.4-84.8) per cent respectively (Table 2). Mean mortality rates for the aetiological subsets in the two groups were 70.8 (35.7-100.0) and 65.7 (17.6-88.4) per cent for arterial embolism, 87.0 (33.3-100.0) and 70.0 (27.3-100.0) per cent for arterial thrombosis, 44.0 (0.0-100.0) and 44.0 (25.0-68.8) per cent for venous thrombosis, and 80.0 (16.7-100.0) and 69.7 (50.0-83.3) per cent for non-occlusive ischaemia, respectively. Mortality rates
Survival after acute mesenteric ischaemia

Table 1. Characteristics of included patient studies dealing with acute mesenteric ischaemia.

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n median age
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### Table 2. In-hospital mortality according to etiology of acute mesenteric ischemia.

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Survival after acute mesenteric ischaemia

Table 2 (previous page). AE, superior mesenteric artery embolism; AT, superior mesenteric artery thrombosis; VT, mesenteric vein thrombosis; NMI, nonocclusive mesenteric ischemia; AE & AT, superior mesenteric artery embolism and superior mesenteric artery thrombosis are included together.
†, mortals; excl, excluded; †† Patients with unknown or other than AE, AT, VT or NMI etiology of acute mesenteric ischemia are included.

within the aetiological subsets of the group of studies that included patients treated with supportive care were higher than those in the group that excluded such patients (except for patients with mesenteric venous thrombosis); however, the latter group comprised solely studies from approximately the past two decades.

The results of quantitative analysis of in-hospital mortality according to disease aetiology, as measured by the pooled relative risks of two aetiological subsets, are presented in Table 3. The pooled mortality risk for mesenteric arterial thrombosis was equivalent to that for non-occlusive mesenteric ischaemia in both sets of studies. The pooled mortality risk from mesenteric arterial emboli was less than that from arterial thrombosis or non-occlusive mesenteric ischaemia. The pooled mortality risk from venous thrombosis was lower than that from arterial causes of acute mesenteric ischaemia in both sets.

The mortality rates for each aetiological subset over the past four decades, obtained from weighted mortality rates and the median year of the inclusion period, are shown in Figure 1. In both groups, overall mortality rate, and also the mortality rates for the individual aetiological subsets of acute mesenteric ischaemia, demonstrated a declining trend over the past four decades.

Surgical treatment

Table 4 shows mortality rates of acute mesenteric ischaemia following surgical and non-surgical treatment according to the aetiological subsets. From the surgical perspective, it is necessary to analyse mortality rates following surgical treatment while excluding moribund patients or patients in whom the diagnosis was made at autopsy. Observational studies that included patients who did not receive surgical treatment may obscure the effectiveness of surgical intervention. Therefore, patients were classified into treatment modalities and categorized into non-surgical or surgical treatment according to disease aetiology.

For non-surgical treatment, the mortality rates of the different aetiological subsets varied between 87.1 and 99.4 per cent. In some patients clinical symptoms improved during supportive care and they did not therefore undergo surgical exploration, which may account for the few survivors. Patients who underwent explorative laparotomy for diagnosis only ("open-and-close procedure") died in almost all cases from massive bowel infarction. Some patients in whom intestinal ischaemia was observed in the absence of mesenteric infarction survived.

Following surgical treatment the mortality rates associated with venous thrombosis and arterial embolism improved from 44.0 to 32.1 per cent and from 70.1 to 54.1 per cent respectively, whereas the mortality rates of mesenteric arterial thrombosis (77.4 per cent) and non-occlusive mesenteric ischaemia (72.7 per cent) remained poor. Mesenteric revascularization conferred no benefit over resection alone, except in patients in whom acute mesenteric ischaemia was caused by venous thrombosis.
Table 3. Pooled relative risks of in-hospital mortality in relation with etiology of acute mesenteric ischemia.

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<th>† / n (right group)</th>
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<td>Prognostic studies 35-66 including supportive care patients</td>
<td>AT vs. AE</td>
<td>619 / 874 vs. 661 / 760</td>
<td>0.85 (0.78–0.92)</td>
<td>&lt;0.001</td>
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<td>AT vs. NMI</td>
<td>414 / 619 vs. 347 / 429</td>
<td>0.86 (0.78–0.95)</td>
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<td>AT vs. VT</td>
<td>402 / 476 vs. 347 / 429</td>
<td>1.01 (0.92–1.10)</td>
<td>0.842</td>
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<tr>
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<td>AT vs. VT</td>
<td>481 / 694 vs. 102 / 232</td>
<td>1.32 (1.05–1.66)</td>
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<tr>
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<td>NMI vs. VT</td>
<td>538 / 627 vs. 102 / 232</td>
<td>1.59 (1.23–2.05)</td>
<td>&lt;0.001</td>
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<tr>
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<td>NMI vs. VT</td>
<td>331 / 423 vs. 97 / 222</td>
<td>1.47 (1.22–1.78)</td>
<td>&lt;0.001</td>
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<tr>
<td>Prognostic studies 67-79 excluding supportive care patients</td>
<td>AT vs. AE</td>
<td>92 / 140 vs. 161 / 230</td>
<td>1.02 (0.90–1.16)</td>
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<td>AT vs. NMI</td>
<td>66 / 97 vs. 62 / 89</td>
<td>1.00 (0.82–1.21)</td>
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<td>AT vs. NMI</td>
<td>126 / 179 vs. 62 / 89</td>
<td>0.99 (0.79–1.24)</td>
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<td>AT vs. VT</td>
<td>87 / 124 vs. 58 / 132</td>
<td>1.56 (1.21–2.03)</td>
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<td>AT vs. VT</td>
<td>149 / 193 vs. 58 / 132</td>
<td>1.62 (1.25–2.09)</td>
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<td>NMI vs. VT</td>
<td>58 / 84 vs. 31 / 83</td>
<td>1.91 (1.36–2.70)</td>
<td>&lt;0.001</td>
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<td>All prognostic studies 35-79</td>
<td>AT vs. AE</td>
<td>711 / 1014 vs. 822 / 990</td>
<td>0.88 (0.82–0.94)</td>
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<td>AE vs. NMI</td>
<td>480 / 716 vs. 409 / 518</td>
<td>0.88 (0.80–0.97)</td>
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<td>AT vs. NMI</td>
<td>528 / 655 vs. 409 / 518</td>
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<td>AT vs. VT</td>
<td>568 / 818 vs. 160 / 364</td>
<td>1.38 (1.16–1.66)</td>
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<td>AT vs. VT</td>
<td>677 / 810 vs. 160 / 364</td>
<td>1.60 (1.32–1.95)</td>
<td>&lt;0.001</td>
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<td>NMI vs. VT</td>
<td>389 / 507 vs. 128 / 305</td>
<td>1.54 (1.32–1.80)</td>
<td>&lt;0.001</td>
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AE, superior mesenteric artery embolism; AT, superior mesenteric artery thrombosis; VT, mesenteric vein thrombosis; NMI, nonocclusive mesenteric ischemia. †, mortals.

Discussion

Because of the low incidence and broad spectrum of acute mesenteric ischaemia, randomized or case–control trials are lacking and preclude an analysis with a higher level of evidence than can be extracted from observational data. A quantitative analysis of observational data was therefore performed to assess mortality and prognosis in relation to aetiological causes of acute mesenteric ischaemia over the past four decades. Most of the studies assessed calculated a mortality rate based on data compiled from all aetiological subsets taken together. This has the drawback of obscuring differences in clinical presentation and characteristics, diagnostic investigation, disease progression, mortality and response to therapeutic modalities that are specific to disease aetiology. Despite the limitations and careful interpretation of observational data, the results of this quantitative analysis of individual aetiological subsets show clearly the better prognosis of acute mesenteric venous thrombosis compared with arterial causes of acute mesenteric ischaemia; the better prognosis of mesenteric arterial embolism compared with arterial
Survival after acute mesenteric ischaemia

Table 4. Treatment and associated in-hospital mortality according to etiology of acute mesenteric ischemia.

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<td>Resection</td>
<td>62 / 109</td>
<td>39 / 81</td>
</tr>
<tr>
<td>Revascularization</td>
<td>14 / 31</td>
<td>40 / 67</td>
</tr>
<tr>
<td>Revascularization + resection</td>
<td>20 / 36</td>
<td>32 / 54</td>
</tr>
<tr>
<td>Resection, revascularization or both (not specified)</td>
<td>31 / 60</td>
<td>14 / 29</td>
</tr>
<tr>
<td>Subtotal</td>
<td>127 / 236</td>
<td>125 / 231</td>
</tr>
<tr>
<td>Total</td>
<td>206 / 317</td>
<td>260 / 371</td>
</tr>
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</table>

thrombosis and non-occlusive ischaemia; the improved prognosis of venous thrombosis and arterial embolism following surgical treatment while the mortality rate associated with mesenteric arterial thrombosis and non-occlusive mesenteric ischaemia remained poor; and the improved overall survival of patients with acute mesenteric ischaemia over the past four decades.

The survival benefit for patients with acute mesenteric venous thrombosis compared with those suffering arterial mesenteric occlusion may be explained by the usually limited segmental bowel infarction and the need for limited intestinal resection. Whether acute arterial occlusion is of embolic or thrombotic origin may not influence the timing of diagnosis and outcome; however, the difference in mortality rate after surgery for embolic and thrombotic arterial occlusion (54.1 versus 77.4 per cent respectively) indicates a far worse prognosis for mesenteric arterial thrombosis. Mesenteric arterial thrombosis is most often superimposed on atherosclerosis at the origin of the superior mesenteric artery. The poor prognosis of patients with mesenteric arterial thrombosis is most likely due to the proximal location of the occlusion that is associated with extensive bowel infarction and the need for extended bowel resection. In contrast, mesenteric arterial embolism occludes the mesenteric vessels at different levels of the mesenteric vascular tree resulting in varying areas of mesenteric infarction. For non-occlusive mesenteric ischaemia, revascularization procedures are not appropriate because the underlying problem is a low-flow state. The mortality rate of these patients will remain high in spite of supportive circulatory treatment and bowel resection, if the underlying cause of mesenteric hypocirculation is not treated adequately.

In addition to distinguishing disease aetiology, therapeutic modalities were categorized into non-surgical or surgical treatment groups. Non-surgical treatment
Chapter 2

Table 4. (Continued)

<table>
<thead>
<tr>
<th></th>
<th>AT</th>
<th>VT</th>
<th>NMI</th>
<th>Overall</th>
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<td></td>
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<td>(%)</td>
<td>t/n</td>
<td>(%)</td>
</tr>
<tr>
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<td>123/124</td>
<td>(99)</td>
<td></td>
<td>16/18</td>
<td>(89)</td>
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<td>(99)</td>
<td></td>
<td>27/31</td>
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<td>65/85</td>
<td>(76)</td>
<td>29/78</td>
<td>(37)</td>
<td>32/44</td>
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<td>22/30</td>
<td>(73)</td>
<td>2/11</td>
<td>(18)</td>
<td>0/0</td>
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<tr>
<td>16/19</td>
<td>(84)</td>
<td>1/2</td>
<td>(50)</td>
<td>0/0</td>
</tr>
<tr>
<td>24/30</td>
<td>(80)</td>
<td>4/21</td>
<td>(19)</td>
<td>0/0</td>
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<tr>
<td>127/164</td>
<td>(77)</td>
<td>36/112</td>
<td>(32)</td>
<td>32/44</td>
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<tr>
<td>286/324</td>
<td>(88)</td>
<td>59/134</td>
<td>(44)</td>
<td>148/172</td>
</tr>
</tbody>
</table>

(supportive care and diagnostic explorative laparotomy) was followed by death in almost all cases, the diagnosis being established when the patient was in a moribund state or at autopsy. This emphasizes the importance of early diagnosis and treatment\(^{1,5}\). Regarding surgical treatment, two findings were notable. As expected, the mortality rates were lower after surgical than non-surgical treatment. However, the mortality from mesenteric arterial thrombosis and non-occlusive mesenteric ischaemia remained high even after surgical treatment (resection, revascularization or both) (77.4 and 72.7 per cent respectively), whereas the in-hospital mortality rate associated with arterial embolism and venous thrombosis had decreased to 54.1 and 32.1 per cent respectively.

Patients who underwent revascularization with or without resection appeared to fare even worse than patients who underwent bowel resection only. This might be explained by the varying prognosis of different revascularization procedures. Relatively small revascularization procedures, for example thrombectomy or embolectomy without subsequent bowel resection, are undertaken when the disease is diagnosed early and there is no transmural bowel necrosis, and may therefore be associated with decreased mortality. On the other hand, large revascularization procedures, such as aortoiliac-mesenteric bypass surgery, are used when there is intestinal vascular insufficiency and may result in increased mortality. Unfortunately, incomplete data made it impossible to distinguish between the various revascularization procedures.

Whether advances in diagnostic tests and therapeutic strategies have improved survival over time is difficult to determine, in part because of the infrequency of acute mesenteric ischaemia and the paucity of data regarding outcome. Over the past decade reviews and expert opinion have disagreed as to whether improvements in mortality from acute mesenteric ischaemia have occurred\(^{1}\). However, in several studies data
Survival after acute mesenteric ischaemia

Studies including supportive care patients

Studies excluding supportive care patients

Mortality (%) vs Year

encompassing two inclusion periods showed improved survival\textsuperscript{63,72,81}. Furthermore, investigation of each aetiological subset in this study, using weighted mortality rates, demonstrated a declining trend in mortality over the past four decades. Whether this trend derives from publication bias, improvements in treatment strategy or from early diagnosis remains a matter of debate. There is no doubt that early diagnosis decreases mortality\textsuperscript{67,54,59,68,71}. The authors were unable to obtain data from published studies on patient and doctor delay, and its relation to mortality. Diagnosis is still often delayed, mainly as a result of the non-specific nature of clinical symptoms during the early phase of acute mesenteric ischaemia and limitations in current diagnostic techniques\textsuperscript{82}. The declining mortality of acute mesenteric ischaemia over the past four decades is therefore probably attributable to better management of the disease, with improvements in surgical interventions and perioperative and postoperative supportive intensive care.

Survival after acute mesenteric ischaemia varied between the different aetiological subsets. The mortality rate after surgical treatment of arterial embolism and venous thrombosis has improved whereas that after surgery for arterial thrombosis and non-occlusive ischaemia remains poor. Although the statement offered by Cokkinis more than 75 years ago may to some degree still apply, there is now room for more optimism and it might be said that ‘the diagnosis of acute mesenteric ischaemia is possible, and, in some cases, the treatment is useful and the prognosis hopeful’.

\textbf{Figure 1.} (previous page) Trends in in-hospital mortality rate with time in relation to aetiology of acute mesenteric ischaemia. The median of the inclusion period of each included study was used in regression analysis, along with in-hospital mortality rates weighted according to the number of patients included in the study. AE, superior mesenteric artery embolism; AT, superior mesenteric artery thrombosis; VT, mesenteric vein thrombosis; NMI, non-occlusive mesenteric ischaemia; overall, mortality rates for all aetiological subsets combined.
References

Survival after acute mesenteric ischaemia


“Opponents of the concept of systematic reviews will cite the usual criticisms regarding the appropriateness of this type of analysis. However I found it to be informative, particularly regarding the differential mortality rates depending on the underlying aetiology.” A reviewer.
### Comparison: 01 Mortality

#### Outcome: 01 AE vs AT

<table>
<thead>
<tr>
<th>Study</th>
<th>Mortality AE</th>
<th>Mortality AT</th>
<th>RR (95%CI Random)</th>
<th>Weight %</th>
<th>RR (95%CI Random)</th>
</tr>
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<td><strong>Studies including supportive care patients</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Voltolini 1996</td>
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<td>18/20</td>
<td>0.8</td>
<td>0.40 [0.19, 0.81]</td>
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<td>88/99</td>
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<td>661/760</td>
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</table>

Test for heterogeneity chi-square=91.71 df=28 p<0.00001
Test for overall effect z=-3.89 p=0.00001

| Studies excluding supportive care patients | | | | | |
| Rius 1979 | 3/5 | 27/27 | 0.8 | 0.60 [0.29, 1.23] |
| Levy 1990 | 3/17 | 3/11 | 0.2 | 0.62 [0.16, 2.65] |
| Stiges 1987 | 4/6 | 16/19 | 1.1 | 0.79 [0.44, 1.44] |
| Grotheus 1986 | 16/21 | 22/27 | 2.9 | 0.94 [0.69, 1.26] |
| Macaroni 1989 | 8/10 | 17/20 | 2.3 | 0.94 [0.66, 1.35] |
| Park 2002 | 5/16 | 12/37 | 0.6 | 0.98 [0.41, 2.28] |
| Rienenschneider 1987 | 23/26 | 32/40 | 3.9 | 1.11 [0.90, 1.36] |
| Luther 2002 | 14/18 | 13/19 | 2.1 | 1.14 [0.77, 1.68] |
| Newman 1999 | 9/11 | 9/13 | 1.7 | 1.19 [0.75, 1.87] |
| Bottger 1991 | 7/10 | 10/17 | 1.2 | 1.19 [0.67, 2.10] |
| **Subtotal(95%CI)** | 92/140 | 161/230 | 16.7 | 1.02 [0.90, 1.16] |

Test for heterogeneity chi-square=4.53 df=9 p=0.79
Test for overall effect z=0.34 p=0.7

| Total(95%CI) | 711/1014 | 822/990 | 100.0 | 0.88 [0.82, 0.94] |

Test for heterogeneity chi-square=91.09 df=38 p<0.00001
Test for overall effect z=-3.77 p=0.0002
### Appendix II

**Comparison: 01 Mortality**

**Outcome:** 02 AE vs NMI

<table>
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<th>Study</th>
<th>Mortality AE n/N</th>
<th>Mortality NMI n/N</th>
<th>RR (95% CI Random)</th>
<th>Weight %</th>
<th>RR (95% CI Random)</th>
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<td>9 / 19</td>
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<td>5 / 7</td>
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<td>2.5</td>
<td>0.65 [0.39, 1.09]</td>
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<td>0.74 [0.60, 0.91]</td>
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<tr>
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<td>67 / 67</td>
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<td>4.3</td>
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<td>57 / 77</td>
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<td>6.6</td>
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<td>5 / 7</td>
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<td>1.9</td>
<td>0.90 [0.49, 1.81]</td>
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<td>0.94 [0.87, 1.02]</td>
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<td>6 / 8</td>
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<td>Veel &amp; Doyle 1977</td>
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<td>Koveker 1985</td>
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<td>4 / 5</td>
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<td>1.05 [0.65, 1.70]</td>
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<td>4 / 16</td>
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<td>Claven 1987</td>
<td>26 / 33</td>
<td>10 / 15</td>
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<td>Kach and Largis 1989</td>
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<td>2 / 4</td>
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<tr>
<td>Boley 1977</td>
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<td>4 / 13</td>
<td></td>
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<td>1.83 [0.73, 1.40]</td>
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<td>6 / 14</td>
<td></td>
<td>1.6</td>
<td>2.12 [1.31, 3.40]</td>
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<tr>
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<td>7 / 7</td>
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<td>347 / 429</td>
<td></td>
<td>82.1</td>
<td>0.86 [0.78, 0.95]</td>
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</table>

Test for heterogeneity chi-square=36.74 df=21 p=0.016

Test for overall effect z=-2.85 p<0.004

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<th>Study</th>
<th>Mortality AE n/N</th>
<th>Mortality NMI n/N</th>
<th>RR (95% CI Random)</th>
<th>Weight %</th>
<th>RR (95% CI Random)</th>
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<tr>
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<td>Sitges 1987</td>
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<td>62 / 89</td>
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<td>17.9</td>
<td>1.00 [0.82, 1.21]</td>
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Test for heterogeneity chi-square=7.39 df=7 p=0.39

Test for overall effect z=0.00 p=1

| Total (95% CI)                     | 480 / 716        | 409 / 516         | 100.0              | 0.88 [0.80, 0.97] |

Test for overall effect z=-2.69 p<0.007

---

59
### Appendix III

#### Comparison: 01 Mortality

**Outcome:** 03 AT vs NMI

<table>
<thead>
<tr>
<th>Study</th>
<th>Mortality AT</th>
<th>Mortality NMI</th>
<th>RR (95%CI Random)</th>
<th>Weight %</th>
<th>RR (95%CI Random)</th>
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<td>57 / 77</td>
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<td>15 / 18</td>
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<td>9 / 9</td>
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<td>6 / 8</td>
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<td>347 / 429</td>
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Test for heterogeneity chi-square=35.77 df=18 p=0.0075
Test for overall effect z=0.20 p=0.8

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<td>4 / 5</td>
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<td>10 / 12</td>
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<td>27 / 43</td>
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<td>10 / 13</td>
<td>3.6</td>
<td>1.10(0.78, 1.57)</td>
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<td>10 / 17</td>
<td>1 / 2</td>
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<td>1.18(0.26, 4.97)</td>
<td>2.0</td>
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<td>2 / 3</td>
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<td>4 / 16</td>
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Test for heterogeneity chi-square=10.94 df=7 p=0.14
Test for overall effect z=0.08 p=0.9

| Total(95%CI) | 528 / 655 | 409 / 518 | 100.0 | 1.01(0.93, 1.09) | 2.0 |

Test for heterogeneity chi-square=45.94 df=26 p=0.0093
Test for overall effect z=0.17 p=0.9

---

Survival after acute mesenteric ischaemia
## Appendix IV

### Comparison: 01 Mortality

**Outcome:** 04 AE vs VT

<table>
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<tr>
<th>Study</th>
<th>Mortality AE n/N</th>
<th>Mortality AT n/N</th>
<th>RR (95%CI Random)</th>
<th>Weight %</th>
<th>RR (95%CI Random)</th>
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<td>Ottinger &amp; Aust 1967</td>
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<td>Havia &amp; Inberg 1975</td>
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Test for heterogeneity chi-square=57.86 df=21 p<0.0001
Test for overall effect z=2.39 p=0.02

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<th>Weight %</th>
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Test for heterogeneity chi-square=9.38 df=8 p=0.31
Test for overall effect z=3.39 p=0.0007

| Total(95%CI)               | 568 / 818        | 160 / 364        | 100.0             |          | 1.38(1.15,1.66)  |

Test for heterogeneity chi-square=69.66 df=30 p=0.0001
Test for overall effect z=3.47 p=0.0005
### Appendix V

#### Comparison: 01 Mortality

**Outcome:** 05 AT vs VT

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Test for heterogeneity chi-square=82.63 df=21 p<0.00001
Test for overall effect z=3.55 p=0.0004

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<td>Bottger 1991</td>
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Test for heterogeneity chi-square=10.24 df=8 p=0.25
Test for overall effect z=3.67 p=0.0002

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Test for overall effect chi-square=89.57 df=30 p<0.00001
Test for overall effect z=4.76 p=0.00001

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## Appendix VI

### Comparison: 01 Mortality

**Outcome:** 06 NMI vs VT

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Test for heterogeneity chi-square=24.80 df=18 p=0.14
Test for overall effect z=3.99 p=0.00007

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Test for heterogeneity chi-square=3.11 df=6 p=0.79
Test for overall effect z=3.71 p=0.00002

Total(95%CI) | 389 / 507 | 128 / 305 | 100.0 | 1.54[1.32,1.80] |

Test for heterogeneity chi-square=29.46 df=25 p=0.00
Test for overall effect z=5.39 p=0.00001

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63
Thrombolytic therapy for acute superior mesenteric artery occlusion: a systematic review

Ivo G. Schoots¹², Marcel Levi², Jim A. Reekers³, Johan S. Lameris³, Thomas M. van Gulik¹

Departments of ¹Surgery and Surgical Laboratory, ²Vascular and Internal Medicine, and ³Radiology, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands

Thrombolysis of mesenteric occlusions – insufficient data but promising results

J Vasc Interv Radiol. 2004 (in press)
Abstract

Aim: The aim of this review is to evaluate thrombolytic therapy for acute superior mesenteric artery occlusion as an alternative or adjunctive treatment modality to surgical therapy and to provide current knowledge for timely and informed decisions regarding treatment of acute mesenteric ischemia.

Methods: A systematic analysis of the available literature from 1966 to 2003 regarding thrombolytic therapy for superior mesenteric artery thromboembolism was performed.

Results: A total of 20 case reports and 7 small series covered 48 patients with acute superior mesenteric artery thromboembolism. In the herein reviewed series, thrombolytic therapy of acute superior mesenteric artery thromboembolism resulted in angiographic resolution of the thromboembolism in 43 patients, in clinical success without requiring additional surgical intervention in 30 patients, and in survival in 43 patients, with similar complication rates as in thrombolytic treatment of peripheral vascular occlusions. Remission of abdominal pain during the first few hours of treatment formed the most important indicator of therapeutic success.

Conclusion: Insufficient evidence from reviewed literature is available to determine the relative effectiveness and safety of thrombolytic treatment for acute superior mesenteric artery thromboembolism, however, initial results appear to be promising. Thrombolytic therapy can be effective relatively quickly, may obviate the need for surgery, and has the potential to resolve the clot completely. In some cases it can be used as an alternative or neo-adjunctive treatment modality to surgery. A treatment guideline for thrombolysis of acute superior mesenteric artery thromboembolism should be developed.
**Introduction**

Despite progress in diagnostic and treatment strategies over the past decades, acute thromboembolic mesenteric ischemia is still a lethal disease with mortality rates ranging from 24%-94% \(^1\). Only an aggressive early therapeutic approach to acute mesenteric ischemia may increase survival \(^2-4\). This aggressive approach constitutes immediate diagnosis, restoration of arterial blood perfusion to the ischemic intestine, resection of the nonviable intestine, second-look procedures and supportive intensive care.

Only surgery includes the assessment of intestinal viability, determination or conformation of the underlying cause, revascularization, and resection of the nonviable intestine. The most useful surgical revascularization techniques for superior mesenteric artery occlusions are the balloon catheter thromboembolectomy in embolism, with or without patch angioplasty, and the aortomesenteric or iliomesenteric bypass grafts in atherosclerotic proximal occlusions or stenoses.

The non-surgical treatment modalities for acute superior mesenteric artery occlusion are catheter-directed vasodilation, endovascular stent placement or thrombolytic therapy. These therapeutic strategies may serve as an alternative or adjunct to surgery for acute superior mesenteric artery occlusion. At present only limited experience of thrombolytic therapy is available in patients with acute superior mesenteric artery thromboembolism. In addition, thrombolysis is sometimes judged unfavorable in the literature \(^5,6\). As such, prolonged infusion of the thrombolytic agent, while ischemia continues, may lead to bowel necrosis and decrease the chance of survival. Thrombolysis also requires intensive clinical and laboratory supervision, and angiography is necessary to confirm the diagnosis, as thrombolysis is neither indicated nor safe in the absence of objective confirmation of vascular obstruction. However, increasing evidence shows that thrombolytic therapy for acute superior mesenteric artery occlusion can be effective within short time, may obviate the need for surgery, and has the potential to resolve the clot completely. Furthermore, increasing data of catheter-directed thrombolytic therapy in peripheral arterial occlusive disease have become available in literature, which demonstrate well-recognized benefits of this treatment modality \(^7-11\). This success of thrombolytic therapy in the peripheral arterial system may also be applicable for the mesenteric circulation.

The relative infrequency of acute mesenteric ischemia (1-2 on 1000 hospital admissions) \(^12\), the variable pathogenesis and the broad spectrum of ischemic injury of the small and large intestines form an almost insurmountable obstacle to the performance of randomized controlled trials. The need for timely and informed decisions regarding treatment of acute mesenteric ischemia in clinical practice necessitates systematic evaluation of previous results, even if only observational data and small case series are available. As the average life expectancy increases and subsequently the number of elderly in our hospitals grows, the need for thrombolytic therapy, but also angioplasty and stenting, in either acute or chronic mesenteric ischemia will increase, especially when surgical therapy in some elderly is neither indicated nor safe.

In this article, the current (observational) data of thrombolytic therapy in patients with acute thromboembolic mesenteric occlusion have been systematically reviewed, in order to evaluate this treatment modality as an alternative or adjunctive therapy to surgery. This compilation of data gives insight into current status of thrombolytic therapy of acute
Thrombolysis of mesenteric occlusions

superior mesenteric artery thromboembolism, may provide questions and answers for clinical investigators to address, and may give rise to directions for future evaluation.

Literature search

In order to evaluate thrombolytic therapy for acute superior mesenteric artery occlusion, a formal computer-assisted search of the medical databases Medline (January 1966 to January 2003), Cochrane Database of Systematic Reviews, Cochrane Clinical Trial Register, and Embase (January 1988 to January 2003) was performed. Keywords and medical subject heading (MeSH) terms used were ‘mesenteric vascular occlusion’, ‘mesentery’ and ‘ischemia’ together with ‘thrombosis’, ‘embolism’, ‘thromboembolism’, ‘thrombolysis’, ‘fibrinolysis’ and ‘thrombolytic therapy’, limited to ‘human’ studies; clinical studies written in English, Spanish, German, French and Italian were identified.

Patient studies were eligible for inclusion if they investigated thrombolytic therapy for acute superior mesenteric artery thromboembolism. In addition, occlusion of the superior mesenteric artery had to be ascertained via angiography. Studies, which reported thrombolytic therapy intra-operatively or thrombolytic therapy with intravascular stent placing in acute superior mesenteric artery occlusion, were excluded from this review. Cases of chronic mesenteric ischemia undergoing elective thrombolytic treatment were also excluded from this study.

The initial search yielded 61 articles, of which 34 did not meet the inclusion criteria. The majority of the excluded papers covered topics such as diagnostic modalities and treatment strategies not under consideration here. Other excluded articles were case reports of patients, treated by angioplasty, stent placement or systemic thrombolysis. Retrieval of candidate papers led to the inclusion of 27 studies in this review; 20 case reports and 7 small series, which are depicted chronologically in Table 1.

Patient characteristics

The studies comprised 48 patients, 17 females and 31 males, with a median age of 69 years, ranging from 41 to 91 years. Risk factors were reported in reviewed case-studies, however, they may be incomplete. 34 out of 48 patients (71%) presenting with acute abdominal pain had atrial fibrillation in their present or previous history. Atherosclerotic and other (cardiovascular) diseases were frequently present, including previous myocardial infarction (13/48), coronary artery disease (5/48), hypertension (8/48), peripheral arterial occlusive disease (2/48), heart valve disease (6/48), and aorta aneurysm (2/48). Other thromboembolic events, excluding acute coronary syndromes but including upper or lower extremity embolism, superior mesenteric thromboembolism and cerebral infarction, occurred in 21 out of 48 patients (44%). 6 of these 21 patients (31%) demonstrated simultaneous occurrence of acute SMA thromboembolism.

Duration of abdominal symptoms, either before hospital presentation or angiographic diagnosis, ranged from 1 to 144 hours (median 8 hours) (Table 1). Bloody diarrhea was reported in 15 out of 48 patients (32%).

Proximal occlusion of the SMA, at various distances from the origin of the SMA and the middle colic artery, was reported in 24 patients (50%) (Table 1). At least 16 patients (33%) demonstrated total or almost total occlusions. Three patients showed
multiple occlusions, and four studies reported six patients demonstrating, not a thromboembolus, but an explicitly obstructing thrombus.

**Technique and Treatment**

Thrombolytic therapy in the management of intestinal ischemia was first reported by Jamieson in 1979, with the successful infusion of streptokinase directly into the superior mesenteric artery. Since then, various thrombolytic treatment strategies have been performed in the therapy of thromboembolic occlusion of the superior mesenteric artery.

**Technique.** - Diagnostic angiography was performed by the standard Seldinger technique via the transfemoral approach into the superior and inferior mesenteric artery and celiac trunk to confirm the diagnosis and to determine the site and the degree of obstruction and collateral circulation. After diagnostic angiography a catheter, depending on artery morphology, was placed in the superior mesenteric artery. Once an adequate guidewire was threaded into the occlusion, a catheter was advanced over the wire into the thromboembolus located in the superior mesenteric artery. End-hole and multiple side-hole infusion catheters (and pulse-spray technique) have been used. Mostly direct infiltration of the thrombus/embolus with a thrombolytic agent was executed during slow withdrawal of the catheter. After thrombus/embolus infiltration the catheter was placed proximally to the occlusion and under close surgical monitoring and surveillance in the intensive care unit a continuous local infusion protocol of a thrombolytic agent with an automated pump was performed.

**Agent.** - In the reviewed series, the most favored thrombolytic agent was urokinase in 38 out of 48 patients (79%), more than streptokinase or rtPA. Intraarterial urokinase has a half-life time of only 16 minutes and its effect can be reversed with intravenous lysine analogues such as e-aminocaproic acid or tranexamic acid. Therefore urokinase administration will not preclude surgery if intestinal resection should be required.

**Dose.** - The available literature shows a great variety in infusion protocols. Urokinase dose demonstrated a large range, though most patients received a relatively high-dose infusion protocol (from 100.000U/h up to 600.000U/h) (Figure 1). High-dose of urokinase therapy resulted in revascularization in less than 3 hours. Low dose streptokinase infusion at 5.000-10.000U/h restored vascularization within 30-60 hours. High-dose infusions have resulted in a higher incidence of complete clot lysis, shorter infusion time and a lower incidence of significant bleeding in peripheral vascular occlusions.

**Anticoagulant treatment.** An adjunctive anticoagulant strategy, implemented in the urokinase treatment, was reported in 30 out of 38 patients. 21 out of 30 patients were intravenously treated with heparin; in 19 out of these 21 patients heparin infusion started during thrombolytic therapy. In only 2 patients (pt 21 and 22) heparin infusion was started following thrombolytic therapy. In another 7 out of 30 patients, heparin was infused intrararterially: in 5 patients heparin was infused simultaneously with the thrombolytic therapy. Only 2 cases did not report the procedure of heparin infusion. Almost all patients were treated with approximately 1000 units of heparin per hour, independently of intra-arterial or intravenous infusion. In most centers heparin was administered through the sheath and was not directly combined with the thrombolytic agent.

There is consensus that heparinization is indicated in acute mesenteric ischemia of arterial origin, but there is controversy as to when it should be initiated. Some investigators consider a delay of 48 hours, because of the risk of intraluminal bleeding of
**Table 1. Case-reports and small series of thrombolytic therapy for acute superior mesenteric artery thromboembolism**

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<tr>
<td>Domínguez et al</td>
<td>2000</td>
<td>45</td>
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<td>Mellander et al</td>
<td>2001</td>
<td>46</td>
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<td>Bakarate et al</td>
<td>2002</td>
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AA, aorta aneurysm; AD, aorta dissection; AF, atrial fibrillation; AMI, acute mesenteric ischemia; AOD, peripheral arterial occlusive disease; CAD, coronary artery disease; CHF, chronic heart failure; CI, cerebral infarction; CM, cardiomyopathy; CML, chronic myeloid leukemia; COPD, chronic obstructive pulmonary disease; DM, diabetes mellitus; EE, embolic event in extremity, EE+, embolic event concomitant with acute mesenteric ischemia; FVIII, factor VIII deficiency; HC, hypercholesterolemia; HT, hypertension; HVD, heart valve disease; MI, myocardial infarction; PTA, percutaneous transluminal arteriogram; PUD, peptic ulcer disease; RF, rheumatic fever; SMAE, superior mesenteric artery embolism; SVT, supraventricular tachyarrhythmia.

P. SMA occlusion proximal to the middle colic artery; D, SMA occlusion distal to the middle colic artery.

T, total occlusion; T (-), almost total occlusion; (+), multiple occlusions; I, incomplete/partial occlusion; T/I, total or incomplete occlusion.

y, year; mo, month; d, day

* bloody diarrhea present, - bloody diarrhea absent

* not reported
Table 1. (continued)

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<tr>
<th>Pt nr.</th>
<th>Duration of presentation (h)/onset angiography</th>
<th>Bloody stools</th>
<th>Site (cm from origin)</th>
<th>Occlusion</th>
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<td>D</td>
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the infracted bowel\(^2\), whereas others recommend immediate heparinization to offset the danger\(^4^6\).
Vasodilative strategy. In the reviewed series only 3 patients underwent additional papaverine infusion, which resulted in two clinical failures, including one technical failure, and one clinical success (pt. 14, 18, and 23). Some authorities suggested routine use of papaverine in cases of persistent vasoconstriction in all patients with superior mesenteric artery emboli. Vasoconstriction in this manner may occur even after the embolus has been removed. It is likely that persistent vasoconstriction is one of the factors that is responsible for failure to restore arterial perfusion to the bowel after embolectomy and for progression from bowel ischemia to infarction even after the blood flow in the superior mesenteric artery was restored. Criteria for the use of papaverine are based on consensus. Peritoneal signs should be absent, a compelling medical reason for the patient not to undergo surgery should be present and a good perfusion of the vascular bed distal to the embolus after a bolus injection of a vasodilator should be sustained.

Initiation. - Initiation of thrombolytic therapy depends on the severity of the intestinal ischemia and duration of abdominal pain. However, duration of abdominal pain and severity of intestinal ischemia are not clearly correlated in all patients. Simo and coworkers demonstrated that response to intraarterial treatment was favorable in two patients who had abdominal pain for 18 hours before treatment (pt. 30 and 31), whereas therapy failed in another two patients with abdominal pain for only 6 hours prior to treatment (pt. 36 and 37). An explanation for this discrepancy could be that the severity of intestinal ischemia depends not only on the duration of arterial occlusion but also on other factors such as the degree of vascular occlusion, the presence or absence of collateral circulation, influence of splanchnic autoregulation and the presence of associated atherosclerotic lesions. Experimental evidence in dogs exists that the degree of superior mesenteric artery occlusion, and thereby the residual blood supply, is of greater importance than the duration of superior mesenteric artery occlusion. Although previous studies advised initiating thrombolytic therapy within 8 to 10 hours of appearance of abdominal complaints, the reviewed reports and experimental data may suggest a longer window of opportunity for initiating thrombolytic therapy of acute superior mesenteric artery occlusion.

Duration. - The most criticized aspect of thrombolytic therapy for acute superior mesenteric artery occlusions is the long infusion time while ischemia continues, which may enhance the chance of bowel necrosis. The reported infusion times varied from bolus injection within minutes to continuous infusion up to 48 hours. Although flow was usually reestablished within the first hours, infusion continued until complete dissolution of the thrombus was achieved. Complete clot lysis may yield better results and aid the identification of underlying lesions. A somewhat arbitrary limit of 48 hours of thrombolytic therapy of lower extremity occlusion is usually applied, based on the observation that the rate of bleeding complications tends to rise exponentially after this point. Infusion was discontinued when abdominal symptoms worsened without evidence of thrombolysis (indicating the need for urgent surgical intervention), when bleeding complications arose, and when no further radiological improvement was achieved in comparison to the previous angiogram.

Efficacy

The primary efficacy variable was technical (angiographic) success, which was defined as resolution of superior mesenteric artery occlusion. Occlusion of the superior mesenteric
artery was distinguished in proximal and distal occlusion, defined as thromboembolism located proximal or distal to the middle colic artery. Resolution of the occlusion was defined as post-procedural, normal angiographic morphology of the vessel or minimal presence of clot remnants without compromising arterial flow. Procedure-related complications were scored separately. The secondary efficacy variable was clinical success, which was defined by the following criteria: angiographically demonstrated resolution of the occlusion, disappearance of abdominal pain during thrombolytic therapy, absence of peritoneal signs at physical examination, treatment without surgical intervention, and hospital discharge. The third efficacy variable was overall survival, which was defined as hospital discharge, with or without additional surgical intervention.

**Technical success.** - In 43 out of 48 patients technical success was obtained (Table 2). Failure of thrombolytic therapy occurred in five patients: 1) bolus infusion of 400,000U urokinase following distal superior mesenteric artery occlusion resulted in increased abdominal pain and subsequent embolectomy and 20 cm intestinal resection (pt. 15); 2) bolus infusion of 250,000U streptokinase followed by 12.5-25 mg/h rtPA infusion after proximal partial superior mesenteric artery occlusion resulted in partial clot resolution and in subsequent multiple mesenteric emboli with increasing abdominal pain and hypotension during the first 24 hours. Subsequently laparotomy and resection was required (pt. 18); 3) bolus infusion of 200,000U urokinase and subsequent continuous intraarterial infusion following distal partial superior mesenteric artery occlusion resulted in partial clot resolution (pt. 34). In this patient infusion was ceased after 15 hours when abdominal pain increased, necessitating laparotomy and resection of necrotic bowel and second look 24 hours later; 4) bolus infusion of 240,000U urokinase and subsequently continuous intraarterial infusion after total distal superior mesenteric artery occlusion resulted in patient deterioration (comatose and hypotensive) and demise (pt. 39); 5) continuous infusion of 50,000U/h urokinase after proximal total superior mesenteric artery occlusion subsequently required resection of necrotic small intestine and ascending colon (pt. 44). Four out of these five patients survived after laparotomy. Technical failure did not appear to be associated with duration of symptoms, localization of occlusion, agent, dose, or infusion protocol (Figure 3).

The chance of successful thrombolysis is dependent on the age of the embolus/thrombus, the best results being obtained within 72 hours of occlusion. All except one patient with
Thrombolysis of mesenteric occlusions

Acute superior mesenteric artery occlusion were treated within 72 hours, which may explain the high technical success rate; however, publication bias should be considered.

Abatement of abdominal pain during the first hour of treatment and a persistently normal abdominal examination may be important factors to indicate the success of thrombolytic therapy. However, despite immediate remission of the abdominal pain during local thrombolytic treatment, bowel necrosis may still occur and necessitate surgical resection, as evidenced in two patients in present data (pt. 2 and 14). Continuous monitoring of patient's clinical status and evaluation of abdominal symptoms for up to 72 hours is mandatory because of unpredictability in disease progression. When symptoms persisted or suspicious peritoneal signs on physical examination were present, intraarterial treatment was suspended despite angiographic improvement, and surgical intervention followed (pt. 7, 36 and 37).

Clinical success. - Despite the technical success in 43 out of 48 patients, complete clinical success was achieved in only 30 patients (Table 2 and 3). Conversely, clinical failure was present in 18 patients. Besides the abovementioned five patients in which thrombolytic treatment failed, another patient had successful thrombolytic therapy, with resolution of abdominal symptoms, but died within seven days because of coronary heart failure (pt 2). A further four patients developed increased abdominal pain, two of which after an initial reduction of abdominal pain. Surgery revealed necrotic bowel requiring bowel resection in all four (pt 2, 14, 32 and 35). In another eight patients, despite the achievement of technical success, exploratory laparotomy or laparoscopy was performed within 72 hours, all in which normal to mild ischemic bowel was found, however, that did not necessitate bowel resection.

Survival. - Thrombolytic therapy with or without additional surgical intervention resulted in survival of 43 out of 48 patients (Table 2 and 3). A total of five patients died as a result of acute thromboembolic mesenteric ischemia and related additional organ failure during hospital stay, only two patients after technical failure. Survival of 43 out of 48 patients with acute superior mesenteric thromboembolism, treated by thrombolytic therapy with or without additional surgery, is in great contrast with the overall reported, in-hospital mortality rates of 24-94% of acute mesenteric ischemia. As previously mentioned only a small number of all patients, suffering from acute mesenteric thromboembolism, is candidate for thrombolytic therapy. The lack of an unbiased control group reveals difficulties to interpret these data, when also considering selection and publication bias and the paucity of data on this subject. However, we may state that thrombolytic therapy in the reviewed patients prevented 30 out of 48 patients from surgery. Furthermore, 13 out of 16 patients survived subsequent surgical treatment. Two patients, in whom surgery was not indicated, died within several days after thrombolytic therapy. Whether unsuccessful thrombolytic therapy poses contra-indications or complications to surgical treatment cannot be drawn from these data.
Table 2. Outcome of thrombolytic therapy for acute superior mesenteric artery thromboembolism

<table>
<thead>
<tr>
<th>Pt. nr.</th>
<th>Drug</th>
<th>Pain relief</th>
<th>Technical success (h)</th>
<th>Complications</th>
<th>Laparotomy; Findings-procedure</th>
<th>Mortality / morbidity</th>
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<td>Yes, resection necrosis, 25cm</td>
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<td>Yes, &lt; 2h</td>
<td>Bleeding (cath.)</td>
<td>No</td>
<td>Died of MI (3d), no abdominal symptoms</td>
</tr>
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<td>3</td>
<td>SK</td>
<td>&lt;</td>
<td>Yes, 34-60h</td>
<td>No</td>
<td>No</td>
<td>Survived</td>
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<td>SK</td>
<td>&lt;</td>
<td>Yes, &lt; 36h</td>
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<td>No</td>
<td>Died of CHF, SVT (7d) no abdominal symptoms</td>
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<td>Yes, &lt; 30h</td>
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<td>Failed, &lt;1h</td>
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<td>Embolectomy, resection necrosis 20cm</td>
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<td>&lt;</td>
<td>Yes, 1-10h</td>
<td>No</td>
<td>No</td>
<td>Survived</td>
</tr>
<tr>
<td>27</td>
<td>UK</td>
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<td>No</td>
<td>No</td>
<td>Survived</td>
</tr>
<tr>
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<td>UK</td>
<td>&lt; 1</td>
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<td>No</td>
<td>No</td>
<td>Survived</td>
</tr>
<tr>
<td>29</td>
<td>UK</td>
<td>&lt; 1</td>
<td>Yes, 6-18h</td>
<td>No</td>
<td>No</td>
<td>Survived</td>
</tr>
<tr>
<td>30</td>
<td>UK</td>
<td>&lt; 1</td>
<td>Yes, 12-18h</td>
<td>No</td>
<td>No</td>
<td>Survived</td>
</tr>
<tr>
<td>31</td>
<td>UK</td>
<td>&lt; 1</td>
<td>Yes, 4-12h</td>
<td>No</td>
<td>No</td>
<td>Survived</td>
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<tr>
<td>32</td>
<td>UK</td>
<td>6</td>
<td>Yes, 24-28h</td>
<td>Yes, resection necrosis 40 cm at 72h</td>
<td>No</td>
<td>Survived, died of shock (20d), no abdominal symptoms</td>
</tr>
<tr>
<td>33</td>
<td>UK</td>
<td>&lt; 1</td>
<td>Yes</td>
<td>No</td>
<td>Yes, normal</td>
<td>Survived, SMA bypass (1 mo)</td>
</tr>
<tr>
<td>34</td>
<td>UK</td>
<td>&gt; 15</td>
<td>Partial</td>
<td>Infusion was stopped at 15h &gt; abd. pain</td>
<td>Yes, resection necrosis, second look 24h</td>
<td>Survived</td>
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<tr>
<td>35</td>
<td>UK</td>
<td>&gt; 8</td>
<td>Yes</td>
<td>Infusion was stopped at 8h &gt; abd. pain</td>
<td>Yes, resection necrosis, second look 24h</td>
<td>Survived</td>
</tr>
<tr>
<td>36</td>
<td>UK</td>
<td>&lt; 1</td>
<td>Yes, 12-20h</td>
<td>No</td>
<td>No</td>
<td>Survived</td>
</tr>
<tr>
<td>37</td>
<td>UK</td>
<td>&lt;</td>
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<td>No</td>
<td>No</td>
<td>Survived</td>
</tr>
<tr>
<td>38</td>
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<td>No</td>
<td>No</td>
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</tr>
<tr>
<td>39</td>
<td>UK</td>
<td>Failed</td>
<td>Shower emboli</td>
<td>No</td>
<td>Yes, atheroma</td>
<td>Died of shock (1d)</td>
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<tr>
<td>40</td>
<td>UK</td>
<td>Failed</td>
<td>Yes</td>
<td>No</td>
<td>Yes, atheroma</td>
<td>Survived, SMA bypass (1 mo)</td>
</tr>
<tr>
<td>41</td>
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<td>&lt;</td>
<td>Yes</td>
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<tr>
<td>42</td>
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<td>&lt;</td>
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<td>No</td>
<td>No</td>
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<tr>
<td>43</td>
<td>UK</td>
<td>&lt;</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>Survived, died of MI (1 mo)</td>
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<tr>
<td>44</td>
<td>UK</td>
<td>&lt;</td>
<td>Failed</td>
<td>Extravasation</td>
<td>Yes, resection necrosis</td>
<td>Survived, died of CI (1 mo)</td>
</tr>
<tr>
<td>45</td>
<td>UK</td>
<td>&lt; 5</td>
<td>Yes, 5-10h</td>
<td>No</td>
<td>No</td>
<td>Survived</td>
</tr>
<tr>
<td>46</td>
<td>rTPA</td>
<td>&lt; 3</td>
<td>Yes, 3-8h</td>
<td>No</td>
<td>No</td>
<td>Survived</td>
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<tr>
<td>47</td>
<td>UK</td>
<td>&lt; hrs</td>
<td>Yes</td>
<td>Reoclusion after 8h, successful thrombolysis, + thrombosis leg (32h)</td>
<td>No</td>
<td>Survived</td>
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<tr>
<td>48</td>
<td>UK</td>
<td>&lt; 1</td>
<td>Yes, 6-18h</td>
<td>No</td>
<td>Yes, exploratory 48h, normal shock, expl. 96h, normal</td>
<td>Survived</td>
</tr>
</tbody>
</table>

SK, streptokinase; UK, urokinase; rTPA, recombinant tissue plasminogen activator; <, decrease (within) / less than; <=, rapid decrease (duration was not reported); >, increase (within); =, equal; y, year; mo, month; d, day; h, hour; __, not reported; x, does not apply.
Thrombolysis of mesenteric occlusions

Survival following surgery. - How the data of survival and complications of acute superior mesenteric artery occlusion following thrombolytic therapy relate to the survival and complications following surgery is unknown. Most reports on acute intestinal ischemia demonstrate data compiled from all etiologic subsets taken together, including thrombotic and embolic arterial occlusion, venous occlusion and non-occlusive mesenteric ischemia. Recently, a systematic review was performed to evaluate survival of these etiological subsets. In addition, the authors have attempted to analyze the relation between survival and surgical treatment of each etiological subset individually. The surgical treatment was grossly divided into bowel resection, revascularization (e.g. thromboembolectomy), or resection and revascularization. Mortality rates of thrombotic and embolic superior mesenteric arterial occlusion, treated with revascularization alone, were 60 and 73%, respectively. Mortality rates in surgical series would anticipate being higher than thrombolytic therapy, as patients with necrotic bowel are included in the surgical series. However, there is neither data available on presence or absence of peritoneal signs, nor on patient characteristics, duration of symptoms, and postoperative complications. No conclusion should be drawn from these data, in comparison to thrombolytic therapy for acute superior mesenteric artery thromboembolism.

Safety

The safety variable was complications that ensued as a result of each procedure.

Bleeding complications. - Complications of thrombolytic therapy were bleeding or hematoma formation at the catheter puncture site occurring in two patients (pt. 2 and 25) (Table 2). Bloody diarrhea was seen in three patients (pt. 4, 5, and 24), hematuria or extravasation of infusion fluid occurred in one patient (pt. 25 and 44). In addition to the presence of bloody diarrhea or positive blood tests in the stools in 15 patients prior to thrombolytic therapy, bloody diarrhea persisted in one patient during thrombolytic treatment (pt. 22). This patient survived without requiring surgical intervention. Another patient (pt. 5), responding to thrombolytic therapy, developed bloody diarrhea, underwent subsequent exploratory laparotomy, which did not demonstrate ischemic or infarcted bowel, and survived.

Minor hemorrhage, mostly at the puncture site, occurs in 10-15% of patients treated with thrombolytic therapy for lower limb arterial occlusion, with a similar complication rate in the here reviewed series. Bloody diarrhea due to mucosal injury was not an indication to stop thrombolytic therapy, unless significant gastro-intestinal bleeding would have occurred. Major hemorrhage (causing hypotension and/or requiring blood transfusion) has not been reported during acute superior mesenteric artery occlusion treatment; however, it occurs in approximately 9% of patients with thrombolytic therapy for lower limb arterial occlusion.

Thromboembolic complications. - Symptomatic cerebral infarction or reversible renal embolization was noted in one patient (pt. 22 and 23) (Table 2). Two other patients died of multiple emboli (shower emboli) within 5 days of treatment (pt. 18 and 39). Recurrence of the superior mesenteric artery occurred in one patient after 8 hours (patient 47). This patient was successfully treated with repeated thrombolysis; however, a leg thrombosis occurred 32 hours later. Recurrence of superior mesenteric artery occlusion in present cases was not observed during follow-up (5 days – 54 months).
Distal embolization as a result of thrombolytic agent infusion has been reported in only four out of 48 patients (8%). This complication caused deterioration of the clinical status abruptly, with worsening of abdominal symptoms; conversion to surgical therapy was considered immediately. Distal embolization of poorly dissolved fragments of thromboemboli has been reported approximately in 12% of the lower extremity occlusions.

Table 3. Factors associated with outcome according to patient characteristics, occlusion and treatment characteristics.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Totals</th>
<th>Technical failure (n = 5)</th>
<th>Clinical failure (n = 18)</th>
<th>Mortality (n = 5)</th>
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<tr>
<td><strong>Patient characteristics</strong></td>
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<tr>
<td>Age &gt; 60 years</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Yes</td>
<td>38*</td>
<td>5</td>
<td>15</td>
<td>4</td>
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<tr>
<td>No</td>
<td>9</td>
<td>0</td>
<td>3</td>
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<td>17</td>
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<td>9</td>
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<td>Occlusion time &gt; 12 hrs</td>
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<td>15</td>
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<td>5*</td>
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<td>Proximal occlusion</td>
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<tr>
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<td>8</td>
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</tbody>
</table>

* missing data from one report
Considerations

Thrombolysis of acute mesenteric artery thromboembolism is a relatively new treatment modality and has potential to be effective and safe. Nevertheless, a few additional considerations should be made concerning this therapy.

Methodological quality. - All of the studies are of relatively low methodological quality (level V evidence), revealing the paucity of evidence on this subject. In case of the present study, there are potentially undesirable consequences when direct treatment actions are taken in response to weak evidence. Furthermore, the included patients are not representative for the whole population of patients with acute superior mesenteric artery thromboembolism. For example, the selection of patients with absent peritoneal findings may influence survival positively comparing data to patients with present peritonitis. Follow-up of included patients was in some cases limited to in-hospital stay. In the reported cases, in-hospital death caused by acute superior mesenteric artery thromboembolism may also be associated with underlying cardiovascular disease or septic shock, in addition to the presence of abdominal symptoms and ischemic or necrotic bowel. In addition, we feel that in the literature not all successes of thrombolysis are reported, nor are all failures. Therefore, data interpretation may carry a high publication and selection bias and caution should be considered to draw any conclusion.

Candidates. - Suspicion on mesenteric infarction is an absolute contraindication for thrombolytic treatment in acute mesenteric embolism or thrombosis when signs of peritonitis are present. Exploratory laparotomy, with subsequent thromboembolectomy and necrotic bowel resection as necessary, is then mandatory. Candidates for thrombolytic therapy of acute superior mesenteric artery thromboembolism will constitute only a small percentage of all cases. Simo and coworkers demonstrated that 18 out of 28 (65%) consecutive patients with angiographically diagnosed superior mesenteric artery embolism presented with clinical signs suggestive of intestinal infarction; consequently, viable intraarterial treatment was only possible in 10 patients (35%). Yamaguchi and coworkers reported only eight out of 30 (27%) consecutive patients who were selected to undergo local intraarterial thrombolysis for superior mesenteric artery embolism. In the reviewed reports, all candidates for thrombolytic therapy of acute superior mesenteric artery thromboembolism were carefully selected, and scrutinized for the absence of peritoneal signs on physical examination and lack of indications of bowel infarction on an abdominal X-ray (no pneumoperitoneum or ileus) or on CT-scan (no intramural air, ileus or ascites). Furthermore, in reviewed data, absolute and relative contraindications, reported for the use of thrombolytic therapy of vascular occlusions in the lower extremity by the Working Party on Thrombolysis in the Management of Limb Ischemia, were not violated.

Early diagnosis. - There is agreement on the importance of early diagnosis of superior mesenteric artery embolism as a means of lowering the high mortality rates associated with acute intestinal ischemia. Early diagnosis will decrease the risk of intestinal necrosis and the presence of peritoneal signs at physical examination. Obviously, early diagnosis will render thrombolytic therapy more effective.

Localization of occlusion. - Thromboembolism, whether localized proximally or distally in the superior mesenteric artery trunk, may result in intestinal necrosis, perforation and eventually peritonitis. Proximal superior mesenteric artery occlusions will, however, affect a greater bowel volume, especially when collateral circulation is absent. The most
serious cases, involving complete proximal superior mesenteric artery occlusion, with no distal vascularization, may nonetheless be treated successfully with intraarterial infusion if the diagnosis is made early (before 8 to 10 hours), as has previously been reported. Moreover, clinical success has also been achieved after the critical period of 8 to 10 hours of abdominal complaints (patient 15, 43, 44 and 48).

**Completeness of occlusion.** - Partial superior mesenteric artery occlusion in contrast to total superior mesenteric artery occlusion may result in less severe intestinal ischemia. Consequently, thrombolytic therapy of a partial superior mesenteric artery occlusion may result in better outcome. However, Yamaguchi and coworkers reported successful thrombolytic treatment of 7 out of 8 patients with total superior mesenteric artery occlusion, which has been supported by other authors. Therefore, the degree of occlusion may not be the most important factor to determine outcome of thrombolytic therapy for acute superior mesenteric artery thromboembolism.

**Embolic or thrombotic occlusion.** - More than half of all emboli are found just distal to the middle colic artery origin affecting a smaller bowel volume than proximal superior mesenteric artery embolism. Thrombotic superior mesenteric artery occlusions, however, are usually superimposed on a prior atherosclerotic stenosis at the origin of the superior mesenteric artery, which may worsen the prognosis. In general, the outcome of thrombotic superior mesenteric artery occlusion is worse compared to embolic superior mesenteric artery occlusion. Furthermore, prior to thrombotic superior mesenteric artery occlusion, patients experienced chronic abdominal pain from preexisting arterial stenosis in 30%, which may delay prompt intervention. Nevertheless, in herein reviewed reports, six patients with acute superior mesenteric artery occlusion of thrombotic origin all responded to thrombolytic therapy within two hours and survived. Acute superior mesenteric artery occlusion of thrombotic or embolic origin may therefore both be considered for thrombolytic treatment.

**Treatment Algorithm.** - The great variety in clinical presentation of acute mesenteric ischemia will always be a challenge for physicians, as early diagnosis plays a pivotal role in surviving intestinal ischemia. The Guidelines on Intestinal Ischemia by the American Gastroenterological Association may help the physician to diagnose and to treat patients with acute mesenteric ischemia. In this review, a suggested modified treatment algorithm for acute superior mesenteric artery thromboembolism is presented in Figure 2, including thrombolytic therapy.

**Conclusion**

Thrombolytic therapy of acute superior mesenteric artery thromboembolism is still to be considered as a relatively new treatment modality. Insufficient evidence is available from reviewed literature to determine the relative effectiveness and safety of thrombolytic treatment for acute superior mesenteric artery thromboembolism, however, initial results appear to be promising. The relative infrequency of acute mesenteric ischemia and the variation in clinical presentation constitute an almost insurmountable obstacle to undertaking randomized or case-control trials. Nevertheless, clinical guidelines (at least based on consensus) for thrombolytic treatment of acute superior mesenteric artery thromboembolism should be developed.
Thrombolysis of mesenteric occlusions

Figure 2: Treatment algorithm for acute superior mesenteric artery thromboembolism, including thrombolytic therapy (modified from the Guidelines on Intestinal Ischemia by the American Gastroenterological Association 79). Solid lines indicate accepted management plan; dashed lines indicate alternate management plan.
Chapter 3

References


Part II

Historical intermezzo
Chapter 4

Riolan’s anastomosis revisited

Thomas M. van Gulik¹ and Ivo G. Schoots¹.

Department of ¹Surgery and Surgical Laboratory, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands

A tribute to Riolan “the prince of the anatomists”
Abstract

Riolan’s anastomosis or arc is eponymously used to indicate the arterial anastomosis between the superior and inferior mesenteric arteries. Vascular as well as gastro-intestinal surgeons are well-acquainted with this collateral mesenteric pathway for retrograde perfusion of the superior mesenteric artery when the origin of the latter is occluded. The eponym suggests that Jean Riolan (1580-1657), a famous 17th century French anatomist, was the first to describe this arterial anastomosis. Riolan was a strong defender of traditional Galenic doctrine in medicine and therefore, proved a vigorous opponent of the new concept of the circulation of the blood as exposed by William Harvey (1578-1657). This makes it unlikely that Riolan would have conceived an arterial collateral pathway in the mesocolon, a notion confirmed by examining his anatomy book published in 1649. He probably had observed vascular arcades running along the inner border of the colon which later associated him with the collateral circulation of the mesentery. Other, well-known anatomical textbooks of his era show that mesenteric circulation was only poorly defined. It was not until 1743, that Albrecht von Haller (1708-1777) gave a detailed description of the anatomy of the mesenteric arteries, referring to the arterial collateral connection between the superior and inferior mesenteric arteries, as the “Arcus Riolani”, in honour of an old master of anatomy.
Introduction

The name of Riolan is eponymously attached to the arterial anastomosis between the superior and inferior mesenteric arteries. Eponyms in medical nomenclature usually bear reference to the author who first described an anatomical structure or functional concept. Hence, the eponym Riolan’s anastomosis suggests that Jean Riolan (1580-1657), a French anatomist, was the first to demonstrate the collateral arterial arcade that may occur in the colonic mesentery in the presence of occlusive abdominal arterial disease. It is paradoxical that this collateral anastomosis of the mesenteric arteries is named after Riolan, since Riolan was famous for his opposition to Harvey’s discovery of the circulation of the blood. This historical note focuses on Jean Riolan, a prominent but controversial 17th century anatomist, and concludes that this anatomist probably never observed the vessel that carries his name.

The “meandering mesenteric artery”

The collateral “meandering mesenteric artery” was described by Moskowitz as a thick wide vessel of uniform caliber with a tortuous course through the mesentery of the left colon, running more or less parallel to the mesenteric border of the colon. Besides Riolan’s anastomosis or arc, a variety of terms have been used to indicate this collateral pathway, including marginal artery, meso-mesenteric artery, middle colic-left colic collateral, artery of Drummond and arc of Treves.

Figure 1. Riolan’s anastomosis constituting the collateral arterial connection between the inferior mesenteric artery and the superior mesenteric artery in the event of occlusion of the origin of the superior mesenteric artery. (Department of Radiology, Academic Medical Center, Amsterdam, the Netherlands)
The “meandering mesenteric artery” that arises from the inferior mesenteric artery for retrograde perfusion of the superior mesenteric artery in the event of occlusion of the origin of the superior mesenteric artery is readily recognized on intestinal arteriograms and is the enlarged branch of the left colic artery connecting centrally with the middle colic artery \(^5\)\(^6\) (Figure 1). Both vascular and gastro-intestinal surgeons are aware of this collateral mesenteric pathway since inadvertent ligation of Riolan’s anastomosis may have disastrous consequences during operative procedures. In the presence of this collateral, reimplantation of the inferior mesenteric artery is recommended during surgery for aortic aneurysm lest blood supply of the superior mesenteric artery is jeopardized. For the same reason, Riolan’s anastomosis should be preserved or reconstructed during left colonic resection.

**Jean Riolan, the anatomist.**

Jean Riolan was born in 1580 in Paris. His father, Jean Riolan the elder (1538-1605), was a famous medical doctor who at one time was the dean of the Faculty of Medicine in Paris \(^7\)\(^8\). Like his father, Jean Riolan was a rigorous defender of traditional medicine and a strong proponent of Galen’s views \(^9\). He became a professor of anatomy and botany at the University of Paris and was appointed physician to the kings Henry IV and Louis XIII, and for 10 years was the physician of Marie de Medici. He was one of the most authoritative anatomists of his time and as a skilled dissector, added much to anatomical and embryological knowledge (Figure 2).

![Figure 2. Portrait of Jean Riolan from the frontispiece of his anatomy textbook, *Encheiridium anatomicum et pathologicum*, 1658.](image)

In anatomical nomenclature, Riolan’s marginal bundle of the orbicularis palpebrarum muscle, Riolan’s cremaster muscle and Riolans’s bouquet (muscles and ligaments arising
from the styloid process) testify to his many contributions to human anatomy\textsuperscript{1,10}. His opus magnum was the \textit{Anthropographia et osteologica}, published in 1618, and with the second edition of this work, Jean Riolan established his fame as a scholar and anatomist. In this book, Jean Riolan tried to reconcile new discoveries with the traditional medical framework as was dictated by Galen. In 1649, Jean Riolan published an anatomical textbook entitled \textit{Encheiridium anatomicum et pathologicum}, famed for its concise presentation of normal and morbid anatomy\textsuperscript{11} (Figure 3).

\textbf{Figure 3.} Title page of Riolan's \textit{Encheiridium anatomicum et pathologicum}, published in 1649 in Leiden, the Netherlands\textsuperscript{11}.

In the fourth edition of this work, published in 1658 in Paris, a chapter is included (\textit{Tractatus de anatome pneumatica}, p603-609) in which he used air injections to explore the topography of blood vessels\textsuperscript{12}. While injecting air through the umbilical vein of a fetus, he noticed that all the organs, arteries and veins swelled with air, from which observation he concluded that all arteries anastomosed with the veins. In this connection, he probably described the mesenteric vascular arcades running along the inner border of the colon, associating his name with the collateral circulation of the mesentery. It was also in this work that Jean Riolan for the first time reacted to the discovery of the circulation of the blood by William Harvey (1578-1657).

Jean Riolan who suffered from troublesome asthma and bladder stones, died at the age of 77, of urinary obstruction\textsuperscript{8}. Although reactionary, he was a great anatomist and was remembered as the last humanist and Galenist of the Paris medical school\textsuperscript{9}.
Riolan's anastomosis revisited

Riolan’s concept of the circulation of the blood

Galen of Pergamon in the second century (AD) was the first to describe arteries as carriers of blood. According to Galen, whose doctrine dictated medical thought for the next 14 centuries, blood passed from the right ventricle through the pulmonary artery to the lungs.

Figure 4. Tabula XII of Riolan's *Encheiridium anatomicum et pathologicum*, showing the venous system (I) and arterial system (IV). Y = celiac trunk, g = inferior mesenteric artery, y = superior mesenteric artery.
in which it mixed with air and became completely purified. It then passed through the pulmonary vein to reach the left chamber of the heart. Blood from the right ventricle passed through pores in the septum to the left ventricle in which blood and air elaborated into the “vital spirit”. The spirituous blood was then distributed throughout the body by the own pulsific properties of aorta and arteries. Riolan almost religiously continued the medicine taught by Galen, as all the philosophers, naturalists and physicians of ancient Greece and Rome governed his thinking. In the context of mesenteric circulation, it is noteworthy that according to Riolan’s views, the blood in the abdomen, whether in the portal vein, the splenic, celiac, or other arteries did not circulate at all. It is therefore unlikely, that Riolan would ever have conceived the presence of a collateral arterial pathway in the mesentery of the colon.

Jean Riolan, Harvey’s foremost adversary.

Jean Riolan was a contemporary of William Harvey, who in his seminal treatise on the circulation of the blood, De motu cordis published in 1628, contradicted Galen’s view on the movement of air and blood to the heart. Riolan was strongly opposed to the principle of the circulation of blood disclosed by Harvey, which according to the latter stemmed from his fear of destroying traditional medicine. Riolan was the only opponent of the new concept of the circulation of blood, whom Harvey deemed worthy of an answer and who he referred to as the “prince of anatomists”.

![Figure 5. Tabula IX-I of Riolan’s Encheiridium anatomicum et pathologicum, showing the mesentery. d = rami arteriarum mesentericarum, c = rami venae portae mesentericae](#)
Even Thomas Bartholin, the Danish anatomist who favored the new ideas on the circulation of the blood, dedicated his book on the discovery of the lymphatic vessels to Riolan, whom he referred to as "the greatest anatomist on earth and in Paris". In response to the theory proposed by Harvey, Riolan came up with his own concept of the circulation of the blood, which he named "circulatio Riolana" as opposed to the "circulatio Harveiana". Riolan’s circulation maintained the Galenic vessels, as the arterio-venous anastomoses and the passages through the cardiac septum. Being a true Galenist, his main concern was to preserve the ancient art of healing while publicly disagreeing with Harvey’s doctrine. The concept of circulation had developed over two millennia, according to Riolan: Hippocrates conceived, Harvey discovered, and Riolan corrected the circulation.

The mesenteric arterial blood supply according to Jean Riolan.

Examination of Riolan’s anatomy textbook, Encheiridium anatomicum, tells us more about Riolan’s perception of the mesenteric vasculature. Figure 4 depicts the venous and arterial system in Riolan’s Encheiridium, showing schematically but very clearly, the celiac trunk, the superior mesenteric artery and the inferior mesenteric artery. These three main intestinal arterial trunks are shown as separate arterial trees, without any arterial interconnection whatsoever. His description of the mesentery is, however, more elaborate than most of the other anatomy textbooks available in the 17th century (Figure 5). Riolan distinguished four types of vessels in the mesentery, i.e. tributaries of the portal vein, lacteals, arteries and nerves. Again there is no mention of any collateral circulation between the superior mesenteric artery and the inferior mesenteric artery.

In the beginning of the 18th century, Galenism had further lost ground in favor of pathological anatomy and physiology. In The Netherlands, the University of Leiden had become one of the leading medical centers in Europe owing to the reputation of the world famous physician and scientist, Herman Boerhaeve (1668-1738). The most important anatomy textbooks of that era were written by the Dutch anatomists Adriaan van der Spiegel (1578-1625), Govert Bidloo (1649-1713) and Bernard Siegfried Albinus (1697-1770). Examination of these anatomy books learns that even in that time of new medical enlightenment, vascularization of the mesentery was only poorly defined, and no reference is made to a collateral arterial pathway in the mesentery of the colon (Figure 6 and 7). (The anatomy book of Albinus only depicts the muscles and bones of the human body.)

Haller’s “arcus magnus mesentericus”

It eventually was a pupil of Herman Boerhaeve, Albrecht von Haller (1708-1777), who would precisely disclose the blood supply of the colon. After Harvey’s principles on the circulation of the blood had become widely accepted, Albrecht von Haller was the first to produce a detailed account of the anatomy of the mesenteric arteries in his monumental work, Icones Anatomicae, published in 1743. Albrecht von Haller, a famous Swiss anatomist and the greatest physiologist in his time, showed that the primary blood supply of the colon is from branches of the superior and inferior mesenteric arteries. He described that both arterial mesenteric trunks were connected by an anastomotic artery, termed the “arcus magnus mesentericus” (Figure 8).
Figure 6. Tabula II (Lib.V) of Adriaan van der Spiegel’s (Adrianus Spigelius) anatomy book De humani corporis fabrica, 1627, showing the greater omentum, the colon and mesocolon held up. The vascular loop (D) with radiating branches depicted in the mesocolon is referred to in the accompanying text as “tributaries of the portal vein, derived from the whole colon” (Venae portae ramus, per totum colum derivatus).
Figure 7. Illustration ("Veertigste aftekenings") from the anatomy book of Govard Bidloo’s *Ontleding des menschelijken lichaams* (Dissection of the human body), 1690 showing the mesentery of the colon reflected open. The capital letters (K, L, M) inserted in the mesocolon refer to fatty and lymphatic structures.
It is assumed that to honor the old anatomist Riolan, who previously pointed out the arterio-venous anastomoses in the mesentery, Haller preferred to use an alternative name, i.e. "Arcus Riolani" to indicate the arterial collateral connection between the superior mesenteric and inferior mesenteric artery, via the middle colic artery. In English nomenclature, this arc is referred to as Riolan’s arc whereas in German and Dutch nomenclature, the arc is usually referred to as Riolan’s anastomosis.

Figure 8. The “arcus magnus mesentericus” as depicted by Albrecht von Haller in his Icones Anatomicae (Tabula arteriarum mesentericarum), published in Leiden in 1743. Note the detailed representation of the middle colic artery, the left colic artery and the collateral vessels that follow the mesenteric border of the colon to interconnect both arterial vessels.
Epilogue

From the aforesaid, it can be concluded that Jean Riolan could not have perceived an arterial collateral arcade in the mesentery of the colon, such as the mesenteric anastomosis named after him. An anastomosis of that kind did not fit with his strong Galenic and anti-Harveian views of the circulation of the blood. He did describe arterio-venous anastomoses in the mesentery and probably noted vascular arcades running along the inner border of the colon. Albrecht von Haller who was the first to establish the arterial collateral pathway between the superior mesenteric and inferior mesenteric artery, being familiar with all the works of Riolan, dedicated this collateral anastomosis to Jean Riolan, who he once called the “prince of anatomists”, in honour of this remarkable old master of anatomy.
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Part III

Experimental studies
Chapter 5

Coagulation and inflammation in ischemia and reperfusion injury

Ivo G. Schoots\textsuperscript{1,2}, Marcel Levi\textsuperscript{2} and Thomas M. van Gulik\textsuperscript{1}

Departments of \textsuperscript{1}Surgery and Surgical Laboratory, and \textsuperscript{2}Vascular and Internal Medicine, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands

Cross-talk between coagulation and inflammation also in ischemia and reperfusion syndromes
1. Ischemia and reperfusion

Oxygen deprivation is a frequently phenomenon in clinical practice. Oxygen deprivation or hypoxia occurs in diseases of pulmonary insufficiency, in vascular disorders, and in cardiogenic/hypovolemic or septic shock. The arterial blood supply suffices the oxygen demanding aerobic cells. Compromising this blood supply profoundly leads to ischemia and tissue injury. Ischemia is defined as a negative imbalance between blood supply and oxygen tissue demand. Increasing degrees of ischemia produce progressive injury over time. While ischemia clearly plays an important role in organ injury, much of the organ injury is sustained not only during the period of ischemia itself, but at reperfusion. 

Microvascular circulation plays an important role in the deleterious consequences of ischemia and reperfusion. Vasoconstriction and increased peripheral resistance in response to ischemia and reperfusion may lead to microvascular dysfunction and endothelial injury. The endothelium, as an interface between blood and the vascular wall, is relatively insensitive to oxygen deprivation; most likely as a result of continuously change in environmental perturbations. However, brief periods of hypoxia result in diminished endothelial homeostatic mechanisms and activation of endothelial signaling pathways. Hypoxia-induced molecular and biochemical changes in the endothelial cell layer simulate a post-ischemic inflammatory response. Simultaneously, these changes affect the balance of the coagulation and fibrinolytic systems leading to a procoagulant state. There are many similarities between the host inflammatory response and the vascular response to hypoxia or ischemia. Endothelial cells subjected to hypoxia or ischemia demonstrate a similar response to infection, such as sepsis. Many pathological conditions relevant to clinical medicine are not primarily driven by a hypoxic or ischemic primary event, but rather by the inflammation triggered as a secondary response. In this review we will briefly outline current knowledge on the crosstalk between coagulation and inflammation, which is associated with microvascular dysfunction following hypoxia or ischemia and reperfusion, and the potentially beneficial effects of restoration of physiological anticoagulant pathways.

2. Endothelium-leukocyte interaction in ischemia and reperfusion

2.1. Differences in endothelial cell dysfunction in microvascular tree

The detrimental effects of ischemia and reperfusion are similarly induced to all endothelial cells in the microvasculature, however, the endothelial dysfunction appears to vary among the vascular tree of arterial, capillary, and venous segments, within the microcirculation.

The ischemia and reperfusion-induced endothelial cell dysfunction in arterioles is primarily characterized by an impaired endothelium-dependent, nitric oxygen-mediated relaxation of smooth muscle cells. The endothelial cell dysfunction in posts ischemic capillaries is manifested by enhanced capillary fluid filtration into the interstitium and reduced number of perfused capillaries. The enhanced fluid filtration is most likely a result of increased hydraulic conductivity, rather than an increased intracapillary pressure. Decreased capillary perfusion appears to result from the obstructive plugging of stiffer activated leukocytes in the capillaries, together with disruption of the endothelial layer, demonstrated by swollen and detached endothelial cells. The disrupted endothelial layer, fastened by the release of endothelial and leukocyte dependent reactive oxygen species.
production, results in a diminished barrier function and subsequent leakage of proteins and fluid into the interstitium (oedema), compromising the microvasculature furthermore. Endothelial cell dysfunction in postcapillary venules accounts for most of the ischemia and reperfusion-induced inflammatory responses. This is characterized by leukocyte-endothelial cell adhesion, platelet-leukocyte aggregation, increased vascular permeability (albumin extravasation) and production of reactive oxygen species. The ischemia and reperfusion-induced activated mast cells and macrophages, localized in the interstitial space adjacent to the postcapillary venules, enhance the inflammatory responses to ischemia and reperfusion.

2.2. Production of reactive oxygen species

The net catabolism of ATP during ischemia results in the accumulation of increased concentrations of purines, hypoxanthine and xanthine. In endothelial cells, hypoxia or ischemia promotes the limited proteolytic conversion of NAD-reducing xanthine dehydrogenase to oxygen-reducing xanthine oxidase (Figure 1). At reperfusion, oxygen is added suddenly and in excess, and the xanthine oxidase-catalyzed reactions proceed rapidly, thereby generating superoxide and triggers a characteristic free radical chain reaction. These radicals and other reactive oxygen species may cause injury directly, but primarily signal the up-regulation of endothelial surface adhesion molecules, especially of P-selectin and intercellular adhesion molecule (ICAM)-1. These reactive oxygen species thereby promote the arrest, adhesion, accumulation, and migration of circulating leukocytes, which amplify the inflammatory response to ischemia-reperfusion only in part through free radical mechanism. Furthermore, the increased vascular permeability in response to ischemia and reperfusion, reflecting the destruction of the endothelial cell-layer in post-ischemic venules, is associated with reactive oxygen species production and leukocyte-endothelial cell adhesion.

2.3. Production of pro- and anti-inflammatory cytokines

Activated endothelium is able to produce many cytokines following oxidative stress. Platelet-activating factor (PAF), which functions as a potent leukocyte activator, was
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identified on hypoxic-stimulated endothelium. Interleukin (IL)-1, as a multifactorial pro-inflammatory cytokine, was also synthesized by endothelial cells during hypoxia. IL-1 induces the expression of adhesion molecules (E-selectin and ICAM-1) on endothelial cells and attracting and activating leukocytes. The expression of hypoxia-induced IL-8 enhances the chemotactic migration and activation of neutrophils, thereby promoting the leukostasis in hypoxic organs. The induction of monocyte chemotactic protein (MCP)-1 in endothelial cells during hypoxia also appears to serve as a strong stimulus for monocytes recruitment. The hypoxia-induced synthesis and release of IL-6 by endothelial cells in vitro appears to have an anti-inflammatory potential in the setting of hypoxia, suppressing IL-1 and tumor necrosis factor (TNF)-α production by macrophages.

2.4. Expression of adhesion receptors

Hypoxia leads to increased expression of adhesion receptors on the surface of both endothelial cells and leukocytes promoting endothelial adheriveness. Leukocyte-influx in post-ischemic tissue is a process of margination, rolling, adherence and migration of leukocytes. Adhesion molecules from the selectin family mediate the margination and rolling of the neutrophils. Within one hour after oxidative stress, the most important receptor of leukocyte rolling, P-selectin (stored in Weibel-Palade bodies), translocates to the surface of endothelial cells. Leukocyte adherence is further established by E-selectins and ICAM-1 on endothelial cells and L-selectins and β2-integrins on activated leukocytes during hypoxia. Activation of leukocytes increases the binding affinity of β2-integrins to endothelial cell receptors, such as ICAM-1, or to fibrinogen, which can bind simultaneously to ICAM-1 and to the β2-integrin Mac-1. Transendothelial migration of leukocytes is modulated by platelet endothelial cell adhesion molecule (PECAM), identified on endothelial cells. Vascular endothelial-cadherin is involved in the regulation of endothelial permeability and transendothelial migration. This adhesion molecule, which is expressed exclusively at inter-endothelial cell-cell junctions, modulates the morphology of the endothelial cell layer. Alterations in inter-endothelial cell-cell junctions have been observed during hypoxia, unfolding interendothelial junctions for leukocyte migration.

2.5. Leukocyte activation at the site of injury

Activated polymorphonuclear leukocytes and monocytes play a central role in the hypoxia-induced tissue injury. After migration into interstitial space, they release cytotoxic enzymes, acids and reactive oxygen species which elicit and amplify a more pronounced inflammatory response. In addition, the local expression of tissue factor (TF) and plasminogen activator inhibitor (PAI)-1 by recruited mononuclear cells contributes to the prothrombotic and fibrinolysis-suppressed environment, which is characteristic for ischemic tissue. This pronounced post-ischemic inflammatory response, which started as a protective mechanism, has become deregulated, and amplifies inflammation in an uncontrolled chain reaction, which further injures endothelium and parenchymal cells.

3. Platelet-endothelium-leukocyte interaction in ischemia and reperfusion

Growing evidence suggests a role for platelets in the pathogenesis of ischemia-reperfusion injury. Platelets are best known for their role in primary hemostasis in injured
endothelium by the formation of aggregates. Activation, adhesion and accumulation of platelets in the post-ischemic microcirculation may generate oxygen radicals and release pro-inflammatory mediators, such as thromboxane A2, leukotrienes, serotonin, platelet factor 4, and platelet derived growth factor (PDGF). In addition, the activation, adhesion and accumulation of platelets to the post-ischemic endothelial surface may initiate endothelial cell damage and contribute to leukocyte activation and recruitment at the site of the injury.

Platelets, similar to leukocytes, role along and adhere to the microvascular endothelium at maximum during the first minutes of post-ischemic reperfusion. Hence, platelets are among the first cells recruited at the site of the injury, and may play a key role in initiating post-ischemic reperfusion injury. Platelet-endothelial cell interactions are prominent in venules as in arterioles, in contrast to leukocyte-endothelial cell interactions which are primarily restricted to venules.

3.1. Platelet thrombus formation on subendothelial surface
Platelet thrombus formation following ischemia and reperfusion occurs on subendothelial surface. Platelets translocating on subendothelial von Willebrand factor (vWF) arrest and recruit additional platelets into growing thrombi. Adhesive bonds between platelets and the post-ischemic endothelial surface are formed by high densities of glycoprotein Ibα (GPIba) on platelets and of vWF on subendothelial tissues. Stable adhesion to the endothelium requires binding of integrin αIIbβ3 to immobilized vWF, fibrinogen, and other ligands, and by binding of integrin α5β1 to fibronectin, leading to platelet cohesion and thrombus growth.

3.2. Platelets adhesion molecules
Platelet adhesion to the exposed subendothelial matrix via integrins, which becomes manifest when the endothelium is denuded, may differ from platelet adhesion to activated endothelial cells following ischemia and reperfusion. Important platelet-adhesion molecules in mediating platelet adhesion to subendothelial matrix proteins are P-selectin, PECAM-1 and several integrins (glycoprotein IIB/IIIa, lymphocyte function antigen (LFA)-1). P-selectin, stored in α-granules of platelets and Weibel Palade bodies of endothelial cells, is rapidly mobilized to the surface, as demonstrated both in vitro and ex vivo experiments following hypoxia and reoxygenation or thrombin that is generated during ischemia and reperfusion. Ischemia and reperfusion-induced platelet-endothelial cell interactions are mediated via endothelial P-selectin, whereas platelet P-selectin promotes platelet interactions with leukocytes. Furthermore, adhesion between platelets of stable platelet aggregates may be explained by enriched P-selectin leading to firm platelet-platelet contacts.

3.3. Platelet-leukocyte aggregation
Platelets and leukocytes colocalize in infarcted tissue, indicating that platelets contribute to the ischemia and reperfusion-induced inflammatory response. Rolling leukocytes may use the β2 integrin Mac-1, and to a lesser extent, LFA-1 (CD11b/CD18), to adhere firmly to and transmigrate across surface-adherent platelets. This indicates that platelets are actually involved in the multi-step process of ischemia/reperfusion-induced leukocyte accumulation and extravasation. Activated platelets adherent to endothelium may recruit
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flowing leukocytes through P-selectin-PSGL-1 interactions, and then transfer these leukocytes to the endothelial cell surface where they can interact with endothelial selectins. Conversely, leukocytes adherent to activated endothelial cells may recruit circulating activated platelets through P-selectin-PSGL-1 interactions. Leukocytes also accumulate on platelet thrombi that have deposited on subendothelial tissues at sites of vascular damage. Platelet microparticles generated at sites of injury express P-selectin, which may bridge adjacent leukocytes and increase accumulation of rolling leukocytes on endothelial selectins at sites of inflammation. Leukocytes also aggregate with circulating platelets after exposure to ischemia and reperfusion. The interplay between platelets and leukocytes induces nuclear translocation of nuclear factor (NF)-κB, which results in enhanced CD11b/CD18 expression and the generation of MCP-1 and superoxide anions. Platelet-secreted pro-inflammatory and chemotactic mediators also contribute to endothelial and leukocyte signalling following ischemia and reperfusion.

3.4. Platelet-coagulation activation
If rolling platelets become activated, they may develop procoagulant surfaces. P-selectin may play an important role in amplifying coagulation, fibrin formation and thrombosis in vivo. The potential recruitment of leukocytes by P-selectin may potentiate the expression of tissue factor. Furthermore, P-selectin may promote the expression of tissue factor on monocytes. Tissue factor activates the extrinsic pathway of coagulation leading to a procoagulant phenotype with fibrin formation.

4. Coagulation and fibrinolysis in ischemia and reperfusion

4.1. Procoagulant state
The endothelium is a dynamic interface that modulates all aspects of vascular homeostasis. In physiological environment, the endothelium is phenotypically a non-thrombogenic surface and is capable of balancing the pro- and anticoagulant mechanisms which prevent intravascular coagulation. However, hypoxia can disturb the endothelial balance from a non-thrombogenic into a prothrombotic state. The coagulant properties, underlying the regulation of vascular homeostasis, may be changed by several mechanisms when vessels are exposed to hypoxia/ischemia, leading to tissue factor expression and fibrin formation (Figure 2).

4.2. Expression of tissue factor
Tissue factor, a strong prothrombotic stimulus, appears to be the initiator of fibrin formation. The normal vessel wall has very low levels of tissue factor (TF), which increases towards the adventitia. Mononuclear cells have been accepted as the cell type most responsible for expressing substantive amounts of TF within the intravascular lumen in response to stress stimuli, although the polymorphonuclear leukocytes and endothelial cells have also been shown to produce tissue factor in selected settings.

Oxygen deprivation results in increased TF expression and subsequently fibrin deposition. Tissue factor appears to be expressed by mononuclear cells. Colocalization of increased tissue factor with mononuclear cells was demonstrated in lungs of hypoxic wild-type mice. Additionally, antibody-induced depletion of monocytes
Ischemia Reperfusion

- Generation of reactive oxygen species
- Production of pro- and antiinflammatory cytokines
- Expression of adhesion receptors
- Activation of neutrophils

Generation of thrombin mediated by tissue factor

- Impairment of anticoagulation pathways

- Suppression of fibrinolysis by PAI-1

- Low levels of antithrombin
- Impaired function of protein C system
- Insufficient TFPI

Formation of fibrin

Inadequate removal of fibrin

Thrombosis of small and midsize vessels

Figure 2. Pathogenetic pathways involved in thrombosis following ischemia-reperfusion. In ischemia-reperfusion, fibrin is formed as a result of the generation of thrombin mediated by tissue factor. Tissue factor, expressed on the surface of activated mononuclear cells and endothelial cells, binds and activates factor VII. The complex of tissue factor and factor VIIa can activate factor X directly or indirectly by means of activated factor IX and factor VIII. Activated factor X, in combination with factor V, can convert prothrombin to thrombin. Simultaneously, all three physiologic means of anticoagulation—antithrombin, protein C, and tissue factor-pathway inhibitor (TFPI)—are impaired. The resulting intravascular formation of fibrin is not balanced by adequate removal of fibrin because endogenous fibrinolysis is suppressed by high plasma levels of plasminogen-activator inhibitor type 1 (PAI-1). The high levels of PAI-1 inhibit plasminogen-activator activity and consequently reduce the rate of formation of plasmin. The combination of increased formation of fibrin and inadequate removal of fibrin results in small and midsize vessel thrombosis.

In cell-blood contact, TF expression by monocytes recruited to the vessel wall is induced by the action of several compounds, including cytokines, C-reactive protein, and advanced glycosylated end products. However, evidence is emerging that endothelial...
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Cells also play an important role in the generation of TF during sepsis. Various cytokines (such as TNF-α and IL-1) have been found to induce TF expression in vascular endothelial cells in vitro. It is likely that in situations of tissue trauma (i.e. extensive surgery, ischemia and reperfusion injury, and burns) TF is expressed constitutively at the site of injury and may contribute to procoagulant stimulation.

4.3. Thrombin generation and fibrin deposition

Ischemia and reperfusion-induced endothelial cell activation promotes the expression of tissue factor and inhibits thrombomodulin activity, leading to thrombin formation and fibrin deposition. Thrombin (factor IIa) is the terminal serine protease of the coagulation pathway, which cleaves fibrinogen into fibrin, and has the ability to activate platelets. The accumulation of fibrin has been demonstrated following myocardial, lung, hepatic, renal, intestinal and cerebral ischemia in different experimental models.

A brief period of ischemia alone in a normal vessel is not sufficient to promote fibrin formation. Therefore, ischemia associated thrombosis is speculated to result from ischemia-induced changes of the microvascular microenvironment, including diminished aerobic metabolism, accumulation of waste products and activated inflammatory response. In the setting of ischemia, the most striking injury occurs during reperfusion. Mechanisms underlying hypoxia-related thrombosis are set in motion during the ischemic period and are exaggerated during reperfusion.

Fibrin deposition on the endothelial surface occurs directly after the onset of reperfusion, as well as the recruitment of platelets. Ischemia and reperfusion-induced fibrinogen degradation, leading to fibrin deposition on the vessel wall might be responsible for post-ischemic platelet adhesion to endothelial surface. The platelet receptor αIIb/β3 integrin appears to bind to fibrinogen and initiates platelet adhesion to the post-ischemic wall. In addition, increased fibrinogen-binding affinity of the GPIIb/IIIa complex of activated platelets initiates firm and irreversible platelet adhesion. As a result of platelet-endothelial aggregates and fibrin deposition, post-ischemic reperfusion may lead luminal narrowing and eventually reocclusion.

4.4. Activation and suppression of fibrinolysis

The physiologic activators of the fibrinolytic system are tissue-type plasminogen activator (t-PA) and urokinase-like plasminogen activator (u-PA). Both serine proteases are capable of converting plasminogen to plasmin.

Inhibition of the fibrinolytic system is an important factor in the pathogenesis of fibrin deposition during ischemia and reperfusion. Fibrinolytic activators and inhibitors are synthesized and stored in endothelial cells. Although the initial response in bacteremia, endotoxemia and recently in ischemia and reperfusion (this thesis) is an increase in fibrinolytic activation (mediated by the almost immediate release of plasminogen activators), this is only short lived and is rapidly shut off by a sustained increase in the main inhibitor of fibrinolysis, plasminogen activator inhibitor-1. TNF-α and IL-1 increase the plasminogen activator inhibitor (PAI)-1 synthesis or release from endothelial cells and also decrease plasminogen activator synthesis.

The appearance of intravascular fibrin deposition following hypoxia is also associated with diminished fibrinolysis. The fibrinolytic system demonstrated increased expression of PAI-1 and suppression of plasminogen activators following hypoxia or
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ischemia, promoting pulmonary or intestinal vascular fibrin deposition. PAI-1 overexpression is likely to be an important factor preventing normally active fibrinolytic mechanisms, which are required to reduce the extent of intravascular fibrin accumulation during hypoxia or ischemia. Monocytes in hypoxic lung pointed to be an important contributor to PAI-1 expression.

4.5. Relevance of fibrin deposition and vascular occlusion

The generation of procoagulant pathways, as well their interactions with platelets and leukocytes, in the microvasculature may lead to intravascular fibrin formation, which in turn, may cause occlusion of the smaller vessels. Local promotion of clotting would serve to isolate an ischemic area. The negative impact on vascular fibrin deposition eventuating in occlusive thrombosis can have obvious deleterious consequences. Although promotion of clotting might wall off a hypoxemic area, vascular fibrin formation could also limit blood flow and promote necrosis in distal tissue. A stronger procoagulant endothelial phenotype, thereby, leads to prolonged microvascular occlusion by fibrin deposition, with enhanced microvascular coagulopathy.

Intravascular fibrin deposition, localized in a specific organ, does not per se lead to overt organ damage except for transient evidence of inflammation. Furthermore, this process is reversible under certain conditions as a result of fibrinolytic clearance of the microvasculature. In addition to intravascular fibrin formation, fibrin may be transferred to extravascular, where it may, in turn, be deposited.

It remains to be seen how the presence of fibrin influences the adjacent tissue and whether inflammation and clotting may facilitate local apoptosis and tissue damage. It is believed that not fibrin formation, but rather the generation of serine proteases and their potential interactions with pro-inflammatory mediators may contribute to post-hypoxic or post-ischemic injury, organ failure and death. Whether preventing fibrin formation per se is helpful in limiting organ damage remains to be established.

5. Anti-coagulant mechanisms in ischemia and reperfusion

TF expressed at the cell surface can interact with factor VII (non-activated) or VIIa (activated). The TF-factor VIIa complex catalyzes the conversion of factors IX and X into IXa and Xa (Figure 3). These factors enhance the activation of factor X and prothrombin, respectively. The initiation of coagulation in many organs during ischemia and reperfusion is primarily mediated by the TF-factor VIIa pathway, leading to thrombin and fibrin generation.

Control of thrombin generation is constrained by three main regulating pathways, i.e. the antithrombin system, protein C system and TF pathway inhibitor (TFPI). However, these three major anticoagulant mechanisms, aiming to reduce the procoagulant state as a result of endothelial dysfunction, appears to be ineffective in inhibiting thrombin generation following ischemia-reperfusion.

5.1. Antithrombin system.

The serine protease inhibitor antithrombin is the principal inhibitor of thrombin (factor IIa) and factor Xa by forming 1:1 inactive protease-protease inhibitor complexes. During severe infection, antithrombin levels are low because of consumption, impaired synthesis,
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Figure 3. Coagulation is initiated by a tissue factor (TF)–factor VIIa complex that can activate factor IX or factor X. At high tissue factor concentrations, factor X is activated primarily by the TF-VIIa complex, whereas at low tissue factor concentrations the contribution of the factor IXa–factor VIIIa complex to the activation of factor X becomes more pronounced. Tissue factor–dependent coagulation is rapidly inhibited by tissue factor–pathway inhibitor (TFPI). Coagulation is maintained through the activation by thrombin of factor XI. Through the intrinsic tenase complex (factors IXa and VIIIa) and the prothrombinase complex (factors Xa and Va), the additional thrombin required to down-regulate fibrinolysis is generated by the activation of thrombin-activatable fibrinolysis inhibitor (TAFI). Activated TAFI down-regulates fibrinolysis by removing from partially degraded fibrin C-terminal lysine residues that are involved in the binding and activation of plasminogen. The coagulation system is regulated by the protein C pathway. Thrombin activates protein C in the presence of thrombomodulin. Together with protein S, activated protein C (APC) is capable of inactivating factors Va and VIIIa, which results in a down-regulation of thrombin generation and consequently in an up-regulation of the fibrinolytic system. The activity of thrombin is controlled by the inhibitor antithrombin.

and degradation by elastase from activated neutrophils. Following ischemia-reperfusion, local sustained reduction of antithrombin has been demonstrated in animal models. The reduction appears to be dependent on the severity of ischemia. Reduced antithrombin levels have been demonstrated in patients with coronary artery disease, unstable angina, and first-event ischemic stroke. Thrombin plays a pivotal role in the activation and propagation of coagulation activation. Hence, antithrombin theoretically plays a major role in containing the derangement of coagulation in inflammation or ischemia and reperfusion. However, there is ample evidence that antithrombin is incapable of adequate regulation of thrombin activity under these pathological circumstances due to low levels of antithrombin in the systemic (in sepsis) or local (ischemia-reperfusion) circulation. Several mechanisms may be responsible for these low antithrombin levels. First, antithrombin levels are continuously consumed by ongoing formation of thrombin and other activated proteases that are susceptible to antithrombin complexation. High circulating levels of thrombin-antithrombin complexes and other protease-antithrombin complexes in patients with generalized intravascular coagulation or acute myocardial
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Infarction support this premise. Second, antithrombin is degraded by elastase, which is released from activated neutrophils. Third, extravascular leakage of this protease inhibitor as a consequence of (ischemia-reperfusion-induced) capillary leakage may further contribute to the reduced levels of antithrombin. Finally, a reduction in heparin sulfate and related glycosaminoglycans in the perturbed matrix surrounding the endothelium following ischemia-reperfusion, may also lead to reduced antithrombin function, because glycosaminoglycans may act as a physiologic heparin-like cofactor of antithrombin.

5.2. Protein C system.
Activated protein C (APC) is formed from protein C when thrombin binds to the endothelial surface-associated thrombomodulin. Endothelial cells, primarily of large blood vessels, express an endothelial protein C receptor (EPCR) that augments the activation of protein C at the endothelial surface. Protein S serves as an essential cofactor when activated protein C decelerates the coagulation cascade by inactivating factors Va and VIIIa by proteolytic cleavage. The protein C system is impaired in situations of endothelial dysfunction. First, enhanced consumption and vascular leakage may result in low localized levels of protein C. In addition, activation of the cytokine network, in particular TNF-α, resulted in a marked down-regulation of thrombomodulin and the protein C receptor on endothelial cells, thereby prohibiting adequate protein C activation. Finally, cofactor protein S may also be affected during endothelial dysfunction. In plasma, 60% of the cofactor protein S is complexed to a complement regulatory protein, C4b binding protein (C4bBP). The anticoagulant capacity of protein C is enhanced by the free fraction of protein S. Increased plasma levels of C4bBP, as a consequence of the acute phase reaction, may result in a relative protein S deficiency contributing to a further procoagulant state. The ability of activated protein C to reduce thrombin generation (e.g. in ischemia-reperfusion) may potentially inhibit the proinflammatory response induced by thrombin.

5.3. Tissue factor Pathway Inhibitor (TFPI).
A third inhibitory mechanism of thrombin generation involves TFPI, which exists in several pools, either endothelial cell associated or lipoprotein bound in plasma. Most TFPI is bound to the vessel wall, and only 10% to 25% is found in circulating blood. This molecule binds to factor Xa within the TF-VIIa-Xa-complex. The relevance of TFPI in the coagulopathy in pathological settings appears to be inhibition of disseminated intravascular coagulation and multi organ failure, most likely due to reduced IL-6 generation.

Although clinical studies in septic patients have not provided clues to its importance because in the majority of patients the levels of TFPI are not diminished compared to control subjects, a recent study in healthy volunteers confirmed the potential of TFPI to block the procoagulant pathway triggered by endotoxin. However there was no reduction in cytokine levels, in contrast to the apparent anti-inflammatory effect of TFPI in a baboon study. Disseminated intravascular coagulation is, in general, associated only with modestly reduced levels or even increased concentration of TFPI plasma levels. It was hypothesized that although circulating TFPI levels increase during inflammatory
reaction, this increase is relatively insufficient during severe sepsis and disseminated intravascular coagulation.

According to ischemia and reperfusion syndromes, increased TF and TFPI plasma levels are associated with patients with ischemic heart disease 121,122, acute myocardial infarction 106,123, unstable angina 124 and stroke 104. However, Abumiya et al. demonstrated decreased plasma TFPI activity in ischemic stroke patients in which influence of cholesterol levels was excluded 125. They suggested that high TFPI levels may have been related to elevated cholesterol levels (which are high in patients with atherothrombotic lesions), which was demonstrated by the increased TFPI activity in patients with hyperlipidemia 126. Furthermore, persistent elevated levels of TF were associated with low TFPI during and after cardiopulmonary arrest in patients with out-of-hospital cardiac arrest 127. These results indicate the activation of the extrinsic coagulation pathway without adequate TFPI generation, which may contribute to thrombin activation and fibrin formation after whole-body ischemia and reperfusion.

6. Crosstalk between coagulation and inflammation in ischemia and reperfusion

There are many similarities between the vascular response to hypoxia or ischemia and the host response to inflammation. Many pathological conditions relevant to clinical medicine, such as coagulation, are not primarily driven by hypoxic or ischemic primary event, but rather by the inflammatory cascade triggered as a secondary response (Figure 4). Microcirculatory dysfunction is probably the chief mechanism underlying subsequent multiple organ system failure.

6.1. Crosstalk between coagulation and inflammation.

Coagulation activation yields proteases that not only interact with coagulation protein zymogens but also with specific cell receptors to induce signaling pathways. Natural anticoagulants, such as tissue factor-factor VIIa, factor Xa, and thrombin have each been shown to elicit proinflammatory activities by activating cells directly 128, probably mediated by the cleavage of cell surface protease activated receptors 129. These receptors signal the intracellular link between coagulation and inflammation at sites of vascular injury, modulating platelet and endothelial cell activation 130-133. Fibrinogen/fibrin is important to the host defense mechanism and probably has an additional role that is not directly related to clotting per se 134.

TF-factor VIIa complex can activate cells 135-137, mediated by a Ca2+-influx, leading to activation of mitogen-activated protein kinase, c-Jun N terminal kinase, and the early growth gene-1 (egr-1) 138. This process potentially modulates the inflammatory mediator release from cells. TF-factor VIIa could elicit a variety of proinflammatory responses in macrophages, including reactive oxygen species and induction of MHC class II and adhesion receptors, possibly by activation of protease activated receptor 2 136. Other inflammatory actions of TF-factor VIIa include reverse migration of monocytes from the basal to the apical surface of the endothelium 139. Furthermore, administration of recombinant TF-factor VIIa induced IL-6 and IL-8 in healthy volunteers (de Jonghe, unpublished observations).

Factor Xa may activate cells via the recently identified effector protease receptor-1 (EPR-1), expressed on e.g. leukocytes 128 and endothelium 140, leading to IL-1 mediated lymphocyte proliferation 141 and edema 142. Furthermore, factor Xa on endothelium may
Figure 4. Proposed actions of activated protein C in modulating the local inflammatory, procoagulant, and fibrinolytic host responses to ischemia and reperfusion. The inflammatory and procoagulant host responses to ischemia and reperfusion are intricately linked. Inflammatory cytokines such as tumor necrosis factor (TNF-α) and interleukin-1 activate coagulation by stimulating the release of tissue factor from monocytes and the endothelium. Tissue factor leads to the formation of thrombin and a fibrin clot. Inflammatory cytokines and thrombin can both impair the endogenous fibrinolytic potential by stimulating the release of plasminogen-activator inhibitor 1 (PAI-1) from platelets and the endothelium. PAI-1 is a potent inhibitor of tissue plasminogen activator, the endogenous pathway for lysing a fibrin clot. In addition, the procoagulant thrombin is capable of stimulating multiple inflammatory pathways and further suppressing the endogenous fibrinolytic system by activating thrombin-activatable fibrinolysis inhibitor (TAFI). The conversion of protein C, by thrombin bound to thrombomodulin, to the serine protease activated protein C is impaired by the inflammatory response. Endothelial injury results in decreased thrombomodulin levels. The end result may be the development of diffuse endovascular injury, microvascular thrombosis, organ ischemia, multiorgan dysfunction, and death. Activated protein C can intervene at multiple points during the systemic response to ischemia and reperfusion. It exerts an antithrombotic effect by inactivating factors Va and VIIIa, limiting the generation of thrombin. As a result of decreased thrombin levels, the inflammatory, procoagulant, and antifibrinolytic response induced by thrombin is reduced. Activated protein C exerts an anti-inflammatory effect by inhibiting the production of inflammatory cytokines (TNF-α, interleukin-1, and interleukin-6) by monocytes and limiting the rolling of monocytes and neutrophils on injured endothelium by binding selectins. Activated protein C indirectly increases the fibrinolytic response by inhibiting PAI-1. Modified from N Engl J Med 2001; 344:699-709.
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Induce synthesis and release of IL-6, IL-8, and MCP-1 by an active site dependent reaction independent of EPR-1.

Thrombin has been shown to induce production of monocyte chemotactic protein-1 (MCP-1) and IL-6 in fibroblasts, epithelial cells, and mononuclear cells in vitro. Thrombin also may induce IL-6 and IL-8 production from endothelial cells in vitro. These effects on cell activation are probably mediated by protease activated receptors.

Thrombin generation following ischemia and reperfusion can induce P-selectin, PAF production, and the expression of ICAM-1, and contribute to rolling and adhesion of neutrophils into the post-ischemic tissue (via surface expression of ICAM-1 and E-selectin) as well as to increased microvascular permeability alterations.

Conversely, activated protein C has the ability to reduce thrombin generation (e.g. in ischemia-reperfusion) and may potentially inhibit the proinflammatory response induced by thrombin, mediated by protease activated receptors. The direct anti-inflammatory activity of activated protein C is elicited by the reduction of plasma cytokines, such as IL-1 and IL-6, tissue factor, and leukocyte cell adhesion. Pro-inflammatory cytokines, such as TNF-α and IL-1, may significantly down-regulate the expression of thrombomodulin, as suggested by cell culture experiments. This latter observation is consistent with many other studies indicating a cross-talk between effect of protein C and inflammation modulation.

Important to mention to the issue of coagulation factor induction of inflammatory mediators is the fact that in many settings the coagulation enzymes are ineffective or require high concentrations to induce cytokine elaboration. Additionally, in many settings these enzymes work synergistically with other stimulants, e.g. endotoxins, to stimulate cytokine elaboration.

6.2. Cytokine mediated stimulation of coagulation activation

The derangement of coagulation and fibrinolysis in sepsis is mediated by several proinflammatory cytokines, such as TNF-α, IL-1, and IL-6. This derangement in ischemia and reperfusion is still under investigation. The principal mediator of coagulation activation in sepsis seems to be IL-6. TNF-α indirectly influences the activation of coagulation because of its effect on IL-6. Anti-inflammatory cytokines, such as IL-10 may modulate the activation of coagulation: administration of recombinant IL-10 to humans completely abrogated endotoxin-induced effects on coagulation.

Taken together, a number of coagulation proteases can induce proinflammatory mediators that have procoagulant effects, which may amplify the cascade that leads to ischemia and reperfusion injury. Effects at the cellular level will be determined by the capacity of the coagulation inhibitors to inactivate these enzymes.

7. Restoration of anticoagulant and fibrinolytic pathways in ischemia and reperfusion

Based on the assumption that defective physiologic anticoagulant mechanisms play a pivotal role in the pathogenesis of coagulation derangement in ischemia and reperfusion syndrome, restoration of these pathways may be a logical approach in the (supportive) treatment of patients with local or generalized posts ischemic reperfusion injury, such as in surgery, thrombolysis, revascularization and shock.
7.1. Antithrombin.
Restoration of the antithrombin pathway may be achieved by administration of antithrombin concentrates. Restoration of antithrombin levels in experimental ischemia and reperfusion models has been demonstrated to adequately block the (systemic or local) activation of coagulation and inflammation and limited reperfused tissue injury.\(^{149,157,158}\) Administration of antithrombin directly inactivates thrombin activity and prevents the sequelae of reperfusion. In addition, post-ischemic treatment with antithrombin reverses the leukocyte responses to thrombin, which may be explained by the interruption of further thrombin/thrombin ligand interaction with P-selectin, which expression is dependent on continued activation of new thrombin receptors.\(^{149}\) Antithrombin inactivates thrombin that has not yet interacted with its receptor and may inhibit P-selectin-dependent rolling of neutrophils. Otrovsky et al suggested that surface P-selectin, which has been rapidly mobilized from Weibel Palade bodies in activated endothelium, is either shed or reinternalized after the activating stimulus has been dissipated.\(^{149}\) Indeed, rapid reinternalization of P-selectin has been demonstrated in activated endothelium.\(^{159}\) In addition, antithrombin has also been shown to release prostacyclin following ischemia-reperfusion,\(^{160,161}\) which has potent anti-adhesive properties.\(^{162,163}\) Furthermore, antithrombin binds to the abundance of glycosaminoglycans, expressed on the endothelium at the site of the reperfusion injury, inhibiting the proteolytic activity of generated thrombin following ischemia-reperfusion. As a result of the anti-coagulant and anti-inflammatory functions of antithrombin, it can be utilized as prophylactic or therapeutic agent in preventing or reversing microvascular dysfunction following ischemia and reperfusion.

7.2. Recombinant-APC.
Restoration of the defective protein C pathway by administration of recombinant APC is another option. Activated protein C administration in a murine model of focal ischemia reduced infarct size and brain edema, suppressed endothelial ICAM-1 and reduced myeloperoxidase in post-ischemic murine brain tissue.\(^{164}\) Activated protein C has also been shown to reduce leukocyte activation and cytokine-induced neutrophil chemoattractant following ischemia-reperfusion in rat kidney, spinal cord and liver. Furthermore, in humans an increased activated protein C to protein C ratio (APC/PC) was demonstrated after bypass cardiac surgery which was negatively correlated to MPO activity and neutrophil L-selectin expression, demonstrating that post-ischemic protein C activation was associated with decreased neutrophil tissue sequestration. This suggests that physiological protein C activation may be involved in regulation of the inflammatory injury during reperfusion of human ischemic coronary circulation.

7.3. Recombinant-TFPI.
The relative insufficiency of endogenous TFPI in ischemia and reperfusion, but even in disseminated intravascular coagulation, may be overcome by the administration of pharmacologic doses of recombinant TFPI (rTFPI). TF-mediated coagulation and microvascular perfusion defects were prevented by anti-TF antibody in a baboon model of cerebral ischemia and reperfusion.\(^{90}\) Furthermore, anti-TF monoclonal antibody inhibited vascular reocclusion after thrombolysis in a rabbit model of carotid artery thrombosis.\(^{168}\)
TF was colocalized with TFPI in post-ischemic tissue. Recombinant-TFPI (rTFPI) has been shown to inhibit ischemia and reperfusion injury in spinal cord ischemia in rabbits and in liver and kidney ischemia in rats by demonstrating increased survival after rTFPI treatment. Thus, rTFPI could potentially be beneficial in the setting of ischemia-reperfusion following surgery, shock or acute thrombosis.

7.4. Fibrinolytic agents

In the development of ischemia and reperfusion injury the abundance of intravascular microthrombi is associated with local or systemic intravascular coagulation. The endogenous fibrinolytic activity, mediated by endothelial release of tissue-type plasminogen activator (t-PA), is then inhibited by the concomitant endothelial or platelet release of plasminogen activator inhibitor (PAI)-1, the physiological, fast-acting inhibitor of t-PA. This hypofibrinolytic state can hypothetically contribute to thrombotic obstruction and may compromise adequate microcirculation, thereby promoting intestinal injury. Recanalization of the thrombotic microvasculature by fibrinolysis may attenuate the sequelae of intestinal post-ischemic reperfusion injury.

Promotion of microvascular fibrinolysis can be achieved by the administration of plasminogen activating drugs, such as streptokinase, recombinant t-PA, or recombinant single-chain urokinase, which all result in plasmin production and, subsequent, enhanced fibrinolytic activation. Such thrombolytic treatments have been demonstrated to reduce mortality in patients with acute myocardial infarction and represent a promising treatment strategy in acute mesenteric thromboembolic occlusion. Furthermore, administration of t-PA has been shown to reduce endotoxin-induced fibrin deposition and concomitant mortality in rabbits.

It should be noted, however, that the success of thrombolytic strategies has been restrained by the frequent occurrence of thrombotic reocclusion of initially reperfused vessels, presumably due to PAI-1-induced inhibition of endogenous fibrinolysis. PAI-1, as a serine protease inhibitor, is present in α-granules in platelets and in endothelial cells and is expressed by monocytes. Previous studies have demonstrated that inhibition of PAI-1 activity promotes endogenous fibrinolysis, inhibits thrombus extension and prevents fibrin deposition in experimental models of thrombosis and disseminated intravascular coagulation.

8. Summary remarks

The endothelium plays a central role in all major pathways involved in the pathogenesis of hemostatic derangement during ischemia and reperfusion. Endothelial cells seem to be directly involved in the initiation and regulation of thrombin generation and the inhibition of fibrin removal. Proinflammatory cytokines are crucial in mediating these effects on endothelial cells, which themselves may also express cytokines, thereby amplifying the coagulative response. Rather than being a unidirectional relationship, the interaction between inflammation and coagulation involves significant cross talk between the respective systems. This could result in inflammation-modifying effects of hemostatic interventions in patients with ischemia/reperfusion-syndromes.
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Local intravascular coagulation and fibrin deposition upon intestinal ischemia and reperfusion in rats

Ivo G. Schoots\textsuperscript{1,2}, Marcel Levi\textsuperscript{2}, E.H. Paulina Roossink\textsuperscript{1}, Pieter B. Bijlsma\textsuperscript{1}, and Thomas M. van Gulik\textsuperscript{1}.

Departments of \textsuperscript{1}Surgery and Surgical Laboratory, and \textsuperscript{2}Vascular Medicine, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands.

\textit{Ischemia-induced microvascular thrombotic obstruction – pivotal role or epiphenomenon?}

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Abstract

**Background:** The aim of this study was to investigate intravascular coagulation and thrombotic obstruction in the splanchnic vasculature after intestinal ischemia in relation to epithelial integrity and function.

**Methods:** Intestinal ischemia was induced in rats by superior mesenteric artery (SMA) occlusion for 20 or 40 minutes. Intestinal injury was assessed by histological analysis, biochemical markers and functional studies. During reperfusion, portal and systemic blood samples were collected to analyse activation of coagulation and fibrinolysis.

**Results:** SMA occlusion resulted in mild to moderate intestinal injury. Twenty and forty minutes of ischemia and 3 hours of reperfusion resulted in local intestinal thrombin generation and conversion of fibrinogen to fibrin, reflected by 3- and 4-fold increases in thrombin-antithrombin complex levels and a 3-fold elevation of fibrin degradation products (D-dimer), respectively. During reperfusion, after a short-lasting, initial activation of local fibrinolysis, plasminogen activator activity was suppressed, as indicated by an almost 4-fold increase in portal plasma levels of the plasminogen activator inhibitor. D-dimer levels showed that activation of coagulation and depression of fibrinolysis resulted in fibrin formation, which was confirmed to be intravascular fibrin deposition by histological examination.

**Conclusions:** Intestinal ischemia and reperfusion results in local intravascular coagulation and fibrin deposition.
Introduction

Intestinal ischemia is a condition that leads to considerable morbidity and mortality and is frequently seen in critically ill patients who suffer from hypovolemic or septic shock.

The endothelium of the splanchnic vasculature is believed to play a major role in the development of intestinal infarction. The endothelium, phenotypically a non-thrombogenic surface, is capable of balancing the pro- and anticoagulant mechanisms that prevent intravascular coagulation. However, ischemia can disturb the endothelial balance of a non-thrombogenic state into a prothrombotic state \(^2\). Ischemia-associated fibrin deposition and thrombosis are speculated to result from ischemia-induced changes of the microvascular microenvironment, including diminished aerobic metabolism, accumulation of waste products and activated inflammatory response.

Fibrin deposition after ischemia and reperfusion injury has received relatively little attention, although it has recently been shown to contribute to microvascular obstructions in early focal cerebral ischemia and reperfusion in rats \(^3\)\(^-\)\(^5\). It was also suggested to be involved in the "no reflow" phenomenon, the paradoxical condition in which no perfusion occurs in the microvasculature during macrovascular reperfusion of ischemic tissue. Fibrin deposition in the microvasculature of the intestine after occlusive intestinal ischemia \(^6\) promoted platelet adhesion, which is likely to contribute to the manifestation of microvascular ischemia and reperfusion injury. Although fibrin deposition and microthrombi in the intestine after ischemia was already reported in the early 1970s \(^7\), the postischemic response and the interaction of the coagulation and fibrinolytic systems in the splanchnic circulation after intestinal ischemia and reperfusion have not been reported so far.

To evaluate the role of intravascular coagulation in microvascular reperfusion injury after acute mesenteric vascular occlusion, we assessed intravascular coagulation, fibrinolysis and subsequent intravascular fibrin deposition and microvascular thrombotic obstructions in the splanchnic circulation after mild and moderate intestinal ischemia and subsequent reperfusion in rats.

Material and Methods

Animals

Adult male Wistar rats (Charles Rivers, Broekman Instituut BV, Someren, The Netherlands), weighing 300-325 g, were fed standard rat chow (Hope Farms, Woerden, The Netherlands) and water ad libitum. The rats were allowed to acclimatize to our laboratory conditions for at least 4 days and were subjected to a regimen of 12:12 h / day-night cycle in mesh stainless-steel cages at constant temperature (22°C). The protocol was approved by the Animal Ethics Committee of the University of Amsterdam (the Netherlands). All animals were handled in accordance with the guidelines prescribed by the Dutch legislation and the International Guidelines on protection, care and handling of laboratory animals. The last 12 hours prior to the experiments, the animals had no access to solid food, but free access to water.

In total, 24 rats were randomly allocated to one sham group and the two experimental groups. Intestinal ischemia and reperfusion was induced by isolating and clamping the superior mesenteric artery (SMA) with an atraumatic clamp for 0 minutes (sham operation, \(n = 7\)), 20 minutes (\(n = 8\)) or 40 minutes (\(n = 9\)), followed by 3 hours of reperfusion. During the sham operation the mesenteric artery was isolated without clamping, followed by 3 hours of sham reperfusion.
To exclude different influences of anaesthesia upon the outcome parameters, all rats were anaesthetized for approximately 4.5 hours, which includes the duration of the surgical procedure and the period of ischemia and reperfusion.

**Surgical procedure**

Under anaesthesia with 1-2% isoflurane, rats were intubated and ventilated. CO₂ levels were kept between 37 and 43 mmHg. Continuous 0.9% saline-glucose solution (5mM) was infused via the tail vein (10ml/hr/kg body weight) to correct for possible fluid loss during the experiment. A canula was inserted into the left carotid artery to measure the mean arterial pressure, which was kept between 90 and 110 mmHg during the experiment. Body temperature was maintained at 37 °C by use of a heating pad and lamp.

A 5.0 cm long midline laparotomy was performed and an intestinal loop of approximately 15 cm (10 cm proximal from the cecum) was isolated and canulated with soft silicon tubes. This loop was gently rinsed with saline prior to connection to a perfusion pump, a heat exchanger and a reservoir to obtain a closed circuit. The reservoir contained freshly made Ringer's solution consisting of (in mmol/L): NaCl, 117.5; KCl, 5.7; NaHCO₃, 25.0; MgSO₄, 1.2; NaH₂PO₄, 1.2; CaCl₂, 2.5 (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands). Glucose (2.5*10⁻³M) and disodiumfluorescein (Na₂Fl, Molecular Probes, Leiden, the Netherlands) (1.0*10⁻⁵M) were added to this solution, in order to evaluate the absorptive and barrier function of the intestinal epithelium.

The SMA was identified after deflecting the intestinal loops to the right side of the abdomen. The SMA was temporarily occluded by an atraumatic clamp at the origin of the aorta, avoiding the accompanying lymphatic trunk. Immediate blanching of the small intestine and cecum confirmed that the blood supply to the intestinal segments had been shut off. The abdomen was then covered with a sterile moist gauze pad. After the period of ischemia, the clamp was removed from the SMA and restoration of blood flow to the gut was verified by returning to its original colour.

During reperfusion, luminal perfusion (1ml*min⁻¹) of the isolated intestinal loop was performed with the Ringer-glucose-Na₂Fl solution. Samples from the perfusate (0.25 ml each) were obtained after 1, 2 and 3 hours of reperfusion and stored at −20 °C until further analysis.

Portal blood samples (1.25 ml each) were collected after 5 minutes, 1 and 3 hours of reperfusion. Three times of portal blood sampling during the experiment was chosen to limit blood extraction (<15% of total blood volume). An equivalent volume of a warmed volume expander of 6% polyhydroxyethyl-starch and 0.9% NaCl (eloHaes, Fresenius Kabi, the Netherlands) was injected through the tail vein to maintain blood volume. After 3 hours of reperfusion, a blood sample (1.25 ml) was also taken from the carotid artery. Blood samples were collected in Na-citrate buffer (final citrate concentration 0.32%) or in EDTA tubes and were centrifuged at 2.000 x g at 4 °C for 20 minutes and the plasma samples were stored at −20 °C until further analysis. At the end of each experiment, a blood-gas analysis was performed.

The rat was sacrificed by bleeding after final blood sampling. Biopsies of the small intestine were collected 5 cm proximal from the isolated rat intestinal loop and were fixed in 10% formaldehyde for histological examination.

**Assessment of intestinal injury**

**Histological analysis:** The formaldehyde fixed jejunal tissues were embedded in paraffin, sectioned, and stained with haematoxylin and eosin (H&E) for histological grading. The histological grading classification of Park-Chiu was used by an independent, non-informed pathologist to assess intestinal injury. Briefly, the scores used were 0: normal mucosa, 1: subepithelial space at villus tips, 2: extension of subepithelial space with moderate lifting, 3: massive lifting down sides the villi, some denuded villi, 4: denuded villi, dilated capillaries, 5: disintegration of the lamina propria, 6: crypt layer injury, 7: transmucosal infarction and 8: transmural infarction.

**Plasma parameters of intestinal injury:** In EDTA plasma samples, lactate dehydrogenase (LDH) and alpha glutathion-S-transferase (αGST) levels were determined to evaluate (intestinal) cell
leakage following ischemia and reperfusion injury. LDH levels were obtained with a cytotoxicity detection kit (Roche Diagnostics GmbH, Mannheim, Germany) and αGST levels by using an enzyme-linked immunoassay (ELISA) (Biotrin, Dublin, Ireland).

**Assessment of intestinal transport and barrier function**

**Intestinal water transport** ($C_{\text{water}}$) was assumed to be reflected by the clearance of water from the total volume of the perfusion solution in the closed circuit (including the reservoir, connecting tubes and isolated intestinal loop) and was used to estimate net intestinal absorption and secretion.

**Glucose transport** ($C_{\text{glucose}}$) was determined to measure the active-transport capacity of the epithelium. The glucose concentration ($C_{\text{Glucose}}$) was determined by a glucose-detection kit (Sigma Diagnostics, St. Louis, MO, USA).

Intestinal epithelial barrier function for small molecules was assumed to be reflected by the clearance of Na2FI ($C_{\text{Na2FI}}$) from the total volume, and was used to measure the passive transport of small water-soluble substances from the intestinal lumen into the tissue. The concentration of Na2FI ($C_{\text{Na2FI}}$) was obtained by using a fluorescence reader (Cytoflour 4000, PerSeptive Biosystems, Framingham, MA, USA) at excitation and emission wave lengths of 485 and 530 nm, respectively; the transport rate was determined as the clearance from the luminal perfusate of the Na2FI probe per minute per g intestine and calculated from the formula:

\[
\text{Clearance (in } \mu\text{L/g.min}) = \left(\frac{C_i V_i - C_f V_f}{0.5 (C_i + C_f) T W}\right)
\]

in which $C$ is the detectable probe concentration of the initial solution (i) and final solution (f), $V$ the volume of the same solutions, $T$ the time in minutes, and $W$ the weight of the intestinal loop in g.

**Assessment of coagulation and fibrinolysis**

**Coagulation and fibrinolysis parameters.** Collected blood samples were centrifuged for 20 minutes at 2,000 x g and plasma was stored at −20°C until assayed. Thrombin generation was assessed by measuring the thrombin-antithrombin (TAT) complexes with an ELISA (Behring, Marburg, Germany). Antithrombin III (ATIII) was measured by an automated amidolytic technique according to methods described by ten Cate et al.7. Fibrin degradation products (D-dimers) were obtained by an ELISA (Asserachrom D-Di, Diagnostica Stago, Asnieres-sur-Seine, France), decribed by Elms et al.1. Plasminogen activator activity (PAA) was measured by an automated amidolytic assay, described by Verheijen et al.11. Briefly, 25 μl of plasma was mixed with 0.1 M TrisHCl, pH 7.5, 0.1% (v/v) Tween-80, 0.3 mM S-2251 (Chromogenix, Mölndal, Sweden), 0.13 M plasminogen and 0.12 mg/ml cyanogen bromide-digested fibrinogen fragments of fibrinogen to a final volume of 250μl. The concentration of PAA under these conditions is proportional to the amount of plasmin formed, which can be spectrophotometrically detected by conversion of the chromogenic substrate. Plasminogen activator inhibitor (PAI) activity was measured with the amidolytic method described by Levi et al.12. Briefly, plasma was incubated with a fixed excess of t-PA (40 IU/ml) for 10 minutes at room temperature. The residual t-PA activity was determined by incubation with 0.13 μM plasminogen (Chromogenix, Sweden), 0.12 mg/ml cyanogen bromide-digested fibrinogen fragments and 0.1 mM S-2251 (Chromogenix, Sweden). Under these circumstances, PAI activity in the sample is inversely proportional to the plasmin generated in the incubation mixture, and can be determined by the conversion of the chromogenic substrate.

**Fibrin deposition.** Immunohistochemical detection of fibrin was performed using a polyclonal biotinylated goat anti-mouse fibrinogen antibody (Accurate chemicals, Boston, USA). Sections of jejunal tissue were washed and bound primary antibodies were detected by successive incubations with streptavidin / horseradish peroxidase (Lab Vision, Fremont, CA) and diaminobenzidine tetrachloride (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands). We performed negative controls with non-specific immunoglobulin for immunohistochemical detection of fibrin. Evaluation of microvascular thrombosis was performed by immunohistochemistry and histological examination.
Intravascular coagulation and fibrin deposition

Statistical analysis
The data analysis was performed using Graphpad Prism version 3.0 (Graphpad Software, Inc) for Windows 95. All quantitative data were presented as mean values ± standard error (SE). Statistical analysis was performed by analysis of variance and subsequent Bonferroni’s post test. Where appropriate, differences between groups were analyzed by the Mann-Whitney test. Differences within groups were obtained by statistical analysis with the Wilcoxon test. *P* values < 0.05 were considered to be statistically significant.

Results

Intestinal injury in response to ischemia-reperfusion
All 24 rats survived the experiment. After 20 and 40 minutes of ischemia and 3 hours of reperfusion, the values for pH, base excess and HCO$_3^-$ concentration in the arterial blood, which reflect the metabolic state of the animals, were within the normal range and did not differ between the groups (results not shown).

Intestinal injury after ischemia and reperfusion, graded according to the Park-Chiu classification was apparent in the biopsies after 20 minutes of ischemia, and was increased after 40 minutes of ischemia, with median scores of 1 (range 0-1) and 2 (range 1-4), respectively. Control rats (sham operation) did not show any intestinal injury.

During reperfusion LDH and α-GST levels in portal plasma were not different after 20 minutes of ischemia, however these levels were increased after 40 minutes of ischemia compared to those in the control group (Table 1).

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<th>Table 1. Portal Plasma Levels of LDH and α-GST during reperfusion after intestinal ischemia.</th>
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<td>Ischemia (min)</td>
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Values are presented as means ± standard error. *p < 0.05 vs 0 minutes of ischemia, †p < 0.05 vs 20 minutes of ischemia

Intestinal transport in response to ischemia-reperfusion
Intestinal clearance of glucose and water is shown in Figure 1. During reperfusion, the isolated intestinal loop in the control rats showed a constant rate of glucose and water clearance. SMA occlusion of 20 minutes did not result in impaired intestinal absorption for glucose and water, whereas 40 minutes of intestinal ischemia resulted in a significantly decreased intestinal absorption.

The absorption capacity of the intestinal loop for glucose was markedly reduced after 40 minutes of vascular occlusion but did not further decrease within 3 hours of reperfusion (Figure 1).
Figure 1. Clearance of intestinal glucose and water (μL/min/g) during 3 hours of reperfusion after 0 minutes (white bars), 20 minutes (grey bars) and 40 minutes (black bars) of intestinal ischemia. Bars are presented as mean values ± SEM. * p < 0.05 vs. control group.

Figure 2. Portal plasma levels of thrombin-antithrombin (TAT) complexes, antithrombin III (ATIII), fibrin degradation products (FDP), plasminogen activator activity (PAA) and plasminogen activator inhibitor-1 (PAI-1) during reperfusion after 0 minutes (control group), 20 minutes and 40 minutes of intestinal ischemia. Data are presented as mean values ± SEM. * p < 0.05 vs. 20 and 40 minutes of intestinal ischemia, # p < 0.05 vs. 20 minutes of intestinal ischemia.
Intravascular coagulation and fibrin deposition

Figure 3. Fibrin deposition in the intestinal submucosal layer. A: Extravascular fibrin deposition after 20 minutes of ischemia and 3 hours of reperfusion. B: Intravascular fibrin deposition after 40 minutes of ischemia and 3 hours of reperfusion.

However, the net water clearance showed a gradual decrease during reperfusion, turning into negative values after 2 and 3 hours of reperfusion.

The epithelial permeability to \( \text{Na}_2\text{Fl} \) did not show any significant change after 20 or 40 minutes of SMA occlusion. The values for \( \text{Na}_2\text{Fl} \) clearance from lumen to tissue after 3 hours of reperfusion were 9.4±4.5 µl/g/min for the control group, 12.8±4.2 µl/g/min after 20 minutes of ischemia, and 12.5±3.7 µl/g/min after 40 minutes of ischemia.

Activation of coagulation and fibrinolysis in response to ischemia-reperfusion

Portal plasma levels of the coagulation parameters TAT, ATIII, FDP (D-dimer), PAA and PAI of the control rats were in the normal range and did not change during the experiment. These sham operated rats also did not show any activation of the coagulation cascade during the whole experiment (Figure 2).

Ischemia and reperfusion resulted in local intravascular coagulation activation. Thrombin generation and conversion of fibrinogen to fibrin occurred as reflected by increase of the portal plasma levels of TAT-complexes and FDP. Portal plasma levels of TAT-complexes increased 3-fold and 4-fold after 20 and 40 minutes of ischemia, respectively. Maximum levels of TAT-complexes were measured 3 hours after the onset of reperfusion and were 16±1 ng/ml \((p<0.001)\) after 20 minutes of ischemia and 22±2 ng/ml \((p<0.001)\) after 40 minutes ischemia (as compared with 4.9±0.8 ng/ml in the control group). Portal FDP (D-dimer) levels increased from 72±8 ng/ml to 155±7 ng/ml \((p<0.05)\) after 20 minutes ischemia and from 62±5 ng/ml to 148±15 ng/ml \((p<0.01)\) after 40 minutes ischemia and 3 hours of reperfusion. Generation of thrombin and formation of TAT-complexes resulted in a consumption of local ATIII levels to 81±4 % \((p<0.001)\) and to 85±3 % \((p<0.001)\) of baseline values after 20 and 40 minutes ischemia, respectively.

After initial local activation of fibrinolysis after 1 hour of reperfusion, as demonstrated by an increase in portal PAA, fibrinolytic activity was subsequently depressed to 63±6 % \((p<0.001)\) and to 74±7 % \((p<0.001)\) of baseline levels 3 h after 20 and 40 minutes of ischemia, respectively. This shut-down of plasminogen activating
activity was associated with an increase in portal plasma levels of PAI, starting at 1 hour of reperfusion and reaching levels of 22±3 IU/ml \((p<0.001)\) and 21±2 IU/ml \((p<0.001)\) after 20 and 40 minutes ischemia and 3 hours of reperfusion, respectively. The reduction in fibrinolytic activity after 1 hour is further reflected by a decrease in fibrin degradation, as evidenced by a reduction in portal FDP levels between 1 and 3 hours, while thrombin generation continued (Figure 2).

The intestinal activation of coagulation and simultaneous depression of fibrinolysis after 20 and 40 minutes of ischemia resulted in intravascular and extravascular deposition of fibrin in the intestine after 3 hours of reperfusion (Figure 3).

In the systemic circulation the markers of coagulation and fibrinolysis were much less affected by intestinal ischemia and reperfusion than those in the local (portal) circulation (Table 2).

Table 2. Local (splanchnic) and systemic (carotid) plasma levels of coagulation and fibrinolysis parameters at 3 hours of reperfusion after intestinal ischemia.

<table>
<thead>
<tr>
<th></th>
<th>TAT (ng/mL)</th>
<th>ATIII (IU/mL)</th>
<th>FDP (ng/mL)</th>
<th>PAA (%)</th>
<th>PAI (IU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>local</td>
<td>syst</td>
<td>local</td>
<td>syst</td>
<td>local</td>
</tr>
<tr>
<td>Pre-ischemia</td>
<td>4.8±0.9</td>
<td>100±1</td>
<td>48±4</td>
<td>100±0</td>
<td>5.9±0.6</td>
</tr>
<tr>
<td>Ischemia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>5.4±0.8</td>
<td>3.7±0.4</td>
<td>99±1</td>
<td>99±1</td>
<td>54±7</td>
</tr>
<tr>
<td>20</td>
<td>15.6±0.7*</td>
<td>5.4±0.6*</td>
<td>80±3*</td>
<td>100±2</td>
<td>155±7#</td>
</tr>
<tr>
<td>40</td>
<td>21.6±2.4*</td>
<td>6.7±0.5*</td>
<td>84±3*</td>
<td>97±2</td>
<td>148±15#</td>
</tr>
</tbody>
</table>

TAT, thrombin-antithrombin complexes; ATIII, antithrombin III; FDP, fibrin degradation products; PAA, plasminogen activator activity; PAI, plasminogen activator inhibitor. Values are presented as means ± SEM. Pre-ischemic levels were only measured systemically. * \(P < 0.05\) vs. systemic plasma levels, *\# \(P < 0.05\) vs. plasma levels of the control group.

Discussion

Intestinal ischemia and reperfusion injury occurs as a continuum ranging from mild reversible post-ischemic organ dysfunction to permanent tissue damage characterized by intestinal necrosis. To evaluate mild and more severe intestinal ischemia, we used a rat model to investigate the effects of 20 and 40 minutes of SMA occlusion upon intestinal function (absorptive capacity and permeability), markers of (intestinal) cellular injury (LDH and αGST) and intestinal morphology. Indeed, as expected, 20 minutes of intestinal ischemia showed only mild structural and functional intestinal changes, whereas 40 minutes of ischemia resulted in more pronounced intestinal injury, functional defects and morphological changes.

The focus of our study was to evaluate local (i.e. portal) activation of coagulation and fibrinolysis during intestinal ischemia and reperfusion. Endothelial injury, after ischemia and reperfusion, changes the vascular endothelium from an anticoagulant surface into a procoagulant surface by changing the synthesis and surface expression of endothelial proteins. Perturbation of endothelial cells results in the induction of tissue-factor expression and suppression of thrombomodulin activity, leading to insufficiently
Intravascular coagulation and fibrin deposition

controlled thrombin generation and fibrin deposition\textsuperscript{13-16}. Indeed, in our experiments, the coagulation system in the intestinal microcirculation was activated after 20 or 40 minutes occlusion of the SMA. Marked thrombin generation, as evidenced by elevated thrombin-antithrombin complex levels and consumption of AT III resulted in fibrinogen-to-fibrin conversion, indicated by high levels of FDP (D-dimer). In addition, fibrin deposits seemed to be inadequately removed, due to a dysfunctional fibrinolytic system caused by high levels of PAI. This inhibition of fibrinolysis was preceded by a short-lasting increase in PAA, most probably released by endothelial cells upon injury. The resulting effect of fibrin generation and inadequate removal indeed resulted in intravascular fibrin deposition in the intestinal microcirculation. The fact that fibrin deposits were only demonstrated in a number of rat intestinal stainings, can be explained by the heterogeneity of intestinal injury after ischemia and reperfusion. This patchy distribution is supported by the variation in grade of intestinal injury (range 1-4) after 40 minutes ischemia and 3 hours of reperfusion, found with the histological grading score of Park-Chiu\textsuperscript{8}. Other authors have shown in mice that accumulation of fibrinogen onto the endothelial cell surface in the post-ischemic microvasculature of the intestine promoted platelet adhesion, early after the onset of reperfusion\textsuperscript{6} which may affect microvascular perfusion in postischemic intestine. Changes in the coagulation balance in portal blood were demonstrated even after 20 minutes ischemia, during which situation intestinal structure and function were largely intact, while 40 minutes of vascular occlusion demonstrated disrupted intestinal function and structure. Whether activation of coagulation and subsequent fibrin deposition after ischemia have any effect on intestinal function and structure is still uncertain, however, these data suggest that endothelial dysfunction precedes epithelial dysfunction. Interestingly, the local intestinal changes in coagulation and fibrinolysis mimic the systemic response upon a generalized inflammatory state\textsuperscript{17}; in this situation tissue-factor-driven thrombin generation is also insufficiently contained by dysfunctional physiological anticoagulant pathways and inadequately balanced by a suppressed fibrinolytic system. This leads to widespread systemic intravascular fibrin deposition, eventually resulting in disseminated intravascular coagulation\textsuperscript{18,19}. In our study, the local intestinal changes in coagulation and fibrinolysis could be set off by endothelial injury and tissue-factor expression alone, or even by local inflammation caused by invasion of endotoxins (Figure 2).

Intestinal ischemia and reperfusion injury in itself may cause an increased permeability of the intestinal epithelial and endothelial barrier\textsuperscript{20,21}, which can lead to endotoxia, thereby promoting the procoagulant state and intravascular fibrin deposition. We did not observe an increase in intestinal permeability after ischemia and reperfusion. In a comparable study of 20 and 40 minutes of intestinal ischemia, Sun and coworkers measured a two-fold increase of blood-to-lumen as well of lumen-to-blood permeability for albumin after 20 and 40 minutes of ischemia and 3 hours of reperfusion\textsuperscript{22-24}. The interpretation of these permeability studies is rather complex, because both the epithelial layer as well as the endothelial layer are restrictive to macromolecules such as albumin\textsuperscript{25,26}. We measured the clearance of Na\textsubscript{2}Fl from the intestinal lumen as a direct marker of epithelial barrier function, and we did not observe any significant change of Na\textsubscript{2}Fl clearance after ischemia. Therefore, in our study it seems unlikely that an intestinal barrier defect induced the procoagulant state. It is more likely, that intravascular coagulation activation is a consequence of endothelial injury.
Previous studies have shown that the activation of coagulation and fibrinolysis in the framework of a systemic inflammatory response are due to activation of pro-inflammatory cytokines. We previously showed that systemic activation of coagulation is mainly driven by interleukin-6 (IL-6), whereas for changes in anticoagulant and fibrinolytic pathways tumour necrosis factor-α (TNFα) can be held responsible. Indeed, activation of the cytokine network (with a prominent role for IL-6 and TNFα) has also been demonstrated by other authors in models of intestinal ischemia and reperfusion. This may demonstrate conjoined pathways in the cascade of inflammation and coagulation activation following intestinal ischemia and reperfusion.

In our model, the changes in coagulation and fibrinolysis in the systemic circulation were much less affected by intestinal ischemia and reperfusion than those in the local splanchnic (portal) circulation. Nevertheless, despite the rapid removal of different coagulation parameters (TAT, PAA and PAI) from the portal blood by the liver, systemic changes of coagulation parameters were detectable. The coagulation parameters (TAT, FDP, PAA and PAI) in the systemic circulation showed significant changes corresponding to the changes found in the portal circulation upon ischemia and reperfusion, however, the changes in these parameters were less pronounced. These observations indicate that a systemic procoagulant state may indeed occur upon intestinal ischemia and reperfusion. This procoagulant state and microvascular obstructions may lead to delayed recovery of damaged tissue, or even may damage tissue at a site remote from the initial ischemic event.

In conclusion, we demonstrated that intestinal ischemia and reperfusion result in local generation of thrombin and subsequent conversion of fibrinogen to fibrin. Simultaneously, intestinal fibrinolysis is impaired, ultimately leading to intravascular fibrin deposition. These findings suggest that microvascular thrombotic obstruction plays a pivotal role in the pathogenesis of structural and functional intestinal injury induced by ischemia and reperfusion.

Acknowledgement
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Intravascular coagulation and fibrin deposition

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Beyond sepsis: activated protein C and ischemia-reperfusion injury

Marcel Levi¹,², Goda Choi²,³, Ivo G. Schoots¹,⁴, Marcus J. Schultz³,⁵, Tom van der Pol²,⁵

Department of ¹Vascular Medicine, ²Internal Medicine, ³Intensive Care, ⁴Surgical Laboratory, and ⁵Laboratory of Experimental Medicine, Academic Medical Center, University of Amsterdam, the Netherlands

Future perspectives: anticoagulation benefits in ischemia and reperfusion syndromes
Abstract

Objective: To review potential clinical situations beyond sepsis in which activated protein C might be effective.

Data Sources: Published articles on experimental and clinical studies of activation of both coagulation and inflammation in various disease states.

Data Synthesis/Conclusion: The efficacy of activated protein C in sepsis may rely on the fact that it can modulate both coagulation and inflammation. Therefore, a potential beneficial effect of activated protein C may be present in disease states that are also characterized by simultaneous activation of these systems. Ischemia-reperfusion injury of various organs may represent such a state. Indeed, in various experimental studies of ischemia-reperfusion, involvement of the protein C system was demonstrated. In some of these models a beneficial effect of administration of activated protein C, or other interventions in the protein C system, was shown.
Introduction

Experimental studies indicate that an impaired function of the protein C pathway plays a major role in the pathogenesis of sepsis and associated organ dysfunction. Administration of activated protein C in a baboon model of intravenous E. coli resulted in survival of all animals whereas control animals in the same experiment all died. A similar beneficial effect was seen in rabbits with meningococcal endotoxin shock. Conversely, experiments in baboons in which the protein C pathway was blocked with monoclonal antibodies resulted in complete lethality of an otherwise sublethal bacteremia model. Also clinical trials in patients with sepsis have shown a beneficial effect of recombinant human activated protein C. Firstly, in a dose-ranging clinical trial, patients with sepsis received activated protein C at doses ranging from 12 µg/kg/hr to 30 µg/kg/hr, or placebo. Based on D-dimer plasma levels the optimal dose of recombinant human activated protein C was determined to be 24 µg/kg/hr. There was a clear trend towards a lower mortality (40% reduction) in patients receiving higher doses of activated protein C, although this was not statistically significant (due to the size of the trial). Subsequently, a large multicenter efficacy trial was prematurely stopped at the second interim analysis because of a significant reduction in mortality in the activated protein C-treated patients. Mortality was 24.7% in the activated protein C group as compared with 30.8% in the placebo group (relative risk reduction 19.4 percent, 95% confidence interval, 6.6 to 30.5). An extensive analysis of subgroups in this trial demonstrated that administration of recombinant human activated protein C proved to be of benefit in virtually all subgroups defined, including age, type and site of infection and disease severity. As expected, patients treated with activated protein C had less organ dysfunction. A recent analysis of long term survival of patients in this trial showed that the benefit of activated protein C was sustained over time.

Mechanisms of the beneficial effect of activated protein C in sepsis

Under physiologic conditions protein C is activated by thrombin bound to the endothelial cell membrane-associated thrombomodulin. Binding of protein C to thrombomodulin not only results in an about 100-fold increase in the activation of protein C, but also blocks the thrombin-mediated conversion of fibrinogen into fibrin and inhibits the binding of thrombin to other cellular receptors on platelets and inflammatory cells. Binding of protein C to the endothelial protein C receptor (EPCR) results in a further 5-fold augmentation of the activation of protein C by the thrombomodulin-thrombin complex. Dysfunction of the protein C system in sepsis is due to a combination of factors, including low levels of zymogen protein C (due to impaired synthesis and degradation by neutrophil elastase) and most notably by downregulation of thrombomodulin by pro-inflammatory cytokines. Observations in patients with severe Gram-negative septicemia indeed confirmed the downregulation of thrombomodulin and impaired activation of protein C in vivo.

Activated protein C is a physiological anticoagulant that modulates coagulation by proteolytically degrading the essential coagulation co-factors Va and VIIIa. Dysfunction of the protein C system may lead to inadequate balancing of tissue factor-mediated thrombin formation and may contribute to the formation of microvascular thrombosis, thereby deteriorating an adequate blood supply to various organs and be a factor in the
occurrence of organ failure. Indeed, low levels of protein C in endotoxemic mice with a one allele targeted deletion of the protein C gene (resulting in a heterozygous protein C deficiency) resulted in more fibrin deposition. In addition, activated protein C may affect endogenous fibrinolysis by inhibiting the fibrinolytic inhibitor PAI-1. In a rat model of disseminated intravascular coagulation, activated protein C was shown to block PAI-1 activity and other experiments showed the ability of activated protein C to enhance clot lysis in vivo. The administration of activated protein C to patients with sepsis indeed results in a modulation of coagulation, as evidenced by a significant decrease in plasma D-dimer levels. Also, the presence of severe coagulation abnormalities in patients with sepsis (classified as disseminated intravascular coagulation) determines a subgroup of patients with the numerically largest benefit of activated protein C. Hence, there is ample evidence that activated protein C exerts its beneficial effects in sepsis through anticoagulant properties.

However, besides its anticoagulant effect activated protein C has important inflammation-modulating properties as well. Indeed, activated protein C has been found to inhibit endotoxin-induced production of TNF-α, IL-1β, IL-6 and IL-8 by cultured monocytes/macrophages. It is likely that the effects of activated protein C on inflammation are mediated by the endothelial protein C receptor (EPCR), that may mediate downstream inflammatory processes. Binding of activated protein C to the endothelial protein C receptor was shown to affect gene expression profiles of cells by inhibiting endotoxin-induced calcium fluxes in the cell and by blocking NFκB nuclear translocation, which is a prerequisite for increases in pro-inflammatory cytokines and adhesion molecules. Blocking the protein C pathway or the protein C receptor by a monoclonal antibody in septic baboons exacerbates the inflammatory response, as evidenced by increased levels of pro-inflammatory cytokines and more leukocyte infiltration and tissue destruction at histological analysis. Conversely, administration of activated protein C ameliorates the inflammatory activation in various models of severe systemic inflammation. Mice with a one-allele targeted disruption of the protein C gene have not only a more severe coagulation response to endotoxin but also demonstrate significant differences in inflammatory responses, as shown by higher levels of circulating pro-inflammatory cytokines. In the clinical trial in septic patients receiving activated protein C, interleukin-6 levels were significantly lower in the treatment group, although the result of other cytokine assays are less unequivocal. Nevertheless, inflammation-modulating properties of activated protein C may certainly be relevant for patients with sepsis.

It is not clear whether the beneficial effect of activated protein C in sepsis is either due to its anticoagulant or its anti-inflammatory effects. It should be remembered that the clinically effective dose of recombinant activated protein C was based on D-dimer levels in the phase II study, suggesting that the treatment is tailored to its anticoagulant effect. If, however, the anticoagulant effect would be most prominent, it is less clear why intervention in other physiological anticoagulant pathways in patients with sepsis was not effective. On the other hand, the significance of the inflammation-modulating properties of activated protein C in vivo are not completely clear and the anti-inflammatory effect of activated protein C in clinical trials is hard to assess. Of note, other potent anti-inflammatory agents were shown not to be effective in patients with sepsis in previous trials. Hence, the benefit of activated protein C is hard to explain on
the basis of anticoagulant or anti-inflammatory effects exclusively. Instead, it is most likely that the beneficial effect is due to a combination of anticoagulant and anti-inflammatory properties. In view of the central role of endothelium in maintaining vascular patency and integrity, the strength of activated protein C may be its capacity to restore the deranged coagulant and inflammatory regulation at the endothelial site.

**Potential benefit of activated protein C in other clinical settings**

It is tempting to speculate that other clinical situations that are characterized by endothelial dysfunction and microvascular failure may benefit of the administration of recombinant activated protein C. Thereby, a prominent role of activated protein C may be envisaged in ischemia-reperfusion syndromes. Virtually all organs may suffer from ischemia-reperfusion injury, which can play an important role in major clinical entities, such as myocardial infarction, acute renal failure, stroke, acute lung injury and intestinal ischemia. Ischemia-reperfusion injury is characterized by a local inflammatory response and local activation of coagulation, reminiscent of the systemic situation in sepsis. Also, besides ischemia-induced cell necrosis, apoptosis may play a role as well. On all these mechanisms, activated protein C may have an effect. In the following we will briefly review the involvement of the protein C system in a selected number of models of ischemia-reperfusion injury. Other potential areas besides ischemia-reperfusion injury-related disease where activated protein C may be effective (such as stroke and other neurological disease, pancreatitis, arterial and venous thromboembolism) fall beyond the scope of this article and will not be discussed.

**Role of the protein C system in ischemia-reperfusion injury**

Renal ischemia-reperfusion syndromes are characterized by tubular necrosis and glomerular thrombosis. An important role of the protein C system in preventing glomerular thrombosis may be inferred from the abundant presence of thrombomodulin expression on endothelial cells in the glomerulus. In inflammatory glomerular disease, such as acute membranoproliferative or lupus glomerulonephritis, an increase in thrombomodulin expression has been implicated. In contrast, in ischemia-reperfusion injury in kidneys, thrombomodulin has been markedly downregulated. Administration of soluble thrombomodulin to rats with renal ischemia-reperfusion injury prevented massive glomerular thrombosis and kidney dysfunction. In another experimental study of renal ischemia and reperfusion administration of activated protein C prevented histological changes and the decrease in renal blood flow, and preserved kidney function, whereas treatment with active site-blocked factor Xa, heparin and inactivated protein C were less effective. Interestingly, a significant effect of ischemia-reperfusion-associated inflammation was observed upon the administration of activated protein C. The increase in renal levels of inflammatory cytokines TNF-α and IL-8 and renal myeloperoxidase activity was significantly reduced in activated protein C-treated animals whereas other interventions had no such effect. Furthermore, in an experimental model of liver ischemia and reperfusion, a similar anti-inflammatory responsiveness was demonstrated. Activated protein C, but also active human urinary thrombomodulin significantly reduced the production of cytokine-induced neutrophil chemoattractant and decreased myeloperoxidase activity and leukocyte accumulation, resulting in attenuation of liver
injury following ischemia and reperfusion. The notion that activated protein C had anti-inflammatory effects in these models of ischemia-reperfusion was supported by the observation that renal injury could also be prevented in rats that were severely leukocytopenic during the experiment \(^{39}\). It is interesting to note that in sepsis-induced renal changes, no effect on thrombomodulin expression and protein C activation was found \(^{40}\).

In coronary arteries of patients with severe atherosclerosis a marked downregulation of thrombomodulin and the protein C receptor was demonstrated on endothelial cells overlying the atherosclerotic plaque, suggesting a role of the protein C system in acute coronary syndromes \(^{41}\). Interestingly, administration of activated protein C prolonged the time to occlusion and improved vessel patency and myocardial blood flow in a canine model of coronary artery thrombosis and reperfusion \(^{42}\). In coronary artery reperfusion during heart surgery, activation of protein C was shown to be related to regulation of inflammatory activity \(^{43}\). Together, it is likely that the protein C system may play a role in coronary artery syndromes and myocardial reperfusion injury.

In acute lung injury the abundant presence of intra- and extravascular fibrin is a histological hallmark \(^{44}\). Experimental and clinical studies have shown that fibrin deposition is due to tissue factor-mediated thrombin generation and suppressed fibrinolysis \(^{45,46}\). In recent experiments, activation of bronchoalveolar coagulation in severe pneumonia was shown to be restricted to the site of acute lung injury \(^{47}\). In these same series of experiments, a significant reduction in bronchoalveolar protein C activation was observed, as evidenced by low levels of protein C and activated protein C in lavage fluids from patients with community acquired and ventilator-associated pneumonia. Similar findings were reported in a study in patients with acute lung injury and adult respiratory distress syndrome (ARDS) \(^{48}\). The decrease in protein C activation in both studies was strongly correlated with high levels of soluble thrombomodulin in the bronchoalveolar fluid, suggesting that shedding of thrombomodulin caused the inability to activate protein C and implicating that the protein C system may be involved in acute lung injury as well.

Acute intestinal ischemia and reperfusion may result in impaired intestinal structure and function, in experimental models characterized by intestinal cell swelling and protein leakage and impaired intestinal absorptive capacity. In addition, intra- and extravascular fibrin deposits may be present, due to activation of mesenteric coagulation and inhibition of fibrinolysis \(^{49}\). Upon 20 to 40 minutes occlusion of the superior mesenteric artery and subsequent reperfusion, portal vein plasma levels of thrombin-antithrombin levels increased, indicating local thrombin generation. This increase in portal coagulation activity is associated with a marked fall in protein C activity levels. Simultaneously, markers for fibrinolysis in portal plasma showed a complete inhibition, due to an increase in levels of plasminogen activator inhibitor, type 1 (PAI-1). This activation of coagulation upon ischemia-reperfusion could be almost completely blocked by systemic administration of activated protein C, whereas heparin and antithrombin were less effective \(^{50}\). Interestingly, amelioration of ischemia-reperfusion-induced intestinal intra- and extravascular fibrin deposition by administration of activated protein C caused a significant improvement in intestinal function and structure.

Taken together, there is interesting evidence to support a role of the protein C system in ischemia-reperfusion injury. Consequently, administration of activated protein C may be a
promising therapeutic option in these situations. The efficacy of the approach deserves further study in experimental and clinical studies.
References


Inhibition of coagulation and inflammation by activated protein C or antithrombin reduces intestinal ischemia/reperfusion injury in rats

Ivo G. Schoots\textsuperscript{1,2}, Marcel Levi\textsuperscript{2}, Arlène K van Vliet\textsuperscript{1}, Adrie M. Maas\textsuperscript{1}, E.H. Paulina Roossink\textsuperscript{1}, and Thomas M. van Gulik\textsuperscript{1}.

Departments of \textsuperscript{1}Surgery and Surgical Laboratory, and \textsuperscript{2}Vascular and Internal Medicine, Academic Medical Centre, University of Amsterdam, Amsterdam, the Netherlands

Anticoagulant therapeutics – anti-inflammatory benefits

AT or APC inhibits intestinal ischemia/reperfusion injury

Abstract

Objective: To examine whether administration of activated protein C or antithrombin reduces local splanchnic derangement of coagulation and inflammation and attenuates intestinal dysfunction and injury following intestinal ischemia/reperfusion.

Design: Randomized prospective animal study.

Setting: University research institute.

Subjects: Adult male Wistar rats, weighing 300-325 g (n = 72).

Interventions: Rats were subjected to superior mesenteric artery occlusion consisting of 20 or 40 minutes ischemia and 3 hours of reperfusion. A randomized intravenous administration of vehicle (0.9% NaCl), heparin, antithrombin, or activated protein C was performed during ischemia, 15 minutes before reperfusion. Coagulation and fibrinolysis parameters obtained from portal blood, were correlated to mucosal fibrin deposition (determined by anti-rat fibrin antibody staining), intestinal function (glucose/water clearance) and intestinal injury (histological evaluation by Park/Chiu score).

Measurements and Main Results. Activated protein C or antithrombin treated animals demonstrated less ischemia/reperfusion-induced intestinal dysfunction and histological changes compared to control animals, whereas intravenous administration of heparin only showed less histological derangement. Activated protein C or antithrombin treated animals showed less thrombin generation, fibrin degradation products and fibrin deposition compared to control animals, as confirmed by histological examination, whereas heparin administration showed only a limited reduction of portal fibrin degradation products levels. Furthermore, activated protein C or antithrombin administration markedly inhibited the inflammatory response, as reflected by reduced interleukin-6 plasma levels to baseline values whereas heparin had no effect.

Conclusions: Administration of activated protein C or antithrombin inhibited local and systemic derangement of coagulation and inflammation following intestinal ischemia/reperfusion, diminished mucosal fibrin deposition and attenuated ischemia/reperfusion induced intestinal injury. These observations suggest that activated protein C or antithrombin reduces ischemia/reperfusion-induced intestinal injury, both through their anticoagulant and anti-inflammatory effects.
Introduction

The clinical course of critically ill patients can be complicated by the development of intestinal ischemia. The mesenteric hemodynamic response to severe sepsis and septic shock diminishes mesenteric blood flow and hence oxygen delivery in combination with an increased metabolic demand and enhances the ischemic state of the gut. Ischemia-reperfusion (I/R)-induced endothelial cell injury results in a procoagulant and fibrinolysis-suppressing environment giving rise to intra- and extravascular fibrin deposition which will further compromise the microcirculation of the intestine and promote necrosis in distal tissue. Mechanisms that have been incriminated to play a role in the procoagulant response are the upregulation of tissue factor in combination with dysfunctional anticoagulant pathways, along with suppression of fibrinolysis mainly due to increased levels of the inhibitor of fibrinolysis: plasminogen activator inhibitor (PAI)-1.

Regulatory anticoagulant pathways, in particular the antithrombin system and the protein C system, appear to be ineffective in inhibiting thrombin generation following I/R. Physiological anticoagulants such as antithrombin (AT) and activated protein C (APC), in addition to reducing thrombin generation, may exert anti-inflammatory properties including modulation of cytokine expression, regulation of cell migration and promotion of apoptosis. Restoration of these defective, physiological anticoagulant mechanisms form a logical approach to the (supportive) treatment of local or remote post-ischemic reperfusion injury.

AT, as a serine protease inhibitor, binds to glycosaminoglycans expressed on the endothelium at the site of the reperfusion injury and inhibits directly the proteolytic activity of generated thrombin. AT and other activated proteases have potent anti-adhesive and anti-inflammatory properties and inhibit the P-selectin-dependent leukocyte rolling and subsequent recruitment of leucocytes into tissues affected by I/R. Administration of AT has been shown to reduce liver and renal I/R injury by reversing this endothelial-leukocyte response to thrombin.

Protein C, as a circulating inactive vitamin K-dependent plasma glycoprotein, is converted to its active form by the endothelial surface-associated thrombin-thrombomodulin complex. APC and its cofactor protein S inactivate the coagulant factors Va and VIIIa. In addition, APC has also potent profibrinolytic and anti-inflammatory properties. APC administration has been shown to reduce cerebral infarct size and brain edema, to suppress endothelial ICAM-1 and to reduce myeloperoxidase formation in a murine model of focal cerebral ischemia. Previous studies have shown that APC inhibits leukocyte activation and cytokine-induced neutrophil chemoattractant expression following I/R in rat kidney, spinal cord and liver.

Owing to the anticoagulant and anti-inflammatory functions described above, AT and APC have a potential as prophylactic or therapeutic agents in the prevention or inhibition of microvascular dysfunction, inflammatory response and reperfusion injury following intestinal I/R. The present study was undertaken to investigate the anticoagulant and anti-inflammatory effects of anticoagulants, including unfractionated heparin, AT and APC, on the derangement of coagulation and inflammation in the splanchnic and systemic circulation following mild and moderate intestinal I/R in rats.
Materials and Methods

Biological and chemical agents

Plasma derived AT concentrate was obtained from Baxter (Vienna, Austria), recombinant-human APC concentrate was provided by Eli Lilly and Company (Indianapolis, IN), and unfractionated heparin was from Leo Pharma B.V. (Ballerup, Denmark). Goat anti-rat fibrin polyclonal antibody was kindly provided by Dr. J.J. Emeis (TNO Prevention and Health, Leiden, the Netherlands) (25). All other reagents were of analytical grade.

Animal model of intestinal I/R

Adult male Wistar rats (Charles Rivers, Broekman Instituut BV, Someren, the Netherlands), weighing 300-325 g, were fed standard rat chow (Hope Farms, Woerden, the Netherlands) and water ad libitum. The rats were allowed to acclimatize to our laboratory conditions for at least 4 days and were subjected to a regimen of 12:12 h / day-night cycle in mesh stainless-steel cages at constant temperature (22°C). The protocol was approved by the Animal Ethics Committee of the University of Amsterdam (the Netherlands). All animals were handled in accordance with the guidelines prescribed by the Dutch legislation and the International Guidelines on protection, care and handling of laboratory animals. The last 12 hours prior to the experiments, the animals had no access to solid food, but free access to water.

Animal model used has been described previously 6. Briefly, under anaesthesia (1-2% isoflurane) and continuous monitoring of mean arterial pressure (90-110 mmHg) and body temperature (37±0.5°C), an intestinal loop of approximately 15 cm (10 cm proximal from the cecum) was isolated and canulated with soft silicon tubes, and connected to a perfusion pump, a heat exchanger and a reservoir to obtain a closed circuit. The reservoir contained freshly made Ringer's solution consisting of glucose (25 mM), in order to evaluate the absorptive function of the intestinal epithelium.

Intestinal ischemia was induced by temporarily occlusion of the superior mesenteric artery (SMA) and confirmed by immediate blanching of the small intestine and cecum. Restoration of blood flow to the gut after declamping of the SMA was confirmed by returning to its original color. During reperfusion, luminal perfusion (1.0 mL/min) of the isolated intestinal loop was performed with the Ringer-glucose solution and samples from the perfusate (0.25 mL each) were obtained after 1, 2 and 3 hours of reperfusion and stored at -20°C until further analysis. Portal blood samples (1.25 mL each) were collected in sodium-citrate buffer (final citrate concentration 0.32%) and in EDTA tubes after 5 minutes, and after 1 and 3 hours of reperfusion, were centrifuged at 2 000g at 4°C for 20 minutes and stored at -80°C until further analysis. Rats were sacrificed by bleeding after final blood sampling and subsequent administration of heparin (2 000 IU/kg) to prevent intravascular clotting. Biopsies of the small intestine were collected 5 cm proximal from the isolated rat intestinal loop and were fixed in 10% formaldehyde for histological examination.

Experimental design of intestinal I/R

In total, 72 rats were randomly allocated to a control group (saline) and three experimental groups (heparin, AT and APC, respectively), consisting of 18 rats each. Saline (0.9% NaCl), heparin (375 U/kg of body weight), AT (250 U/kg) or APC (100 µg/kg) was administered intravenously into the penile vein during the ischemic period, 15 minutes before reperfusion.

There is no single test that can directly match the anticoagulant effect of heparin, antithrombin and activated protein C. Therefore, we have chosen doses of each of these three agents that will result in plasma levels that are comparable to those achieved in clinical practice and have been shown to possess therapeutic antithrombotic potential in previous studies 15,16,20,22,26,27. Therapeutic doses of heparin in clinical studies result in anti-factor Xa levels of 0.5-1.0 IU/ml. Previous studies from our group have shown that a bolus subcutaneous injection of 375 IU/kg in a rat results in such plasma levels 28 and therefore this dose was chosen. Recent trials with antithrombin concentrate used dosages of approximately 250 to 300 IU/kg. Studies with 250 IU/kg
antithrombin concentrate have shown efficacy in animal models of endotoxemia and ischemia/reperfusion, whereas 50 or 100 IU/kg did not. The therapeutic dose of recombinant activated protein C in clinical studies is 24 μg/kg/hr. For technical reasons, we have chosen to administer activated protein C as a bolus and in view of the half-life of this agent a dose of 100 μg/kg was selected. In other studies the administration of this dose has demonstrated attenuation of liver and renal ischemia/reperfusion injury in rats. Based on the pharmacokinetics of this dose, therapeutic plasma levels during 2-4 hours of the experiment can be expected.

Each bolus injection of 1 mL/kg vehicle or anticoagulant was administered within seconds. Before reperfusion was induced by declamping the superior mesenteric artery, the anticoagulant equilibrated in the systemic circulation during 15 minutes, affecting the groups of 20 and 40 minutes of intestinal ischemia equally.

In each group, intestinal ischemia and reperfusion was induced by isolating and clamping the SMA with an atraumatic clamp for 0 minutes (sham operation, n = 6), 20 minutes (n = 6) or 40 minutes (n = 6), followed by 3 hours of reperfusion.

**Histological assessment of intestinal injury**

Histological grading of intestinal injury of formaldehyde fixed jejunal tissues, counterstained with haematoxylin and eosin, was performed by two independent, non-informed examiners, using the Park-Chiu classification.

**Assessment of intestinal transport**

Intestinal water transport (Cl_water) was assumed to be reflected by the clearance of water from the total volume of the perfusion solution in the closed circuit (including the reservoir, connecting tubes and isolated intestinal loop) and was used to estimate net intestinal absorption and secretion. Glucose transport (Cl_glucose) was determined to measure the active-transport capacity of the epithelium. The glucose concentration (C_glucose) was determined by a ‘Glucose Assay Reagent’ utilizing the hexokinase-glucose 6-phosphate dehydrogenase enzymatic assay (Sigma Diagnostics, St. Louis, MO, USA). The transport rate of water and glucose was determined as the clearance from the luminal perfusate per minute per gram intestine and calculated from the formula:

\[
\text{Clearance (in μL/g.min)} = \frac{(C_i*V_i-C_f*V_f)}{(0.5*(C_i+C_f)*T*W)}
\]

in which C is the detectable glucose concentration of the initial solution (i) and final solution (f), V the volume of the same solutions, T the time in minutes (min), and W the weight of the intestinal loop in gram (g).

**Assessment of coagulation and fibrinolysis**

Plasma samples in sodium-citrate buffer, stored at -80 °C were utilized. Thrombin generation was assessed by measuring the thrombin-antithrombin (TAT) complexes with an enzyme-linked immunosororbent assay (ELISA) kit (Behring, Marburg, Germany). AT was measured by an automated amidolytic technique according to methods previously described. Fibrin degradation products (D-dimers) were obtained by an ELISA (Asserachrom D-Di, Diagnostica Stago, Asnieres-sur-Seine, France). Plasminogen activator activity (PAA) and plasminogen activator inhibitor (PAI)-l activity were measured by amidolytic assays previously described.

**Immunohistochemical assessment of fibrin deposition**

Fibrin deposition was detected on formaldehyde-fixed tissue sections using immunohistochemistry according to standard procedures, described previously. As negative controls, parallel sections consisted of the omission of the primary antibody and yielded no immunohistochemical reaction. Microscopical evaluation of fibrin deposition was performed by two blinded examiners.
Measurement of cytokines
Levels of rat tumor necrosis factor-α, cytokine induced neutrophil chemoattractant, interleukin-1β and interleukin-6 were determined with the use of rat ELISA kits (R&D Systems, Minneapolis, MN) in EDTA plasma samples, stored at -80 °C.

Statistical analysis
The data analysis was performed using Graphpad Prism version 3.0 (Graphpad Software, Inc) for Windows 95. Quantitative data were presented as median values and interquartiles or as mean values ± standard error of the mean (SEM). Differences between experimental groups for repeated measurements were analyzed by analysis of variance (ANOVA) and subsequent Bonferroni’s post-hoc test. Differences between experimental groups for single measurements were analyzed by the unpaired student-t test. Mann-Whitney U test was only used for analysis of the histology scores because equal variance and normal distribution conditions were violated. P values <0.05 were considered to be statistically significant.

Results
Effects of heparin, AT and APC on morphology of I/R-induced intestinal injury
Microscopical examination of intestinal tissue revealed subepithelial space in the villus tips with moderate to massive lifting, together with villus denudation in saline treated animals after 20 minutes of ischemia and 3 hours of reperfusion, showing a median score of 3.5 (range 2-4, \( P=0.007 \)) (Figure 1). Intestinal injury was higher to the extent of disintegration of the lamina propria in most intestinal tissues of saline treated animals after 40 minutes of ischemia, yielding a median score of 5 (range 4-5, \( P=0.001 \)); such changes were not observed in sham-operated animals. Intravenous administration of heparin, AT or APC demonstrated significantly less intestinal injury after 40 minutes of ischemia and 3 hours of reperfusion to median scores of 3 (heparin, \( P=0.021 \)), 4 (AT, \( P=0.046 \)) and 3.5 (APC, \( P=0.032 \)), respectively. There was a non-significant trend towards a lower score after administration of heparin, AT and APC after 20 minutes of SMA occlusion.

Figure 1. Effects of heparin, antithrombin and activated protein C on morphology of I/R-induced intestinal injury
Histological analysis of intestinal tissues was performed after 3 hours of reperfusion and assessed according to the Park-Chiu classification \(^{30}\). Data of animals treated with saline (closed bars), heparin (hatched bars), antithrombin (AT) (crossed bars), or activated protein C (APC) (blocked bars) are presented as median values and interquartiles (n = 6, in each group). *\( P<0.05 \) compared with the sham-operated group; †\( P<0.05 \) compared with 20 minutes I/R plus saline group; ‡\( P<0.05 \) compared with 40 minutes I/R plus saline group.
Effects of heparin, AT and APC on I/R-induced intestinal dysfunction

Intestinal clearances of glucose and water during 3 hours of reperfusion were significantly and dose-dependently decreased after 20 and 40 minutes of intestinal ischemia in saline treated animals (Figure 2). Administration of heparin, AT or APC did not improve intestinal dysfunction after 40 minutes of ischemia and 3 hours of reperfusion; however, administration of AT or APC significantly attenuated intestinal dysfunction after 20 minutes of ischemia and 3 hours of reperfusion to baseline glucose and water clearance levels. This was in particular the case for glucose clearance. Heparin failed to show any improvement of intestinal dysfunction after 20 minutes of ischemia and 3 hours of reperfusion.

Figure 2. Effects of heparin, antithrombin and activated protein C on I/R-induced intestinal dysfunction

Intestinal clearances of glucose and water during 3 hours of reperfusion were determined using an intestinal loop of approximately 15 cm. Values represent the total clearance following 3 hours of reperfusion. Data of animals treated with saline (closed bars), heparin (hatched bars), antithrombin (AT) (crossed bars), or activated protein C (APC) (blocked bars) are expressed as mean values ± SEM (n = 6, in each group). *P<0.05 compared with the sham-operated group.

Effects of heparin, AT and APC on intestinal I/R-induced activation of coagulation and fibrinolysis

Portal plasma levels of the coagulation parameters TAT, AT, FDP (D-dimer), PAA and PAI-1 in the sham-operated animals were in the normal range and did not change during the whole experiment.

Intestinal ischemia and reperfusion resulted in local activation of coagulation. Thrombin generation and conversion of fibrinogen to fibrin occurred as reflected by
AT or APC inhibits intestinal ischemia/reperfusion injury
Figure 3. Effects of heparin, antithrombin and activated protein C on intestinal I/R-induced activation of coagulation and suppression of fibrinolysis

Activation of coagulation and suppression of fibrinolysis were determined by the measurement of portal plasma levels of thrombin-antithrombin (TAT)-complexes (A,B), antithrombin (AT) (C,D), fibrin degradation products (FDP) (E,F), plasminogen activator activity (PAA) (G,H) and plasminogen activator inhibitor (PAI)-1 (I,J). Animals were intravenously administered 0.9% saline, heparin, antithrombin (AT), or activated protein C (APC), 15 minutes before reperfusion. Data (n = 6, in each group) are expressed as mean values ± SEM. Portal plasma levels of repeated measurements during 3 hours of reperfusion in saline treated animals subjected to sham operation ( ), or to 20 minutes ( ) or 40 minutes ( ) of ischemia are depicted in A, C, E, G and I. Portal plasma levels of animals treated with saline (closed bars), heparin (hatched bars), antithrombin (AT) (crossed bars), or activated protein C (APC) (blocked bars), subjected to 20 minutes or 40 minutes of ischemia after 3 hours of reperfusion are depicted in B, D, F, H and J. Values shown represent the 3 hour reperfusion time-point. *P<0.05 compared with the sham-operated group; †P<0.05 compared with 20 minutes I/R plus saline group; ‡P<0.05 compared with 40 minutes I/R plus saline group.
Figure 4. Effects of heparin, antithrombin and activated protein C on intestinal I/R-induced mucosal fibrin deposition and microvascular thrombosis

Representative results of mucosal fibrin deposition after 3 hours of reperfusion are shown of animals in the sham-operated group (A and B), and of animals subjected to 40 minutes of ischemia, treated with saline (C and D), heparin (E and F), antithrombin (G and H), or activated protein C (I and J). For A, C, E, G and I, original magnification x200 (bar in A represents 50 μm); for B, D, F, H and J, original magnification x500 (bar in B represents 20 μm). Location of enlargement (asterisk (*)), lumen (L), villus (V), and epithelium (E) are indicated. Arrows indicate fibrin deposition (brown-stained areas).
increase of the portal plasma levels of TAT-complexes and FDP (Figure 3). During 3 hours of reperfusion portal plasma levels of TAT-complexes increased 3-fold and 5-fold after 20 and 40 minutes of ischemia, respectively. Generation of thrombin and formation of TAT-complexes resulted in local consumption of AT to 92±1% \((P<0.001)\) and to 86±1% \((P<0.001)\) of baseline values after 20 and 40 minutes of ischemia, respectively.

Administration of AT demonstrated significant lower mesenteric thrombin generation after mesenteric I/R, as reflected by a 2-fold and 1.5-fold reduction of portal plasma levels of TAT-complexes and FDP, respectively, after 40 minutes of ischemia and 3 hours of reperfusion. Similar results were obtained after administration of APC. Administration of heparin resulted in a relatively small reduction of FDP levels \((P=0.049)\) after 40 minutes of ischemia and 3 hours of reperfusion, whereas thrombin generation was not reduced, as reflected by comparable levels of TAT-complexes and AT values in portal plasma compared to plasma levels in saline treated animals.

After initial local activation of fibrinolysis after 1 hour of reperfusion, as demonstrated by an increase in portal PAA, fibrinolytic activity was subsequently depressed to 91±2\% \((P<0.002)\) and to 81±2\% \((P<0.001)\) of baseline levels 3 hours after 20 and 40 minutes of ischemia, respectively. This decrease of plasminogen activating activity was associated with an increase in portal plasma levels of PAI-1, reaching levels of 17±1 IU/mL \((P<0.001)\) and 25±2 IU/mL \((P<0.001)\) after 20 and 40 minutes ischemia and 3 hours of reperfusion, respectively.
Administration of APC significantly depressed plasma levels of PAI-1 after ischemia and reperfusion, which resulted in increased fibrinolytic activity. Neither heparin nor AT administration showed reduced PAI-1 levels, although heparin administration demonstrated a small increase in fibrinolytic activity after 40 minutes of mesenteric ischemia and 3 hours of reperfusion.

**Effects of heparin, AT and APC on intestinal I/R-induced fibrin deposition and microvascular thrombosis**

Microscopical assessment of intestinal tissues of saline treated animals revealed mucosal fibrin deposits and microvascular thrombotic obstructions after 20 (data not shown) and 40 minutes of intestinal ischemia and 3 hours of reperfusion (Figure 4C and 4D), whereas histological examination after sham operation did not reveal mucosal fibrin deposits (Figure 4A and 4B). Either AT or APC administration showed markedly less mucosal fibrin deposits (Figure 4G-J) after 40 minutes of ischemia and 3 hours of reperfusion, compared to saline administration.

**Effects of heparin, AT and APC on intestinal I/R-induced inflammation**

Portal plasma levels of tumor necrosis factor-α, cytokine induced neutrophil chemoattractant and interleukin-1β were below the 15 pg/mL detection limit. Portal plasma concentrations of interleukin-6 were significantly higher after reperfusion in rats subjected to 20 (P=0.046) and 40 (P=0.002) minutes of intestinal ischemia than after

![Graph](image-url)
reperfusion in sham-operated rats (Figure 5). The increases of portal plasma interleukin-6 were significantly inhibited by either AT or APC administration, but not by the administration of heparin after 40 minutes of intestinal ischemia and 3 hours of reperfusion.

Effects of heparin, AT and APC on intestinal I/R-induced splanchnic and systemic coagulation, fibrinolysis and inflammation

In the systemic circulation, the markers of coagulation, fibrinolysis and inflammation were much less affected by intestinal I/R than those in the splanchnic (portal) circulation in the saline treated animals (Table 1); however, the systemic changes in coagulation, fibrinolysis, and inflammation mimicked the local splanchnic response. The markers measured in the systemic circulation did not evidence any systemic derangement of coagulation and inflammation after either AT or APC administration in rats subjected to 40 minutes of ischemia and 3 hours of reperfusion as baseline values were detected.

Table 1. Effects of heparin, antithrombin or activated protein C on splanchnic and systemic plasma levels of parameters for coagulation, fibrinolysis and inflammation.

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Sham</th>
<th>I/R+ NaCl</th>
<th>I/R+ Heparin</th>
<th>I/R+ AT</th>
<th>I/R+ APC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Coagulation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TAT (ng/mL)</td>
<td>Splanchnic</td>
<td>5±1</td>
<td>25±2(^a)</td>
<td>27±1(^a)</td>
<td>10±0(^a)</td>
</tr>
<tr>
<td></td>
<td>Systemic</td>
<td>5±1</td>
<td>10±1(^b)</td>
<td>9±1(^b)</td>
<td>6±1</td>
</tr>
<tr>
<td>AT (IU/mL)</td>
<td>Splanchnic</td>
<td>100±1</td>
<td>85±1(^a)</td>
<td>84±1(^a)</td>
<td>340±10</td>
</tr>
<tr>
<td></td>
<td>Systemic</td>
<td>101±2</td>
<td>92±1(^b)</td>
<td>94±2(^b)</td>
<td>345±10(^b)</td>
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<tr>
<td>FDP (ng/mL)</td>
<td>Splanchnic</td>
<td>75±4</td>
<td>233±9(^a)</td>
<td>196±13(^a)</td>
<td>142±9(^a)</td>
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<tr>
<td></td>
<td>Systemic</td>
<td>73±6</td>
<td>118±3(^b)</td>
<td>101±3(^b)</td>
<td>84±4</td>
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<td><strong>Fibrinolysis</strong></td>
<td></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>PAA (%)</td>
<td>Splanchnic</td>
<td>102±2</td>
<td>81±2(^a)</td>
<td>94±3</td>
<td>85±4(^a)</td>
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<tr>
<td></td>
<td>Systemic</td>
<td>101±1</td>
<td>91±1(^b)</td>
<td>98±1</td>
<td>100±2</td>
</tr>
<tr>
<td>PAI-1 (IU/mL)</td>
<td>Splanchnic</td>
<td>6±1</td>
<td>25±2(^a)</td>
<td>27±2(^a)</td>
<td>29±2(^a)</td>
</tr>
<tr>
<td></td>
<td>Systemic</td>
<td>6±1</td>
<td>11±1(^b)</td>
<td>13±0(^b)</td>
<td>14±0(^b)</td>
</tr>
<tr>
<td><strong>Inflammation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>Splanchnic</td>
<td>78±10</td>
<td>175±24(^a)</td>
<td>171±29(^a)</td>
<td>60±6(^a)</td>
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<tr>
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<td>Systemic</td>
<td>59±9</td>
<td>147±20(^b)</td>
<td>148±29(^b)</td>
<td>49±5</td>
</tr>
</tbody>
</table>

TAT, trombin-antithrombin complex; AT, antithrombin; FDP, fibrin degradation products; PAA, plasminogen activator activity; PAI-1, plasminogen activator inhibitor-1; IL-6, interleukin-6. Values shown represent the 3 hour reperfusion time-point. \(^a\)P<0.05 compared with systemic plasma levels of the same group. \(^b\)P<0.05 compared with the systemic plasma levels of sham-operated group.
Discussion

Splanchnic ischemia and gut mucosal injury have been incriminated to play a role in the development and maintenance of the systemic inflammatory response, a key factor causing multiple organ failure. APC and AT exert anticoagulant and anti-inflammatory properties and may therefore be utilized as prophylactic or therapeutic agents in the prevention or inhibition of microvascular dysfunction and inflammatory response following intestinal I/R.

In saline treated animals, 20 or 40 minutes occlusion of the SMA induced a procoagulation diathesis in the intestinal microcirculation during reperfusion. Marked thrombin generation, as evidenced by elevated thrombin-antithrombin complex levels and consumption of AT resulted in fibrinogen-to-fibrin conversion, indicated by high levels of FDP (D-dimer). In addition, fibrin deposits seemed to be inadequately removed, due to a dysfunctional fibrinolytic system caused by high levels of PAI-1. This inhibition of fibrinolysis was preceded by a short-lasting increase in PAA, most probably caused by immediate release of plasminogen activator by endothelial cells upon injury. The resulting effect of fibrin generation and inadequate fibrin removal resulted in demonstrable intravascular fibrin deposition in the intestinal microcirculation following I/R.

Pharmacological doses of either APC or AT, both physiological anticoagulants significantly inhibited I/R-induced intestinal thrombin generation, fibrin formation and fibrin deposition after mild and moderate intestinal ischemia, whereas heparin showed only a limited reduction of plasma FDP levels. Restoration of the antithrombin system following AT administration reduced plasma levels of TAT-complexes, indicating reduced thrombin and fibrin generation. Interestingly, therapeutic administration of heparin was ineffective to decrease the coagulation activation following intestinal I/R, indicating that the endogenous amount of AT, available at the mesenteric endothelium, was not capable of inhibiting factors Xa and thrombin by heparin. Alternatively, it has been shown that the heparin-antithrombin complex is not able to block surface bound coagulation activation, which may also explain the failure of heparin to reduce coagulation activation. Considering combined treatment of heparin and antithrombin in this study, heparin may have resulted in adverse effects of heparin on the microcirculatory actions of antithrombin as shown during endotoxemia. It has been demonstrated that heparin competitively inhibits the binding of antithrombin to other glycosaminoglycans. Restoration of the protein C system following APC administration resulted in an equivalent decrease of TAT-complex formation and AT consumption, indicating increased inhibition of generated factor Xa by inactivation of factor Va and VIIa, and attenuation of thrombin and fibrin generation after mild and moderate intestinal I/R. In addition, APC increased fibrinolytic activity as reflected by elevated plasminogen activator activity and reduced plasma levels of its inhibitor PAI-1. Both, AT and APC administration significantly diminished mucosal fibrin generation and deposition.

Fibrin deposition and microvascular thrombosis play a pivotal role in disseminated intravascular coagulation-associated multiple organ failure and I/R-induced injury, by compromising microcirculatory blood flow. I/R-induced intestinal dysfunction and histological changes after mild and moderate intestinal ischemia were less after either APC or AT administration compared to saline infusion, whereas heparin only reduced the histological sequelae of intestinal I/R. Heparin has been demonstrated to preserve intestinal perfusion and gut mucosal pO2 levels after hemorrhage and resuscitation.
rats, which may account for the observed reduction in intestinal I/R injury. However, histological changes only reflect part of the damage caused by I/R and the ensuing coagulation and inflammatory activation. In fact, it has been shown that functional changes upon I/R do not always strongly correlate with structural changes. The attenuation of intestinal I/R injury following anticoagulant administration may result from increased microvascular perfusion owing to diminished fibrin deposits and microthrombotic obstructions, however, may also result from the anti-inflammatory properties mentioned above.

In support of the latter, the inflammatory response after intestinal I/R was lower following AT infusion compared to vehicle administration, as reflected by the reduction of interleukin-6 plasma levels to baseline values. The administration of APC also significantly inhibited the inflammatory response, whereas administration of heparin did not decrease post-ischemic interleukin-6. We have previously shown that systemic activation of coagulation is mainly driven by interleukin-6, whereas for changes in the anticoagulant and fibrinolytic pathways, tumor necrosis factor-α can be held responsible. Although in our model of mild to moderate intestinal I/R, local plasma levels of tumor necrosis factor-α, cytokine induced neutrophil chemoattractant and interleukin-1β were not detectable, previous studies have shown that tumor necrosis factor-α and cytokine-induced neutrophil chemoattractant following ‘severe’ I/R were suppressed after APC or AT administration.

Interestingly, the local splanchnic changes in coagulation and fibrinolysis mimic the systemic response upon a generalized inflammatory state; in this situation tissue-factor-driven thrombin generation is also insufficiently contained by dysfunctional anticoagulant pathways and inadequately balanced by a suppressed fibrinolytic system. This leads to widespread systemic intravascular fibrin deposition, eventually resulting in disseminated intravascular coagulation and subsequent organ failure in critically ill patients. Either APC or AT administration has been shown to effectively alleviate the coagulation abnormalities in patients with disseminated intravascular coagulation. As a result of the protective properties of these anticoagulants to I/R injury, we may speculate that APC and AT are beneficial not only to reduce coagulation abnormalities, but also to counteract intestinal injury in patients with disseminated intravascular coagulation associated with shock or sepsis in critically ill patients. In our model, the systemic circulatory events concerning coagulation and fibrinolysis were much less influenced by intestinal ischemia and reperfusion than those in the local, splanchnic (portal) circulation. Systemic monitoring of coagulation parameters (D-dimer) may not reveal splanchnic coagulation abnormalities after mild intestinal I/R. However, after 40 minutes of intestinal ischemia, systemic circulatory changes in coagulation and fibrinolysis showed significant alterations corresponding to the changes found in the portal circulation upon I/R. These observations indicate that a systemic procoagulant state may indeed occur upon intestinal I/R, which may damage tissue at a site remote from the initial ischemic event. Acute respiratory failure is the most important sequel in this clinical scenario. The administration of APC or AT concentrates completely reversed the systemic changes of coagulation activation after intestinal I/R in the present study, and therefore, may also diminish remote activation of coagulation and inflammatory response.
Conclusion

Intestinal I/R resulted in considerable local and systemic derangement of the coagulation and inflammatory system, compromising mucosal and submucosal microcirculation by widespread microthrombosis and deposition of fibrin. Administration of the physiological anticoagulants APC or AT inhibited the derangement of coagulation and inflammation following intestinal I/R, diminished mucosal fibrin deposition and decreased histological changes of intestinal injury. Although the early stage of treatment initiated and the short time window of this experimental study do not reflect the broad treatment spectrum of intestinal ischemia in a large variety of clinical settings, these observations may still be relevant for analyzing pathways in the pathogenesis of I/R injury and for the potential treatment of critically ill patients with intestinal ischemia. The present results may support the initiation of future clinical studies to investigate the potential benefit of this treatment.

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References


Enhancement of endogenous fibrinolysis does not reduce local fibrin deposition but modulates inflammation upon intestinal ischemia and reperfusion

Ivo G. Schoots¹,², Marcel Levi², Arlène K van Vliet¹, Adrie M. Maas¹, Paul J. Declerck³, and Thomas M. van Gulik¹.

Departments of ¹Surgery and Surgical Laboratory, ²Vascular and Internal Medicine, Academic Medical Centre, University of Amsterdam, Amsterdam, the Netherlands, and ³Laboratory for Pharmaceutical Biology and Phytopharmacology, Faculty of Pharmaceutical Sciences, University of Leuven, Belgium.

Microcirculatory failure – a limited role for suppressed endogenous fibrinolysis

Abstract

**Background:** Intestinal ischemia/reperfusion (I/R) causes local inhibition of endogenous fibrinolysis in combination with activation of coagulation. This may lead to thrombotic obstructions that compromise microcirculation and promote intestinal injury. Inhibited fibrinolysis following intestinal I/R is related to increased plasminogen activator inhibitor (PAI)-1. This study examined the effect of enhanced fibrinolysis in a rat model of intestinal I/R that results in local activation of coagulation, suppression of endogenous fibrinolysis and mucosal fibrin deposition.

**Methods & Results:** Fibrinolysis was enhanced by intravenous administration of recombinant tissue plasminogen activator (rt-PA) or by inhibition of PAI-1 by administration of MA-33H1F7. Although anti-PAI-1 antibody or rt-PA administration enhanced circulatory fibrinolytic activity, as evidenced by increased portal plasma plasminogen activator activity, elevation of fibrin degradation products and decreased levels of PAI-1, mucosal fibrin deposition and microthrombosis were not reduced in postischemic intestinal tissue. Furthermore, enhanced fibrinolysis did not attenuate I/R-induced intestinal injury or dysfunction, as demonstrated by morphological and functional analysis. However, both interventions resulted in decreased levels of interleukin-6, which indicates fibrin-induced modulation of inflammation.

**Conclusions:** Enhancement of fibrinolytic activity neither increased removal of mucosal fibrin deposition nor attenuated intestinal I/R injury. These results suggest a limited role of suppressed endogenous fibrinolysis in microcirculatory failure and consequent deterioration of intestinal function and structure following intestinal I/R.
Introduction

Patients subjected to circulatory shock, hypoxia and sepsis may sustain ischemia-reperfusion (I/R)-induced intestinal injury, due to decreased blood flow and oxygen delivery in combination with increased metabolic demand in sepsis. This results in (often clinically unrecognized) mucosal dysfunction and in local inflammatory response. In this regard, the gut plays a pivotal role in the development and maintenance of the systemic inflammatory response syndrome and has been speculated to be “one of the motors of multiple organ injury”.

An important factor in the development of organ failure is the abundance of intravascular microthrombi associated with local or systemic intravascular coagulation. This widespread occurrence of microthrombi has also been demonstrated in ischemic intestinal tissue. Recently, we demonstrated fibrin deposits and microthrombotic obstructions in the intestinal microcirculation following experimental intestinal I/R, as a result of ongoing activation of coagulation and suppression of endogenous fibrinolysis. The endogenous fibrinolytic activity, mediated by endothelial release of tissue-type plasminogen activator (t-PA), was inhibited by the concomitant endothelial or platelet release of plasminogen activator inhibitor (PAI)-1, the physiological, fast-acting inhibitor of t-PA. This hypofibrinolytic state can hypothetically contribute to thrombotic obstruction and may compromise adequate microcirculation, thereby promoting intestinal injury. This led to the hypothesis that “recanalization” of the thrombotic microvasculature by fibrinolysis may attenuate the sequelae of intestinal post-ischemic, reperfusion injury.

Promotion of microvascular fibrinolysis can be achieved by the administration of plasminogen activating drugs, such as streptokinase, recombinant t-PA, or recombinant single-chain urokinase, which all result in plasmin production and, subsequent, enhanced fibrinolytic activation. Such thrombolytic treatments have been demonstrated to reduce mortality in patients with acute myocardial infarction and represent a promising treatment strategy in acute mesenteric thromboembolic occlusion. Furthermore, administration of t-PA has been shown to reduce endotoxin-induced fibrin deposition and concomitant mortality in rabbits.

It should be noted, however, that the success of thrombolytic strategies has been restrained by the frequent occurrence of thrombotic reocclusion of initially reperfused vessels, presumably due to PAI-1-induced inhibition of endogenous fibrinolysis. PAI-1, as a serine protease inhibitor, is present in α-granules in platelets and in endothelial cells and is expressed by monocytes. In previous studies, we have demonstrated that inhibition of PAI-1 activity promotes endogenous fibrinolysis, inhibits thrombus extension and prevents fibrin deposition in experimental models of thrombosis and disseminated intravascular coagulation.

The aim of the present study was to investigate the contribution of endogenous suppression of fibrinolysis and increased fibrin deposition to intestinal dysfunction and injury in a rat model of intestinal I/R. Enhancement of fibrinolytic activity in the mesenteric circulation was induced by administration of recombinant t-PA. The role of PAI-1 in preventing t-PA-mediated fibrinolysis prompted us to investigate also the effect of inhibition of PAI-1 activity, using an anti-rat PAI-1 monoclonal antibody (MA-33H1F7).
Material and Methods

**Biological and chemical agents**

Recombinant tissue plasminogen activator (rt-PA) concentrate (Alteplase) was purchased from Genentech Inc. (San Francisco, CA) and anti-PAI-1 monoclonal antibody (MA)-33H1F7 was produced as described \(^{27}\). Goat anti-rat fibrin polyclonal antibody \(^{28}\) was kindly provided by Dr. J.J. Emeis (TNO Prevention and Health, Leiden, The Netherlands). All other reagents were of analytical grade.

**Animal model of intestinal I/R**

Adult male wistar rats (Charles Rivers, Broekman Instituut BV, Someren, The Netherlands), weighing 300-325 g, were fed standard rat chow (Hope Farms, Woerden, The Netherlands) and water ad libitum. The rats were allowed to acclimatize to our laboratory conditions for at least 4 days and were subjected to a regimen of 12:12 h / day-night cycle in mesh stainless-steel cages at constant temperature (22°C). The protocol was approved by the Animal Ethics Committee of the University of Amsterdam (The Netherlands). All animals were handled in accordance with the guidelines prescribed by the Dutch legislation and the International Guidelines on protection, care and handling of laboratory animals. The last 12 hours prior to the experiments, the animals had no access to solid food, but had free access to water.

Under anaesthesia with 1-2% isoflurane, rats were intubated and ventilated with air, O\(_2\), and 1-2% isoflurane. CO\(_2\) levels were kept between 37 and 43 mmHg. Continuous Ringer’s lactate solution was infused via the tail vein (10mL/hr.kg body weight) to correct for possible fluid loss during the experiment. A canula was inserted into the left carotid artery to measure the mean arterial pressure, which was kept between 90 and 110 mmHg during the experiment. Body temperature was maintained at 37 °C by use of a heating pad and lamp.

A 5 cm long midline laparotomy was performed and an intestinal loop of approximately 15 cm (10 cm proximal from the cecum) was isolated and cannulated with soft silicon tubes. This loop was gently rinsed with saline prior to connection to a perfusion pump, a heat exchanger and a reservoir to obtain a closed circuit. The reservoir contained freshly made Ringer’s solution consisting of (in mmol/L): 117.5 NaCl; 5.7 KCl; 25.0 NaHCO\(_3\); 1.2 mM MgSO\(_4\); 1.2 NaH\(_2\)PO\(_4\); 2.5 CaCl\(_2\) (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands). Glucose (25 mM) was added to this solution, in order to evaluate the absorptive function of the intestinal epithelium.

The superior mesenteric artery (SMA) was identified after deflecting the intestinal loops to the right side of the abdomen. The SMA was temporarily occluded by anatraumatic clamp at the origin of the aorta, avoiding the accompanying lymphatic trunk. Immediate blanching of the small intestine and cecum confirmed that the blood supply to the intestinal segments had been shut off. The abdomen was then covered with a sterile moist gauze pad. After the period of ischemia, the clamp was removed from the SMA and restoration of blood flow to the gut was confirmed by returning to its original colour. The rats in the sham-operation group underwent the same procedure except that clamping of the SMA was omitted. During reperfusion, luminal perfusion (1.0 mL/min) of the isolated intestinal loop was performed with the Ringer-glucose solution. Samples from the perfusate (0.25 mL each) were obtained after 1, 2 and 3 hours of reperfusion and stored at -20 °C until further analysis.

Portion blood samples (1.25 mL each) were collected after 5 minutes, and after 1 and 3 hours of reperfusion. Three times of portal blood sampling during the experiment was chosen to limit blood extraction (<15% of total blood volume). An equivalent volume of a warmed volume expander of 6% polyhydroxyethyl-starch and 0.9% NaCl (eloHaes, Fresenius Kabi, The Netherlands) was injected through the tail vein to maintain blood volume. Blood samples were collected in sodium-citrate buffer (final citrate concentration 0.32%) and in EDTA tubes and were centrifuged at 2,000g at 4 °C for 20 minutes and the plasma samples were stored at −80 °C until further analysis.
Rats were sacrificed by bleeding after final blood sampling and subsequent administration of heparin (2000 IU/kg) to prevent intravascular clotting. Biopsies of the small intestine were collected 5 cm proximal from the isolated rat intestinal loop and were fixed in 10% formaldehyde for histological examination.

To exclude different influences of anaesthesia upon the outcome parameters, all rats were anaesthetized for approximately 4.5 hours, which includes the duration of the surgical procedure and the period of ischemia and reperfusion.

**Experimental design of intestinal I/R**

In total, 54 rats were randomly allocated to one control group (saline) and two experimental groups (rt-PA and anti-PAI-1 monoclonal antibody, respectively), consisting of 18 rats each. rt-PA (1.0 mg/kg of body weight), anti-PAI-1 MA-33H1F7 (1.5 mg/kg) or 0.9% saline was administered intravenously into the penal vein during ischemia, 15 minutes before reperfusion. In each group, intestinal ischemia and reperfusion was induced by isolating and clamping the SMA with an atraumatic clamp for 0 minutes (sham operation, n = 6), 20 minutes (n = 6) or 40 minutes (n = 6), followed by 3 hours of reperfusion.

**Assessment of coagulation and fibrinolysis**

Plasma samples in sodium-citrate buffer, stored at -80 °C were utilized. Thrombin generation was assessed by measuring the thrombin-antithrombin (TAT) complexes with an enzyme-linked immunosorbent assay (ELISA) kit (Behring, Marburg, Germany). ATIII was measured by an automated amidolytic technique according to methods previously described. Fibrin degradation products (D-dimers) were assessed by an ELISA (Asserachrom D-Di, Diagnostica Stago, Asnières-sur-Seine, France). Plasminogen activator activity (PAA) was measured by an automated amidolytic assay. Briefly, 25 μL of plasma was mixed with 0.1 M TrisHCl, pH 7.5, 0.1% (v/v) Tween-80, 0.3 mM S-2251 (Chromogenix, Mölndal, Sweden), 0.13 M plasminogen and 0.12 mg/mL cyanogen bromide-digested fibrinogen fragments of fibrinogen to a final volume of 250μL. The amount of plasmin formed under these conditions is proportional to the concentration of PAA present, and can be spectrophotometrically detected by conversion of the chromogenic substrate. Plasminogen activator inhibitor (PAI)-1 activity was measured with the amidolytic method. Briefly, plasma was incubated with a fixed excess of t-PA (40 IU/mL) for 10 min at room temperature. The residual t-PA activity was determined by incubation with 0.13 μM plasminogen (Chromogenix, Sweden), 0.12 mg/mL cyanogen bromide-digested fibrinogen fragments and 0.1 mM S-2251 (Chromogenix, Sweden). Under these circumstances, the plasmin generated is inversely proportional to the amount of PAI-1 present.

**Immunohistochemical assessment of fibrin deposition**

Fibrin deposition was detected on formaldehyde-fixed tissue sections using immunohistochemistry according to standard procedures. Briefly, paraffin sections of jejunal tissue (4 μm) were deparaffinized and rehydrated through a graded series of xylene-ethanol. Endogenous peroxidase activity was blocked by treatment with 1.5% (v/v) H2O2 in phosphate buffered saline (PBS) for 30 minutes. Non-specific protein binding was blocked using Blocking Agent. All sections were incubated overnight at 4°C using polyclonal goat anti-rat fibrin antibody (TNO Prevention and Health, Leiden, The Netherlands) diluted in PBS (1:1600). Immunoreaction was detected using the Vectastain ABC Elite Kit (Vector Laboratories, Burlingame, England) and staining was developed using 0.5 mg/mL 3,3'-diaminobenzidine (DAB) (Sigma, Zwijndrecht, The Netherlands), 0.02% (v/v) H2O2 in 30 mM imidazole and 1mM EDTA (pH 7.0). The time of development was equal for all the tissues. Finally, sections were counterstained with hematoxylin, dehydrated and mounted. As negative controls, parallel sections consisted of the omission of the primary antibody and yielded no immunohistochemical reaction. Evaluation of fibrin deposition was performed by microscopical examination.
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Measurement of cytokines
In EDTA plasma samples, stored at -80 °C, levels of tumor necrosis factor-α, cytokine induced neutrophil chemoattractant, interleukin-1β and interleukin-6 were determined with the use of rat-ELISA kits (RnD Systems, Minneapolis, MN).

Histological assessment of intestinal injury
The in formaldehyde fixed jejunal tissues were embedded in paraffin, sectioned, and stained with haematoxylin and eosin for histological grading. The histological grading classification of Park-Chiu was used by two independent, non-informed examiners to assess intestinal injury. Briefly, the scores used were 0: normal mucosa, 1: subepithelial space at villus tips, 2: extension of subepithelial space with moderate lifting, 3: massive lifting down sides the villi, some denuded villi, 4: denuded villi, dilated capillaries, 5: disintegration of the lamina propria, 6: crypt layer injury, 7: transmucosal infarction and 8: transmural infarction.

Assessment of intestinal transport
Intestinal water transport (Clwater) was assumed to be reflected by the clearance of water from the total volume of the perfusion solution in the closed circuit (including the reservoir, connecting tubes and isolated intestinal loop) and was used to estimate net intestinal absorption and secretion. Glucose transport (Clglucose) was determined to measure the active-transport capacity of the epithelium. The glucose concentration (CGlucose) was determined by a glucose-detection kit (Sigma Diagnostics, St. Louis, MO, USA). The transport rate of water and glucose was determined as the clearance from the luminal perfusate per minute per gram intestine and calculated from the formula: Clearance (in μL/g.min) = (Ci*Vi-Cf*Vf) / (0.5*(Ci+Cf)*T*W) in which C is the detectable glucose concentration of the initial solution (i) and final solution (f), V the volume of the same solutions, T the time in minutes (min), and W the weight of the intestinal loop in grams (g).

Statistical analysis
The data analysis was performed using Graphpad Prism version 3.0 (Graphpad Software, Inc) for Windows 95. All quantitative data were presented as mean values ± standard error of the mean (SEM). Statistical analysis for repeated measurements was performed by analysis of variance and subsequent Bonferroni’s post-hoc test. Differences between experimental groups were analyzed by the unpaired student-t and where appropriate by the Mann-Whitney U test. P values <.05 were considered to be statistically significant.

Results
Effects of rt-PA and MA-33H1F7 on intestinal I/R-induced activation of coagulation and fibrinolysis
Intestinal I/R resulted in local intravascular coagulation activation and suppression of fibrinolysis. Thrombin generation and conversion of fibrinogen to fibrin was demonstrated by increase of the portal plasma levels of TAT-complexes and FDP (Figure 1). During 3 hours of reperfusion this thrombin generation resulted in local consumption of ATIII to 92±1% (P<.001) and 86±1% (P<.001) of baseline values after 20 and 40 minutes of ischemia, respectively, which was associated with a 3-fold and 5-fold rise of TAT-complexes, respectively. The initial rise of plasminogen activating activity after 1 hour of reperfusion, demonstrating local activation of fibrinolysis, was blunted by fibrinolytic inhibition to 91±2% (P<.002) and to 81±2% (P<.001) of baseline levels, 3 hours after 20 and 40 minutes of ischemia, respectively. This decrease of plasminogen activating activity was accompanied by an increase in portal plasma levels of PAI-1, starting at 1 hour of reperfusion and reaching levels of 17±1 IU/mL (P<.001) and 25±2
Figure 2. Effects of rt-PA and MA-33H1F7 on intestinal I/R-induced fibrin deposition and microvascular thrombosis

Representative results of fibrin deposition after 3 hours of reperfusion are shown of animals in the sham-operated group (A,B), and of animals subjected to 40 minutes of ischemia, treated with saline (C,D), rt-PA (E and F), or MA-33H1F7 (G,H). For A, C, E, and G, original magnification x200 (bar in A represents 50 μm); for B, D, F and H, original magnification x500 (bar in B represents 20 μm).
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A. TAT (ng/mL) vs. Time of reperfusion (min)

B. TAT (ng/mL) vs. Time of reperfusion (min)

C. ATIII (%) vs. Time of reperfusion (min)

D. ATIII (%) vs. Time of reperfusion (min)

E. FDP (ng/mL) vs. Time of reperfusion (min)

F. FDP (ng/mL) vs. Time of reperfusion (min)
Figure 1. Effects of rt-PA and MA-33H1F7 on intestinal I/R-induced activation of coagulation and fibrinolysis

Activation of coagulation and suppression of fibrinolysis were determined by the measurement of portal plasma levels of thrombin-antithrombin (TAT)-complexes (A,B), antithrombin III (ATIII) (C,D), fibrin degradation products (FDP) (E,F), plasminogen activator activity (PAA) (G,H) and plasminogen activator inhibitor (PAI)-1 (I,J). Animals were intravenously administered saline, rt-PA (1.0 mg/kg of body weight) or anti-PAI-1 MA-33H1F7 (1.5 mg/kg), 15 minutes before reperfusion. Data (n = 6, in each group) are expressed as mean values ± SEM. Portal plasma levels of repeated measurements during 3 hours of reperfusion in saline treated animals subjected to sham operation ( ), or to 20 minutes ( ) or 40 minutes ( ) of ischemia are depicted in A, C, E, G and I. Portal plasma levels of animals treated with saline (closed bars), rt-PA (crossed bars), or MA-33H1F7 (blocked bars), subjected to 20 minutes or 40 minutes of ischemia after 3 hours of reperfusion are depicted in B, D, F, H and J. *P<.05 compared with the sham-operated group; †P<.05 compared with 20 minutes I/R plus saline group; ‡P<.05 compared with 40 minutes I/R plus saline group.

IU/mL (P<.001) after 20 and 40 minutes ischemia and 3 hours of reperfusion, respectively.

Administration of rt-PA enhanced circulatory plasminogen activator activity (PAA exceeded beyond detection limit) (Figure 1H). This increased fibrinolytic activity resulted in a 2.5-fold and 4-fold increase of baseline FDP portal plasma levels (272±22 ng/mL)
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after 20 and 40 minutes of ischemia and 3 hours of reperfusion, respectively (Figure 1F). Administration of rt-PA eliminated approximately all free active PAI-1 (Figure 1J).

Administration of anti-PAI-1 monoclonal antibody abolished the I/R-induced suppression of fibrinolysis by decreasing PAI-1 activity resulting in approximately a 2-fold increase of plasminogen activating activity after 20 and 40 minutes of ischemia (Figure 1H). This increased fibrinolytic activity led to a 3-fold and 4.5-fold rise of baseline FDP portal plasma levels (266±23 ng/mL) after 20 and 40 minutes of ischemia and 3 hours of reperfusion, respectively (Figure 1F).

Effects of rt-PA and MA-33H1F7 on intestinal I/R-induced fibrin deposition and microvascular thrombosis

Microscopical assessment of intestinal stainings of saline treated rats revealed mucosal and submucosal fibrin deposits, microvascular thrombotic obstructions and villus microvessel hemorrhage after 20 (data not shown) and 40 minutes of intestinal ischemia (Figure 2C and 2D) and 3 hours of reperfusion, whereas histological examination of intestinal stainings after sham operation did not reveal any mucosal fibrin deposits, microvascular thrombotic obstructions or villus microvessel hemorrhage (Figure 2A and 2B).

Administration of rt-PA and anti-PAI-1 antibody had no effect at all on mucosal and submucosal deposition of fibrin, microvascular thrombotic obstructions or villus microvessel hemorrhage after 20 and 40 minutes of intestinal ischemia and 3 hours of reperfusion (Figure 2E-H).

Figure 3. Effects of rt-PA and MA-33H1F7 on intestinal I/R-induced inflammation

The local pro-inflammatory response to intestinal I/R was determined by the detection of interleukin-6 in portal plasma. Data (n = 6, in each group) are expressed as mean values ± SEM. Interleukin-6 portal plasma levels of saline treated, sham operated animals ( ), or subjected to 20 minutes ( ) or 40 minutes ( ) of ischemia during 3 hours of reperfusion are depicted in A. Interleukin-6 portal plasma levels of animals treated with saline (closed bars), rt-PA (crossed bars), or MA-33H1F7 (blocked bars), subjected to 20 minutes or 40 minutes of ischemia after 3 hours of reperfusion are depicted in B. *P<.05 compared with the sham-operated group; †P<.05 compared with 20 minutes I/R plus saline group; ‡P<.05 compared with 40 minutes I/R plus saline group.
**Effects of rt-PA and MA-33H1F7 on intestinal I/R-induced inflammation**

Portal plasma levels of tumor necrosis factor-α, cytokine induced neutrophil chemoattractant and interleukin-1β were below the 15 pg/mL detection limit. Portal plasma concentrations of interleukin-6 were significantly higher in rats subjected to 20 (P=.046) and 40 (P=.002) minutes of intestinal ischemia as compared to sham-operated rats after 3 hours of reperfusion (Figure 3). The increases of portal plasma interleukin-6 after 20 and 40 minutes of ischemia followed by reperfusion were significantly reduced to sham-operated values by rt-PA administration (P=.028 and P=.049, respectively) and anti-PAI-1 antibody administration (P=.022 and P=.010, respectively).

**Effects of rt-PA and MA-33H1F7 on I/R-induced intestinal injury and dysfunction**

Histological analysis of rat intestinal tissues, subjected to sham operation, or to 20 or 40 minutes of intestinal ischemia, was performed after 3 hours of reperfusion according to the Park-Chiu classification. Microscopical assessment of intestinal injury revealed subepithelial spaces in the villus tips with moderate to massive lifting, together with villus denudation in saline treated animals after 20 minutes of ischemia with a mean score of 3.2±0.4 (range 2-4, P=.007) (Figure 4A). Intestinal injury was increased to disintegration of the lamina propria in most intestinal tissues of saline treated animals after 40 minutes of ischemia, with a mean score of 4.7±0.2 (range 4-5, P=.001); such changes were not observed in sham-operated animals. Neither rt-PA nor anti-PAI-1 monoclonal antibody administration reduced I/R-induced intestinal injury.

Intestinal clearances of glucose and water during 3 hours of reperfusion were significantly and dose-dependently decreased after 20 and 40 minutes of intestinal ischemia in saline treated animals (Figure 4B and 4C). Administration of rt-PA or anti-PAI-1 antibody did not attenuate I/R-induced intestinal dysfunction.

**Discussion**

Intestinal I/R-induced endothelial cell injury results in a procoagulant and fibrinolysis-suppressing environment giving rise to fibrin deposition, which may further compromise the microcirculation of the intestine and promote necrosis in distal intestinal tissue. Mechanisms that play a role in the procoagulant response are the upregulation of tissue factor in combination with dysfunctional anticoagulant pathways, along with suppression of fibrinolysis mainly due to increased levels of the inhibitor of fibrinolysis: PAI-1. In the present study we investigated the relative contribution of suppressed endogenous fibrinolysis to the development of fibrin deposition and microthrombosis following intestinal reperfusion injury, by restoring the suppressed fibrinolytic activity either by intravenous administration of rt-PA or by inhibition of PAI-1 by monoclonal antibody 33H1F7.

Intestinal I/R resulted in inadequate removal of mucosal and submucosal fibrin deposits and microthrombotic obstructions in postischemic intestinal tissue in saline treated animals. This was presumably the result of a dysfunctional fibrinolytic system, as reflected by the suppression of plasminogen activator activity following increased plasma levels of PAI-1. Restoration of the dysfunctional fibrinolytic system by rt-PA administration or PAI-1 inhibition enhanced intravascular fibrinolytic activity, as reflected by elevated plasminogen activating activity and total abolishment of PAI-1 in the
Figure 4. Effects of rt-PA and MA-33H1F7 on I/R-induced intestinal injury and dysfunction

Histological analysis of intestinal tissues was performed after 3 hours of reperfusion and assessed according to the Park-Chiu classification. Intestinal clearances of glucose and water during 3 hours of reperfusion were determined in an intestinal loop of approximately 15 cm. Data of animals treated with saline (closed bars), rt-PA (crossed bars), or MA-33H1F7 (blocked bars) are expressed as means ± SEM (n = 6, in each group). *P<.05 compared with the sham-operated group.
mesenteric circulation. Subsequent increased levels of FDP indicated augmented removal of intravascular fibrin in both groups, which is supported by a previous study, in which administration of anti-PAI-1 antibody reduced thrombosis and restored postsischemic reperfusion in mesenteric arterioles. Although in the current study adequate intravascular, thrombolytic activity increased intravascular fibrin removal and restored reperfusion in arterioles, mucosal and submucosal fibrin deposition and microthrombosis in combination with continuing activated coagulation in postsischemic intestinal tissue was not reduced either after rt-PA administration or after PAI-1 inhibition. Also, the enhanced fibrinolytic activity in our model did not attenuate I/R-induced intestinal injury, as demonstrated by morphological and functional analysis. Postischemic intestinal structure and water and glucose clearances were equally affected in saline, rt-PA or anti-PAI-1 treated animals. Although a hypofibrinolytic state may hypothetically contribute to thrombotic obstruction and may hamper adequate microcirculation thereby promoting distal injury, “recanalization” of the thrombotic microvasculature by fibrinolysis did not attenuate the sequelae of intestinal post-ischemic, reperfusion injury.

Modulation of fibrinolysis was shown to cause similar results in experimental models of acute myocardial ischemia and reperfusion. Administration of rt-PA or streptokinase did not benefit ventricular function and structure in canine models of coronary I/R. The results of these studies suggest that fibrin deposition and associated injury is of limited importance for the total amount of necrosis. Consequently, based on these reports and our present observations, it may be suggested that the relative contribution of suppressed endogenous fibrinolysis to microcirculatory fibrin deposition and I/R-injury is of limited importance.

As the inflammatory response plays a pivotal role in I/R injury, inhibition of inflammatory cell recruitment/migration and cytokine release has demonstrated to reduce I/R-injury. It is therefore noteworthy that, in addition to their effects on local fibrinolytic activity, administration of rt-PA or anti-PAI-1 antibody significantly inhibited the inflammatory response following intestinal I/R, as demonstrated by the reduction of interleukin-6 portal plasma levels to baseline values. Interestingly, these results illustrate the tight cross-talk between coagulation and fibrinolysis on the one hand and activation of inflammation on the other hand.

Previous studies have shown that tumor necrosis factor-α and interleukin-1β are pivotal regulators of plasminogen activators and inhibitors. Inhibition of these inflammatory mediators by monoclonal antibodies, soluble receptors, or receptor antagonists resulted in an abolishment of endotoxin-induced effects on fibrinolysis. Experiments in mice with targeted disruptions of genes encoding components of the plasminogen-plasmin system confirm that fibrinolysis itself may also affect inflammation. Mice with a deficiency of plasminogen activators have more extensive inflammation in various tissues when challenged with endotoxin, whereas PAI-1 knockout mice, in contrast to wild-type controls, display a reduction of inflammatory activity. It is thought that fibrinolytic activators and inhibitors may modulate the inflammatory response by their effect on inflammatory cell recruitment and migration. In particular the plasminogen activator u-PA and its receptor (u-PAR) play a central role in this respect. Taken together, it seems that the fibrinolytic shutdown as occurs during intestinal I/R, may contribute to the inflammatory activity and results in enhanced release of interleukin-6, whereas promotion of endogenous fibrinolysis as achieved in our experiments by
administration of r-tPA or inhibition of PAI-1, may modulate this inflammatory activity and result in lower interleukin-6 expression. However, in spite of inflammatory response modulation, administration of r-tPA or inhibition of PAI-1 did not attenuate I/R-induced intestinal injury.

In conclusion, intestinal I/R resulted in considerable derangement of the coagulation and inflammatory system, and compromised the enteric microcirculatory system by widespread deposition of fibrin and microthrombosis. Despite enhancement of fibrinolytic activity, administration of rt-PA or anti-PAI-1 antibody neither increased removal of mucosal and submucosal fibrin deposition nor attenuated intestinal I/R injury. These results suggest a limited role of suppressed fibrinolysis in compromising enteric microcirculation with subsequent deterioration of intestinal function and structure following intestinal I/R.

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References


Role of fibrinolysis in intestinal ischemia/reperfusion injury


Hypoxia/reoxygenation impairs glucose absorption and cAMP-mediated secretion more profoundly than glutamine absorption and Ca\(^{2+}\)/PKC-mediated secretion in rat ileum *in vitro*.

Ivo G. Schoots\(^1\), Pieter B. Bijlsma\(^1\), Geert I. Koffeman\(^1\), Thomas M. van Gulik\(^1\)

Departments of \(^1\)Surgery and Surgical Laboratory, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands

*Intraluminal fluid sequestration following intestinal ischemia – a better understanding*
Differential effects of hypoxia on intestinal absorption and secretion

Abstract

Background: Intestinal ischemia and reperfusion may lead to profuse secretion of water and electrolytes. The underlying mechanisms have been related to increased hydrostatic pressure, to denudation of intestinal villi and recently, to adenosine-mediated enhancement of chloride secretion.

Methods: We studied the effects of hypoxia and reoxygenation on baseline electrophysiological parameters, on glucose- and glutamine-induced absorption, on secretion induced by carbachol, histamine and forskolin, and on epithelial barrier function to disodium-fluorescein and horseradish peroxidase, in rat ileum mounted in Ussing chambers.

Results: We observed that 30 minutes of hypoxia followed by 60 minutes of reoxygenation differentially affected glucose- and glutamine-absorption to respectively 11 % and 42 % of control values. cAMP-mediated secretion induced by forskolin was reduced to 9 % of controls. In contrast, Ca$^{2+}$/PKC-mediated secretion induced by carbachol or histamine was only reduced to 35-48 % of controls. Furthermore, the epithelium was fully capable to maintain its barrier function to small and large permeability probes, even after 90 minutes of hypoxia.

Conclusion: We conclude that hypoxia and reoxygenation differentially impairs nutrient absorption, corroborating recent absorption data in \textit{in vivo} models of ischemia, and that it differentially affects secretory capacity in crypts, dependent on the intracellular messenger pathway. The relative persistence of Ca$^{2+}$/PKC-mediated secretion to hypoxia and reoxygenation indicates that secretagogues that activate this pathway play a significant role in the intraluminal fluid sequestration and diarrhea observed after intestinal ischemia and reperfusion.
Chapter 10

Introduction

Intestinal ischemia and reperfusion injury may occur in a variety of pathophysiologic conditions, e.g. hemorrhage, trauma, and sepsis, and can instigate multiple organ failure. Ischemia and reperfusion result in intestinal epithelial dysfunction which can lead to the disruption of the physiological barrier between the intestinal lumen and the internal milieu, and therefore result in increased exposure to pro-inflammatory cytokines and susceptibility to infection.

An early manifestation of epithelial dysfunction due to ischemia/reperfusion is the switch from net intestinal absorption to net secretion leading to intraluminal fluid sequestration and diarrhea. At the microscopical level, the first visible damage consists of detachment of the villus epithelial cells, with the formation of subepithelial blebs. Salt and nutrient uptake, with fluid co-transport, is localized in the villi. This active transport may be decreased after deprivation of oxygen due to depletion of ATP reserves and loss of Na⁺-K⁺-ATPase activity. Under normal physiological conditions active anion secretion and associated water secretion is mainly confined to the intestinal crypts. Immediately after acute ischemia, a profuse secretion of water and electrolytes develops during reperfusion. The underlying mechanisms causing this net secretion are still incompletely understood. It has been attributed to an increase in "filtration flow" into the lumen as a result of increased hydrostatic pressure in the subepithelial tissue, combined with an increased hydraulic conductivity of the intestinal epithelium due to a loss of villus epithelial integrity following ischemia. Robinson and coworkers questioned this hypothesis, because after ischemia the capillary circulation is obstructed, so that movement of fluid across the capillaries of the villus core is rather unlikely. Instead, they proposed that the net secretion results from the observed epithelial cell loss of the villi (villus denudation) after ischemia, leading to an arrest of the absorptive process, while the less damaged crypt epithelium still continues to secrete, thus causing an imbalance between absorption and secretion after ischemia. More recently, it was hypothesized that ischemia may actually stimulate secretion, based on the observation that chemical hypoxia induced adenosine-mediated anion secretion in T84 colon carcinoma cell monolayers. This hypoxia-induced secretory response in cell lines is not observed in in vivo animal models, where secretion is not apparent during the ischemic episode, but manifest after the initiation of reperfusion.

Another manifestation of epithelial dysfunction following ischemia is the increase of epithelial permeability. This has also been attributed to the desquamation of villus intestinal cells and the resulting villus denudation as observed in in vivo studies. Alternatively, the loss of intestinal barrier function may be caused by increased tight junctional permeability, which can be induced by chemical hypoxia in T84 colon carcinoma cell monolayers. The intestinal permeability to small (e.g. Cr-EDTA) and large (Bovine Serum Albumin) probe molecules after ischemia and reperfusion has been widely investigated in vivo, and intestinal permeability increments in various animal models are detectable after ischemic episodes of 20 to 60 minutes. In contrast, T84 and Caco-2BBe intestinal monolayers are extremely resistant to true hypoxia, and in the latter cell line the earliest permeability increases to small probe molecules like fluorescein sulfonic acid appeared after 12 hours of true hypoxia, and such increments were still indetectable for fluorescein-isothiocyanate dextran 70 (MW 70 kD) after 48 hours of hypoxia. On the other hand, interpretation of particularly macromolecular
permeability measurements from blood-to-lumen or lumen-to-blood as shown in vivo animal studies \cite{19,21,22} is complicated, as also the vascular endothelial barrier is restrictive to macromolecules and may be affected by ischemia \cite{23,24}.

Considering the complex interplay between vascular, subepithelial and epithelial factors in in vivo models of ischemia and reperfusion, and taking into account that compared to in vivo studies, monolayers of intestinal cell-lines show a notably different response to hypoxia, it is of interest to study the effects of hypoxia and reoxygenation in in vitro small intestinal preparations. We determined baseline electrophysiological parameters, glucose absorption, glutamine absorption, cAMP-mediated secretion induced by forskolin, Ca\(^{2+}\)/PKC-mediated secretion induced by carbachol or histamine, and epithelial barrier function using disodium-fluorescein and horseradish peroxidase as permeability probes, during varying periods of hypoxia and reoxygenation in rat ileum mounted in Ussing chambers.

**Material & Methods**

**Animals.** All experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee of the Academic Medical Centre and the University of Amsterdam. Adult, male Wistar rats (Charles Rivers, Broekean Instituut BV, Someren, the Netherlands), weighing 300-350 g, were fed standard rat chow (Hope Farms, Woerden, the Netherlands) and water ad libitum. Rats were acclimatized to laboratory conditions for at least 7 days prior to experiments. Animals were housed in stainless-steel cages at a constant temperature (22°C) and subjected to a regimen of 12:12 hours light-dark cycle regimen.

**Experimental procedure and electrophysiological measurements.** Rats were anaesthetized with a mixture of ketamine (90 mg/kg) and xylazine (10 mg/kg) via intramuscular injection. Laparotomy was performed by midline incision. A segment of distal ileum was isolated 3 to 5 cm from the coecum, and the lumen was rinsed with Ringer’s solution (37°C) to remove intestinal contents. After ligating the blood supply, the ileal segment was rapidly excised and placed in ice-cold Ringer’s solution. Animals were then killed by intracardial injection of sodium-pentobarbital. The isolated segments were stripped of muscle layers and mounted in Ussing-chambers within 10 minutes after excision. Inclusion of Peyers patches was avoided and silicone grease was used to minimize edge damage. The exposed serosal tissue surface area was 50 mm\(^2\). Mucosal and serosal compartments contained 10 ml of Ringer’s solution, which was thermostated to 37°C, carbogenated (humidified 95% O\(_2\), 5% CO\(_2\); pH 7.3) and circulated by gas-lifting. The serosal solution also contained 2.0 mM L-Glutamine.

Transepithelial potential difference was measured with Ag/AgCl-electrodes. Transepithelial resistance (R\(_t\)) was determined every 30 seconds from voltage deflections induced by 10 \(\mu\)A bipolar current pulses through platinum wires. Electrodes and platinum wires were connected to the perfusion solution via Ringer-agar bridges. The equivalent short circuit current (I\(_{sc}\)) and R\(_t\) were calculated according to Ohm’s law. Continuous monitoring of the transepithelial potential difference was performed by a customized computer program using Lab View (National Instruments, USA). Electrophysiological parameters were monitored during the entire experimental time course.

**Secretory and absorptive function.** The viability of the ileal epithelium to react to a secretory stimulus during the time course of the experiments was judged by the measurements of carbachol-induced I\(_{sc}\)-changes (10\(^{-5}\) M, added serosal), determined at 30, 60, 90 or 120 minutes in four different control tissues per animal. The viability to react to an absorptive stimulus was judged in the same tissues by determination of I\(_{sc}\)-changes, induced by mucosal addition of 20 mM D-glucose at 10 minutes after carbachol administration (20 mM of mannitol was added concomittantly to the serosal side to maintain iso-osmolality).
**Barrier function.** After mounting the tissues, the permeability probes disodium-fluorescein (Na$_2$Fl) and horseradish peroxidase (HRP) were added mucosally to a final concentration of $10^{-5}$ M. Serosal samples of 500 µL were taken every 30 min for two hours and were replaced by an equal volume of Ringer’s solution. Permeability of the ileal epithelium was determined by the serosal appearance of Na$_2$Fl and HRP. Na$_2$Fl was detected in a fluorescence-reader (Cytoflour®, Series 4000, PerSeptive Biosystems, Inc., Framingham, MA, USA) at excitation and emission wavelengths of 485/20 and 530/25 nm, respectively. HRP was measured enzymatically. Samples of 100µl were mixed in a 96 wells plate with 100µl phosphate buffer (0.1 M, pH 6.0) containing 0.003% H$_2$O$_2$ and 0.009% ortho-dianisidine dihydrochloride (Sigma-Aldrich Chemie BV, Zwijndrecht, the Netherlands). After 15 minutes the reaction was halted by adding a 50 µl 2.0 M H$_2$SO$_4$ solution. The HRP concentration-dependent optical absorption rate was determined with a spectrophotometer at 450-490 nm wavelength (Thermo Max, Molecular Devices Co., Sunnyvale, CA, USA).

**Hypoxia and reoxygenation.** The effects of hypoxia and reoxygenation on nutrient and electrolyte transport were studied after 30 min equilibrium time. Hypoxia was induced by ending carbogenation and perfusing both compartments with 95% N$_2$ and 5% CO$_2$. As a result oxygen pressure decreased from 540-560 mmHg to 45-50 mmHg within 2-3 minutes. Reoxygenation was achieved by returning to carbogen perfusion. After 30 min of hypoxia and 60 min of reoxygenation of tissues the $I_{sc}$-changes induced by glucose, glutamine, carbachol, histamine and forskolin were measured. Baseline electrophysiological parameters were monitored during the entire time course of the experiments. The barrier function was studied in control tissues during 120 minutes, and in the hypoxia-reoxygenation experiments mentioned above, using the permeability probes Na$_2$Fl and HRP. The epithelial barrier function was furthermore studied in a second series of experiments, in which hypoxia was induced not only for 30 minutes, but also for 60 and 90 minutes. In these experiments, hypoxia was induced at $t = 0$ minutes. The equilibrium time was omitted to ensure a 90, 60 or 30 minute reoxygenation period, respectively.

**Histology.** Following the experiments, the ileal tissues were fixed in 10% formaldehyde, embedded in paraffin, sectioned, and stained with haematoxylin and eosin (H&E) for histological examination. Test solution composition, chemicals. Krebs-Ringer’s solution contained 117.5 mM NaCl, 5.7 mM KCl, 25.0 mM NaHCO$_3$, 1.2 mM MgSO$_4$, 1.2 mM NaH$_2$PO$_4$, and 2.5 mM CaCl$_2$. All chemicals, with HRP being type II were obtained from Sigma-Aldrich Chemie BV (Zwijndrecht, the Netherlands), except L-Glutamine (Biowittaker, Verviers, Belgium) and disodium-fluorescein (Molecular Probes, Leiden, The Netherlands). Forskolin and indomethacin were dissolved in ethanol, and final concentrations of ethanol in the serosal compartment of the Ussing chambers were 0.1%. This concentration was without detectable effect on all measured parameters.

**Statistical analysis.** Results reported represent mean values ± standard error of the mean (SEM). Statistical analysis was performed by the Mann-Whitney U test. A value of $P < 0.05$ (two-tailed) was considered statistically significant.

**Results**

All investigated tissues demonstrated a serosa-positive transepithelial potential difference after mounting tissues in the Ussing chambers. After 30 min of equilibrium time, the $I_{sc}$ of control experiments decreased from 60.0±7.0 to 32.5±4.0 µA/cm$^2$ ($n = 16$) during the experimental time course (*Figure 1A*). $R_t$ decreased from 42.5±1.9 to 22.1±1.7 Ω·cm$^2$ at $t = 120$ min (*Figure 1B*). Thereafter, both the $I_{sc}$ and $R_t$ stabilized in control tissues monitored for 150 min. Serosally applied to control tissues at 30 min, tetrodotoxin (TTX, $10^{-6}$ M), atropine ($10^{-5}$ M) or indomethacin ($10^{-5}$ M) had no detectable effect on spontaneous $I_{sc}$ or $R_t$ (all tested at $n = 6$, data not shown).
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Effects of hypoxia and reoxygenation on short circuit current (I_sc) and transepithelial resistance (Rt). During 30 min of hypoxia the I_sc declined sharply from 65.8±7.5 to 2.8±2.8 μA/cm² (n = 6) within 10 min of hypoxia (Figure 1A), whereas R_t did not change compared to control epithelia (Figure 1B). Within minutes, the I_sc showed a rapid rise during reoxygenation. A peak value of I_sc was invariably observed, immediately following reoxygenation, after which I_sc levels increased further to control values during reoxygenation.

![Figure 1 A, B. Short circuit current (I_sc) (Figure 1A) and trans-epithelial resistance (R_t) (Figure 1B) in rat ileum control tissues (n = 16) and in tissues exposed to hypoxia from t = 30 min to t = 60 min (n = 6). During hypoxia the I_sc declined significantly from 65.8±7.5 to 2.8±2.8 μA/cm² within 10 min of hypoxia (* P < 0.05). A rapid increase of I_sc was observed immediately after reoxygenation. This increase of I_sc was followed by a limited decline, after which I_sc levels regained initial control values. R_t remained unchanged throughout hypoxia and reoxygenation. Values represent means ± SEM.](image)

Effects of hypoxia and reoxygenation on nutrient-induced absorption and carbachol-induced secretion. Changes in the I_sc (ΔI_sc), induced by serosal addition of 10⁻⁵ M carbachol and by mucosal administration of 20 mM D-glucose remained constant throughout the time course of control experiments, with no significant differences between additions at 30, 60, 90 or 120 minutes (n = 5 - 6, Table 1).

Changes in I_sc, induced by carbachol or by D-glucose after 30 min hypoxia and 60 min of reoxygenation (Figure 2A), showed significant reductions compared to I_sc-changes in control tissues. Carbachol-induced I_sc-changes decreased 2-fold, from 72.9±17.4 to 33.8±11.4 μA/cm² (n = 6, P = 0.041), and D-glucose-induced I_sc-changes decreased 10-fold, from 17.6±2.7 to 1.8±0.8 μA/cm² (n = 6, P = 0.002). The decrease of I_sc-changes after D-glucose stimulation to 11±6 % of matched controls (=100 %) was significantly different from the decrease of I_sc-changes after carbachol stimulation to 47±8 % of matched controls (P = 0.015).
Table 1. $I_{sc}$-changes induced by addition of carbachol and glucose at 30, 60, 90 or 120 minutes after tissue mounting.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Glucose $\Delta I_{sc}$</th>
<th>Carbachol $\Delta I_{sc}$</th>
<th>Number (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>20.7 ± 3.7</td>
<td>87.7 ± 2.5</td>
<td>5</td>
</tr>
<tr>
<td>60</td>
<td>23.5 ± 6.4</td>
<td>79.8 ± 17.7</td>
<td>5</td>
</tr>
<tr>
<td>90</td>
<td>17.0 ± 2.1</td>
<td>83.7 ± 10.2</td>
<td>6</td>
</tr>
<tr>
<td>120</td>
<td>17.6 ± 2.7</td>
<td>72.9 ± 17.4</td>
<td>6</td>
</tr>
</tbody>
</table>

$I_{sc}$-changes ($\Delta I_{sc}$) induced by serosal addition of $10^{-5}$ M carbachol at 30, 60, 90 or 120 minutes after tissue mounting, and $I_{sc}$-changes, induced by mucosal addition of 20 mM D-glucose at 10 minutes after carbachol administration. No significant differences in $I_{sc}$-changes were observed between different timepoints of addition of either carbachol or D-glucose.

After 30 min hypoxia and 60 min of reoxygenation, changes in $I_{sc}$, induced by L-glutamine or by carbachol (Figure 2B) also showed significant reductions compared to $I_{sc}$-changes in control tissues. Carbachol-induced $I_{sc}$-changes decreased 3-fold, from 51.7±8.5 to 15.6±2.3 $\mu$A/cm$^2$ ($n = 7$, $P = 0.002$), and L-glutamine-induced $I_{sc}$-changes also decreased 3-fold, from 22.6±4.5 to 7.3±1.3 $\mu$A/cm$^2$ ($n = 7$, $P = 0.002$). The decrease of $I_{sc}$-changes after L-glutamine stimulation to 42±10 % of matched controls (=100 %) was comparable to the decrease of $I_{sc}$-changes after carbachol stimulation to 35±7 % of matched controls ($P = 0.63$).

Figure 2 A. Changes in short circuit current ($\Delta I_{sc}$) in rat ileum ($n = 6$), induced by 20 mM D-glucose or by $10^{-5}$M carbachol after 30 min hypoxia and 60 min reoxygenation (hatched bars), were significantly (* $P < 0.05$) decreased compared to control values (open bars), but to a different extent, respectively 11±6 % and 47±8 % of matched controls ($P = 0.015$). Values represent means ± SEM. Inserts: typical recording of control responses to D-glucose and to carbachol.

Figure 2 B. Changes in short circuit current ($\Delta I_{sc}$) in rat ileum ($n = 7$), induced by 20 mM L-glutamine or by $10^{-5}$M carbachol after 30 min hypoxia and 60 min reoxygenation (hatched bars), were significantly (* $P < 0.05$) decreased compared to control values (open bars), to a comparable extent, respectively 42±10 % and 35±7 % of matched controls ($P = 0.63$). Values represent means ± SEM. Inserts: typical recording of control responses to L-glutamine and to carbachol.

Effects of hypoxia and reoxygenation on forskolin- and histamine-induced secretion compared to carbachol-induced secretion. Control values of $10^{-5}$M forskolin-induced secretion at $t = 120$ minutes were 49.0±14.7 $\mu$A/cm$^2$ (Figure 3A). Control values of $10^{-5}$M carbachol-induced secretion, added 10 minutes later, were 93.8±21.6 $\mu$A/cm$^2$ (Figure 3A),
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not significantly different from control values in Figure 2A and 2B, indicating that prior addition of forskolin did not decrease $I_{sc}$-responses to carbachol. After 30 min hypoxia and 60 min of reoxygenation changes in $I_{sc}$, induced by forskolin or by carbachol (Figure 3A), showed significant reductions compared to $I_{sc}$-changes in control tissues. Forskolin-induced $I_{sc}$-changes decreased 10-fold to $3.0 \pm 1.2 \mu A/cm^2$ ($n = 6, P = 0.002$), and carbachol-induced $I_{sc}$-changes decreased 2-fold to $38.4 \pm 6.1 \mu A/cm^2$ ($n = 6, P = 0.026$). The decrease of $I_{sc}$-changes after forskolin addition to 9±3 % of matched controls (=100 %) was significantly different from the decrease of $I_{sc}$-changes after carbachol administration to 49±11 % of matched controls ($P = 0.004$).

Figure 3 A. Changes in short circuit current ($\Delta I_{sc}$) in rat ileum (n = 6), induced by $10^{-5}$M forskolin or by $10^{-5}$M carbachol after 30 min hypoxia and 60 min reoxygenation (hatched bars), were significantly (* $P < 0.05$) decreased compared to control values (open bars), but to a different extent, respectively 9±3 % and 49±11 % of matched controls ($P = 0.004$). Values represent means ± SEM. Inserts: typical recording of control responses to forskolin and to carbachol.

Figure 3 B. Changes in short circuit current ($\Delta I_{sc}$) in rat ileum (n = 9), induced by $10^{-4}$M histamine or by $10^{-5}$M carbachol after 30 min hypoxia and 60 min reoxygenation (hatched bars), were significantly (* $P < 0.05$) decreased compared to control values (open bars), to a comparable extent, respectively 44±14 % and 47±15 % of matched controls ($P = 1.0$). Values represent means ± SEM. Inserts: typical recording of control responses to histamine and to carbachol.

Control values of $10^{-4}$M histamine-induced secretion at t = 120 minutes were 56.4±9.3 $\mu A/cm^2$ (Figure 3B). Control values of $10^{-5}$M carbachol-induced secretion, added 10 minutes later, were 70.5±9.1 $\mu A/cm^2$ (Figure 3B), not significantly different from control values in Figure 2A and 2B, indicating that prior addition of histamine also did not decrease $I_{sc}$-responses to carbachol. After 30 min hypoxia and 60 min of reoxygenation changes in $I_{sc}$, induced by histamine or by carbachol (Figure 3B) showed significant reductions compared to $I_{sc}$-changes in control tissues. Histamine-induced $I_{sc}$-changes decreased 2-fold to $26.0 \pm 9.2 \mu A/cm^2$ ($n = 9, P = 0.024$), and carbachol-induced $I_{sc}$-changes decreased once more 2-fold to $32.4 \pm 9.3 \mu A/cm^2$ ($n = 9, P = 0.014$). The decrease of $I_{sc}$-changes after histamine addition to 44±14 % of matched controls (=100 %) was comparable to the decrease of $I_{sc}$-changes after carbachol administration to 47±15 % of matched controls ($P = 1.0$).

Effects of hypoxia and reoxygenation on barrier function. $Na_2Fl$-fluxes reached steady-state values after 60 to 90 min (Figure 4A). The permeability of rat ileal epithelium for $Na_2Fl$ did not change when subjected to 30 min of hypoxia and reoxygenation,
irrespective of whether the hypoxia was introduced after a 30 min equilibrium period (with 60 min reoxygenation time, data not shown), or at the onset of the experiment at \( t = 0 \) min (without an equilibrium period, with 90 min reoxygenation time) (Figure 4A). Even after prolonged periods of hypoxia, 60 and 90 min, no change in \( \text{Na}_2 \text{Fl} \) permeability was observed compared to controls (\( n = 6 - 10 \), see legend Figure 4). HRP-flux reached steady-state values at 90 min in control experiments (Figure 4B). Also the HRP-fluxes at the three different periods of hypoxia and reoxygenation were comparable to control values.

**Figure 4 A-B.** Mucosal to serosal flux of \( \text{Na}_2 \text{Fl} \) (Figure 5A) and HRP (Figure 5B) in rat ileum *in vitro* in control tissues (open bars; \( n = 10 \)), or exposed to 30 min (forward hatched bars; \( n = 6 \)), 60 min (backwards hatched bars; \( n = 6 \)) or 90 min (crossed bars; \( n = 6 \)) of hypoxia, which were introduced at \( t = 0 \) min. No significant differences were observed between hypoxia-exposed tissues as compared to controls. Values represent means ± SEM.

**Effects of hypoxia and reoxygenation on morphology.** Histological appearance of the tissues at the end of the hypoxia-reoxygenation experiments as determined by light microscopy was unchanged compared to control tissues. In both control and hypoxia-reoxygenated tissues a shortening of the villi was observed, with some cell sloughing at the tips of the villi, as well as widened intercellular spaces between and occasionally under villus epithelial cells, corresponding to previously reported data on morphology of oxygenated rat ileum mounted in Ussing chambers at a 120 min time course.

**Discussion**

This *in vitro* study demonstrates the effects of a graded level of hypoxia, (with oxygen pressures of 45 - 50 mm Hg), and reoxygenation on baseline electrophysiological parameters, induced nutrient and electrolyte transport and barrier function in rat ileal epithelium. We observed three notable findings: a) A differential effect of hypoxia and reoxygenation upon the absorptive capacity of the small intestinal epithelium. Sodium-coupled glucose transport showed a substantial reduction after hypoxia and reoxygenation to 11 % of control values, whereas sodium-coupled glutamine transport was less affected, to 42 % of controls. b) A differential effect of hypoxia and reoxygenation upon the two main secretory pathways \(^{25,26}\) of the small intestinal epithelium was demonstrated: The cAMP-mediated anion secretion, as induced by forskolin, decreased to 9 % of control values, comparable to the reduction in glucose transport. In contrast, the \( \text{Ca}^{2+}/\text{PKC-} \)}
mediated secretory capacity, reflected by cholinergic or histaminergic stimulation of anion secretion, decreased to only 35 - 49% of controls, comparable to the reduction of glutamine uptake. c) A lack of effect of hypoxia and reoxygenation was observed on the small intestinal barrier function to substances with molecular weights ranging from 376 Da to 40 kDa, even after prolonged episodes of graded hypoxia lasting up to 90 minutes.

A differential influence of hypoxia and reoxygenation on the absorptive and the secretory functions of the small intestine may give rise to a pro-secretory imbalance, leading to intraluminal fluid sequestration and diarrhea. This hypothesis, already put forward by Robinson and coworkers in the late seventies, was based upon in vivo studies of ischemia and reperfusion in canine small intestine. Following ischemia and reperfusion glucose and water absorption were completely abolished, while electrolyte and water secretion persisted. They explained this by the observation of extensive villus denudation during ischemia and reperfusion.

The present study demonstrates that differential influences of hypoxia and reoxygenation on the absorptive and the secretory functions of the small intestine are already regulated at the level of intracellular messenger pathways, instead of villus denudation alone. Hypoxia and reoxygenation in our in vitro model did not lead to extensive villus denudation. A considerable transport capacity of glutamine was still measured after hypoxia and reoxygenation. Furthermore, the intestinal specimen maintained their macromolecular barrier function and structure following hypoxia and reoxygenation as evidenced by permeability and morphological examination.

We observed that the villus enterocytes were still capable to maintain a substantial part of their absorptive capacity for glutamine after a relatively short period of graded hypoxia, while in contrast glucose absorption was almost abolished. This differential effect of hypoxia upon glutamine- versus glucose absorption qualitatively resembles recent findings by Kles et al. in Ussing chamber studies of rat small intestine, after in situ luminal perfusion of jejunal loops during one hour of mesenteric ischemia. They reported a 70 to 90% reduction in glucose transport, while glutamine transport was fully preserved, and subsequently showed that the ischemia-induced reduction in glucose absorptive capacity was caused by trafficking of functional SGLT-1 protein from the brush border membrane to intracellular pools. Also their model of ischemia did not result in marked villus denudation, which may be related to a protective effect of luminal perfusion during ischemia. Thus our in vitro model of hypoxia qualitatively appears to reflect an in vivo animal model of ischemia with a relatively mild degree of epithelial damage.

Besides this differential effect on nutrient absorption, we furthermore observed differential effects of hypoxia and reoxygenation upon the cAMP-mediated and the Ca\(^{2+}\)/PKC-mediated secretory pathways. It is unlikely that the 10-fold reduction in forskolin-induced, cAMP-mediated secretion after hypoxia and reoxygenation is simply due to ATP depletion in crypt enterocytes, because carbachol addition after forskolin to the same tissues still showed a marked secretory response. The relatively small, 2- to 3-fold reduction of Ca\(^{2+}\)/PKC-mediated anion secretion after hypoxia and reoxygenation was observed both after cholinergic stimulation and after histaminergic stimulation of this intracellular messenger pathway. In particular the relative persistence of histamine-induced anion secretion after hypoxia may be relevant to the secretory response of the small intestine after ischemia and reperfusion in vivo: 1) Barret and coworkers have
shown that in contrast to cholinergic stimulation, histaminergic stimulation appears to be far less effective in generating negative second messengers, such as inositol 3,4,5,6, tetrakisphosphate. This can block the ability of the epithelium to respond to a second Ca\(^2+\)/PKC-dependent agonist \(^{29}\). This implies that histamine-dependent activation of epithelial chloride secretion may be permissive for ongoing responsiveness, whereas cholinergic stimulation is not. 2) Plasma levels of histamine showed little change during ischemia in rabbit small intestine, but promptly increased during reperfusion \(^{30}\), thus parallel to the appearance of secretion. Moreover, pretreatment of rats with the histamine-degrading enzyme diamine-oxidase largely prevented the intraluminal fluid accumulation induced by small intestinal ischemia-reperfusion \(^{31}\). The released histamine may originate from mucosal and mesenteric mast cells, as both types of mast cells degranulate after ischemia-reperfusion \(^{23,32}\).

The high spontaneous $I_{sc}$ in the rat ileum in our in vitro study (Figure 1A) could not be attenuated by serosally applied TTX, atropine or indomethacin, indicating that it was not caused by ongoing neural activity, a high cholinergic tone or by prostaglandin release. After the induction of hypoxia, an immediate decrease of the spontaneous $I_{sc}$ was observed, and a rapid recovery was determined after reoxygenation. These phenomena were previously observed in an elegant study by Munck \(^{35}\), in which rat jejunum was exposed to five minutes of unilateral or bilateral hypoxia in Ussing chambers. He demonstrated that about 80 % of the spontaneous $I_{sc}$ was caused by chloride secretion and the remaining 20 % was due to sodium uptake. Both serosal and bilateral hypoxia caused a similar drop in $I_{sc}$ as seen in our present experiments, followed by rapid recovery after reoxygenation. Comparable observations have been reported in rat colon \(^{34}\). Chloride secretion is driven by the ongoing activity of basolateral Na\(^+-\)K\(^+\)-ATPase in combination with basolateral Na\(^+-\)K\(^+\)-2Cl\(^-\) co-transport, basolateral K\(^+\) conductance and the Cl\(^-\) conductance in apical cell membranes. The high spontaneous $I_{sc}$ in rat small intestine in vitro indicates a high activity of basolateral Na\(^+-\)K\(^+\)-ATPase, and during hypoxia intracellular ATP stores may thus rapidly deplete, leading to the fast reduction of spontaneous chloride secretion. In contrast, in T84 colon crypt cell line monolayers, chemical depletion of ATP stores led to a transient, adenosine-mediated activation of chloride secretion, which disappeared when ATP-levels reached 5% of control values \(^{11-13}\). Moreover, chemical hypoxia decreased the $R_t$ of T84 colon cell monolayers from 1500 $\Omega \cdot cm^2$ to approximately 700 $\Omega \cdot cm^2$, while in our study in rat ileum no effect of hypoxia upon $R_t$ could be detected.

The differences in $I_{sc}$ response to hypoxia between the T84 colon cell monolayers and rat ileum may be related to the very low spontaneous $I_{sc}$ (2 $\mu$A/cm\(^2\)) of the T84 colon cell monolayers, or may be caused by differences between chemical hypoxia and true hypoxia, to which T84 monolayers are extremely resistant \(^{11}\). This may also explain the differences in effects of hypoxia upon the $R_t$ of the T84 monolayers and the $R_t$ of rat ileal tissues. Moreover, there are large differences in the $R_t$ in both tissue preparations and in their morphology. In unstripped rat small intestine, 80% of the $R_t$ is formed by the subepithelial layer ($R_{se}$) \(^{35}\). In stripped tissue, the $R_{se}$ still forms about 65% of $R_t$ \(^{36}\) and 15% of the $R_t$ is caused by the lateral intercellular spaces \(^{37}\). Thus, in a stripped small intestinal tissue only 20 % of $R_t$ is located in the tight junctions. In comparison, in the T84 colon cell monolayers this value is close to 100 %, because one may assume that in an intestinal monolayer with a $R_t$ of 1500 $\Omega \cdot cm^2$ the relative contribution of the lateral intercellular
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spaces to $R_t$ will be much smaller than 15%. Therefore, in contrast to the $R_t$ of T84 monolayers, the $R_t$ of rat ileal tissue may be a poor indicator of its barrier function, which may be measured more appropriately by quantification of epithelial permeability to small and/or large probe molecules.

In our *in vitro* study, neither hypoxia, even after 90 min, nor reoxygenation influenced the ileal permeability to disodium-fluorescein or to horseradish peroxidase. This observation is somewhat puzzling, considering the numerous reports from *in vivo* animal models, in which intestinal ischemia and reperfusion resulted in decreased intestinal barrier function. The most likely explanation is that the increased intestinal epithelial permeability after ischemia and reperfusion *in vivo* is not caused by epithelial hypoxia *per se*, but is a secondary effect induced by agents of vascular endothelial and/or submucosal origin, which may initially be activated or released by ischemia and reperfusion. Kubers and coworkers have provided evidence in support of this interpretation in *in vivo* animal models of ischemia and reperfusion. They used the blood-to-lumen clearance of $^51$Cr-EDTA (MW 243 D), which rapidly equilibrates completely between plasma and interstitium, to assess intestinal permeability in feline ileal loops exposed to ischemia and reperfusion. The increased intestinal permeability, induced by ischemia and reperfusion, was reduced by the inhibition of granulocyte recruitment, by constitutive endothelial nitric oxide production and by nitric oxide donors, and it was enhanced via mast cell activation using nitric oxide synthesis inhibitors. Moreover, ischemia and reperfusion did not induce an increase in intestinal permeability to $^51$Cr-EDTA in mast cell-deficient mice, in contrast to ischemia and reperfusion in wild-type mice, and mast cell stabilizers completely prevented the increased mucosal permeability to $^51$Cr-EDTA after ischemia and reperfusion in rat small intestine. These findings are consistent with the concept that microvascular dysfunction with increased vascular albumin leak and associated mast cell degranulation are initial events in ischemia and reperfusion. This may be a prerequisite for the impairment of intestinal barrier function that occurs in ischemia and reperfusion *in vivo*.

In conclusion, we have shown that hypoxiareoxygenation in rat small intestine *in vitro* has a differential effect on glutamine- and glucose-absorption, confirming recent reports of differential nutrient absorption in *in vivo* models of intestinal ischemia. Furthermore the present study is the first report, to our knowledge, that indicates that hypoxiareoxygenation affects the cAMP-mediated secretory capacity of enterocytes to a much stronger extent than the Ca$^{2+}$/PKC-mediated secretory capacity, which may be relevant for the understanding of the mechanisms involved in the development of intraluminal fluid sequestration and diarrhea after intestinal ischemia.
References


Differential effects of hypoxia on intestinal absorption and secretion


Part IV

Experimental studies (USA)
VEGF-mediated induction of MnSOD occurs through redox-dependent regulation of forkhead and IκB/NF-κB

Md. Ruhul Abid ¹, Ivo G. Schoots ¹,², Katherine C. Spokes ¹, Christina Mawhinney ¹, and William C. Aird ¹

From the ¹ Division of Molecular and Vascular Medicine and the Center for Vascular Biology Research, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts 02215, USA, and the ²Department of Surgery and Vascular Medicine, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands.

Abstract

The mitochondrial antioxidant, manganese superoxide dismutase (MnSOD), plays a critical cytoprotective role against oxidative stress. Vascular endothelial growth factor (VEGF) was previously shown to induce expression of MnSOD in endothelial cells by a NADPH oxidase-dependent mechanism. The goal of the current study was to determine the transcriptional mechanisms underlying this phenomenon. VEGF resulted in PKC-dependent phosphorylation of IκB, and subsequent translocation of p65 NF-κB into the nucleus. Overexpression of constitutively active IκB blocked VEGF stimulation of MnSOD. In transient transfection assays, VEGF increased MnSOD promoter activity, an effect that was dependent on a second intronic NF-κB consensus motif. In contrast, VEGF-mediated induction of MnSOD was enhanced by the PI3K inhibitor, LY294002, and by DN-Akt, and decreased by CA-Akt. Overexpression of a constitutively active (phosphorylation resistant) form of FKHRL1 (TM-FKHRL1) resulted in increased MnSOD expression, suggesting that the negative effect of PI3K-Akt involves attenuation of forkhead activity. In co-transfection assays, the MnSOD promoter was transactivated by TM-FKHRL1. Flavoenzyme inhibitor, diphenyleneiodonium (DPI), and antisense oligonucleotides against p47 phox (AS-p47 phox) inhibited VEGF stimulation of IκB/NF-κB and forkhead phosphorylation, supporting a role for NADPH oxidase activity in both signalling pathways. Like VEGF, hepatocyte growth factor (HGF) activated the PI3K-Akt-forkhead pathway. However, HGF-PI3K-Akt-forkhead signalling was insensitive to DPI and AS-p47 phox. Moreover, HGF failed to induce phosphorylation of IκB/NF-κB or nuclear translocation of NF-κB, and had no effect on MnSOD expression. Together, these data suggest that VEGF is uniquely coupled to MnSOD expression through growth factor-specific ROS-sensitive positive (PKC-NF-κB) and negative (PI3K-Akt-forkhead) signalling pathways.
Introduction

VEGF is an endothelial cell-specific mitogen \(^1\textendash}^3\), and chemotactic agent \(^4,5\), which is involved in wound repair \(^6\), angiogenesis of ischemic tissue \(^7,8\), tumor growth \(^9\textendash}^14\), microvascular permeability \(^15\textendash}^17\), vascular protection \(^18,19\), and hemostasis \(^20,21\). In addition to its mitogenic and chemotactic effects, VEGF also acts to promote endothelial cell survival \(^22\textendash}^24\). The VEGF family of proteins binds to three receptor-type tyrosine kinases, Flt-1 (VEGF receptor-1), KDR/Flk-1 (VEGF receptor-2) and VEGFR-3. VEGFR-1 and -2 are normally expressed in vascular endothelial cells, whereas VEGFR-3 is expressed in the lymphatic endothelium. Of the VEGF receptors, KDR/Flk-1 is believed to play the most important role in mediating endothelial cell proliferation, migration and permeability. VEGF has been shown to activate a number of different intracellular signalling pathways, including PKC \(^25\textendash}^29\), PI3K and Akt \(^25,27,30,31\), MEK1/2 \(^32,36\), p38 MAPK \(^29,34,35,37,38\), and phospholipase C\(\gamma\) \(^27\). VEGF may alter endothelial cell phenotype through transcriptional and/or post-transcriptional mechanisms. Among the transcription factors that have been implicated in VEGF signalling are NF-\(\kappa\)B, Egr-1, NFAT-1, Ets-1 and Stat-3/5 \(^39\textendash}^48\). More recently, we reported that VEGF results in PI3K-Akt-dependent phosphorylation and nuclear exclusion of forkhead family of proteins \(^49\). In previous studies, we provided evidence for a link between VEGF signalling and the redox state of the cell \(^50,51\). Specifically, we demonstrated that VEGF induces the activity of NADPH oxidase and that NADPH oxidase-derived reactive oxygen species (ROS) are, in turn, required for VEGF-mediated induction of cell migration, proliferation and MnSOD expression. Subsequent studies have confirmed the importance of ROS in VEGF signal transduction \(^52,53\). The superoxide dismutase (SOD) family includes cytosolic Cu, Zn-SOD, mitochondrial manganese SOD (MnSOD), and extracellular Cu, Zn-SOD. By converting superoxide \((O_2\cdot^-)\) to \(H_2O_2\) and \(O_2\), these enzymes inhibit free radical reactions that lead to oxidative damage. MnSOD is encoded by the nuclear \(SOD2\) gene and is localized in mitochondria, the major site for oxidative phosphorylation. ROS have been shown to induce mitochondrial damage and dysfunction \(^54,55\), leading to impaired Krebs’ cycle \(^56\) and induction of apoptotic pathways \(^57\textendash}^63\). MnSOD plays a critical cytoprotective role against oxidative stress \(^64\textendash}^66\). Its importance in homeostasis is evidenced by the lethal cardiomyopathy that develops in neonatal mice that are null for MnSOD \(^67\). Conversely, overexpression of MnSOD in transgenic mice protects against oxidative injuries caused by ischemia-reperfusion in brain \(^68\), oxygen therapy-induced inflammation in the lungs \(^69\), and drug-induced toxicity in heart \(^70\). In the present study, we wished to extend our previous findings by elucidating the mechanisms underlying VEGF stimulation of MnSOD. We show that the effect of VEGF on MnSOD expression involves a negative PI3K-Akt-forkhead pathway, and a positive PKC-NF-\(\kappa\)B pathway, both of which are sensitive to NADPH oxidase activity. The presence of two opposing pathways, one negative and the other positive, is likely to render the VEGF-MnSOD axis highly modulatable by the extracellular environment.
Material and Methods

Cell culture and Reagents. Human coronary artery endothelial cells (HCAEC) and human umbilical vein endothelial cells (HUVEC) were grown in Endothelial Growth Medium-2-MV (EGM-2-MV) BulletKit (Clonetics, San Diego, CA) at 37°C and 5% CO₂. Endothelial cells from passage 3 to 6 were used for all experiments. Cells were serum starved in 0.5% FBS prior to treatment with 50 ng/ml human VEGF165 or 40 U/ml HGF (Pepro Tech Inc, Rocky Hill, NJ). Where indicated, cells were pre-incubated for 30 min with 50 μM LY294002, 1 μM GF109203X, 50 μM PD98059, or 10 μM diphenyleneiodonium (DPI) (Biomol, Plymouth Meeting, PA).

Western and Northern blot analyses. Endothelial cells were harvested for total protein and Western blots were carried out as previously described. The following phosphospecific antibodies were used: Ser-256 FKHR, Ser-473 Akt, ERK1/2, JNK, IKB, NF-κB, and CREB from Cell Signalling (Beverly, MA); anti-pTyr 4G10 from UBI (Waltham, MA); and anti-β-actin from Sigma (St. Louis, MO). Anti-ERK1/2, anti-Akt, anti-FKHR, anti-hcB, anti-NF-κB (p65), and anti-CREB/ATF antibodies were obtained from Cell Signalling. RNA extraction and northern blot assays were performed as described previously.

Immunolocalization studies. HCAEC were plated onto 4-well chamber slides (Lab-Tek, Christchurch, New Zealand) at a density of 30,000 cells/well. The cells were grown in EGM-2 MV medium for 48 h and fixed in ice-cold 3.7% paraformaldehyde for 10 min, washed with PBS, and subsequently incubated with primary anti-p65 antibody (1:100 dilution) for 2h. Following extensive washes in PBS, the cells were incubated with a FITC-labelled secondary antibody (1:200 dilution) for 1 h. Following additional PBS washes, the slides were mounted in Aquamount (Vector, Burlingame, CA) and viewed under confocal fluorescence microscopy. To-Pro (Molecular Probes, Eugene, OR) was used for identification of nuclear co-localization.

Adenoviruses. HCAEC were infected with adenoviruses encoding the cDNAs of β-galactosidase (β-gal or Adv), dominant negative T308A, S473A-Akt (DN-Akt), constitutively active Gag-Akt (CA-Akt), wild type (WT)-FKHRli and triple mutant (TM)-FKHRli. The triple mutant version of FKHRl contains T32A, S253A, and S315A and is resistant to agonist-induced phosphorylation. WT and DN-PKC viruses (a generous gift from George King, Joslin Diabetes Center, Harvard Medical School, Boston, MA) were used as previously described. Adenovirus expressing degradation-resistant, constitutively active IκBα (IκB S32/36A) was a generous gift from Kaikobad Irani (Johns Hopkins University School of Medicine, Baltimore, MD).

Co-transfection assays. Control vector (pECE) and vector expressing FKHRl (pECE-FKHRl) were provided by Michael Greenberg, Children’s Hospital, MA. The pGL3-based construct containing 3340-bp promoter of human MnSOD (pGL3-Mn-Luc) was a generous gift from Moon Yim (Laboratory of Biochemistry, NHLBI, NIH, Bethesda, MD). A 369-bp fragment (from nt. 2410 to nt. 2778) of the 2nd intron of MnSOD containing the putative NF-κB, C/EBP and NF-1 consensus sites was generated by PCR using human genomic DNA as template, the sense primer: 5’-TCTTGTCACCGTTAGTGGTTTGCACAAGGAAGATAATCG-3’, and the reverse primer: 5’-AATAGTCGACTTCACAACTGGGTATTCCCCAGTCTCTCC-3’ (Sall site underlined). The resulting fragment was digested with Sall and inserted into the Sall site of the pGL3-Mn-Luc plasmid in forward and reverse orientations, giving rise to pGL3-Mn2-Luc, and pGL3-Mn2R-Luc, respectively. To generate the NF-κB mutation (pGL3-Mn2(mt-NF-κB)-Luc), a 369-bp fragment was regenerated with the same forward primer as above, but with a different reverse primer containing the mutations (in lower case): 5’-AATAGTCGACCTCCACAACCTGGGTATTCCCCAGTCTCTCC-3’ (Sall site underlined). A mutation in the C/EBP site (pGL3-Mn2(mt-CEBP)-Luc) was obtained using a two-step mutagenesis protocol with the primers: 5’-GATTAAAAGAGGAGGAAGGTAATCATCCTGGAAGATTTAC-3’ and 5’-GTAAATCTTCCAGAATGTaaTAACTTCCTCCTTTTAATC-3’ (mutations in lower case). A total of 0.05 pmol of the MnSOD reporter plasmid, 50 ng of a control plasmid containing the Renilla luciferase reporter gene under the control of a cytomegalovirus enhancer/promoter (pRL-CMV), and 0.075 pmol of the forkhead expression vector (or control vector, pECE) were
incubated with 2 μl of FuGENE 6 (Roche, Nutley, NJ).Twenty-four h later, the cells were washed with phosphate-buffered saline and cultured for 12 h in EBM plus 0.5% FBS. The cells were then incubated in the presence or absence of VEGF for 6 h, at which time they were lysed and assayed for luciferase activity using the dual-luciferase reporter assay system (Promega, Madison, WI) and a Lumat LB 9507 luminometer (Berthold, Bad Wildbad, Germany). Experiments were performed in triplicates and repeated at least three times.

**Transfection with antisense oligonucleotide p47 phox.** HCAEC were grown to 70-80% confluency in 10-cm plates and transfected with 200 nM phosphorothioated antisense-p47 phox oligonucleotide in Opti-MEM containing lipofectin (10 μg/ml) for 4 h. The cells were then incubated in EGM-2 medium for 24 h and serum starved in 0.5% serum for 12-16 h before VEGF treatment for the times indicated. Antisense sequence (5’- TTTGTCTGGTTGTGTGGG-3’) was complementary to nucleotides 394-413 of human p47phox mRNA, and was phosphorothioate-modified and HPLC purified (Sequitur, Natick, MA).

**Assay for NADPH oxidase activity in HCAEC and HUVEC.** The cells were washed with ice-cold PBS, collected by a cell scraper and dounce homogenized in a buffer containing 20 mM KH2PO4 (pH 7.0), 1X protease cocktail inhibitor (Sigma), 1 mM EGTA, 10 μg/ml aprotinin, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, 0.5 mM PMSF. NADPH oxidase activity of the cell lysate was measured using a modified assay as described. Briefly, photon emission from the chromogenic substrate lucigenin as a function of acceptance of electron/O-2 generated by the NADPH oxidase complex was measured every 15 s for 20 min in a Berthold luminometer. NADPH oxidase assay buffer containing 250 mM HEPES (pH 7.4), 120 mM NaCl, 5.9 mM KCl, 1.2 mM MgSO4 (7H2O), 1.75 mM CaCl2 (2H2O), 11 mM glucose, 0.5 mM EDTA, 100 μM NADH and 5 μM lucigenin was used. The data was transformed to relative light units/min/mg of protein, using a standard curve generated with xanthine/xanthine oxidase.

**Measurement of intracellular ROS generation.** The changes in intracellular ROS levels by measuring the oxidative conversion of cell-permeable 2’,7’-dichlorofluorescein diacetate (DCFH-DA; Molecular Probes Inc., Eugene, OR) to fluorescent dichlorogluorescein (DCF) by FACS analysis as previously described.

**Results**

**VEGF-mediated induction of MnSOD is attenuated by PI3K-Akt-forkhead signalling pathway.** Previous studies in non-endothelial cells have demonstrated that insulin inhibits MnSOD expression via a PI3K-Akt-forkhead-dependent pathway. In contrast, we have shown that VEGF, while capable of inducing phosphorylation and nuclear exclusion of forkhead in endothelial cells in a PI3K-Akt-dependent manner, results in a net increase in MnSOD mRNA and protein levels. Together, these findings suggest that VEGF-mediated induction of MnSOD involves interplay between positive and negative pathways. To test this hypothesis, HCAEC and HUVEC were pre-incubated for 30 min in the absence or presence of the PI3K-inhibitor, LY294002 (50 μM) or wortmannin (100 nM), treated with VEGF (50 ng/ml) for 4 h, and then processed for Northern blot analysis of MnSOD. Inhibition of PI3K increased basal and VEGF-induced MnSOD expression in both types of endothelial cells (Figure IA shows the effect of LY294002 in HCAEC). To determine whether PI3K exerts its negative effect on MnSOD expression through Akt, HCAEC and HUVEC were infected with adenoviruses expressing either β-galactosidase (Ad-β Gal), constitutively active (Gag) Akt (CA-Akt) or dominant negative (T308A, S473A) Akt (DN-Akt), and incubated in the absence or presence of VEGF.
Figure 1. VEGF-mediated activation of PI3K-Akt-forkhead attenuates MnSOD expression in endothelial cells. (A) Northern blot analysis of MnSOD was carried out using total RNA from HCAEC treated with or without 50 ng/ml VEGF for 4 h in the absence or presence of pre-treatment with LY294002 (50 μM). 28S RNA was used as a loading control (lower panel). Both 4-kb and 1-kb MnSOD transcripts are shown. (B) Northern blot analysis of MnSOD was carried out using total RNA from HCAEC infected with 20 MOI adenoviruses expressing control (Ad-β Gal), dominant negative (DN) or constitutively active (CA) Akt and treated in the absence or presence of 50 ng/ml VEGF for 4 h. (C) Same as in (B) except HCAEC were infected with 3 MOI adenoviruses expressing control P-gal (Adv), phosphorylation-resistant triple-mutant (TM) or wild-type (WT)-FKHRL1. (D) Northern blot analysis of MnSOD mRNA from HCAEC infected with adenoviruses expressing either control β-gal (Adv), TM-FKHRL1, or WT-FKHRL1, and grown in medium containing 5% FBS. (E) Co-transfection of HCAEC with pGL3-Mn-Luc (Mn) and either an empty expression vector (pECE) or an expression vector encoding TM-FKHRL1 (FKHRL1). The expression levels were normalized to pRL-CMV activity and expressed as fold induction relative to Mn plus pECE co-transfection alone. Experiments were carried out in triplicate. Mean ± s.d. of three independent experiments are shown.

Overexpression of DN-Akt accentuated the effect of VEGF on MnSOD expression, whereas CA-Akt had the opposite effect (Figure 1B shows HCAEC). Together, these findings suggest that PI3K-Akt attenuates VEGF-mediated induction of MnSOD in endothelial cells. Previous studies of C. elegans and mice have implicated the daf-2/insulin-forkhead signalling pathway in regulating MnSOD expression 76-78. The human
MnSOD gene contains two forkhead consensus sites in the upstream promoter region—one at −1249 (GTAAACAA; inverse of TTGTTTAC) and another at −997 (TTGTTTTAA) 75. In non-endothelial cells, insulin has been shown to induce PI3K-Akt-dependent phosphorylation and nuclear exclusion of FKHRL1, and to inhibit MnSOD expression 75. To determine whether MnSOD lies downstream of VEGF-PI3K-Akt-forkhead in endothelial cells, HCAEC and HUVEC were infected with adenoviruses overexpressing β-galactosidase (Adv), wild-type (WT)-FKHRL1, or a constitutively activated phosphorylation-resistant triple-mutant (TM)-FKHRL1. The overexpressing cells were serum-starved and then treated in the presence or absence of VEGF for 4 h. VEGF-mediated induction of MnSOD was accentuated by overexpression TM-FKHRL1, but not Adv or WT-FKHRL1 (Figure 1C shows HCAEC). In the absence of VEGF treatment, TM-FKHRL1 alone had no effect on MnSOD expression in serum-starved cells (Figure 1C). However, in the presence of serum-rich medium, TM-FKHRL1-infected cells demonstrated increased MnSOD mRNA levels (Figure 1D). Together, these data suggest that forkhead-mediated induction of MnSOD is dependent on VEGF- or serum-mediated activation of positive pathway(s). In co-transfection assays, the overexpression of FKHRL1 resulted in a 6.6 ±0.3-fold induction of the 3340-bp human MnSOD promoter (Figure 1E). These findings support the conclusion that the PI3K-Akt signalling pathway negatively regulates expression of MnSOD via phosphorylation and nuclear exclusion of forkhead.

VEGF-mediated induction of MnSOD is positively regulated through PKC and IκB/NF-κB.

Although VEGF triggers a negative PI3K-Akt-forkhead signalling pathway, VEGF signalling results in the net induction of MnSOD mRNA and protein. To identify the positive pathway(s) responsible for this effect, endothelial cells were pre-incubated with other inhibitors of signalling and then treated in the absence or presence of VEGF. As shown in Figure 2A, pre-incubation of endothelial cells with the PKC inhibitor, GF109203X (1 μM) resulted in marked inhibition of VEGF induction of MnSOD, whereas the MEK1/2 inhibitor, PD98059 (50 μM), had no such effect. To determine which PKC isoforms are involved in mediating the VEGF response, HCAEC were infected with either wild type (WT) or dominant negative (DN) isoforms of PKC and treated in the absence or presence of VEGF. VEGF induction of MnSOD was completely abrogated by DN-PKCζ, significantly inhibited by DN-PKCδ, and unchanged by DN-PKCa or DN-PKC ε (data not shown). These findings suggest that VEGF-mediated induction of MnSOD is dependent on novel and atypical PKC isoforms. Previous studies have demonstrated a link between PKC-δ/ζ signalling and NF-κB activity in endothelial cells 71,79,80. Moreover, NF-κB has been shown to mediate inducible expression of MnSOD in a variety of cell types 65,81-91. Therefore, we hypothesized that VEGF induces MnSOD via a PKC-NF-κB-dependent signalling pathway. Consistent with this hypothesis, VEGF-mediated induction of MnSOD was blocked by over-expression of a constitutively active, phosphorylation/ubiquitination-resistant form of IκB (Figure 2B and 2C). Previous studies have pointed to the importance of an NF-κB consensus element in the second intron (at +2758 nt, relative to start site of the 2nd intron). In transient transfection assays, VEGF induced MnSOD promoter activity in the presence, but not the absence of second intronic sequence (Figure 2D). Mutation of the NF-κB motif (mt-NF-κB) resulted
Redox regulation of forkhead and IκB/NF-κB by VEGF

Figure 2. VEGF-mediated induction of MnSOD in endothelial cells is dependent on PKC and IκB/NF-κB. (A) Northern blot analysis of MnSOD. HCAEC were serum-starved overnight, pretreated with or without PD98059 (50 μM) or GF109203X (1 μM) for 30 min, and then incubated in the absence or presence of 50 ng/ml VEGF for 4 h. The same blot was stripped and reprobed for 18S RNA as a loading control (lower panel). (B) Northern blot analysis of MnSOD from HCAEC infected with adenoviruses expressing β-gal (Ad-β Gal), wild-type (WT) or dominant negative (DN) PKC-δ or PKC-ζ, or constitutively active IκB (CA-IκB), and treated with or without VEGF. (C) Same as in (B) except the cells were infected with adenoviruses expressing β-gal (Adv), TM-FKHRL1, WT-FKHRL1 or CA-IκB. (D) Transfection of HCAEC with a 3340-bp MnSOD promoter without (Mn) or with a 369-bp 2nd intronic sequence (Mn2; reverse orientation Mn2R) coupled to a luciferase reporter gene. The transfected cells were grown for 36 h and then incubated for 12 h either in serum starved (S/S) or serum rich (S/R) medium. The expression levels were normalized to pRL-CMV activity and expressed as fold induction relative to pGL3-Mn-Luc or pGL3-Mn2-Luc alone. Experiments were carried out in triplicate. Mean ± s.d. of three independent experiments are shown. (E) Same as in (D) except the reporter used were Mn2, Mn2 with CEB/P site mutated (mt-CEBP) or Mn2 with NF-κB site mutated (mt-NF-κB).

in a loss of induction, whereas mutation of the C/EBP site (at +2648 nt; mt-CEBP) had no such effect (Figure 2E). The transcription factor NF-κB is normally sequestered in the cytoplasm by IκB. NF-κB activation requires phosphorylation-induced ubiquitination and degradation of cytoplasmic IκB, with subsequent release and nuclear translocation of NF-κB. Incubation of HCAEC with VEGF resulted in increased phosphorylation of IκB in the cytoplasm at 15 min, and decreased total cytoplasmic IκB at 30 and 60 min (Figure 3A). Moreover, VEGF promoted the nuclear translocation of p65 NF-κB at 15 and 30 min (Figure 3B). Pre-incubation with the PKC inhibitor, GF109203X, blocked VEGF-mediated phosphorylation of IκB (Figure 3C) and nuclear translocation of NF-κB.
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Figure 3. VEGF induces phosphorylation of IκB and subsequent nuclear translocation of NF-κB. (A) Western blot analysis of IκB in the cytoplasmic fraction of HCAEC. Cells were serum starved overnight and treated with VEGF (50 ng/ml) for the times indicated. After transfer, the membrane was probed with anti-phospho-IκB antibody, and then stripped and probed for total IκB. Anti-SOD1 (cytoplasmic Cu, Zn-SOD) antibody was used as a loading control for cytoplasmic protein. (B) Western blot analyses of p65 NF-κB in paired cytoplasmic and nuclear fractions from VEGF-treated HCAEC. IκB levels do not change in the nuclear fraction, and were therefore used as an internal control. (C) Western blot analyses of phospho-IκB in cytoplasmic fractions from VEGF-treated HCAEC pre-incubated in the absence or presence of vehicle or PKC inhibitor GF109203X (GF109). The membrane was stripped and re-probed for total IκB and SOD1. (D) Corresponding nuclear fractions of HCAEC cell lysates that were used in (C) were analyzed first for p65 NF-κB protein, and then for IκB. The bottom panel shows that the nuclear fraction is completely devoid of the cytoplasmic protein, SOD1. All experiments were carried out in triplicate and a representative blot is shown.

(Figure 3D). In immunofluorescent assays, VEGF induced the nuclear translocation of p65 NF-κB, an effect that was similarly blocked by GF109203X (Figure 4). Taken together, these results suggest that VEGF-mediated induction of MnSOD is dependent on a PKC-NF-κB signalling pathway.

VEGF-forkhead-mediated attenuation and VEGF-NF-κB-mediated induction of MnSOD are dependent on NADPH oxidase activity

We previously reported that VEGF signalling induces NADPH oxidase activity in endothelial cells and that VEGF-mediated induction of MnSOD mRNA and protein is dependent on NADPH oxidase-derived ROS. In the next series of experiments, we examined the role of NADPH oxidase in regulating the VEGF-forkhead-MnSOD and VEGF-NF-κB-MnSOD pathways. We generated antisense to the p47 phox component of NADPH oxidase (AS-p47 phox). To confirm efficacy of the antisense, HCAEC and HUVEC were either pretreated with the flavoenzyme inhibitor DPI, or transfected with...
Redox regulation of forkhead and IκB/NF-κB by VEGF

Figure 5. NADPH oxidase activity is required for VEGF-mediated phosphorylation of forkhead and IκB, and subsequent nuclear translocation of NF-κB. (A) HCAEC and HUVEC were transfected with scrambled (ScramAS) or p47 phox antisense oligonucleotides (AS-p47 phox ), and assayed for NADPH oxidase activity by lucigenin-based photon emission (see Materials and Methods). (B) Northern blot analysis of MnSOD in control or VEGF-treated HCAEC transfected with ScramAS or AS-p47 phox . (C) Western blots analysis of phospho-specific and total FKHR in control- or VEGF-treated HCAEC pre-incubated with DPI, or transfected with ScramAS or AS-p47 phox. Western blot analysis of phosphorylated IκB (D) or nuclear p65 NF-κ B (E) in control- or VEGF-treated HCAEC pre-incubated with DPI, or transfected with ScramAS or AS-p47 phox. All experiments were carried out at least in duplicate and a representative blot is shown.

AS-p47 phox . As shown in Figure 5A, DPI and AS-p47 phox each resulted in significant inhibition of NADPH oxidase activity in HCAEC and HUVEC (Figure 5A). Moreover, consistent with our previous results using DPI and DN-Racl (50), AS-p47 phox blocked VEGF-mediated induction of MnSOD in both endothelial cell types (Figure 5B shows HCAEC). As shown in Figure 5C, VEGF-mediated phosphorylation of FKHR was inhibited by AS-p47 phox or DPI. Moreover, VEGF-mediated phosphorylation of IκB and nuclear translocation of p65 NF-κB was blocked by AS-p47 phox or DPI (Figure 4 and 5D). Taken together, the above data suggest that NADPH-oxidase activity is required for VEGF-induced forkhead and IκB/NF-κB signalling in endothelial cells.

HGF signalling is coupled to forkhead, but not to NF-κB activity in endothelial cells

Previous studies in non-endothelial cells have shown that activation of the insulin receptor results in forkhead-dependent downregulation of MnSOD 75. These results raise the question of whether the induction of MnSOD observed with VEGF is specific to this growth factor, or is a general property of endothelial cells. We have consistently failed to
Figure 4. VEGF induces nuclear translocation of p65 NF-κB in intact HCAEC. HCAEC were grown in Lab-Tek chamber slides for 48 h and serum-starved overnight, pretreated with or without GF109293X (1 μM) or DPI (10 μM) for 30 min and then incubated with 50 ng/ml VEGF for 30 min. The cells were then fixed, immunostained with anti-p65 NF-κB antibody followed by FITC-labeled secondary antibody (left panel). For co-localization, the nuclei were stained with To-Pro (middle panel). The right panel shows superimposed images from the corresponding left and middle panels.
Redox regulation of forkhead and IκB/NF-κB by VEGF

Figure 6. HGF is coupled to PI3K-Akt-forkhead, but not to IκB/NF-κB signalling in endothelial cells. (A) Northern blot analyses of HCAEC RNA for MnSOD expression levels. Serum starved cells were pre-treated with or without PI3K-inhibitor, LY294002, for 30 min (L), and then treated without (C) or with VEGF (V), EGF (E), HGF (H). 28S RNA was used a loading control. (B) Western blot analyses of total cell lysates of HCAEC treated with VEGF or HGF for the times indicated. The membranes were subsequently stripped and treated with the antibodies for phospho-specific and total proteins as indicated. (C) Western blot analysis of phospho-IκB from total cell lysates of serum starved HCAEC treated either with VEGF or HGF for the times indicated. The membrane was stripped and subsequently probed for anti-IκB and anti-β-actin antibodies, respectively. (D) Western blot analysis of cell lysates from HCAEC that were pre-treated with DPI, PD98059 or LY294002 for 30 min and treated with or without HGF for 15 min. The blot was processed for anti-phospho-Akt, anti-phospho-FKHR and anti-Akt antibodies. (E) HCAECs were transfected with either Scram AS or AS-p47 phox and treated with or without HGF for 15 min. The blots were processed for anti-phospho-Akt, anti-phospho-FKHR and anti-Akt antibodies. All experiments were carried out in triplicates and a representative blot is shown.

demonstrate an effect of insulin or insulin-like growth factor on forkhead phosphorylation and activity in endothelial cells (data not shown). Thus, we decided to study hepatocyte growth factor (HGF)/scatter factor. HGF plays a role in endothelial cell proliferation, migration and angiogenesis. The addition of HGF (40 ng/ml) to HCAEC and HUVEC did not result in significant changes in MnSOD expression (Figure 6A shows
HCAEC). In Western blot analyses, HGF treatment resulted in increased phosphorylation of CREB, Erk1/2, JNK, Akt and forkhead proteins (Figure 6B). However, in contrast to VEGF, HGF failed to induce IκB phosphorylation in HCAEC (Figure 6C). These data suggest that while both VEGF and HGF activate a number of common downstream signalling pathways, only VEGF induces NF-κB activity and MnSOD expression. Finally, pretreatment of HCAEC with DPI or transfection with p47 phox failed to block HGF-mediated phosphorylation of Akt and forkhead (Figure 6D and 6E). These data suggest that the requirement for NADPH oxidase activity is a function of the growth factor (VEGF) and not the downstream pathway per se (Akt-forkhead).

Discussion

Although VEGF plays a prominent role in endothelial cell migration, proliferation and barrier function, it also functions in other important ways to maintain homeostasis. For example, VEGF enhances the survival of endothelial cells, alters the expression of hemostatic factors, and modulates selectin levels. Recent findings from our lab, as well as others, suggests that VEGF signalling is coupled to the redox state of the cell . VEGF induces NADPH oxidase activity, which in turn is necessary for VEGF function and induction of MnSOD expression .

Several lines of evidence suggest that protein kinase receptor signalling normally serves to inhibit MnSOD mRNA and protein. First, forkhead factors have been shown to activate MnSOD expression . Second, many growth factors, including insulin, IGF-1, EGF, VEGF, and erythropoietin have been shown to phosphorylate forkhead proteins, resulting in their exclusion from the nucleus and secondary reduction in the expression of forkhead-responsive target genes . Finally, in non-endothelial cells, insulin suppresses MnSOD levels via a PI3K-Akt-forkhead-dependent signalling pathway . In the current study, we have shown that while VEGF does indeed trigger PI3K-Akt-forkhead signalling, the tendency to repress MnSOD expression is overridden by a positive PKC-NF-κB signalling axis. The observation that HGF neither activates NF-κB nor induces MnSOD expression suggests that the interplay between positive and negative pathways is more a function of the mediator, than it is of the cell type.

An interesting question is why VEGF, as distinct from other growth factors, results in a net increase in MnSOD. Given that VEGF signalling, but not HGF signalling, is critically coupled to the redox state of the cell, perhaps the increased levels of MnSOD are important for modulating ROS levels and/or protecting the endothelial cell against excessive oxidative stress. Alternatively, by converting short-lived superoxide in the mitochondria into membrane permeable hydrogen peroxide, increased levels of MnSOD may ultimately link signalling between mitochondria and cytosol. Finally, both NF-κB and MnSOD have been implicated in the control of cell survival , raising the possibility that the VEGF-PKC-NF-κB-MnSOD axis plays an important role in inhibiting endothelial cell apoptosis.

It is also interesting to speculate whether the VEGF PKC-NF-κB pathway evolved to overcome a “default” PI3K-Akt-forkhead pathway, or whether the presence of positive and negative signalling provides the system with an additional level of regulation. For
Redox regulation of forkhead and IκB/NF-κB by VEGF

Figure 7. Model. Schematic of VEGF-MnSOD signalling axis: 1) VEGF induces NADPH oxidase activity and NADPH oxidase-derived ROS are in turn necessary for VEGF signalling, 2) VEGF induces MnSOD mRNA, 3) VEGF-mediated activation of PI3K-Akt results in phosphorylation and nuclear exclusion of forkhead proteins, thus exerting a negative effect on MnSOD expression, 4) VEGF-mediated activation of PKC results in phosphorylation and degradation of IκB, subsequent nuclear translocation of NF-κB, and induction of MnSOD expression, and 5) MnSOD protein, which is localized in the mitochondria, results in conversion of free oxygen radical to hydrogen peroxide, which may then diffuse into the cytosol and modulate signalling.

For example, it is possible that extracellular mediators that are comparatively more efficient in activating PI3K-Akt (e.g. angiopoietin-1) will inhibit VEGF-mediated induction of MnSOD, whereas endogenous inhibitors of PI3K-Akt may have the opposite effect. Alternatively, extracellular signals that preferentially affect NF-κB nuclear translocation and/or DNA binding activity may influence the VEGF PKC-NF-κB axis. In other words, at any given point in time and space, the net effect of VEGF on MnSOD expression will depend on the relative activity or set point of the negative and positive pathways.

There is conflicting data in the literature regarding the role of NF-κB in VEGF signalling. One group has been unable to demonstrate an effect of VEGF on nuclear translocation of p65 NF-κB, DNA-protein binding, or IκB protein levels in HUVEC. Others have shown that VEGF does indeed induce NF-κB binding in HUVEC. A role for NF-κB in VEGF signalling is further supported by studies employing non-specific inhibitors of the transcription factor. In this report, we have shown unequivocally...
that VEGF induces the phosphorylation of IκB and the nuclear translocation of p65 NF-κB. Moreover, VEGF-mediated induction of the MnSOD gene was blocked by a repressor of NF-κB, and VEGF stimulation of MnSOD promoter activity was ablated by mutation of the second intron NF-κB consensus element. Taken together, these findings support a functional role for NF-κB in the VEGF signalling pathway.

A previous study demonstrated that VEGF induces the expression of intercellular adhesion molecule (ICAM)-1, vascular cellular adhesion molecule (VCAM)-1 and E-selectin in endothelial cells. The data supported a role for NF-κB in mediating this effect. Interestingly, chemical inhibition of PI3K resulted in super-induction of the selectin genes, pointing to a negative role for PI3K-Akt. In another report, VEGF-mediated induction of tissue factor was enhanced by PI3K inhibition. Although these latter studies did not address the role for forkhead transcription factors in this process, they suggest that VEGF target genes other than MnSOD are regulated through the coordinate action of positive and negative pathways.

The finding that TM-FKHRL1 induces MnSOD expression only in the presence of VEGF or serum contrasts with our previous observations that TM-FKHRL1 alone is sufficient for inducing p27kip1. These data suggest that TM-FKHRL1-mediated transactivation of MnSOD requires the presence of additional positive factor(s), such as NF-κB and/or other transcription factors or co-activators. In co-transfection assays, the forced over-expression of TM-FKHRL1 resulted in increased promoter activity, whether or not the cells were serum starved. At the present time, we cannot explain the discrepancy in results between the endogenous gene and the promoter—namely that in the absence of VEGF or serum, over-expression of TM-FKHRL1 induces promoter activity, but not the endogenous gene. Possibilities include, but are not limited to, differences in duration of exposure to TM-FKHRL1 (48 h in Adenovirus experiments, 24 h in transfections), differences in levels of TM-FKHRL1 expression in the two assays, or the unique behavior of an episomally integrated plasmid construct containing a defined fragment of the promoter.

Compared with DN-Akt, chemical inhibition of PI3K with LY294002 resulted in a more pronounced effect on basal and VEGF stimulated levels of MnSOD. These results may be attributable to non-specific effects of the chemical inhibitor. Alternatively, the differences may reflect the involvement of another PI3K inhibitory pathway that is independent of Akt-forkhead. Further studies are required to address this question.

We previously reported that VEGF signalling is coupled to the redox state of endothelial cells. Specifically, inhibition of NADPH-oxidase activity blocks the effect of VEGF on endothelial cell migration, proliferation and MnSOD expression. In the present study, we have extended these findings by demonstrating a critical role for NADPH oxidase in mediating both the negative (VEGF-PI3K-Akt-forkhead) and positive (VEGF-PKC-NF-κB) signalling pathways. In contrast, NADPH oxidase inhibition had little or no effect on HGF signalling at the level of Akt and forkhead phosphorylation. These results suggest that the sensitivity of VEGF signalling to NADPH oxidase activity is not common to all protein tyrosine kinase receptors.
Conclusion

We have demonstrated that: 1) VEGF, but not HGF, induces MnSOD expression in endothelial cells; 2) such discordance may be explained by the unique capacity of VEGF to activate NF-κB; and 3) NADPH oxidase activity is required for VEGF-mediated, but not HGF-mediated activation of PI3K-Akt-forkhead. The results raise several interesting questions. For example, how and at what level of the signal transduction cascade do ROS modulate VEGF signalling? Are any VEGF signalling pathway(s) NADPH oxidase-independent? Do the effects of VEGF on ROS and NF-κB (in addition to its reported effects on adhesion molecule and tissue factor expression/activity) reflect an important pro-inflammatory role for this molecule? The answers to these questions should provide important new insights into the mechanisms of VEGF signalling in the endothelium.

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References


Redox regulation of forkhead and IkB/NF-κB by VEGF

Redox regulation of forkhead and IκB/NF-κB by VEGF

Summary and conclusions
This thesis focuses on several issues regarding treatment of acute mesenteric ischemia.

**Chapter 1** provides an introduction into the lethal condition of acute mesenteric ischemia and gives an overview of the surgical revascularization procedures in acute mesenteric ischemia. The rapid onset of acute mesenteric ischemia and the potential rapidity with which bowel infarction may occur explain the lethality of this disease. Despite considerable advances in medical diagnosis and treatment over the past four decades, mesenteric vascular occlusion still has a poor prognosis with an in-hospital mortality rate of 59–93 per cent. The relative infrequency of acute mesenteric ischemia, the variable pathogenesis and the broad spectrum of ischemic injury of the small and large intestines make it almost impossible to study this disease and its diagnostic and therapeutic strategies in clinical randomized or case-controlled trials. Surgery is by far the most favorable treatment, as it includes the assessment of intestinal viability, determination or confirmation of the underlying cause, revascularization, and resection of the nonviable intestine. However, in the last decades of the previous century, a number of new treatment modalities have been introduced, which may benefit a selection of patients with acute mesenteric artery occlusion and may improve outcome of this lethal disease.

**Chapter 2** discusses the differentiation of acute mesenteric ischemia on the basis of etiology (arterial embolism, arterial thrombosis, venous thrombosis and non-occlusive mesenteric ischemia). This differentiation is of great importance because of variation in disease progression, response to treatment and outcome. However most of the previous retrospective studies assessed calculated a mortality rate based on data compiled from all etiological subsets taken together, and therefore obscure differences in clinical presentation and characteristics, diagnostic investigation, disease progression, mortality and response to therapeutic modalities that are specific to disease etiology. We therefore performed a systematic review of the available literature, which concludes: 1) the prognosis after acute mesenteric venous thrombosis is better than that following acute arterial mesenteric ischemia; 2) the prognosis after mesenteric arterial embolism is better than that after arterial thrombosis or non-occlusive ischemia; 3) the mortality rate following surgical treatment of arterial embolism and venous thrombosis (54.1 and 32.1 per cent respectively) is less than that after surgery for arterial thrombosis and non-occlusive ischemia (77.4 and 72.7 per cent respectively); and 4) the overall survival after acute mesenteric ischemia has improved over the past four decades. Taken together, there are large differences in prognosis after acute mesenteric ischemia depending on etiology. Surgical treatment of arterial embolism has improved outcome whereas the mortality rate following surgery for arterial thrombosis and non-occlusive ischaemia remains poor.

**Chapter 3** systematically reviews the current (observational) data of thrombolytic therapy in patients with acute thromboembolic mesenteric occlusion, in order to evaluate this treatment modality as an alternative or adjunctive therapy to surgery. Thrombolytic therapy of acute superior mesenteric artery thromboembolism is still to be considered as a relatively new treatment modality. Insufficient evidence is available from reviewed literature to determine the relative effectiveness and safety of thrombolytic treatment for acute superior mesenteric artery thromboembolism, however, initial results appear to be promising. The relative infrequency of acute mesenteric ischemia and the variation in
clinical presentation constitute an almost insurmountable obstacle to undertaking randomized or case-control trials. Nevertheless, this compilation of data gives insight into current status of thrombolytic therapy of acute superior mesenteric artery thromboembolism, may provide questions and answers for clinical investigators to address, and may give rise to directions for future evaluation and clinical guidelines (at least based on consensus).

Chapter 4 questions the idea that Riolan would have conceived an arterial collateral pathway in the mesocolon. Riolan’s anastomosis or arc is eponymously used to indicate the arterial anastomosis between the superior and inferior mesenteric arteries. Vascular as well as gastro-intestinal surgeons are well-acquainted with this collateral mesenteric pathway for retrograde perfusion of the superior mesenteric artery when the origin of the latter is occluded. The eponym suggests that Jean Riolan (1580-1657), a famous 17th century French anatomist, was the first to describe this arterial anastomosis. Riolan was a strong defender of traditional Galenic doctrine in medicine and therefore, proved a vigorous opponent of the new concept of the circulation of the blood as exposed by William Harvey (1578-1657). This makes it unlikely that Riolan would have conceived an arterial collateral pathway in the mesocolon, a notion confirmed by examining his anatomy book published in 1649. He probably had observed vascular arcades running along the inner border of the colon which later associated him with the collateral circulation of the mesentery. It was not until 1743, that Albrecht von Haller (1708-1777) gave a detailed description of the anatomy of the mesenteric arteries, referring to the arterial collateral connection between the superior and inferior mesenteric arteries, as the “Arcus Riolani”, in honour of an old master of anatomy.

Chapter 5 outlines current knowledge on the crosstalk between coagulation and inflammation and the potentially beneficial effects of restoration of the dysfunctional physiological anticoagulant pathways in the microvasculature, following hypoxia or ischemia and reperfusion. The endothelium plays a central role in all major pathways involved in the pathogenesis of hemostatic derangement during ischemia and reperfusion. Endothelial cells seem to be directly involved in the initiation and regulation of thrombin generation and the inhibition of fibrin removal. Proinflammatory cytokines are crucial in mediating these effects on endothelial cells, which themselves may also express cytokines, thereby amplifying the coagulative response. Rather than being a unidirectional relationship, the interaction between inflammation and coagulation involves significant cross-talk between the systems. This could result in inflammation-modifying effects of hemostatic interventions in patients with ischemia and reperfusion-syndromes.

In chapter 6 we evaluate the role of intravascular coagulation in microvascular reperfusion injury after acute mesenteric occlusion. We demonstrated that intestinal ischemia and reperfusion result in local generation of thrombin and subsequent conversion of fibrinogen to fibrin. Simultaneously, intestinal fibrinolysis is impaired, ultimately leading to intravascular fibrin deposition. These findings suggest that microvascular thrombotic obstruction plays a role in the pathogenesis of structural and functional intestinal injury induced by ischemia and reperfusion.
In chapter 7 we briefly review the involvement of the protein C system in a selected number of models of ischemia-reperfusion injury. Experimental studies indicate that an impaired function of the protein C pathway plays a major role in the pathogenesis of sepsis and associated organ dysfunction. Also clinical trials in patients with sepsis have shown a beneficial effect of recombinant human activated protein C. It is tempting to speculate that other clinical situations that are characterized by endothelial dysfunction and microvascular failure may benefit from the administration of recombinant activated protein C. Thereby, a prominent role of activated protein C may be envisaged in ischemia-reperfusion syndromes. Ischemia-reperfusion injury is characterized by a local inflammatory response and local activation of coagulation, reminiscent of the systemic situation in sepsis. Virtually all organs may suffer from ischemia-reperfusion injury, which can play an important role in major clinical entities, such as myocardial infarction, acute renal failure, stroke, acute lung injury and intestinal ischemia. There is interesting evidence to support a role of the protein C system in ischemia-reperfusion injury. Consequently, administration of activated protein C may be a promising therapeutic option in these situations. The efficacy of this approach deserves further study in experimental and clinical studies.

In chapter 8 we show that activated protein C or antithrombin inhibits local and systemic derangement of coagulation and inflammation following intestinal ischemia and reperfusion in rats, diminishes mucosal fibrin deposition and attenuates ischemia/reperfusion-induced intestinal injury. Intestinal ischemia and reperfusion resulted in considerable local and systemic derangement of the coagulation and inflammatory system, compromising mucosal and submucosal microcirculation by widespread microthrombosis and deposition of fibrin. Activated protein C or antithrombin treated animals showed less thrombin generation, fibrin degradation products and fibrin deposition compared to control animals, as confirmed by histological examination, whereas heparin administration showed only a limited reduction of portal fibrin degradation products levels. Furthermore, activated protein C or antithrombin administration markedly inhibited the inflammatory response, as reflected by reduced interleukin-6 plasma levels to baseline values whereas heparin had no effect. Furthermore, activated protein C or antithrombin treated animals demonstrated less ischemia/reperfusion-induced intestinal dysfunction and histological changes, compared to control animals. These observations suggest that activated protein C or antithrombin reduces ischemia and reperfusion-induced intestinal injury, both through their anticoagulant and anti-inflammatory effects.

In chapter 9 we demonstrate that enhancement of fibrinolytic activity by intravenous administration of recombinant tissue plasminogen activator (rt-PA) or by inhibition of PAI-1 by administration of MA-33H1F7 neither increased removal of mucosal fibrin deposition nor attenuated intestinal ischemia and reperfusion injury in a rat model of acute mesenteric occlusion. Intestinal ischemia/reperfusion causes local inhibition of endogenous fibrinolysis in combination with activation of coagulation. This may lead to thrombotic obstructions that compromise microcirculation and promote intestinal injury. However, enhanced fibrinolysis did not result in improvement of any of the measured parameters. Although anti-PAI-1 antibody or rt-PA administration enhanced circulatory
Summary and conclusions

Fibrinolytic activity, as evidenced by increased portal plasma plasminogen activator activity, elevation of fibrin degradation products and decreased levels of PAI-1, mucosal fibrin deposition and microthrombosis were not reduced in postischemic intestinal tissue. Furthermore, enhanced fibrinolysis did not attenuate ischemia and reperfusion-induced intestinal injury or dysfunction, as demonstrated by morphological and functional analysis. However, both interventions resulted in decreased levels of interleukin-6, which indicates fibrin-induced modulation of inflammation. These results suggest a limited role of suppressed endogenous fibrinolysis in microcirculatory failure and consequent deterioration of intestinal function and structure following intestinal ischemia and reperfusion.

In contrast to the previous in vivo studies, in chapter 10 we developed an in vitro Ussing chamber model to study the effect of hypoxia and reoxygenation on the functional characteristics of the intestinal epithelium, focusing on the dysfunctional properties of barrier function and absorptive and secretory capacity of the intestinal epithelial layer. Intestinal ischemia and reperfusion may lead to profuse secretion of water and electrolytes. The underlying mechanisms have been related to increased hydrostatic pressure, to denudation of intestinal villi and recently, to adenosine-mediated enhancement of chloride secretion. By studying these mechanisms in an in vitro system, we avoid the complex interplay between vascular, subepithelial and epithelial factors in in vivo models of ischemia and reperfusion. We conclude that hypoxia and reoxygenation differentially impair nutrient absorption, corroborating recent absorption data in in vivo models of ischemia, and that it differentially affects secretory capacity in crypts, dependent on the intracellular messenger pathway. The relative persistence of Ca^{2+}/PKC-mediated secretion to hypoxia and reoxygenation indicates that secretagogues that activate this pathway play a significant role in the intraluminal fluid sequestration and diarrhea observed after intestinal ischemia and reperfusion.

In addition to the studies focusing on mesenteric ischemia, we fundamentally analyze the role of reactive oxygen species in intracellular mechanisms of the endothelial cell in response to vascular endothelial growth factor (VEGF) in chapter 11. Reactive oxygen species (ROS) have traditionally been viewed as cytotoxic molecules, predominantly generated in pathological conditions such as ischemia and reperfusion syndromes, but they are now recognized to play a critical role in signal transduction and transcriptional regulation within the vascular tree. It has recently been shown that VEGF-induced proliferation, migration, and downstream expression of some but not all genes in endothelial cells are dependent upon a Rac1-regulated NADPH oxidase-derived ROS, suggesting that VEGF signaling in the endothelium is tightly coupled to NADPH oxidase activity. We demonstrate that NADPH oxidase-derived ROS serve to modulate selective VEGF-dependent signaling pathways, including PI3-kinase/Akt, MAPK, and PKC, transcriptional profiles and biological functions in endothelial cells.
Samenvatting en conclusies
Dit proefschrift beschrijft verschillende aspecten van de behandeling van acute mesenteriale ischemie.

**Hoofdstuk 1** introduceert de letale aandoening van acute mesenteriale ischemie en geeft een overzicht van de chirurgische revascularisatie mogelijkheden na acute afsluiting van de arterie mesenterica superior. Het acute beloop van een darminfarct verklaart de letaliteit van deze aandoening. Binnen enkele uren kunnen de dunne darmen volledig afsterven. Ondanks de grote vooruitgang in het stellen van de diagnose en de behandeling van acute mesenteriale ischemie in de afgelopen decennia blijft afsluiting van de mesenteriale vasculatuur een slechte prognose hebben met overlijdenspercentages in het ziekenhuis van 59-93 procent.

Het relatief weinig voorkomen van acute mesenteriale ischemie, de verschillende pathogenese en het brede spectrum van ischemische schade van de dunne en dikke darm maken het bijna onmogelijk deze aandoening en haar diagnose en behandeling strategieën in klinische gerandomiseerde of case-control studies te bestuderen. Chirurgie is de meest gebruikelijke behandeling voor deze aandoening vanwege de mogelijkheid tot het vaststellen van het onderliggende lijden, de levensvatbaarheid van de aangedane darm te bestuderen, de aangedane darm direct te revasculariseren, en indien nodig niet vitaal darmweefsel te reseceren. Ondanks deze voordelen van de chirurgie zijn er in de afgelopen jaren nieuwe behandelingsmethoden ontwikkeld die mogelijk een geselecteerde patiëntenpopulatie met acute occlusie van de mesenteriale arteriën kunnen dienen, en zodoende de overleving van deze letale aandoening kunnen verbeteren.

**Hoofdstuk 2** bediscussieert de etiologische verschillen van het acute mesenteriale ischemie syndroom. Acute mesenteriale ischemie kan ontstaan door arteriële embolieën, arteriële trombose, veneuze trombose en non-occlusieve mesenteriale ischemie. Het is van groot belang onderscheid te maken tussen de verschillende ontstaansvormen van acute mesenteriale ischemie aangezien de variatie in ziekteverloop kan leiden tot verschillen in behandeling en overleving. Toch worden in de meeste retrospectieve studies de mortaliteitspercentages berekend op basis van gecompileerde datasets van alle etiologische subgroepen tezamen, en daardoor worden karakteristieken van de subgroepen gemaskeerd, zoals verschillen in klinische presentatie en patiëntenpopulatie, diagnostiek, ziekteverloop, mortaliteitscijfers, en respons op therapeutische strategieën. Daarom hebben wij van de bestaande literatuur van de afgelopen 40 jaar een systematische analyse verricht, die concludeert dat 1) de prognose van acute veneuze mesenteriale ischemie beter is dan dat van acute arteriële mesenteriale ischemie; 2) de prognose van arteriële mesenteriale embolieën beter is dan dat van arteriële trombose of non-occlusieve ischemie; 3) het mortaliteitspercentage na chirurgische behandeling van arteriële embolieën en veneuze trombose (respectievelijk 54.1 en 32.1 procent) minder is dan dat chirurgie van arteriële trombose en non-occlusieve ischemie (respectievelijk 77.4 en 72.7 procent); en 4) de totale overleving van acute mesenteriale ischemie verbeterd is gedurende de afgelopen 4 decennia. Samenvattend bestaan er grote verschillen in prognose van acute mesenteriale ischemie op basis van verschil in etiologie. De prognose van de chirurgische behandeling van arteriële embolieën is in de afgelopen decennia verbeterd terwijl prognose van arteriële trombose en non-occlusieve ischemie slechts blijft.
Hoofdstuk 3 geeft een overzicht van de huidige (observationele) data van de trombolytische behandeling van patiënten met tromboembolische mesenteriale occlusie, met als doel deze nieuwe behandelingsstrategie als een alternatieve of additionele therapie van de chirurgische behandeling te evalueren. Trombolyse van een acute afsluiting van de arterie mesenterica superior wordt gezien als een relatief nieuwe behandelingsmethode. Er is momenteel onvoldoende bewijs voor handen om de effectiviteit en veiligheid van de trombolytische behandeling voor arterie mesenterica superior afsluiting te bepalen. Toch lijken de eerste resultaten veelbelovend. Het relatief weinig voorkomen van acute mesenteriale ischemie en de variatie in klinische presentatie houdt het vrijwel voor onmogelijk om gerandomiseerde of case-gecontroleerde studies te verrichten. Desalniettemin, deze analyse van gecompileerde data geeft inzicht in de huidige status van de trombolytische behandeling van acute tromboembolische afsluiting van de arterie mesenterica superior, voorziet in vragen van en antwoorden voor de clinicus, en geeft aanzet tot het opstellen van klinische richtlijnen (gebaseerd op consensus).

Hoofdstuk 4 plaats een kritische noot bij de ontdekking van de “arcus Riolani” door Riolan zelf. Riolan’s anastomosis of arcus Riolani is een eponiem voor de arteriële anastomose tussen de arterie mesenterica superior en inferior. Zowel vaat als gastro-intestinaal chirurgen zijn zeer bekend met deze collaterale mesenteriale circulatie voor retrograde perfusie van de arterie mesenterica superior wanneer deze is geoccludeerd. Het eponiem suggereert dat Jean Riolan (1580-1657), een beroemde Franse anatoom, als eerste deze arteriële anastomose heeft beschreven. Riolan was een grote voorstander van de traditionele Galenische doctrine binnen de geneeskunde en daardoor een uitgesproken tegenstander van de nieuwe denkwijze over de circulatie van bloed die werd uitgedragen door William Harvey (1578-1657). Daarom is het onwaarschijnlijk dat Riolan zelf deze arteriële collaterale circulatie in het mesocolon heeft ontdekt, hetgeen is aangetoond door zijn anatomieboek uit 1649 te bestuderen. Riolan heeft waarschijnlijk de vasculaire arcades geobserveerd, die lopen langs de binnenkant van het colon, waardoor hij later geassocieerd werd met de arteriële collaterale circulatie in het mesenterium. Het duurde tot 1743 alvorens Albrecht von Haller (1708-1777) een gedetailleerde beschrijving gaf van de anatomie van de mesenteriale circulatie, die refereert naar de arteriële collaterale verbinding tussen de arterie mesenterica superior en inferior, en als de “Arcus Riolani” genoemd is, als eerbetoon aan een grootmeester in de anatomie.

Hoofdstuk 5 schets de huidige kennis aangaande de “crosstalk” tussen stolling/coagulatie en ontsteking/inflammatie en de mogelijke voordelen van het herstellen van disfunctionele fysiologische antistollingsroutes in de microcirculatie, na hypoxie of ischemie en reperfusie. Het endotheel speelt een centrale rol in alle grote routes die betrokken zijn bij de pathogenese van hemostase gedurende ischemie en reperfusie. Endotheel cellen lijken direct betrokken bij de initiatie en regulatie van trombine generatie en de inhibitie van fibrine degeneratie. Proinflammatoire cytokines zijn cruciaal in het aanzetten van deze effecten in endotheel cellen. Tevens hebben endotheel cellen ook de mogelijkheid tot het produceren van cytokines en kunnen daarmee het stollingsproces vermenigvuldigen. De communicatie van endotheel cellen met andere cellen en organen over ischemie en reperfusie betreft een complexe samenhang (“crosstalk”) tussen meerdere systemen, vell meer dan een eenzijdige en directe interactie. Deze crosstalk kan grote betekenis hebben.
in ontstekingsgestuurde effecten van antistollingsinterventies in patiënten met ischemie en reperfusie syndemen.

In hoofdstuk 6 evalueren we de rol van intravasculaire stollingsactivatie in experimentele mesenteriale ischemie en reperfusie schade. We hebben aangetoond dat darmischemie en reperfusie resulteerde in locale generatie van trombine en vervolgens omzetting van fibrinogeen in fibrine. Hierbij is tevens de intestinale fibrinolyse verminderd, wat uiteindelijk leidde tot intravasculaire fibrine depositie. Deze resultaten suggereren dat microvasculaire obstructie een rol speelt in de door ischemie en reperfusie geïnduceerde pathogenese van structurele en functionele darmschade.

In hoofdstuk 7 bespreken we kort de betrokkenheid van het proteïne C systeem in een geselecteerd aantal modellen van ischemie en reperfusie schade. Verschillende experimentele studies hebben aangetoond dat een verminderde functie van de proteïne C route een belangrijke rol speelt in de pathogenese van sepsis en het hieraan gekoppelde orgaan falen. Ook klinische studies in patiënten met sepsis hebben de voordelen van recombinant humaan geactiveerd proteïne C aangetoond. Het is dan ook verleidelijk te speculeren dat andere klinische situaties, die gekarakteriseerd zijn door endotheliale en microvasculaire disfunctie (zoals ischemie en reperfusie syndemen), baat hebben bij de toediening van gerecombineerd geactiveerd proteïne C. Sepsis-reperfusie schade wordt gekarakteriseerd door een lokale ontstekingsreactie en een lokale stollingsactivatie die vergelijkbaar is met de situatie in sepsis. Sepsis-reperfusie schade speelt een belangrijke rol in grote klinische problemen, zoals hartinfarct, acuut nieren falen, acuut long falen, herseninfarct en darminfarct. Er is voldoende bewijs dat de rol van het proteïne C systeem in ischemie-reperfusie schade ondersteunt. Tevens kan de toediening van geactiveerd proteïne C een veelbelovende therapeutische optie betekenen in deze situaties. De effectiviteit van deze benadering verdient verdere analyse in experimentele en klinische studies.

In hoofdstuk 8 tonen wij aan dat geactiveerd proteïne C of antitrombine de door ischemie-reperfusie geïnduceerde lokale (mesenteriale) en systemische activatie van stolling en ontsteking vermindert, de mucosale fibrine depositie remt, en de ischemie-reperfusie geïnduceerde darmschade verbetert. Intestinale ischemie-reperfusie in deze experimentele studie resulteerde in een forse lokale en systemische verstoring van het stollingssysteem en verschillende ontstekingsroutes met als gevolg dat de mucosale en submucosale microcirculatie door uitgebreide microtrombose en fibrine depositie werd gecomprimeerd. Toediening van geactiveerd proteïne C of antitrombine verminderde trombine vorming, fibrine degradatie producten en fibrine depositie (na histologische evaluatie), terwijl toediening van heparine alleen een kleine daling in portale spiegels van fibrine degradatie producten liet zien. Verder, toediening van geactiveerd proteïne C of antitrombine verminderde de ontstekingsreactie, hetgeen werd weerspiegeld door de daling van interleukine-6 levels tot normaal waarden, waarbij toediening van heparine geen effect liet zien. Tevens gaf de behandeling van geactiveerd proteïne C of antitrombine minder ischemie-reperfusie geïnduceerde darmdisfunctie en histologische veranderingen, in vergelijking met de controle behandelingen. Deze bevindingen
suggereerden dat geactiveerd proteïne C of antitrombine de ischemie-reperfusie geïnduceerde darmschade reduceert door antistollings- en ontstekingsremmende effecten.

In hoofdstuk 9 demonstreren wij in een experimentele studie van mesenteriële occlusie dat toename van fibrinolytische activiteit door 1) intraveneuze toediening van recombinant tissue plasminogeen activator (rt-PA) of door 2) inhibitie van plasminogeen activator inhibitor (PAI)-1 door intraveneuze toediening van MA-33H1F7 (anti-PAI-1), dat nôch de afbraak van mucosale fibrine depositie verhoogt, nôch de door ischemie-reperfusie geïnduceerde darmschade verbetert. Intestinale ischemie-reperfusie veroorzaakte locale inhibitie van endogene fibrinolyse in combinatie met stollingsactivatie. Dit kan leiden tot microtrombotische verstoppingen wat de microcirculatio verder kan comprimeren met toename van de darmschade tot gevolg. Maar verhoogde fibrinolyse resulteerde niet in verbetering van één van de gemeten parameters. Hoewel de toediening van anti-PAI-1 of rt-PA de circulatoire fibrinolytische activiteit verhoogde (hetgeen werd aangetoond door een stijging in de portaal gemeten activiteit van plasma plasminogeen activator, een toename van fibrine degradatie producten en een daling van PAI-1 plasma spiegels), namen mucosale fibrine depositie en microtrombose niet af in het postischemische darmweefsel. Tevens verbeterde een toename in fibrinolytische activiteit de ischemie-reperfusie geïnduceerde darmschade en -disfunctie niet, wat werd aangetoond door histologische en functionele analyse. Desalniettemin, beide interventies resulteerde in een daling van de plasma spiegels van interleukine-6, hetgeen wijst op fibrine geïnduceerde modulatie van de ischemie-reperfusie uitgelokte ontstekingsreactie. Deze resultaten suggereren een beperkte rol voor de onderdrukte endogene fibrinolyse in microcirculatoire disfunctie na ischemie en reperfusie, en de vervolgens verslechtering van de darmfunctie en -structuur.

In tegenstelling tot de voorgaande in vivo studies ontwikkelden wij in hoofdstuk 10 een in vitro Ussing kamer model om het effect van hypoxie en reoxygenatie op de functionele karakteristieken van het darmepitheel te bestuderen, waarbij wij ons concentreerden op de disfunctionele kenmerken van de barrière functie en absorptieve en secretoire capaciteit van het darmepitheel. Intestinale ischemie-reperfusie kan leiden tot forse secretie van water en elektrolyten. De onderliggende mechanismen worden gerelateerd aan verhoogde hydrostatische druk, aan afbrokkelen van de darmvilli en recent aan adenosine gemedieerde toename van chloride secretie. Door deze mechanismen te bestuderen in een in vitro systeem te bestuderen vermijden we de complexe interactie tussen de endotheliale, subepitheliale en epitheliale factoren in in vivo modellen van ischemie en reperfusie. Wij concluderen dat hypoxie en reoxygenatie de absorptie van nutriënten in de villi differentieel verminderen, dat overeenkomt met recente data van darmischemie in in vivo modellen. Tevens demonstreren wij dat de secretoire capaciteit in de crypten differentieel worden beïnvloed, en dat deze afhankelijk is van de intracellulaire messenger pathways. Het relatief aanwezig blijven van de Ca\textsuperscript{2+}/PKC gemedieerde secretie na hypoxie and reoxygenatie geeft aan dat secretagogen die deze route activeren een belangrijke rol spelen in de intraluminale secretie en diarree aangetroffen na intestinal ischemie en reperfusie.
In navolging van de eerdere studies die zich concentreren op acute mesenteriale ischemie hebben wij in hoofdstuk 11 de rol bestudeerd van zuurstof radicalen in intracellulaire mechanismen van endotheel cellen in reactie op 'vascular endothelial growth factor' (VEGF). Zuurstof radicalen worden met name gezien als schadelijke moleculen voor de cel, die voornamelijk worden gegenereerd in pathologische situaties, zoals ischemie en reperfusie syndromen. Echter recent worden zuurstof radicalen een cruciale rol toegedicht in signaal transductie and transcriptionele regulatie binnen het vasculaire stelsel. Recent is aangetoond dat VEGF geïnduceerde proliferatie, migratie, en downstream expressie van enkele genen in endotheel cellen afhankelijk zijn van Rac1-gereguleerde NADPH-oxidase verkregen zuurstof radicalen. Dit suggereert dat VEGF signaal transductie in de endotheel cel nauw gerelateerd is aan NADPH-oxidase activiteit. NADPH-NADP systeem speelt een belangrijke rol in detoxificatie processen binnen de endotheel cel. Wij hebben aangetoond dat NADPH-oxidase verkregen zuurstof radicalen de taak hebben selectieve VEGF-afhankelijke signaal routes (zoals PI3-kinase/Akt, MAPK, en PKC), transcriptionele profielen en biologische functies in endotheel cellen te moduleren.
Dankwoord / Acknowledgements

"Het komt allemaal goed …"

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Curriculum Vitae


Ivo G. Schoots was born on April 25th, 1973 in Schiedam, the Netherlands. In 1991 he graduated from high school (Atheneum) at the Sint Willibrordus College in Goes. After that he attended medical school, the first year at the University of Antwerp, Belgium, than at the University of Amsterdam, the Netherlands, where he graduated in 1996. He finished his internships in 1999. During medical school he performed additional clinical internships in Cambridge (England), Curacao, en Seattle (WA), and a research internship at the department of Surgery (dr. M.P. Simons and prof. H. Obertop). In 1999 he started a PhD-programme at the departments of Surgery (prof. T.M. van Gulik) and Vascular Medicine (prof. M.M. Levi) at the Academic Medical Center, Amsterdam. The research performed led to this thesis. In 2003 he started as ‘postdoctoral fellow’ (Fulbright scholarship) at the laboratory of Molecular & Vascular Medicine, BIDMC, Harvard Medical School, Boston (prof. W.C. Aird). Since July 2004 he works as a surgical (non)trainee at the Onze Lieve Vrouwe Hospital (dr. Out). From January 2005 he will start his surgical training at the University Medical Center Utrecht (prof. I.H. Borel Rinkes).
Stellingen behorend bij het proefschrift:

“Clinical and experimental studies on treatment of acute mesenteric ischemia”

1. Afsluiting van de mesenteriale vaten moet worden gezien als één van die aandoeningen waarvan de diagnose onmogelijk, de prognose hopeloos en de behandeling zinloos is. (A.J. Cokkinis, 1926)

2. De diagnose van acute mesenteriale afsluiting is mogelijk, en in sommige gevallen is de behandeling zinvol en de prognose hoopvol. (dit proefschrift)

3. Systemische evaluatie van onderzoeksresultaten is noodzakelijk om vooruitgang te boeken in de fast-moving industrie van de gezondheidszorg, zelfs als alleén observationele data en kleine case-series beschikbaar zijn. (dit proefschrift)

4. Trombolyse van acute mesenteriale afsluitingen kan snel en effectief zijn, en kan een chirurgische ingreep bij een patiënt voorkomen. (dit proefschrift)

5. Riolan, een beroemde 17e eeuwse anatoom, heeft nooit het mesenteriale bloedvat geobserveerd dat zijn naam draagt: de ‘arc of Riolan’. (dit proefschrift)

6. Het mesenteriale ischemie/reperfusie syndroom en de systemische inflammatie in sepsis tonen dat er vergelijkbare routes in de cascade van ontstekings- en stollingsactivatie zijn. (dit proefschrift)

7. De antistollingsmiddelen activated protein C en antithrombin herstellen de verstoorde stollings- en ontstekingsreactie in mesenteriale ischemie en reperfusie. (dit proefschrift)

8. Een goed functionerende partij darmen is meer waard dan welke hoeveelheid hersenen dan ook.

9. In de gezondheidszorg moet het niet de vraag zijn hoe door slechte clinici schade aan patiënten kan worden voorkomen, maar juist hoe door goede clinici schade aan patiënten kan worden voorkomen.

10. De classificatie van chirurgie als 'snijdend specialisme' doet tekort aan de contemplatieve aspecten van het vak in de pre-, per- en postoperatieve situatie.

11. De schaarste aan onzekerheid in dagelijks chirurgisch handelen wordt gerechtvaardigd door ‘Sometimes wrong; never in doubt’.

12. De windsurfsprong Table-Top is (als) omgekeerd opereren.

13. Liever 10 knopen in de lucht dan 1 met de hand.

Ivo Schoots (11 november 2004)