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Coagulation and inflammation in ischemia and reperfusion injury

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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 postischemic 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 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
identified on hypoxic-stimulated endothelium \(^{17}\). 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 \(^{18}\) and ICAM-1 \(^{19}\)) on endothelial cells and attracting and activating leukocytes \(^{20}\). The expression of hypoxia-induced IL-8 \(^{21}\) enhances the chemotactic migration and activation of neutrophils, thereby promoting the leukostasis in hypoxic organs \(^{22,23}\). 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 \(\text{in vitro}\) appears to have an anti-inflammatory potential in the setting of hypoxia, suppressing IL-1 and tumor necrosis factor (TNF)-\(\alpha\) production by macrophages \(^{24,25}\).

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 adhesiveness \(^{26}\). 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 \(^{27}\). Leukocyte adherence is further established by E-selectins and ICAM-1 on endothelial cells and \(\text{L-selectins and } \beta_2\text{-integrins on activated leukocytes during hypoxia}^{28}\). Activation of leukocytes increases the binding affinity of \(\beta_2\text{-integrins to endothelial cell receptors, such as ICAM-1, or to fibrinogen, which can bind simultaneously to ICAM-1 and to the } \beta_2\text{-integrin Mac-1}^{29}\). 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 \(^{30,31}\). 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 \(^{32}\).

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 \(^{33}\) 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)-kB, 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
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- Generation of reactive oxygen species
- Generation of thrombin mediated by tissue factor
- Production of pro- and antiinflammatory cytokines
- Impairment of anticoagulation pathways
- Expression of adhesion receptors
- Activation of neutrophils
- 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.

did suppress fibrin deposition in hypoxic lungs. Furthermore, monocytes stimulated by hypoxic stress in vitro demonstrated transcriptional upregulation of TF mRNA. The hypothesis of P-selectin- or ICAM-1-mediated adherence of polymorphonuclear leukocytes to the hypoxic vessel wall and subsequent reactive oxygen species production, causing endothelial injury and exposure of TF, was dismissed because antibody-induced depletion of polymorphonuclear neutrophils had no effect on fibrin accumulation. Oxygen deprivation enhances de novo early growth response (Egr)-1 synthesis, which might be a primary driving motif underlying hypoxia-induced tissue factor transcription, and can initiate the local procoagulant response.

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 72,79-81. 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 73-77.

A brief period of ischemia alone in a normal vessel is not sufficient to promote fibrin formation 82,83. 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 84.

Fibrin deposition on the endothelial surface occurs directly after the onset of reperfusion, as well as the recruitment of platelets 35,76. 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 76,85. 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 86.

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 87. 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
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 VIIa 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 VIIa) 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 V 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 dependant 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
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 \textsuperscript{121,122}, acute myocardial infarction \textsuperscript{106,123}, unstable angina \textsuperscript{124} and stroke \textsuperscript{104}. However, Abumiya et al. demonstrated decreased plasma TFPI activity in ischemic stroke patients in which influence of cholesterol levels was excluded \textsuperscript{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 \textsuperscript{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 \textsuperscript{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. Cross talk 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 \textsuperscript{128}, probably mediated by the cleavage of cell surface protease activated receptors \textsuperscript{129}. These receptors signal the intracellular link between coagulation and inflammation at sites of vascular injury, modulating platelet and endothelial cell activation \textsuperscript{130-133}. Fibrinogen/fibrin is important to the host defense mechanism and probably has an additional role that is not directly related to clotting \textit{per se} \textsuperscript{134}.

TF-factor VIIa complex can activate cells \textsuperscript{135-137}, mediated by a Ca\textsuperscript{2+}-influx, leading to activation of mitogen-activated protein kinase, c-Jun N terminal kinase, and the early growth gene-1 (egr-1) \textsuperscript{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 \textsuperscript{136}. Other inflammatory actions of TF-factor VIIa include reverse migration of monocytes from the basal to the apical surface of the endothelium \textsuperscript{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 \textsuperscript{128} and endothelium \textsuperscript{140}, leading to IL-1 mediated lymphocyte proliferation \textsuperscript{141} and edema \textsuperscript{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-activable 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 antiinflammatory 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
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 \textit{in vitro}.\textsuperscript{143} Thrombin also may induce IL-6 and IL-8 production from endothelial cells \textit{in vitro}.\textsuperscript{144} These effects on cell activation are probably mediated by protease activated receptors 1, 3, and 4.\textsuperscript{145} Furthermore, 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)\textsuperscript{148} as well as to increased microvascular permeability alterations.\textsuperscript{149}

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.\textsuperscript{150} Pro-inflammatory cytokines, such as TNF-α and IL-1, may significantly down-regulate the expression of thrombomodulin, as suggested by cell culture experiments.\textsuperscript{112,151} This latter observation is consistent with many other studies indicating a cross-talk between effect of protein C and inflammation modulation.\textsuperscript{152}

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.

\textbf{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.\textsuperscript{153} This derangement in ischemia and reperfusion is still under investigation. The principal mediator of coagulation activation in sepsis seems to be IL-6.\textsuperscript{154} TNF-α indirectly influences the activation of coagulation because of its effect on IL-6.\textsuperscript{153,155} 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.\textsuperscript{156} 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.

\textbf{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{149} Indeed, rapid reinternalization of P-selectin has been demonstrated in activated endothelium.\textsuperscript{159} In addition, antithrombin has also been shown to release prostacyclin following ischemia-reperfusion,\textsuperscript{160,161} which has potent anti-adhesive properties.\textsuperscript{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.\textsuperscript{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.\textsuperscript{90} Furthermore, anti-TF monoclonal antibody inhibited vascular reocclusion after thrombolysis in a rabbit model of carotid artery thrombosis.\textsuperscript{168}
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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 than 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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