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INHIBITION OF THROMBIN IN CARDIAC SURGERY – EXPERIMENTS IN A PORCINE MODEL

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Academic Dissertation

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To my wife Saija and children Antti, Eero, and Saana

To my mother

Contents

LIST OF ORIGINAL PUBLICATIONS

I Jormalainen M, Vento AE, Wartiovaara-Kautto U, Suojaranta-Ylinen R, Rämö OJ, Petäjä J. Recombinant hirudin enhances cardiac output and decreases systemic vascular resistance during reperfusion after cardiopulmonary bypass in a porcine model. J Thorac Cardiovasc Surg 128:189–96, 2004.

II Jormalainen M, Vento AE, Lukkarinen H, Kääpä P, Kytö V, Lauronen J, Paavonen T, Suojaranta-Ylinen R, Petäjä J. Inhibition of thrombin during reperfusion improves immediate postischemic myocardial function and modulates apoptosis in a porcine model of cardiopulmonary bypass. J Cardiothorac Vasc Anesth 21:224–31, 2007.

III Jormalainen M, Vento AE, Wartiovaara-Kautto U, Suojaranta-Ylinen R, Lauronen J, Paavonen T, Petäjä J. Antithrombin reduces pulmonary hypertension during reperfusion after cardiopulmonary bypass in a pig. Acta Anaesthesiol Scand 51:178–88, 2007.

IV Jormalainen M, Vento AE, Wartiovaara-Kautto U, Suojaranta-Ylinen R, Lauronen J, Paavonen T, Petäjä J. Ischemic intestinal injury during cardiopulmonary bypass does not show an association with neutrophil activation: a porcine study. Eur Surg Res 42:59–69, 2009.

ABBREVIATIONS

ACT	activated coagulation time
AT	antithrombin
CPB	cardiopulmonary bypass
F	coagulation factor
GAG	glycosaminoglycan
I/R	ischemia and reperfusion
IL	interleukin
MPO	myeloperoxidase
NO	nitric oxide
PAR	protease activated receptor
PGI_2	prostacyclin
pHa	arterial pH
pHi	intramucosal pH
$paCO_2$	arterial CO_2 pressure
$piCO_2$	intramucosal CO_2 pressure
PMN	polymorphonuclear leukocyte
ROS	reactive oxygen species
TAT	thrombin antithrombin complex
TF	tissue factor
TNF	tumor necrosis factor
TnT	troponin T

ABSTRACT

Background and objectives

Cardiac surgery involving cardiopulmonary bypass (CPB) induces activation of inflammation and coagulation system and is associated with ischemia-reperfusion injury (I/R injury) in various organs including the myocardium, lungs, and intestine. I/R injury is manifested as organ dysfunction. Thrombin, the key enzyme of coagulation, plays a central role also in inflammation and contributes to regulation of apoptosis as well. Therefore, we wanted to study if recombinant hirudin, a direct inhibitor of thrombin, could attenuate reperfusion induced generation of thrombin and whether the direct inhibition of thrombin would affect general hemodynamics and intestinal microcirculation. We also studied the effects of thrombin inhibition on early functional recovery of the post-ischemic myocardium and explored potential mechanisms of thrombin activity on myocardial I/R injury. Further, we wanted to study the effects of supplementary antithrombin on myocardial and lung I/R injury. Finally, we studied intestinal I/R injury after CPB and more specifically whether local post CPB inflammatory response in the gut wall would associate with intestinal mucosal perfusion.

Material and methods

Forty five pigs were used for the studies. Animals underwent 75 min of normothermic CPB, 60 min of aortic clamping, and 120 min of reperfusion period. Two randomized blinded studies were performed (study I, III). Twenty animals received an iv-bolus of recombinant hirudin (r-hirudin) lepirudin (n=10) or placebo (n=10) 15 minutes before the aortic clamp was released and then continued with a 135 min intravenous infusion of r-hirudin or placebo (study I). Twenty animals in a similar setting received an iv-bolus of AT (250 IU/kg) (AT group, n=10) or placebo (n=10) 15 min before aortic declamping. An additional group of 5 animals received 500 IU/kg of AT (AT+ group) in an open label setting to test dose response (study III). In both studies, thrombin-antithrombin complexes (TAT), activated clotting times (ACT), and several hemodynamic parameters were measured before CPB, after weaning from CPB, and during the reperfusion period. In addition, antithrombin (AT) activity and troponin T were measured in study III. Intramucosal pH and pCO_2 were measured from the luminal surface of ileum simultaneously with arterial gas analysis at 30 min intervals (Study I, III). In study II, serial myocardial biopsies and a larger sample of myocardium, which were taken from the animals in study I, were analyzed to quantitate leukocyte infiltration (myeloperoxidase activity, MPO), histological alterations, and apoptosis using the caspase 3 and TUNEL-method. In study III, in addition to measuring myocardial MPO activity and histological alterations, a larger sample of lung was taken also for histological evaluation.

Study IV included placebo animals from studies I and III. Based on ileal myeloperoxidase activity (MPO) the animals were divided into two groups (CPB induced increase in MPO ("MPO+") vs. no such increase ("MPO-")) for comparison of the parameters measuring gut mucosal perfusion. Intestinal biopsies taken after 120 min of reperfusion were analyzed for histological alterations. Additionally, several hemodynamic parameters and blood thrombin-antithrombin complexes (TAT) were calculated according to the group division.

Results. R-hirudin inhibited thrombin formation after aortic declamping; at 120 min TAT levels (μg/L, mean ± SD) were 75 ± 21 and 29 ± 44 ($p < .001$) for placebo and r-hirudin receiving pigs, respectively. When compared with placebo-group, r-hirudin receiving pigs showed significantly higher stroke volume, cardiac output, and lower systemic vascular resistance, at 60 min and at 90 min after aortic declamping ($p < .05$). Based on arterio-mucosal pCO_2 and pH difference, progressive worsening of intestinal microcirculatory perfusion occurred in the placebo group but not in the r-hirudin group (Study I). Microthrombosis was not observed in either group indicating sufficient anticoagulation and excluding intravascular clots as an explanation for LV dysfunction in the current experiment. Instead, ample myocardial activation of inflammation was present but only a trend of r-hirudin associated anti-inflammatory effect was observed. Compared with the controls, TUNEL-positive myocytes were detected significantly less frequently in the r-hirudin group (0.05 ± 0.06 vs. 0.13 ± 0.07 TUNEL- positive nuclei %, $p = .042$) (Study II).

AT effectively inhibited coagulation as assessed by ACT but AT did not prevent thrombin generation during CPB. In AT an AT+ groups only, cardiac output (CO) and stroke volume (SV) showed a trend of post ischemic recovery during the first 15 min after CPB. AT attenuated reperfusion induced increase in pulmonary arterial diastolic pressure (PAPD) but did not have significant effects on systemic or pulmonary vascular resistance. The effects of AT on SV, CO, and PAPD were fortified in AT+ group. AT did not show effects on inflammatory changes in either myocardial or pulmonary tissue specimens. AT did not reduce post-ischemic troponin T release.

In study IV, myocyte degeneration, endothelial activation, and vasculitis were more pronounced in the MPO+ group ($p<0.05$). Instead, the MPO- group showed significantly increased $piCO_2$ and lower mucosal pH values during reperfusion. Hemodynamics or TAT levels did not differ between the groups.

Conclusions

In conclusion, our data suggest that r-hirudin may be an effective inhibitor of reperfusion induced thrombin generation in addition to being a direct inhibitor of preformed thrombin. Overall, the results suggest that inhibition of thrombin, beyond what is needed for efficient anticoagulation by heparin, has beneficial effects on myocardial I/R injury and hemodynamics during cardiac surgery and CPB. We showed that infusion of the thrombin inhibitor r-hirudin during reperfusion was

associated with attenuated post ischemia left ventricular dysfunction and decreased systemic vascular resistance. Consequently microvascular flow was improved during ischemia-reperfusion injury. Improved recovery of myocardium during the post-ischemic reperfusion period was associated with significantly less cardiomyocyte apoptosis and with a trend in anti-inflammatory effects. Thus, inhibition of reperfusion induced thrombin may offer beneficial effects by mechanisms other than direct anticoagulant effects. AT, in doses with a significant anticoagulant effect, did not alleviate myocardial I/R injury in terms of myocardial recovery, histological inflammatory changes or post-ischemic troponin T release. Instead, AT attenuated reperfusion induced increase in pulmonary pressure after CPB. Taken the clinical significance of postoperative pulmonary hemodynamics in patients undergoing cardiopulmonary bypass, the potential positive regulatory role of AT and clinical implications needs to be studied further.

Inflammatory response in the gut wall proved to be poorly associated with perturbed mucosal perfusion and the animals with the least neutrophil tissue sequestration and I/R related histological alterations tended to have the most progressive mucosal hypoperfusion. Thus, mechanisms of low-flow reperfusion injury during CPB can differ from the mechanisms seen in total ischemia reperfusion injury.

INTRODUCTION

Cardiac surgery using cardiopulmonary bypass (CPB) provokes both a systemic and local activation of inflammation and coagulation systems. This is caused by the contact of blood with the artificial, non-endothelial, surfaces of the CPB circuit, surgical trauma, the release and reinfusion of highly active blood from the surgical wound, and the reperfusion of ischemic tissue. As a result, the complement system, leukocytes and platelets are activated, and cytokines are released. Despite the use of heparin the plasma coagulation cascades are activated and thrombin is generated. Clinical manifestations are disturbed hemostasis and organ dysfunction.

The myocardium suffers global ischemia during aortic clamping and CPB can cause hypoperfusion of various organs, including the lungs and intestine. After aortic clamping and CPB, restoration of circulation results in reperfusion injury in post ischemic tissues. The I/R-induced myocardial dysfunction is of particular clinical interest.

Thrombin, the key enzyme of coagulation, is a multifunctional protease playing a pivotal role between coagulation and inflammation and it contributes to regulation of apoptosis as well. A link between ischemia-reperfusion injury and apoptosis suggests that myocardial apoptosis may be involved in the pathogenesis of myocardial dysfunction during cardiac surgery.

Hirudin is a selective and effective inhibitor of thrombin. R-hirudin has been shown to have beneficial effects on the myocardial I/R injury in various experimental models. However, the effects of hirudin on CPB induced ischemia-reperfusion model have not been studied. Antithrombin is a major physiological anticoagulant inhibiting thrombin and other proteases of the coagulation but possess anti-inflammatory actions independently of its effects on coagulation as well. Supplementation of AT has alleviated I/R injury in various organs but only scarce and controversial data of AT effects on myocardial I/R injury are available.

The general aim of this study was to evaluate the potential of thrombin inhibition in reducing the adverse effects of ischemia-reperfusion injury in myocardium, lungs, and intestine associated with the use of CPB and cardiac surgery.

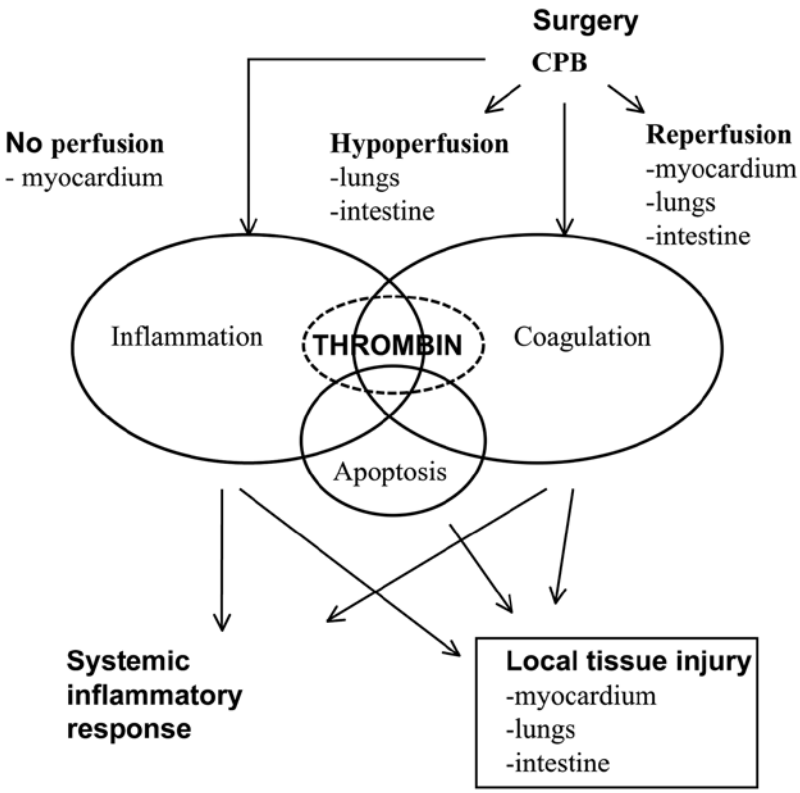

Figure 1. The rationale of testing thrombin inhibition as a means to improve recovery from cardiac surgery. The central position of thrombin in controlling crossroads of inflammation, coagulation, and apoptosis renders diverse organ and vascular bed specific functional end results possible. Further, thrombin's complex mechanisms of action indicate potential differences in the functional results depending on by how thrombin functions are downregulated.

REVIEW OF THE LITERATURE

1. Inflammatory response during cardiac surgery and CPB

Cardiac surgery with cardiopulmonary bypass (CPB) and cardioplegic cardiac arrest is known to cause systemic activation and complex interactions of several inflammatory mediators involving complement, cytokines, endotoxin, and leukocytes. In addition to systemic inflammatory response, reperfusion of ischemic heart and other organs suffering from CPB-related hypoperfusion activates local inflammatory process.

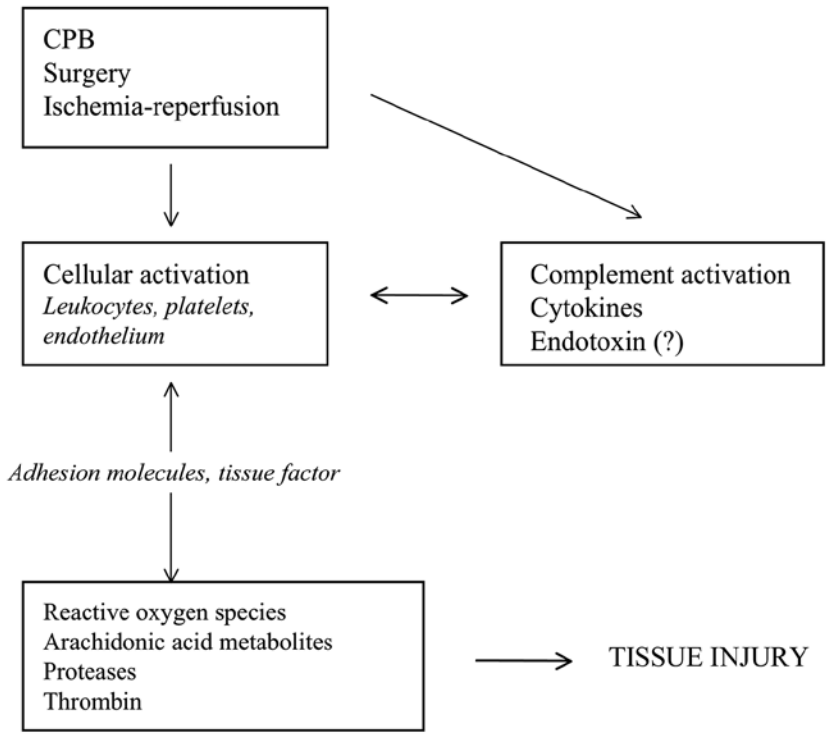

Figure 2. Schematic representation of the inflammatory response to cardiac surgery and CPB.

1.1. Complement activation

The activation of inflammatory response during CPB is an extremely complex process. Contact of blood components with non-physiological surfaces of the oxygenator, reservoir, and tubing activates the complement system. The exposure of blood to CPB circuit activates the alternative pathway, resulting in the formation

of C3a and C5a with anaphylactic and chemotactic activity (Chenoweth et al. 1981, Utley 1990). C3a stimulates platelet aggregation, while C5a stimulates neutrophil activation and adherence to endothelial cells (Utley 1990). The classical pathway is activated by reversal of heparin with protamine and is associated with a rise in C4a levels and a further rise in C3a levels (Cavarocchi et al. 1985, Kirklin et al. 1986). The C3a levels remain elevated after CPB in association with the duration of CPB (Kirklin et al. 1983). Steinberg et al. (1993) demonstrated significantly elevated levels of complement components C3a, C4a, and terminal membrane attack complex C5b-9 during CPB. C5b-9 may activate endothelial cell leukocyte adhesion molecule transcription and expression and also promote leukocyte activation and chemotaxis by inducing endothelial cytokine and monocyte chemoattractant protein 1 secretion (Collard et al. 1999).

1.2. Cytokines

Cytokines are intercellular messengers produced by leukocytes, platelets, and endothelial cells in response to various stimuli including complement activation, endotoxin release, I/R, and by other cytokines (Paparella et al. 2002, Wan et al. 1997a). The release of proinflammatory cytokines, such as interleukin-6 (IL-6) and interleukin-8 (IL-8), has been shown to associate with CPB (Piglioli et al. 2003, Wei et al. 2001, Wei et al. 2003). Increased levels of tumor necrosis α (TNF-α) during and after CPB have been measured in many studies (Biglioli et al. 2003) but not in all (Brix-Christensen et al. 2001, Wei et al. 2001, Wei et al. 2003). TNF-α and IL-6 may be important as they have been associated with myocardial dysfunction (Chain et al. 1999, Deng et al. 1996). Brix-Chistensen et al. (2001) demonstrated significantly elevated plasma levels of IL-8 and IL-10 in piglets undergoing CPB. IL-8 is a potent chemotactic factor that activates neutrophils and is central to neutrophil accumulation in tissues (Williams et al. 1999). In addition, IL-8 has been found to associate with hemodynamic instability after CPB (Wei et al. 2003). The proinflammatory cytokine response to cardiac surgery is balanced by the release of anti-inflammatory cytokines, such as IL-10. Il-10 is a potent inhibitor of the production of proinflammatory cytokines TNF-α, IL-1, IL-6, and IL-8 (Journois et al. 1996).

1.3. Endotoxin

Circulating levels of endotoxin, a lipopolysaccharide found in the outer membrane of various gram-negative bacteria, have been demonstrated during and after CPB in many studies (Andersen et al. 1993, Jansen et al. 1992, Mollhof et al. 1999, Nilsson et al. 1990, Rocke et al. 1987). There are many possible sources of endotoxin during CPB but the gut is probably the most important (Andersen et al. 1993, Baue 1993, Mollhof et al. 1999). Many authors suggest that compromised splanchnic perfusion during CPB results in intestinal mucosal injury and increased permeability allowing

translocation of intestinal bacteria and endotoxemia (Anderssen et al. 1993, Mollhof et al. 1990, Ohri et al. 1994b). However, Anderssen et al. (1993) could not find significant relationship between an elevation of endotoxin levels and a fall in gastric intramucosal pH (pHi). In addition, Myles et al. (1996) could not demonstrate any evidence that intestinal ischemia during CPB, measured by gastric mucosal pH, predisposes to endotoxemia. Endotoxinemia may be partly responsible for the activation of complement via alternative pathway (Jansen et al. 1992) and for the release of cytokines, including TNF-α and IL-6 (Giroir et al. 1993, Jirik et al. 1989).

1.4. Endothelium

The vascular endothelium plays a pivotal role maintaining homeostasis during surgery and CPB associated inflammatory response. Under normal physiologic conditions resting but still active vascular endothelium regulates the balance between vasodilatation and vasoconstriction, thrombosis and anticoagulation, transport of fluid and solutes between the intravascular and extravascular space, and blood cell adherence to the endothelium (Verrier 1996). In response to inflammatory mediators, such as cytokines, endotoxin, complement activation products (C5a), and oxygen free radicals, endothelium is shifted to its activated form resulting in changes in gene expression and cellular functions. Once activated, endothelial cells further promote inflammatory reactions and thrombosis by releasing cytokines, nitric oxide (NO), and by expressing different leukocyte adhesion molecules and tissue factor (TF) on their surface (Virkhaus et al. 1995).

Endothelial cell activation can be divided into two different types. In the hypoxic type, in response to abrupt reperfusion of ischemic tissue, stimuli, such as reactive oxygen species and activated complement fragments, induce the transient expression of preformed proteins stored in the endothelial cells promoting leukocyte-endothelial interactions and coagulation within seconds to minutes. Alternatively, in response to TNF-α, IL-1, and IL-6, several transcriptional genes are activated and production of proteins on the endothelial cells is completed over the course of several hours. These proteins include leukocyte adhesion molecules that mediate leukocyte recruitment to the sites of inflammation early in the course of tissue reaction and TF that initiates the intravascular formation of thrombin (Boyle et al. 1998, Pober and Cotran 1990).

Direct evidence for CPB associated endothelial activation and injury has been shown by measuring increased concentrations of soluble endothelial adhesion molecules (sICAM, sVCAM, sE-selectin) and circulating endothelial cell (CECs) numbers during reperfusion after CPB (Schmidt et al. 2006).

1.5. Leukocytes

The clinical importance of leukocyte activation, most importantly neutrophils and monocytes, during cardiac surgery and CPB is widely accepted. Leukocyte activation

during CPB has been demonstrated by elevated levels of leukocyte adhesion molecules (Gilliland et al. 1999) and neutrophil derived enzyme activities, including neutrophil elastase and myeloperoxidase (Larson et al. 1996). Several pathways lead to leukocyte activation. These include complement C5a, proinflammatory cytokines and thrombin. Fung et al. (2001) demonstrated in a simulated CPB model that upregulation of neutrophil adhesion molecule (CD11b) as well as the release of neutrophil specific myeloperoxidase and elastase was effectively inhibited by anti-factor D monoclonal directed to inhibit the activation of alternative complement cascade. Further, neutrophil complement C5a receptor blockade during simulated extracorporeal circulation completely blocked neutrophil adhesion molecule (β2 integrin) upregulation and induction of plasma IL-8 release (Rinder et al. 2007). Schwartz et al (1998) demonstrated that CPB primes neutrophils to produce reactive oxygen species (superoxide $\cdot O^-_2$). It has also been suggested that neutrophil priming occurs early before CPB in cardiac surgical patients indicating that anesthesia, surgical trauma, or other events may be involved (Gu et al. 2002).

The peripheral monocytes produce numerous proinflammatory and anti-inflammatory cytokines (Zimmermann et al. 2003). In addition, activated monocytes are procongulant as they can express tissue factor that initiates the activation of extrinsic coagulation pathway (Shibamiya et al. 2004). Monocytes are activated during CPB and recruited to the inflammatory site by increased monocyte chemoattractant factors and possibly by complement factor C5a (Wehlin et al. 2005). Monocyte activation is thought to take place in both the surgical wound and CPB circuit, although this tends to occur over a slower time course compared to activation of complement or neutrophils (Kappelmayer et al. 1993, Steinberg et al. 1993). In contrast to rise in neutrophils associated with CPB, an acute reduction of circulating monocytes has been observed and suggested to be secondary to complement activation and monocyte adherence to activated endothelium and the CPB circuit (Diegeler et al. 1998, Hiesmayr et al. 1999, Wehlin et al 2005)

1.6. The role of surgical trauma

The evidence shows that surgical trauma itself during cardiac surgery contributes to the inflammatory response. Czerny et al. (2000) suggested that the impact of CPB on inflammatory response during coronary artery bypass surgery (CABG) is smaller than has been expected and demonstrated that surgical access itself markedly contributes to the release of inflammatory mediators. The release of inflammatory cytokine IL-6 as well as adhesion molecules P-selectin and ICAM-1 were comparable between the groups with or without CPB (Cherny et al. 2000). Wehlin et al. (2004) measured less complement activation but similarly increased interleukin and leukocyte activation markers between the groups operated with or without CPB.

Studies investigating the influence of CPB on inflammatory response during cardiac surgery demonstrate that some inflammatory makers (complement factors,

TNF-α, IL-8, IL-10, leukocyte elastase) increase from baseline values in surgery with or without CPB, but the peak levels are highest in association with CPB (Piglioli et al. 2003). However, the differences in these inflammatory markers progressively decrease and finally fade out during early and late postoperative period (Piglioli et al. 2003). Leukocyte counts are increased and peak 24–48 hours postoperatively in both operative strategies but such increases are slightly more elevated in surgery with CPB (Ascione et al. 2000). Neutrophils and neutrophil activation marker elastase levels increase during during the first 12 hours of CPB surgery, whereas elastase rises in later phases (12–24 hours) in surgery without CPB (Ascione et al. 2000). The evidence of other inflammatory markers (IL-1, IL-6) is less consistent (Piglioli et al. 2003).

Retransfusion of suctioned blood from surgical wound may also contribute to the systemic inflammatory response. Skrabal et al. (2006) demonstrated that the levels of inflammatory markers PMN-elastase, IL-6, and C-reactive protein were significantly higher in retransfusion patients compared to no-retransfusion patients after CPB.

Overall, although CPB's proinflammatory role remains significant, surgical trauma and I/R injury of various organs during CPB may be likely the major contributors to the inflammatory response.

2. Coagulation response

Cardiac surgery with CPB activates coagulation system and disturbs hemostasis markedly in many ways. Surcigal trauma, blood contact with artificial surfaces of CPB circuit, CPB related hemodilution, systemic heparinization, hypothermia, and CPB-induced inflammatory response are all possible triggers of coagulopathy, which may lead to excessive bleeding or promote thrombosis (Paparella et al. 2004). Hemostatic disturbances include a decrease in platelet counts and dysfunction (Bevan 1999, Wahba et al. 2000), consumption and dilution of coagulation factors, fibrinolysis, and increased thrombin generation.

2.1. Platelets

CPB induces platelet activation that subsequently leads to significant decrease in platelet counts and functional defects, which contributes to bleeding diathesis after CPB surgery (Bevan 1999, Ray et al. 1994). Heparin, hypothermia, and contact with CPB circuit are considered the major triggers for platelet activation (Bevan 1999, Wahba et al. 2000). In addition, the duration of CPB affects platelet count and function. (Wahba et al 2001). At the site of injury small amount of platelets adhere to exposed collagen and von Willebrand factor (vWF) and subsequently activate to form a platelet monolayer and to express TF (Brass 2003). Thrombin formed locally

at the site of injury further activates platelets. In the extension phase more platelets, expressing surface receptors that can rapidly respond to thrombin, accumulate on the top of initial platelet monolayer to build a growing plug. Plug stabilization occurs by direct interactions of platelets and by fibrin fibrils, which form an extensive meshwork around the aggregated platelets (Brass 2003). Platelet activation results in platelet-platelet aggregation, degranulation of vasoactive substances (adenosine diphosphate, thromboxane, serotonine, epinephrine), and the surface expression of P-selectin thereby promoting platelet-neutrophil aggregation (Boyle et al. 1996). Thus, platelet activation is closely regulated by endothelial cell activation in response to inflammation and injury, and especially the thrombin mediated activation is involved (Boyle et al. 1996, Brass 2003, Mackman 2007).

2.2. Fibrinolysis

The fibrinolytic system is activated during cardiac surgery (Chandler et al. 1995, Chandler and Velan 2003) which may contribute to postoperative bleeding (Ray et al. 1994). It is a complex cascade of serine proteases and their inhibitors which are activated during cardiac surgery and CPB. Plasmin, which is produced when tissue plasminogen activator (t-PA) interacts with circulating plasminogen, controls the runaway reaction of clot acceleration by degrading fibrinogen and fibrin. T-PA levels rapidly increase during CPB followed by postoperatively elevated plasminogen activator inhibitor (PAI-1) and reduced t-PA levels shifting fibrinolytic balance towards procoaguable state (Boyle et al. 1996, Chandler et al. 1995, Valen et al. 1994). However, the fibrinolytic response to CPB is patient specific. Chandler et al. (1995) found that there is at least a 400-fold variability in t-PA release and 50-fold variability in PAI-1 expression, which may explain why some patients have bleeding diathesis and others a propensity to thrombosis. The mechanisms that result in t-PA release in CPB patients are largely unknown, but endothelium activated by the inflammatory response and generation of thrombin have been proposed to be involved (Valen et al. 1994, Boyle et al. 1996).

2.3. Thrombin during cardiac surgery and CPB

2.3.1. Generation of thrombin

The coagulation system has traditionally been divided into the intrinsic and extrinsic pathways, both of which lead to a final common pathway, resulting in thrombin generation and formation of an insoluble fibrin clot. The intrinsic pathway begins when blood comes into contact with an artificial or negatively charged surface resulting in the activation of factor XII to XIIa, and proceeds with the presence of prekallikrein and high-molecular-weight-kininogen (HMWK), which results in the

activation of factor XI (Figure 3). However, while factor XII, prekallikrein, and HMWK are no longer thought to be fundamental to blood coagulation in vivo, the contribution of intrinsic pathway on hemostasis remains an open question (Bevan 1999, Mann 2003a). The current knowledge of the blood coagulation system and thrombin formation has led to the proposal that physiologically relevant coagulation mechanism is primarily composed of three procoagulant enzyme complexes, named the extrinsic tenase complex, intrinsic tenase complex, and prothrombinase complex (Mann 2003a) (Figure 3).

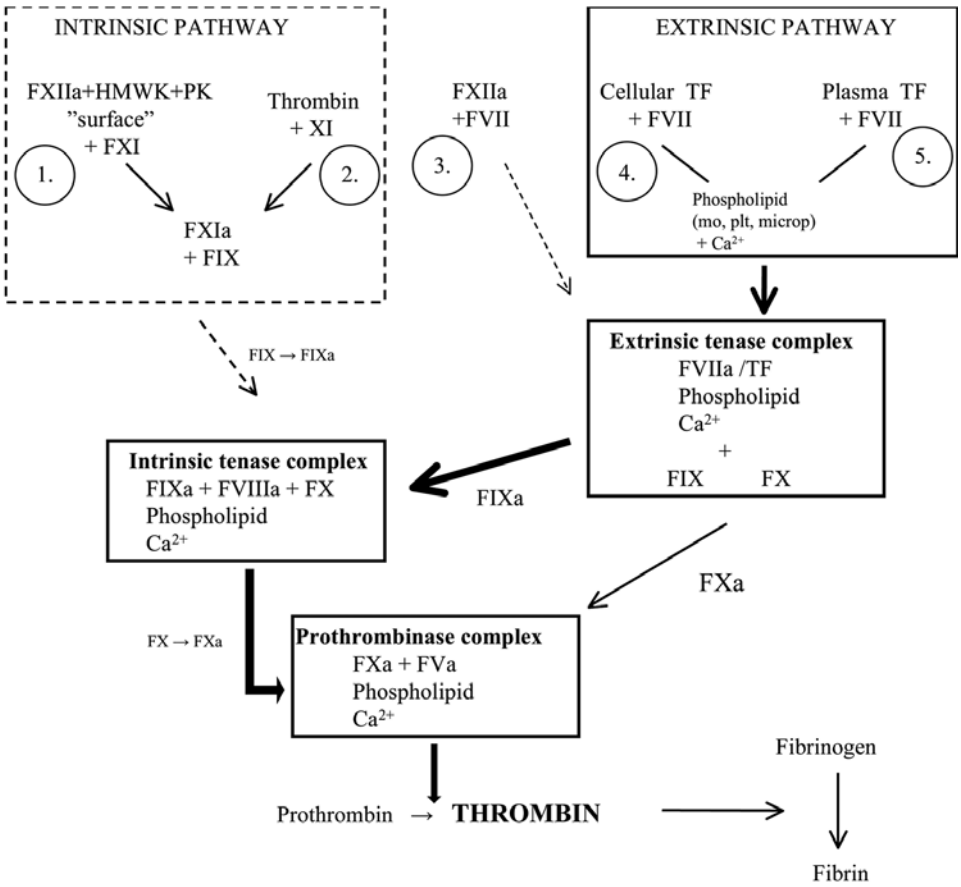

Figure 3. Diagram illustrates the revised pathway of coagulation and the five possible pathways for formation of thrombin during cardiac surgery and CPB. 1. The intrinsic pathway is initiated by activation of factor XII, which with the cofactors high molecular weight kininogen (HMWK) and prekallikrein (PK) activates factor XI. 2. Thrombin, once formed, directly activates factor XI. Factor XIa activates factor FIX, which forms part of the intrinsic tenase complex. 3. Animal studies indicate that factor FXIIa activates factor FVII, but this has not been studied in cardiac patients on CPB. Presence of FXIIa makes this, however, a feasible route of activation of coagulation during

CPB. 4. and 5. In the extrinsic coagulation pathway, both cellular tissue factor (TF) and soluble plasma TF activate factor FVII to form factor FVIIa/TF complex of the extrinsic tenase. Plasma TF requires negatively charged phospholipid surface cofactor (monocyte (mo), platelet (plt), or microparticle (microp)) and the presence of Ca ions. Extrinsic tenase activates both factor IX and X. Factor Xa complexes with factor FVa to form the prothrominase complex, which cleaves prothrombin to thrombin. Factor Xa produced initially by the tissue factor pathway and extrinsic tenase complex activates small amounts of prothrombin to thrombin. Following the initial formation, thrombin propagates its formation by activating FVIII and FV, nonenzymatic cofactors in intrinsic tenase and prothrombinase complex. The intrinsic tenase complex activates further FX and, as a result bypasses the dependence on TF-FVII complex as a source of FXa. Intrinsic tenase complex, aided by activated platelets, is 50 times more efficient in activating factor FX than the extrinsic tenase complex. The preferential in vivo route of thrombin generation is indicated by bold arrows. Modified from Edmunds and Coleman 2006 and Mann 2003b.

The key event initiating the formation of thrombin is binding of factor VII or VIIa and TF. Plasma factor VIIa pre-exists in the blood at approximately 1–2 % of the total factor VII concentration (Nemerson 1988). Cell-bound TF is normally constitutively expressed only in extravascular locations, but can be expressed on endothelial cells, neutrophils, monocytes, and platelets in response to various stimuli, such as inflammatory mediators (Mackman et al. 2007, Steffel et al. 2006). TF can also be detected in the bloodstream, referred to as circulating or blood-borne TF. It is associated with mircoparticles originating from endothelial cells, leukocytes, or platelets (Steffel et al. 2006). Recently, a distinct form of circulating TF has been discovered. It is soluble, exhibits procoagulant activities, and is expressed and released among others from endothelial cells in response to cytokine stimulus (Bogdanov et al. 2003, Szotowski et al. 2005). However, the relative contribution of different forms of TFs in the initiation and propagation of coagulation is unclear (Steffel et al. 2006).

2.3.2. Generation of thrombin during CPB

Progressive generation of thrombin and activity during cardiac surgery with CPB has been demonstrated by measurements of total thrombin generation marker prothrombin fragment F1+2, inhibition of free thrombin by AT (thrombin- antithrombin complexes, TAT), markers thrombin fibrinogen- cleaving activity (FPA), and a marker of specific fibrin breakdown (D-dimer) (Boisclair et al 1993a, Boisclair et al. 1993b, Brister et al. 1993, Eisses et al. 2004, Raivio et al. 2006). Instead of a steady continuous increase in thrombin generation, thrombin is generated in distinctive bursts during surgery and CPB. Surgery before CPB induces a slight increase in markers of thrombin generation (Boisclair et al. 1993a, Eisses et al. 2004) followed by a more pronounced increase in thrombin generation after the initiation of CPB and during CPB (Boisclair et al. 1993a, Chandler and Velan 2003, Eisses et al. 2004, Raivio et

al. 2006). Reperfusion of the ischemic heart results in a distinct burst of thrombin generation (Chandler and Velan 2003, Eisses et al. 2004, Raivio et al. 2006). Chandler and Velan (2003) demonstrated in a computer model of the patients vascular system taking into account marker clearance, hemodilution, blood loss, and transfusions, that CPB and reperfusion of ischemic heart results in distinct bursts of nonhemostatic thrombin generation and dysregulated fibrin formation.

2.3.3. Mechanisms of thrombin generation

There are multiple possible triggers and sites of thrombin generation during cardiac surgery with CPB. Thrombin is normally formed and acts locally at the sites of tissue injury but continuous exposure of blood to the surgical wound and foreign materials of CPB converts a local reaction to systemic, whole body reaction. Historically, CPB induced activation of coagulation was thought to be predominantly due to contact activation of the intrinsic pathway requiring the activation of factor XII (FXII). However, clinical evidence suggests that the intrinsic contact activation pathway plays less of a role than extrinsic TF-pathway in the patients on CPB. For example, a patient with congenital deficiency of FXII still generated thrombin following CPB (Burman at al. 1994). In addition, Boisclair et al. (1993b) could not find an association between factor XIIa levels and thrombin generation during CPB.

Monocytes have been implicated in the thrombin generation during cardiac surgery and CPB. TF expression by the circulating monocytes was found to be induced during prolonged simulated extracorporeal circulation (Kappelmayer et al. 1993). Also, during clinical CPB, increased TF expression of circulating monocytes has been shown (Chung et al. 1996, Shibamiya et al. 2004) but not by all (Barstad et al 1996, Parrat and Hunt 1998). However, both circulating monocytes and monocytes adherent to the CPB circuit showed increased procoagulant activity (Barstad et al 1996, Parrat and Hunt 1996). Interestingly, an increased TF independent direct monocyte surface receptor CD11b mediated factor Xa generation was detected (Parrat and Hunt 1998).

Procoagulant microparticles, derived mainly from platelet but also from erythrocytes and other cells provide a platform of negatively charged phospholipids with TF for thrombin generation during CPB. Nieuwland et al. (1997) demonstrated a significantly elevated concentration of platelet- derived microparticles in systemic circulation during clinical CPB. These microparticles generated thrombin via the TF/FVIIa mediated pathway in vitro (Nieuwland et al.1997). However, it was speculated to what extent the procogulant activity of microparticles was dependent on the negatively charged phospholipids.

In addition to the activating mechanisms, the location of thrombin generation is important. The current evidence indicates that thrombin is primarily generated in the surgical wound by the TF (extrinsic) coagulation pathway during cardiac surgery and CPB. Blood, rich in activated coagulation components, is suctioned from surgical

wound and retransfused into the systemic circulation. Cellular or plasma TF is an essential receptor and cofactor for factor VII in the initiation of thrombin formation. Cell bound TF is present on many cells, but not on the pericardium. Concentrations of soluble plasma TF increases markedly during cardiac surgery with CPB (Edmunds and Coleman 2006, Hattori et al. 2005). Plasma tissue factor requires monocytes, platelets, or microparticles to provide a phospholipid surface for activating factor VII (Edmunds and Coleman 2006).

Thrombin generation in pericardial blood during CABG is profuse with over a 30-fold increase in concentration of F1+2 and up to a 50-fold concentration of TAT in pericardial blood in comparison to systemic blood (Sturk-Maquelin et al. 2003). In the pericardial wound soluble TF concentration is increased several fold compared to the concentration in peripheral plasma (Hattori et al. 2005, Philippou et al. 2000). Pericardial blood contains also procoagulant microparticles derived from platelets and other cellular sources in higher concentration than found in systemic blood (Nieuwland et al. 1997) and microparticle-bound TF obtained from pericardial blood stimulates thrombin generation (Sturk-Maquelin et al. 2003). Wound monocytes alone weakly activate FVII (Hattori et al. 2005). However, it has been shown that activated monocytes with soluble TF in the wound activates coagulation factors VII and X to generate thrombin more efficiently than microparticle TF (Hattori et al. 2005). Furthermore, rapid appearance of TF- bearing monocytes and neutrophils with high procoagulant activity in pericardial blood has been shown (Shibamiya et al. 2004). A significant proportion of these cells formed complexes with platelets (Shibamiya et al. 2004). In vitro experiments demonstrated collagen fibers as stimuli that rapidly induced the appearance of TF on leukocytes. Thus, microparticle derived and monocyte TF act in concert in the surgical wound to promote thrombin generation.

Blood collecting into the surgical field (mediastinum, pericardial and throracic cavities) is routinely suctioned (cardiotomy suction) and returned to CPB circuit, thus contributing to the activation of coagulation in systemic blood. Tabuchi et al. (1993) observed increased systemic levels of TAT, fibrinogen and fibrin degradation products after the suctioned blood was returned to the CPB circuit (Tabuchi et al. 1993). De Haan et al. (1995) concluded that retransfusion of highly activated pericardial blood renewed systemic clotting and fibrinolysis and increased postoperative blood loss. Vice versa, the elimination of cardiotomy suction has been shown to attenuate thrombin generation and activation of coagulation during CPB (Aldea et al. 2002, De Somer et al 2002).

Reperfusion of the ischemic heart results in a distinct burst of thrombin generation (Chandler and Velan 2003, Eisses et al. 2004, Kalweit et al 2005, Raivio et al. 2006). This suggests that myocardial ischemia and reperfusion induces a local formation of thrombin and contributes, at least in part, to the increase in thrombin markers at systemic levels during the reperfusion period. However, other post ischemic organs, such as the lungs and intestine, may be involved as well.

Taken all together, the relative contribution of systemic and local presentation of

TF for thrombin generation and blood coagulation during cardiac surgery and CPB is extremely complex and not fully known.

2.4. Thrombin and its interactions

Thrombin is a multifunctional proteolytic enzyme (serine protease) formed during blood coagulation from its inactive precursor prothrombin. Once formed, thrombin can freely diffuse from the surface it was formed to encounter at least a dozen potential substrates and cofactors. Many of its downstream reactions are largely directed by cofactors that act by localizing thrombin to various substrates, blocking substrate binding to critical exocites, producing new exocites for substrate recognition, and allosterically modulating the properties of the active site of thrombin. Depending on how thrombin activity is directed by cofactors, the net effect is either procoagulant or anticoagulant (Lane et al. 2005).

Thrombin acts as a procoagulant when it facilitates the clotting of blood by catalyzing conversion of fibrinogen to fibrin. This action is fortified by activation of factor XIII that covalently stabilizes the fibrin clot, inhibition of fibrinolysis by activation of thrombin activatable fibrinolysis inhibitor (TAFI), and by amplifying its own generation by activating factors VIII and XI (Di Cera 2007). In addition, thrombin activates platelets and promotes platelet adhesion (Brass 2003).

Thrombin acts as an anticoagulant through activation of protein C that adds negative regulation to the coagulation cascade and is a link to the anti-inflammatory cascade (Esmon 2003). Thrombin activates protein C through binding to thrombomodulin on the endothelial cell surface, concurrently losing its clotting potential. Reaction is enhanced approximately 20- fold when protein C is bound to the endothelial cell protein C receptor (EPCR). Once activated protein C dissociates from EPCR it binds to protein S. This complex then inactivates factors Va and VIII leading to downregulation of thrombin generation (Esmon 2003).

Thrombin is an important link between coagulation and inflammation. The proinflammatory effects of thrombin are mediated through activation of endothelial cells, leukocytes, smooth muscle cells, and platelets, as well as through the release of cellular mediators. Thrombin activates endothelial cells to express several leukocyte adhesion molecules (Esmon 2005). In addition, thrombin activates endothelial cells and monocytes to release various chemokines and cytokines and is a direct activator of P-selectin, which recruits neutrophils to the endothelial surface, initiates neutrophil-endothelial cell interactions and thus promotes inflammation (Esmon 2005). On the other hand, downregulation of endothelial antithrombotic mechanisms, such as thrombomodulin-protein C and fibrinolytic pathways, during inflammation may alter the coagulation/anticoagulation balance in favor of the procoagulant state (Boyle et al. 1996a, Esmon 2005). The cellular effects of thrombin are mainly triggered by cleavage of protease- activated receptors (PARs), members of the G-protein-coupled receptor superfamily (Coughlin 2000, Huntington 2005).

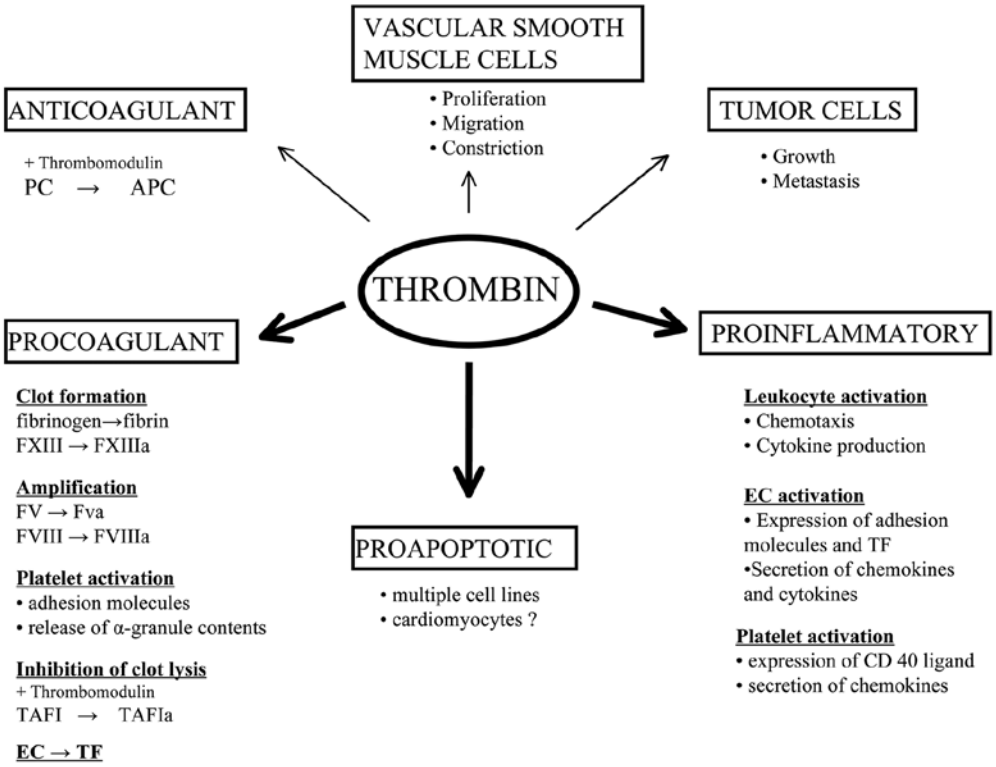

Figure 4. Multiple actions of thrombin. Actions pointed out with bolded arrows are discussed in more detailed in this review, see text. PC = protein C, APC = activated protein C, F = factor, TAFI = thrombin-activatable fibrinolysis inhibitor, TAFIa = activated TAFI, EC = endothelial cell, TF = tissue factor.

Physiologically, the half-time of thrombin in plasma is very short, from seconds to a minute, mostly due to the direct inhibitory effect of a natural plasma protease, antithrombin (AT). The thrombin-antithrombin complex formation is accelerated by 1000-fold by glycosaminoglycans and is irreversible (Lane et al. 2005). Heparin cofactor II (HCII) is another natural serine protease inhibitor inhibiting thrombin but is considered secondary to antithrombin (Huntington 2005).

2.5. The role of heparin

Anticoagulation is used to prevent immediate blood clotting within the CPB-circuit and to minimize excessive CPB related activation of the hemostatic system. Unfractionated heparin is a polysaccharide mixture of low- and high-molecular-weight fractions and catalyses thrombin inhibition by binding to AT. While being an effective inhibitor of systemic thrombin heparin is a poor inhibitor of clot-

bound thrombin (Weitz et al. 1990), which remains active and capable of cleaving fibrinogen (Weitz et al. 1990). A substantially higher heparin concentration is required to effectively inhibit clot-bound thrombin (Weitz et al 1990). The anticoagulant effects of heparin are predominantly mediated by a heparin-antithrombin complex which most effectively inactivates thrombin but also inactivates factors Xa, IXa, XIa, and XIIa (Hirsh and Raschke 2004). Heparin also attenuates the extrinsic pathway of coagulation by the release of tissue factor pathway inhibitor (TFPI), but the TFPI response is heterogenous (Adams et al. 2002). In addition, heparin in high concentrations inihibits thrombin by activating heparin cofactor II (HCII) and independently of AT and HCII-mechanisms, modulates factor Xa generation (Hirsh and Raschke 2004). As AT is required for an effective heparin anticoagulant effect, decreased plasma levels of AT may result in impaired responsiveness to heparin. Plasma AT levels may be decreased in response to preoperative heparin management (Dietrich et al. 1991) or decrease during CPB as a result of hemodilution or consumption (Hashimoto et al. 1994, Ranucci et al. 2004).

The standard dose of heparin administered prior to CPB in the majority of cardiac centers is 300–400 U/kg aiming to activated coagulation time (ACT) over 400 s. However, an ACT value measured either by a kaolin- or celite-method which gives only general information of the blood clotting state, is not correlated with the plasma heparin concentration, and is influenced by hemodilution, hypothermia, and platelet abnormalities (Despotis et al. 1999).

Heparin administered during CPB may significantly affect the postoperative hemostatic status. In a randomized study of patients undergoing CABG with CPB, subgroup analysis based on the plasma heparin concentration indicated that a higher dose of heparin (mean 678 U/kg) compared to 479 U/kg, resulted in better inhibition of thrombin activation and fibrinolysis, higher levels of factors V and III, fibrinogen, and AT, and as a consequence, less postoperative bleeding (Despotis et al. 1996). Similarly, Raivio et al. (2008) confirmed that higher heparin levels were associated with lower levels of thrombin generation and reduced transfusion requirements during CPB. Koster et al. (2002) demonstrated that a higher heparin concentration caused significant reduction of thrombin generation and fibrinolysis without increasing postoperative blood loss. They also suggested that other than heparin-antithrombin mechanism, such as TFPI-pathway, may have been involved. However, in contrast, Boldt et al. (1995) found an increased blood loss in a high-heparin (600 U/kg) group compared to a low-heparin (300 U/kg) group. Thus, the optimal heparin dosage during CPB remains debatable.

Heparin exhibits anti-inflammatory properties. An increased concentration of heparin administered during CPB was associated with a significant reduction in the concentrations of neutrophil elastase and a trend toward lower concentrations of a soluble adhesion molecule P-selectin and complement C5b-9, which indicates that heparin attenuates neutrophil activation and the inflammatory response (Koster et al. 2002). Experimental studies have demonstrated that heparin has a cardioprotective

effect in myocardial I/R injury. Heparin and N- acetyl heparin, a derivative of heparin sulfate without anticoagulant effects, reduced complement activation induced myocardial injury in a rabbit isolated heart model (Friedrichs et al. 1994) and myocardial infarct size in coronary occlusion model in dogs (Black et al. 1995). These effects were suggested to be independent of the anticoagulation effects of heparin as N- acetyl heparin had similar effects even though it lacks anticoagulant activity. The cardioprotective mechanism may involve the ability of heparins to inhibit complement activation in response to tissue I/R injury (Black et al. 1995, Friedrichs et al. 1994).

3. Apoptosis

Apoptosis, a mechanism of programmed cell death, is a highly regulated, genetically determined and energy requiring process that is active both in physiological and pathophysiological conditions. Apoptosis allows the organ or tissue dispose cells which are dysfunctional or no longer needed. Ischemic necrosis and apoptosis are two distinct mechanisms of cell death often coexisting in I/R injury. Ischemic necrosis is characterized by adenosine triphosphate depletion, cell swelling, and loss of cell membrane integrity, thereby initiating the inflammatory reaction. Apoptosis, in contrast, is characterized by cell shrinkage, membrane blebbing, nuclear condensation and DNA fragmentation, without loss of membrane integrity. The cell is eventually broken into small membrane-enclosed pieces (apoptotic bodies) which are phagocytoced into neighboring cells, including macrophages and parenchymal cells. This prevents the release of cellular compounds, sparing the adjacent tissue from inflammation.

3.1. Mechanisms of apoptosis

Generally, apoptosis proceeds in two separate phases: the decision/initiation phase and the execution phase. In the initiation phase proapoptotic signals trigger activation of the molecular machinery of apoptosis and interact with the intracellular antiapoptotic proteins. Only if the balance favors apoptosis does the execution phase take place and the molecular execution machinery becomes fully activated.

Three distinct cellular pathways may lead to apoptosis. First, apoptosis can be initiated by stimulation of the membrane-bound death receptors of the tumor necrosis receptor family (TNF-R), such as Fas (CD95), TNF-R1, or death receptors (DR) 3-6 (extrinsic pathway). The second main pathway of apoptosis in myocytes is stress-induced activation of the specific intracellular proteins (intrinsic pathway), especially the Bcl-2 family consisting of both proapoptotic and antiapoptotic members. The antiapoptotic Bcl-2 family members stabilize the mitochondrial

membrane, while proapoptotic members permeabilize it and induce the release of mitochondrial mediators of apoptosis such as cytochrome C. Both main pathways lead first to the activation of upstream cysteine proteases (caspases) cascade, and eventually to the activation of downstream/terminal caspases. Terminal effector caspases, such as capase 3, are responsible for cleavage of intracellular substrates required for cellular survival, architecture, and metabolic function leading to DNA fragmentation and apoptotic cell death. A third pathway, which is caspase independent, is stress-induced release of apoptosis-inducing factor (AIF) from mitochondria (Valen 2003).

3.2. The time course of apoptotic process

The in vivo time course of apoptosis is relevant for the current study setting but remains poorly known. The estimated duration of the apoptotic process from first stimulus to fragmentation of DNA is from 12 to 24 hours but cellular morphological changes are visible in less than 2 hours. In an in vitro model of cardiomyocyte apoptosis, DNA fragmentation was completed 14 hours after stimulation (Suzuki et al. 2001). The earliest signs of apoptosis were detected at 2 hours after the apoptotic stimuli but the activation of caspase 3 was not significantly increased until 4 hours after the stimuli (Suzuki et al. 2001). In the porcine model of cardiac surgery with CPB, cardiomyocytes containing active caspase 3 and also apoptotic cardiomyocytes were detected at 2 hours after cardioplegic ischemia. (Malmberg et al. 2006). In an in vivo coronary ligation model in dogs progressive cardiomyocyte apoptosis was still seen at 72 h after reperfusion. (Zhao et al. 2001).

3.3. Detection of apoptosis

Many of the characteristic biochemical events are useful in detecting apoptotic cells. The presence of ongoing apoptotic processes can be studied by demonstrating the activation of downstream caspases, such as caspase 3, by western blotting of target proteins or by demonstrating caspase activity by enzyme assay (Saraste and Pulkki 2000). The current method of choice for quantification of apoptotic cardiomyocytes is the TUNEL-assay (in situ terminal deoxynucleotidyl transferase-labeled dUTP nick end labeling (Saraste and Pulkki, 2000). The disadvantage with the TUNEL-assay is that it may overestimate apoptotic nuclei, as non-apoptotic viable cells undergoing DNA repair (Ansari et al. 1993, Kanoh M et al.1999), active gene transcription (Kockx et al. 1998), as well as necrotic cells are labelled (Ohno et al. 1998). The most reliable evidence of apoptosis is based on the analysis of the morphological features. Nuclear condensation, shrinkage of the cell and fragmentation into apoptotic bodies can be visualised using light microscopy. Electron microscopy is required for demonstrating the loss of intracellular structures.

3.4. Apoptosis and thrombin

Thrombin has been shown to induce apoptosis in several cultured cell lines including myocytes (Ahmad et al. 2000, Choi et al. 2003, Donovan et al. 1997, Turgeon et al. 1998). Thrombin acts via activation of three (PAR-1,-3, -4) of the four cell surface protease-activated receptors (PARs) (Coughlin 2000). PAR-1 has been shown to mediate thrombin induced apoptosis in cultured motoneurons, astrocytes, and tumorigenic cell lines (Ahmad et al. 2000, Donovan et al. 1997, Turgeon et al. 1998). The effects of thrombin or activation of PAR-1 on motoneurons were completely prevented by cotreatment of the cultures with hirudin or caspase inhibitors (Turgeon et al. 1998) However, thrombin was shown to induce apoptosis in dopaminergic neurons independently of PAR-1 (Choi et al. 2003). Also in that setting apoptosis was effectively inhibited by hirudin. The cardiovascular signaling properties of PAR-1 have been studied in platelets and the vessel wall. One recent study indicated that PAR-1 may mediate cellular effects of thrombin also in the myocardium (Strande et al. 2007). However, in myocardium, also other receptors may contribute as cultured cardiomyocytes co-express PAR-1, PAR-2, and PAR-4 (Sabri et al. 2000, Sabri et al. 2003). On the other hand, thrombin has shown to increase acute cell death in cultured cardiomyocytes subjected to I/R by a mechanism that involves activation of PAR-1 (Mirabet et al. 2005). Thus, due to the lack of definite studies on thrombin and myocardial apoptosis during I/R, it can only be suggested that thrombin may induce either acute cell death or apoptosis depending on the complex cellular and molecular environment of myocardial I/R injury.

4. Ischemia-reperfusion injury, general considerations

4.1. Endothelium

Endothelium seems to be a stage for the initial and crucial events in ischemia reperfusion injury. The endothelial dysfunction appears to be manifested in a site-specific manner in the microvaculature. Ischemia is known to alter endothelial cell membrane function and cell morphology accompanied by depletion of energy stores and a diminished production of some bioactive agents (prostacyclin and NO) while the production of others is increased (endothelin, thromboxane A_2) (Carden and Granger 2000). Likewise, ischemia promotes expression of certain proinflammatory gene products (leukocyte adhesion molecules, cytokines) while it suppreses others (cNOS, thrombomodulin) (Carden and Granger 2000). Many of these endothelial cell responses to ischemia are exacerbated by reperfusion resulting in endothelial dysfunction (Carden and Granger 2000).

Reperfusion of ischemic tissues results in a profound increase in the production of reactive oxygen species (ROS) within minutes by endothelial xantine oxidase and a corresponding decline in the synthesis of nitric oxide (NO) by endothelial nitric oxide

synthase (NOS). The imbalance between the production of ROS and NO manifests as impaired endothelium-dependent vasodilatation in arterioles and in capillaries it manifests as increased fluid infiltration and leukocyte capillary plugging. In addition, increased production of endothelin-1 increases vasoconstriction and reduces blood flow (Carteaux et al. 1999). ROS can rapidly initiate the inflammatory reaction especially in the venules by eliciting the production of platelet activating factor, promoting the complement activation, and mobilizing the stored pool of P-selectin on the endothelial cell surface (Carteaux et al. 1999).

Furthermore, the inflammatory reaction can activate endothelium to express TF, thus potentially promoting microvascular thrombosis in the reperfused tissues (Boyle et al. 1996).

4.2. Complement and cytokines

Reperfusion of ischemic tissue results in complement activation and the formation of several proinflammatory mediators (Collard et al. 1999). Biologically active complement components include anaphylatoxins C3a and C5a and components iC3b, and C5b-9. Particularly important is C5a that stimulates leukocyte activation and chemotaxis and further amplifies the inflammatory response by inducing TNFα, IL- 1, and IL- 6 production (Collard et al. 1999). C5b-9 may activate endothelial cells to increase leukocyte adhesion molecule expression and also promote leukocyte activation and chemotaxis by inducing endothelial IL- 8 and monocyte chemoatractant protein 1 secretion (Collard et al. 1999). Finally, C5b-9 may alter vascular tone by inhibiting endothelial- dependent relaxation (Collard et al. 1999). Thus, the complement system may compromise blood flow to an ischemic organ by altering vascular homeostasis and increasing leukocyte-endothelial adherence.

Cytokines released by activated leukocytes and endothelium play a pivotal role in I/R injury as they can further activate endothelium and platelets, and attract and activate leukocytes. Thus, cytokines are likely to act both individually and in a complex meshwork of signal interactions between the cells (Wan and Yim 1999).

4.3. The role of neutrophils

Neutrophil-endothelial interaction is a key process leading to widespread endothelial and tissue damage. ROS, cytokines, thrombin and other inflammatory mediators first activate both the neutrophils and vascular endothelium. Activation of these cells promotes the expression of adhesion molecules on both the neutrophils and endothelium, which recruits neutrophils to the surface of endothelium and initiates a specific multi-step cascade of rolling, adhesion, and transmigration of neutrophils into the tissue. Here they release cytotoxic proteases, such as collagenase, elastase and myeloperoxidase, and ROS causing damage to the vascular endothelium and surrounding tissues (Figure 5.) (Boyle et al. 1998, Jordan et al. 1999, Park and Lucchesi 1999).

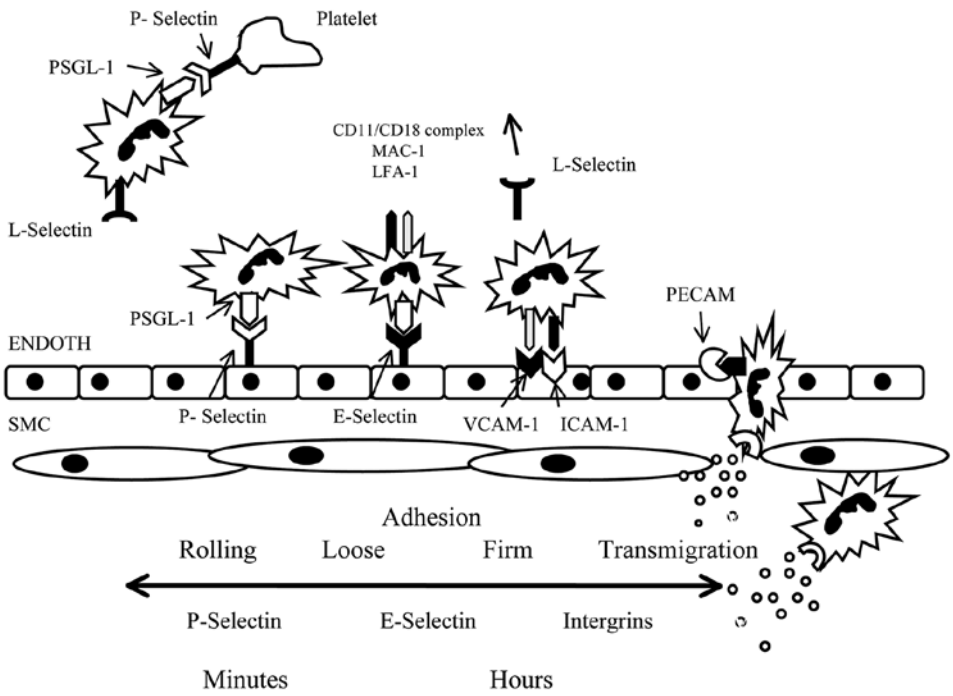

Figure 5. Illustration of the interactions between neutrophils and endothelium. Rolling and loose adhesion is mediated by selectins (P-Selectin, E-Selectin). P-selectin is constitutively synthesized and stored in the Weibel-Palade bodies of the endothelial cells from which it is mobilized rapidly to the endothelial cells in response to inflammatory stimuli. P-Selectin is expressed also by activated platelets, which contributes to neutrophil-platelet conjugate formation by binding P-Selectin glycoprotein-1 (PSGL-1). E-Selectin becomes available only after lag time required for gene transcription, protein synthesis and expression on the endothelial cell. Platelet activation factor (PAF) together with P-selectin stimulate neutrophils to shed L-selectin and upregulate CD11/CD18 complex (β-integrin), which initiates the transition state of the neutrophil from rolling to firm adhesion. Firm adhesion and transmigration is mediated by integrins (CD11/CD18, ICAM-1, VCAM, PCAM). ENDOTH = endothelium, VSM = vascular smooth cells, ICAM-1 = intercellular adhesion molecule-1, VCAM-1 = vascular adhesion molecule-1, PECAM = platelet/endothelial cell adhesion molecule. See reviews by Boyle et al. 1998, Day and Taylor 2005, Jordan et al. 1999, Park and Lucchesi 1999.

Myeloperoxidase (MPO), belonging to the family of peroxidases, is abundantly present in neutrophils and to lesser degree in monocytes and tissue macrophages (Lau and Baldhus 2006). During neutrophil activation and degranulation, MPO is released into phagocytic vacuoles and the extracellular space. The complete MPO-system consists of the enzyme MPO, hydrogen peroxide (H_2O_2), and oxidizable cofactors. MPO acts as a catalytically active protein for many substrates generating

reactive oxidants, such as hypochlorous acid (HOCl) and tyrosyl radicals, causing vascular dysfunction and tissue damage (Lau and Baldhus 2006). During I/R injury, measurement of MPO activity has been used as a marker for neutrophil accumulation in various tissues, including the heart and intestine (Fernandez et al. 2006, Grisham et al. 1986, Hayward and Lefer 1998, Oktar et al. 2002, Sun et al. 1999, Wilson et al. 1993, Özden et al. 1999).

5. Myocardial ischemia-reperfusion injury

Postoperative myocardial dysfunction is a result of the deleterious effects initiated by a period of global ischemia and exacerbated by reperfusion of oxygenated blood. Ischemia and reperfusion may cause an irreversible cell necrosis or, if less severe, may result in a reversible depression of myocardial function due to disturbances of myocyte physiology.

Sudden cessation of blood flow to the myocardium causes decreased oxygen and metabolic substrates supply, as well as accumulation of metabolic by- products. Aerobic metabolism is turned to anaerobic metabolism within seconds, which results in rapid failure to resynthesize energy rich phosphates, including adenosine 5'- triphosphate (ATP) and phosphocreatine, and intracellular accumulation of protons, lactate and inorganic phosphates. This leads to membrane ATP- dependent ionic pump dysfunction, favoring the entry of Ca^{2+}, sodium, and water into the cell. Low levels of intracellular ATP, Ca^{2+} overload, and acidosis act to inhibit myocyte function resulting in decreased myocardial contraction without cell ultrastructural changes. Reperfusion at this point results in restoration of normal myocyte function. With continued ischemia, ATP levels fall further as ATP is degraded to ADP, AMP, inosine, and finally hypoxanthine. If ischemia is allowed to continue beyond 30–40 minutes, total adenosine nucleotide pool becomes depleted and metabolic and morphological changes begin to occur rapidly. At this point, cellular death may occur irrespective of reperfusion conditions. Reperfusion of ischemic myocardium results in cellular injury characterized by cell swelling, intracellular and mitochondrial calcium accumulation, an impaired ability to utilize oxygen, disruption of cellular enzyme activity, and loss of normal cell membrane function.

However, myocardial injury in clinical cardiac surgery and in experimental settings with global cardiac arrest is significantly modified by the protective effects of hypothermia and cardioplegic solutions. Thus, the relative roles of the different inflammatory mediators, adhesion molecules, and cellular responses observed in the experimental studies of regional ischemia reperfusion injury, have remained unclear in the heart suffering I/R injury after cardioplegic arrest.

5.1. Myocardial protection

I/R injury is associated with myocardial dysfunction or myonecrosis, which results from sudden cessation of coronary blood flow to the extent that oxygen delivery to the myocardium is insufficient to meet basal myocardial requirements to preserve cellular membrane stability and myocyte viability. Basic principles of myocardial protection include: rapid cardiac arrest, since oxidative metabolism is rapidly lost and anaerobic metabolism is inadequate to supply energy stores, hypothermia to decrease myocardial oxygen consumption and prevent the depletion of high energy phosphates, and avoidance of myocardial edema related to cardioplegic infusion (Levitsky 2006). The question, whether it is necessary to add metabolic substrates into the cardioplegic solution, remain unclear.

Since the concept of "elective cardiac arrest" was introduced 1955, a variety of cardioprotection strategies have evolved. The cold crystalloid cardioplegia delivered intermittently via the antegrade route through aortic root cannulation is commonly considered as the golden standard in clinical cardiac surgery and has been used successfully in many experimental studies (Fischer et al. 2003, Freude et al.2000, Eising et al. 2000, Malmberg et al. 2006, Schreiber et al. 2006, Vähäsilta et al. 2005). Although cardioplegia is generally accepted to be mandatory for appropriate cardioprotection during cardiac surgery with CPB, there is still controversy concerning different aspects of cardioplegia composition (crystalloid or blood), temperature (cold, tepid, or warm), and the mode of delivery (intermittent or continuous, antegrade or retrograde). Blood is thought to offer benefits over crystalloid cardioplegia as it provides closer characteristics of normal physiology including in part its oxygen carrying capacity, buffering capacity, and less associated hemodilution. Indeed, there are experimental studies and clinical prospective and randomized studies indicating the benefit of blood cardioplegia when investigating release of cardiac enzymes, metabolic response, and other laboratory test results (Barner 1991, Cohen et al. 1999, Guru et al. 2006). However, evidence demonstrating no difference between cold blood and cold crystalloid also exists (Övrum et al. 2004).

5.2. Potential mechanisms of myocardial dysfunction

The pathophysiology of myocardial I/R injury during cardiac surgery is complex and still not fully understood. The post-ischemic myocardium may function normally or become dysfunctional by several mechanisms including Ca^{2+}- overload, ROS, inflammatory reaction, no-reflow phenomena, and apoptosis. These mechanisms are discussed separately.

5.2.1. Calcium and myocardial contractile dysfunction

Alterations in the availability or homeostasis of Ca^{2+} or sensitivity of contractile apparatus to Ca^{2+} are candidates for mechanism of myocardial dysfunction. "Calcium

paradox" suggests that cellular dysfunction during myocardial I/R causes increased intracellular calcium concentration possibly due to extracellular calcium influx or as a result of dysfunctional sarcoplasmic retinuculum to adequately sequester and store intracellular ionized calcium (Yellon and Hausenloy 2007). Intracellular Ca^{2+} overload results in myocardial excitation-contraction uncoupling at the cellular level, which may lead to myocardial dysfunction at functional level.

5.2.2. The oxygen paradox and reactive oxygen species

"The oxygen paradox" is based on the premise that oxygen, although essential for cell survival, can be injurious during reperfusion of the previously ischemic myocardium. Upon reperfusion, molecular oxygen undergoes sequential reduction to form ROS. ROS are unstable and highly reactive molecules. Endothelial cells, myocytes, and neutrophils are indentified as an important source of ROS (Zweier and Talukder 2006). ROS can directly damage cellular membranes and proteins if the level of oxidative stress exceeds the capacity of the endogenous free radical scavengers (Kevin et al. 2005, Zweier and Talukder 2006).

5.2.3. Local inflammatory response

I/R-induced acute inflammatory response can damage post-ischemic myocardium. Most of the evidence between local inflammation and myocardial I/R injury comes from the studies in regional ischemia. However, there is evidence that a local inflammatory reaction is also induced in the myocardium subjected to cardioplegic ischemia and reperfusion. For example, transcardiac veno-arterial increase in proinflammatory cytokine IL-6 levels and trapping of activated neutrophils and leukocyte-platelet aggregates have been shown during initial reperfusion after cardioplegic arrest (Zahler et al. 1999). Furthermore, gene expression of leukocyte adhesion molecules (E-selectin, ICAM-1), proinflammatory cytokines (IL-I, IL-2, TNF-α), and vasoactive mediators (eNOS, endothelin-1) in the human heart during cardiac surgery have been demonstrated (Valen et al. 2001).

5.2.3.1. The role of neutrophils

The neutrophil has a central role in the local inflammatory reaction contributing to myocardial I/R injury (Jordan et al. 1999). Early histological studies showed a direct correlation between the duration of ischemia and infarct size with the extent of neutrophil accumulation within myocardial tissue (Romson et al. 1983). Since then, results from many experimental and clinical studies in regional ischemia and reperfusion models suggest that neutrophils contribute to the myocardial reperfusion injury (Lefer 2002). Also several experimental studies in the models of CPB suggest that neutrophils have a role in myocardial I/R injury. In a pig model of CPB neutrophils

accumulated into the myocardium in parallel with the impairment of hemodynamic function during reperfusion (Brix-Christensen et al. 2002). Perfusion with activated neutrophils before hypothermic global ischemia significantly attenuated functional recovery of rabbit hearts in the experimental Langendorff- model (Myers et al. 1992). In the experimental pig model of CPB and cardioplegic arrest, inhibition of neutrophil surface adhesion receptor CD11/CD18 upregulation attenuated the accumulation of neutrophils within the myocardium, which was associated with the decreased biochemical and functional evidence of I/R injury (Wilson et al. 1993).

In a clinical study, leukocyte depletion of blood cardioplegia with filters attenuated myocardial injury and improved early myocardial function (Roth et al. 2000). However, the benefits of leukocyte depletion with different filters have been inconsistent (Lim et al. 2007).

Although endothelial proinflammatory changes initiate during ischemia, it is not until reperfusion that neutrophil-mediated damage take place. Once neutrophils make contact with the endothelium, they become further activated in a multistep process of rolling, adhesion, and transmigration into the extracellular space (Figure 5.) and consequently release ROS, proteases, myeloperoxidase, and other inflammatory products that amplify the recruitment and activation of greater numbers of neutrophils into the myocardium (Jordan et al. 1999, Boyle et al. 1998, Park and Lucchesi 1999). Cytotoxic ROS react and damage endothelial cells and myocyte membrane lipids and nucleic acids, resulting in cellular dysfunction, edema, and cell death. ROS can also cause damage by reacting with polyunsaturated fatty acids, resulting in formation of lipid peroxidases and hydroperoxidases, which damage the sarcolemma and impair the function of membrane-bound enzyme systems (Zweier and Talukder 2006). Proteases and MPO may destroy the supporting extracelluar matrix, break down the barrier function of the endothelium, leading to swelling and impaired cardiomyocyte function (Boyle et al. 1998, Jordan et al. 1999).

5.2.4. The no-reflow phenomenon

The no-reflow phenomena is defined as the inability to fully reperfuse previously ischemic myocardium after the blood flow has been restored to the arteries supplying the tissue. There are several possible mechanisms involved. I/R-induced endothelial activation promotes neutrophil- endothelial cell adhesion and capillary plugging, which together with formation of ROS and neutrophil degranulation products, leads to impaired endothelial-dependent vasorelaxation, endothelial cell and interstitial edema, and, finally, capillary obstruction (Jordan et al 1999, Sellke et al. 1993, Zweier and Talukder 2006). Neutrophil-platelet aggregates may also contribute to mechanical obstruction. Zahler et al. (1999) demonstrated intracoronary neutrophil and neutrophil-platelet sequestration during early reperfusion after global cardiac arrest. Activation of intracoronary coagulation and microthrombosis has been shown in a pig I/R model (Mrak 1990). Inhibition of the extrinsic coagulation

pathway resulted in a significant reduction of platelet and fibrin deposition within the myocardium, which was associated with decreased no-reflow area (Golino et al. 2000). Furthermore, global cardioplegic arrest has been shown to induce a significant myocardial production of endothelin, a highly potent vasoconstrictor, which was associated with myocardial dysfunction (Dorman et al. 2000).

5.2.5. Thrombin in myocardial I/R injury

Local coagulation response is induced in myocardial ischemia-reperfusion injury during CPB. Kalweit et al (2005) measured increased transcoronary gradient of activated coagulation factor XII and thrombin generation marker F1+2 during global myocardial ischemia in humans. Petäjä et al. (1999) demonstrated increased thrombin activity (FPA) and a significant activation of antithrombotic protein C pathway in the ischemic coronary beds early after global myocardial ischemia in humans. Furthermore, thrombin was present in myocardium subjected to I/R (Strande et al. 2007).

The TF-FVII mediated pathway to thrombin is involved in myocardial I/R injury. Isolated rabbit hearts subjected to global ischemia and reperfusion showed a marked TF activity when exposed to oxygen free radicals (Golino et al. 1996). In *in vivo* experiments, rabbit hearts subjected to regional myocardial ischemia showed a marked increase in TF activity, which was not detected in animals receiving either a monoclonal antibody against rabbit TF or the oxygen free radical scavenger, superoxide dismutase (Golino et al. 1996). Furthermore, in a rabbit model of regional myocardial ischemia and reperfusion, blockade of the TF-mediated activation of the extrinsic coagulation pathway by human recombinant site-blocked activated factor VII significantly reduced infarct size, the size of the no-reflow area, and reduced platelet and fibrin accumulation in the coronary circulation of ischemic but viable myocardium (Golino et al. 2000). However, the predominant site of TF expression in myocardial I/R injury is controversial. The argument mainly involves whether the expression of TF by cardiac myocytes (Drake et al. 1989) or the inducible expression of TF by vascular endothelium is involved. Golino et al. (1996) demonstrated *in vitro* an increased TF messenger RNA expression and TF-activity in endothelial cells in culture when exposed to exogenously generated oxygen free radicals. In a rabbit in situ coronary ligation model TF expression was up-regulated in cardiomyocytes but not in endothelial cells in the ischemic but viable areas of myocardium and administration of an inhibitory anti-TF antibody either before or after ischemia significantly reduced infarct size after I/R injury (Erlich et al 2000). Structural and functional disruption of the endothelium was observed, which would permit plasma clotting factors to gain access to TF expressed by extravascular myocytes, thereby allowing the formation of thrombin in extravascular site (Erlich et al. 2000). Indeed, instead of no intravascular fibrin deposition or microvascular thrombosis, a low level of extravascular fibrin deposition was observed (Erlich et al. 2000). In the

same study, admistration of hirudin, a direct inhibitor of thrombin, significantly reduced myocardial infarct size but the treatment with ancrod, a defibrinogenating agent, had no effect on infarct size, which suggest that the TF-thrombin pathway contributes to myocardial I/R injury by other mechanisms than simply initiating fibrin deposition (Erlich et al 2000). Instead, they proposed that cardiomyocyte TF intiates extravascular thrombin generation, which has a proinflammatory role in myocardial I/R injury by increasing chemokine expression and enhancing leukocyte recruitment (Erlich et al. 2000).

Thrombin has direct effects on cardiomyocytes as it has been shown to depress myocyte contractile function (Hird et al. 1995) and to increase acute cell death in dose-dependent manner, when cultured cardiomyocytes were subjected to simulated ischemia and reperfusion (Mirabet et al. 2005). Thrombin enhanced the cytosolic Ca^{2+} overload by a mechanism that involves thrombin PAR-1 receptors (Mirabet et al. 2005). In addition, an inhibitor of the PAR-1 receptor reduced myocardial infarct size in rat models of myocardial I/R injury (Strande et al. 2007).

However, whether thrombin mediates its deleterious effects in myocardial I/R injury via thrombosis, inflammation or other molecular mechanisms, such as apoptosis, is not fully clear.

5.2.6. Apoptosis and myocardial I/R injury

Apoptosis has been observed in the animal models of regional myocardial I/R injury (Fliss and Gattinger 1996, Gottlieb et al. 1994) and in the border zones of histologically infracted human myocardium (Saraste et al. 1997). Cardiomyocyte apoptosis has been shown to be present in the myocardium of animals and humans also during reperfusion after CPB and cardioplegic arrest (Fischer et al. 2003, Freude et al.2000, Malmberg et al. 2006, Schmitt et al. 2002, Vähäsilta et al. 2005, Yeh et al. 2003). In the model of porcine open heart surgery, apoptotic myocytes were significantly increased after 30 min of cardioplegic ischemia and 90 min of reperfusion, when detected with TUNEL method and caspase 3 (Vähäsilta et al. 2005). Malmberg et al. (2006) demonstrated with a similar porcine model that 90 min ischemia results in a higher amount of TUNEL positive cardiomyocytes and cardiomyocytes containing active caspase 3 than 60 min ischemia and 120 min of reperfusion.

Apoptosis seems to be initiated during myocardial ischemia and completed during reperfusion (Fischer et al. 2003, Freude et al.2000). The exact stimuli and mechanisms inducing cardiac apoptosis during CPB and cardiac arrest are not completely known. However, mechanical stress, hypoxia, ROS, calcium overload, nitric oxide especially through inducible nitric oxide synthase with peroxynitrate formation, or humoral factors, such as TNFα, and angiotensin II are thought to be most important stimuli involved (Anselmi et al. 2004, Fisher et al. 2003, Valen 2003, von Harsdorf et al. 1999).

5.2.6.1. Apoptosis and myocardial dysfunction

Apoptosis may be involved in myocardial dysfunction after cardiac arrest (Anselmi et al. 2004). In a rabbit model of 60 min myocardial ischemia and 30 min reperfusion, intermitted cold blood cardioplegia was superior to cold crystalloid cardioplegia in preventing apoptosis, which was associated with preserved left ventricular function (Feng et al. 2005). Schmitt et al. (2002) studied 11 patients undergoing elective CABG. The heart was arrested with cold crystalloid cardioplegia. Electron microscopy revealed no signs of terminal apoptotic process, probably due to the short interval between induction of ischemia and tissue sampling, but some nuclei displaying early apoptotic changes and the release of cytochrome c, which is an early event in the apoptotic cascade, showed a significant increase. This increase correlated positively with the time of the cardioplegic arrest and reperfusion, positively with pulmonary capillary wedge pressure, and negatively with cardiac index, thus showing a correlation between apoptosis and myocardial dysfunction (Schmitt et al. 2002).

Even a small number of myocytes affected by apoptosis may have a significant impact on cardiac contractility, because single cell death may impinge upon the force generating ability of neighbouring cells depressing more severely overall muscle performance (Cheng et al. 1995).

5.3. Myocardial dysfunction and post-CPB hemodynamics

Clinical myocardial dysfunction in varying degrees occurs in nearly all patients undergoing cardiac surgery with cardiac arrest and CPB (Cohen et al. 1999, Guru et al. 2006, Kloner et al. 1994). Several studies have analyzed myocardial function in the hours after surgery and CPB but only few have focused on myocardial function immediately after separation from CPB (De Hert et al. 1996, Gorcsan et al. 1994, Mavi et al. 2005). Myocardial function was depressed during the immediate post CPB period but recovered to pre- CPB levels during the next 5 to 30 minutes (DeHert et al. 1996, Mavi et al. 2005). In the study of Weng et al. (2003) postoperatively decreased myocardial function reached a nadir 1 hour after CPB but recovered to pre- CPB level during the next postoperative hours.

In contrast, experimental studies show significantly decreased myocardial function and deteriorating hemodynamics after cardiac arrest and CPB without recovery to the preoperative situation. In the porcine model of 90 min cardioplegic arrest and 120 min CPB, CPB was followed by significantly reduced mean aortic pressure, left ventricular function, and cardiac output (CO) and increased heart rate without subsequent recovery during the seven hour follow-up (Eising et al. 2000). Schreiber et al. (2006) observed a significantly decreased left ventricular contractility, ejection fraction (EF), stroke volume (SV), and CO at 60 min and 120 min after one hour cardioplegic arrest and CPB in pigs. Similarly, Vähäsilta et al (2001) observed 44 % loss of CO at 60 min and 120 min after 30 min of cold crystalloid cardioplegic arrest CPB in pigs. In addition to significantly decreased CO, Mayers et al. (1996) observed

increased systemic and pulmonary vascular resistance (SVR and PVR, respectively) four hours after 80 min cardioplegic arrest and CPB in dogs.

CPB alone has been demonstrated to affect postoperative myocardial function and hemodynamics in experimental settings. Aybek et al. (2003) showed significant depression of left ventricular contractility, cardiac index (CI), and EF and significantly increased SVR and PVR 15 min after weaning from one hour CPB in piglets. Similar myocardial dysfunction and hemodynamic changes were detected during 120 min follow-up after 90 min CPB in pigs (Carteaux et al. 1999).

6. Lung injury after CPB

Pulmonary dysfunction after cardiac surgery and CPB is a significant and common problem ranging from subclinical functional changes in most cases to full-blown acute respiratory distress syndrome (ARDS). "The postpump lung syndrome" is a well known phenomenon and is characterized by increased pulmonary permeability, lung edema, and hypoxemia, increased alveolo-arterial oxygen gradient, intrapulmonary shunt, decreased pulmonary compliance, and increased pulmonary vascular resistance (Ng et al. 2002, Riedel 1999). The pathophysiology of CPB-induced lung injury is complex and poorly understood but two important mechanisms, systemic inflammatory response and local lung I/R injury, are likely to be responsible (Asimakopoulus et al. 1999, Ng et al. 2002, Ng et al. 2006, Riedel 1999).

6.1. Local inflammatory response

CPB-induced lung injury associates with the local activation of an inflammatory response (Asimakopoulus et al. 1999, Giomarelli et al. 2003, Massoudy et al. 2001, Ng et al. 2002). Massoudy et al. (2001) studied the role of the lungs themselves in the release of the proinflammatory cytokines and showed a significant increase in the levels of IL-6 and IL-8, on the pulmonary venous side in patients undergoing CABG with CPB. They also showed a decrease in the counts of activated platelet-leukocyte coaggregates and activated neutrophils across the lung reflecting sequestration of these cells to the pulmonary vascular endothelium. An experimental study with piglets placed on CPB demonstrated that neutrophil adhesion blockade reduced lung injury, manifested by less sequestration of neutrophils in the lung and almost no increase in pulmonary vascular resistance (Gillinov et al. 1994).

Histological changes indicating I/R injury after CPB include alveolar edema, alveolar capillary interstitial edema, detachment and swelling of endothelial cells, detachment of pneumocytes, extravasation of erythrocytes, alveolar erythrocytes, and infiltration of inflammatory cells (Carney et al. 1999, Ege et al. 2004, Ng et al.2002, Ng et al.2006)

6.2. Low flow I/R injury

The blood supply and oxygenation of lung tissue is provided by pulmonary artery and bronchial artery with extensive capillary connections, and by alveolar ventilation. Their relative contributions for the delivery of oxygen to maintain lung tissue viability during CPB remains unclear. When decompressing the heart with venous cannulae placed in the right atrium, only a little blood reaches the lungs via the pulmonary artery. In the experimental study with neonatal piglets undergoing 90 min of CPB, exposure to CPB alone caused pulmonary injury but cessation of pulmonary artery flow further contributed to the pulmonary dysfunction (Chai et al. 1999). Ege et al. (2004) studied the importance of pulmonary artery perfusion during cardiac surgery in humans and found that cross-clamping of both pulmonary artery and aorta resulted in more severe lung injury in terms of longer leukocyte sequestration, histological injury, and increased alveolo-arterial oxygen gradient when compared to aortic occlusion alone. They also suggested that oxygenated blood delivery via the bronchial artery is insufficient to prevent lung injury during total CPB. To this end, the experimental study in piglets showed that CPB (pulmonary artery clamping without aortic clamping) results in significantly decreased bronchial artery blood flow despite adequate perfusion pressure and eventually histological tissue injury and reduced blood oxygenation capacity after reperfusion (Schlensak et al 2001). Thus, lung perfusion is transiently compromised during CPB leading to low flow ischemia followed by reperfusion, which results in lung injury in various degrees. Furthermore, alveolar ventilation may contribute to lung tissue oxygenation and viability, as continued ventilation during CPB has been shown to result in lesser inflammatory and proteolytic responses, and may better preserve pulmonary function than CPB without ventilation (Ng et al. 2008).

6.3. Thrombin in lung injury

Only few studies defining correlates of the local activation of coagulation and inflammation in lung I/R injury exist. In experimental in situ left lung I/R rat model thrombin was localized to alveolar macrophages, pulmonary artery endothelial cells, and alveolar epithelial cells after ischemia and 15 minutes of reperfusion (Farivar et al. 2006). Later in reperfusion (4 hours) thrombin was localized also to neutrophils (Farivar et al. 2006). Ischemia and four hours of reperfusion of the lung induced a significant increase in cytokine and chemokine levels and leukocyte counts in brochoalveolar lavage samples, increase in tissue MPO levels, and derangements in vascular permeability (Farivar et al. 2006). Inhibition of thrombin with hirudin, significantly reduced derangements in vascular permeability and intra-alveolar inflammatory cell sequestration (Farivar et al. 2006).

6.4. Functional changes

The functional changes following lung I/R injury can grossly be divided into increased microvascular permeability, gas exchange abnormalities from pulmonary edema, and increased pulmonary vascular resistance (PVR). Pulmonary hypertension including increased pulmonary artery pressure (PAP) and PVR is a well documented phenomena in experimental studies (Carteaux et al. 1999, Chai et al. 1999, Salvatierra et al. 2001). Pulmonary endothelium produces both vasodilatators (NO, prostacyclin PGI_2) and vasoconstrictors (thromboxane, endothelin-1) whose balance determines the pulmonary vascular tone. CPB and I/R- induced perturbations in endothelial function probably contribute to the reversible pulmonary hypertension during post bypass period (Carteaux et al. 1999, Fortier et al. 2004, Wan et al. 1997b, Wessel et al. 1993). Previous studies with pigs and humans have shown that the pulmonary endothelial dysfunction after CPB is manifested as an impaired endothelium dependent response to acetylcholine induced NO release (Chai et al. 1999, Wessel et al. 1993). NO is thought to produce relaxation of underlying smooth muscle cells via the cyclic GMP-pathway (Wessel et al. 1993). PGI_2 is an endogenous arachidonic acid metabolism derivative in the vascular endothelium acting via Gs-protein-related receptor to increase cyclic adenosine monophosphate (cAMP) (Scheeren et al. 1997). PGI_2 may also activate cGMP production. In the experimental study with pigs, Fortier et al. (2004) demonstrated pulmonary endothelial dysfunction and decreased cAMP after CPB. In this setting prophylactic use of inhaled PGI_2 was associated with preserved pulmonary intravascular cAMP levels and the pulmonary vascular tone. Besides its vasodilatatory effects, other physiological PGI_2 effects observed include inhibition of platelet aggregation and inhibition of leukocyte activation and adhesion to endothelium (Scheeren and Radermacher 1997). Thus, PGI_2 effects may be important in the context of CPB induced pulmonary I/R injury and dysfunction.

7. Intestinal hypoperfusion during and after CPB

Overall intestinal blood flow has been shown to be normal or increase during CPB as measured by flow in the superior mesenteric artery (Ohri et al. 1994a, Stamler et al. 1998, Tao et al. 1995). In contrast, mucosal hypoperfusion was detected by laser flowmetry and the mucosa remained ischemic, which was evidenced by mucosal acidosis in tonometric measurements (Ohri et al. 1994a, Stamler et al. 1998, Tao et al. 1995).

The non-physiological blood flow during CPB is associated with a variety of pathophysiological mechanisms, including hypothermia, systemic inflammatory response, and local inflammation due to I/R injury, which may impair intestinal blood flow regulation and intestinal perfusion. In addition, hypovolemia, hemodilution, and decreased cardiac output during reperfusion after CPB may contribute to

intestinal hypoperfusion. As a result, loss of gut barrier function allows bacterial translocation which may lead to endotoxemia and multiple organ dysfunction (Ohri and Velissaris 2006). Attempts to alleviate the CPB associated gut hypoperfusion have shown only modest success.

Animal and clinical studies indicate that both hypothermic and normothermic CPB is associated with impaired intestinal perfusion (Doguet et al. 2004, Ohri et al. 1994a, Ohri et al. 1994b, Ohri et al. 1997, Stamler et al. 1998, Tao et al. 1995, Tofukuji et al. 2000). However, one study comparing different CPB protocols (four combinations of hypothermic, normothermic, pulsatile, and non pulsatile) found only modest differences in gastric blood flow and mucosal hypoperfusion during and after CPB, which indicates that also other factors must be involved (Ohri et al. 1997).

Mucosal hypoperfusion is likely to be result of mucosal vasoconstriction and blood redistribution away from the mucosa (Tao et al. 1995, Schwarte et al. 2005). Sack et al (2002), using intravital microscopy, demonstrated arteriolar vasoconstriction and reduction in blood cell velocities at the microvascular level as well as increased permeability of the vessel wall despite the normal and stable macrohemodynamics during CPB. Functional capillary density was reduced by 30 % as compared to pre-CPB values, but the underlying mechanism remains an open question (Sack et al. 2002).

The state of intestinal blood pressure-flow autoregulation during CPB is poorly known. In an experimental pig model of CPB, increased perfusion pressure caused significant increment in intestinal perfusion, but, in contrast, cerebral perfusion pressure increased only minimally, which indicates intact autoregulation in the brain but lower limits of autoregulation in the splanchnic vascular beds during CPB (Mackay et al. 1995). Because the CPB is associated with increased sympathetic nervous system tone, arteriolar and pre-capillary sphincter tone may be increased by the action of endogenously released cathecolamines. In addition, CPB and systemic inflammatory state is associated with mesenterial endothelial injury and dysfunction with abnormal vascular reactivity, which may further contribute to the impaired regulation of intestinal microcirculation (Doguet et al. 2004, Tokufuji et al. 2000).

Several studies have shown progressively increased intestinal oxygen demand and the decrease in oxygen delivery during and after CPB (oxygen delivery/demand mismatch) (Ohri et al. 1994a, Stamler et al. 1998, Tao et al. 1995). Oxygen delivery, in addition to the perfusion flow, is dependent on the hemoglobin concentration, hemoglobin oxygen saturation, and the amount of dissolved oxygen. Both CPB and post-CPB periods are notable for reductions in haematocrit and post-CPB period for reduced cardiac output. Although blood during CPB is well oxygenated, a decrease in jejunal mucosal oxygen tension and in mucosal microvascular hemoglobin oxygen saturation during CPB has been reported in pigs (Haisjackl et al. 1999). This may be due to the countercurrent exchange of oxygen, which results in decreased oxygen tension at the tip of the villus. It may also be caused by regional blood redistribution (Haisjackl et al. 1999). Increased oxygen demand suggests higher gut tissue

metabolism, which may occur as a result of a local inflammatory response (Ohri and Velissaris 2006, Tao et al. 1995).

7.1. Intestinal I/R injury

While several mechanisms lead to gut wall hypoperfusion during CPB, restoration of normal circulation conditions to hypoperfused areas after CPB may result in I/R injury. Among the myriad of pathophysiological factors involved in intestinal I/R injury (Mallick et al. 2004, Schoenberg and Beger 1993) the role of activated neutrophils has repeatedly been put forward (Cooper et al. 2004, Granger 1988, Grisham et al. 1986, Hayward and Lefer 1998, Hernandez et al. 1987, Oktar et al. 2002, Schoenberg et al. 1991, Sun et al. 1999, Özden et al. 1999). However, in contrast to the blood flow conditions during CPB, one feature in common in these studies is total ischemia followed by "true" reperfusion when neutrophil tissue sequestration, measured by MPO, typically increases several fold (Grisham et al. 1986, Hayward and Lefer 1998, Oktar et al. 2002, Sun et al. 1999, Özden et al. 1999). Only a few studies defining correlates of the CPB related systemic inflammatory response with the local inflammatory response of the gut wall have been reported (Doguet et al. 2004, Tokufuji et al. 20005). In a pig model of CPB functional and selective inhibition of complement C5a significantly limited mesenteric neutrophil sequestration, measured by MPO, and attenuated neutrophil-mediated impairment of microvascular regulation (Tokufuji et al. 20005). However, complement inhibition did not show any effect on the reduced mucosal blood flow, mucosal acidosis, increased epithelial permeability and mesenteric edema during CPB (Tofukuji et al. 2000).

7.2. Histological changes

Intestinal I/R injury is characterized by a number of histological changes including mucosal damage, leukocyte infiltration, tissue edema, capillary plugging (Schoenberg and Beger 1993). For this purpose, many different grading systems have described but no consensus how intestinal I/R injury should be graded exists (Quaedackers et al. 2000). Only sparse data exists on histological injury in the small intestine during CPB-surgery.

7.3. Thrombin in intestinal I/R injury

Previous experimental studies strongly suggest that the cross-talk between inflammation and coagulation systems takes place also in intestinal I/R injury (Olanders et al. 2005, Schoots et al. 2004, Tsuboi et.al 2007). Schoots et al (2004) demonstrated with the rat mesentery artery occlusion model that intestinal ischemia and reperfusion resulted in local activation of coagulation as reflected by an increase of the portal vein plasma concentration of TAT complexes and fibrinogen

degradation products. Microscopic evaluation of intestinal samples showed mucosal fibrin deposits and microvascular thrombosis (Schoots et al. 2004). These results are consistent with the report by Tsuboi et al. (2007), who demonstrated using a similar animal model that the increased serum level of TAT complexes and the intestinal mRNA level of PAR1 coincided with increased mucosal cytokine levels and MPO activity during reperfusion.

7.4. Intestinal tonometry

Gastrointestinal tonometry is a minimally invasive indirect method to assess the adequacy of aerobic metabolism of the gut mucosa. Hypoperfusion of the gut below a critical level causes mucosal accumulation of CO_2 and acidosis. The saline filled tonometric method was adopted for assessing intestinal luminal pCO_2 and arterial bicarbonate for the calculation of intestinal pH (pHi) (Fiddian-Green et al. 1982). However, because of several confounding factors, quantitating intestinal perfusion should be based on the intestinal pCO_2 measurement or on the gap between arterial and intestinal pCO_2 rather than on the intramucosal pH alone (Heino et al. 1998, Kolkman et al. 2000, Knichwitz et al. 1998, Vincent and Creteur 1998). If pHi is used, more than a single pHi value, a trend evaluation is recommended (Doglio et al. 1992). During metabolic acidosis, a high arterial-intramucosal pH difference may be used as an evidence for local hypoperfusion (Boyd et al. 1993).

Although the tonometric measurements have correlated with hypoperfusion in the different parts of the gastrointestinal tract (Montgomery et al. 1989), small bowel tonometry was found to be more accurate than gastric tonometry in detecting intestinal hypoperfusion during burn and hemorrhagic shock in pigs (Cancio et al. 2007, Walley et al. 1998). An air-automated tonometry catheter connected to a tonometry computer module seems to eliminate many of the technical problems inherent in the traditional manual saline filled tonometry, including protracted pCO_2 equilibration time, inaccurate pCO_2 detection, and methodological errors during injection and sampling of saline (Barry et al. 1998, Creteur et al. 1997, Kolkman et al. 2000).

8. Inhibition of thrombin

8.1. Hirudin

Hirudin, a naturally occurring anticoagulant derived from the medicinal leech, is a selective and effective inhibitor of thrombin. Hirudin directly inhibits the active site pocket and fibrinogen binding site of free and clot bound thrombin (Weitz et al. 1990, Weitz et al. 1998). Hirudin, in relative difference to heparin, primarily inhibits thrombin activity instead of thrombin generation (Eichinger et al. 1995, Zoldehyi et al. 1994). However, thrombin activates several clotting factors and amplifies

its own formation. Thus, blocking thrombin activity by hirudin might secondarily inhibit further thrombin formation (Pernerstorfer et al. 2000). Recombinant hirudin (r-hirudin), as an alternative to heparin, has proven to be a safe and effective anticoagulant in animals and patients undergoing CPB (Greinacher and Lubenow 2001, Riess et al 1997, Walenga et al. 1994). Hemodynamic or hematologic adverse effects of r-hirudin treatment were not noticed in dogs undergoing CPB (Walenga et al. 1994). Difficulties in monitoring, a rather long half-life, possible enhanced bleeding tendency, and lack of specific antidote are clear disadvantages of its use in clinical practice (Greinacher and Lubenow 2001).

8.1.1. Experimental and clinical evidence of selective inhibition of thrombin

In previous studies of inflammation associated coagulopathy r-hirudin blunted endotoxin-induced thrombin activity and fibrin generation in humans (Pernerstorfer et al. 2000), and attenuated liver injury in rats (Pearson et al. 1996). In rabbits, r-hirudin significantly reduced renal fibrin deposits in an endotoxin-induced DIC model (Munoz et al. 1999).

Despite the use of heparin, the generation of thrombin and the activation of coagulation occurs during CPB. Thus, additional inhibition of thrombin could be beneficial. Bivalirudin, a synthetic polypeptide with a short half life of 30 min, is another direct thrombin inhibitor used safely in clinical cardiac surgery (Dyke et al. 2006). In an experimental CPB rat model, combination of low dose bivalirudin and standard dose of heparin reduced significantly thrombin generation (TAT) and attenuated the increase of inflammatory markers (IL-6, IL-10) when compared to standard dose heparin alone (Welsby et al. 2007). The anti-inflammatory effect of bivalirudin is feasible as direct thrombin inhibition may reduce the activation of monocytes and endothelial cells, in turn reducing the release of inflammatory mediators from these cells (Johnson et al. 1998).

In the context of I/R, inhibition of thrombin with r-hirudin has been shown to have beneficial effects on the myocardial I/R injury. In cell cultures subjected to simulated ischemia and reperfusion lepirudin prevented thrombin induced acute cardiomyocyte death (Mirabet et al. 2005). In the experimental coronary ligation models, functional inhibition of thrombin with r-hirudin reduced myocardial infarct size (Erlich et al. 2000, Strande et al. 2007). However, the effects of hirudin on CPB induced myocardial ischemia-reperfusion injury have not been studied.

8.2. Antithrombin

Antithrombin (AT) is a major physiological anticoagulant inhibiting thrombin and other proteases of the coagulation, mainly FXa (Roemisch et al. 2002, Rosenberg 1989). The effect of AT on these coagulation proteases is accelerated about a 1000-fold by heparin. Therapeutic AT concentrates contain human plasma-derived inhibitor

or recombinant human AT. The latter product has been used safely even in high doses (200 IU/kg) in clinical cardiac surgery (Levy et al. 2002).

In addition to its anticoagulant activity, AT exhibits anti-inflammatory effects. Studies on experimental animal models of sepsis and septic shock have shown that high dose (250 IU/kg) supplementary AT can reduce the mortality and organ injuries by attenuating coagulation disorders and inflammatory response (Dickneite and Leithauser 1999, Minnema et al. 2000, Okajima 1998, Uchiba et al. 1998). Lower doses (50 and 100 IU/kg) of AT significantly inhibited coagulation abnormalities but did not prevent pulmonary vascular injury in an animal model of sepsis (Uchiba et al. 1998). More importantly, supplementation of AT (250 IU/kg) alleviated I/R injury in the liver (Okano et al. 1995), kidney (Mizutani et al. 2003), lung (Salvatierra et al. 2001), and intestine (Özden et al. 1999). These effects has been largely attributed to the anti-inflammatory property of AT. In an experimental lung transplantation dog model, AT supplementation reduced the expression of monocyte adhesion molecules, neutrophil sequestration, tissue edema, and reduced pulmonary vascular resistance (Salvatierra et al. 2001). Ostrovsky et al. (1997) showed that AT supplementation significantly reduced neutrophil rolling and adhesion in a feline mesentery I/R model. Supplementary AT has also been shown to reduce tissue MPO activity, neutrophil sequestration, histological damage, and tissue cytokine levels in intestinal I /R injury in rats (Tsuboi et al. 2007, Özden et al. 1999).

However, only scarce and controversial data of AT effects on myocardial I/R injury are available. In an isolated rat heart model physiological AT levels had no influence on myocardial I/R injury but high AT levels actually worsened tissue injury (Margreiter et al. 2003).

AT has been shown to reduce both hemostatic activation and inflammatory response during CPB. In a simulated CPB model, high dose AT (5 U/mL) but not low dose (1 U/mL) supplementation to minimally heparinized human blood blunted thrombin generation (F1+2), inhibited platelet activation and reduced neutrophil and monocyte activation (Rinder et al. 2006). Koster et al. (2003) demonstrated in cardiac patients that heparin and additional bolus of AT (50 IU/kg) given before CPB reduced hemostatic activation, as shown by a significant decrease in thrombin generation and activity when compared to heparin alone. They also showed that AT attenuated leukocyte activation, which was evidenced by decreased elevation of neutrophil- derived cytokine IL-6 and protease elastase (Koster et al. 2003).

8.2.1. Mechanisms of anti-inflammatory effects

The main mechanism suggested for AT's anti-inflammatory, non-anticoagulant, effects has been the endothelial release of prostacyclin (PGI_2) mediated by AT interaction with endothelial cell surface glycosaminoglycans (GAG) (Mizutani et al. 2003, Salvatierra et al. 2001, Uchiba et al. 1995, Uchiba et al. 1998). The effects of PGI_2, in turn, include vasodilatation, inhibition of platelet aggregation, inhibition

of neutrophil activation and adhesion, and suppression of proinflammatory cytokine production (Amstrong et al. 1977, Mizutani et al. 2003, Salvatierra et al. 2001). However, there is increasing evidence for cellular receptors of AT and intracellular events modulated by such interaction. Syndecan-4 has been identified as a heparin sulfate proteoglycan, when acting as an AT receptor, modulates the regulation of adherence and migration of leukocytes to the endothelium and into the tissue (Dunzendorfer et al. 2001, Kaneider et al. 2001). It has been shown that, AT dose dependently inhibited the expression of IL-6, TNF-α, and TF genes in endotoxin stimulated cultured monocytes and endothelial cells (Oelschläger et al. 2002).

Thus, beyond the control of coagulation, AT exhibits anti-inflammatory effects through interactions with cells by reducing the synthesis and release of proinflammatory mediators and by modulating leukocyte activation and their interaction with the vessel wall.

8.2.2. Heparin and antihrombin supplementation

Heparin may block the anti-inflammatory actions of AT. Simultaneous infusion of heparin and AT could, while enhancing AT-heparin complex formation in plasma, paradoxically reduce AT-GAG coupling on endothelial surface resulting ultimately in reduced instead of enhanced AT functionality (Pulletz et al. 2000, Roemisch et al. 2002, Uchiba et al. 1995). It has been suggested that soluble heparin and GAGs compete for AT's binding site, thus reducing its ability to interact with cells. Although anticoagulant properties of AT are potentiated by heparin in terms of thrombin inhibition, its interaction with endothelial cells is diminished and anti-inflammatory effects are significantly impaired.

AIMS OF THE PRESENT STUDY

The general aim of this study was to evaluate the potential of thrombin inhibition in reducing the adverse effects of ischemia-reperfusion injury in the myocardium, lungs, and intestine associated with the use of CPB and cardiac surgery.

The specific aims were:

1. to test if r-hirudin, selective and effective inhibitor of thrombin, could attenuate reperfusion-induced generation of thrombin.
2. to study whether the direct inhibition of thrombin would affect general hemodynamics and intestinal microcirculation.
3. to study the effects of thrombin inhibition on early functional recovery of the post-ischemic myocardium and to explore potential mechanisms of thrombin activity on myocardial I/R injury.
4. to test the effects of supplementary antithrombin on myocardial and lung I/R-injury.
5. to study whether local post CPB inflammatory response in the gut wall would associate with intestinal mucosal perfusion.

MATERIAL AND METHODS

1. Animals

A total of 45 2.5 months old pigs (cross-breed of Landrace and Yorkshire) of both sexes, weighing approximately 25 kg, were used as experimental animals (Figure 1). They were allowed at least three days of in-house acclimatization with ad libitum access to standard laboratory food and water. All animals received humane care in compliance with the "Principles of Laboratory Animal Care" formatted by the National society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1985). All experiments were approved by the Local Ethical Committee of Helsinki University Central Hospital.

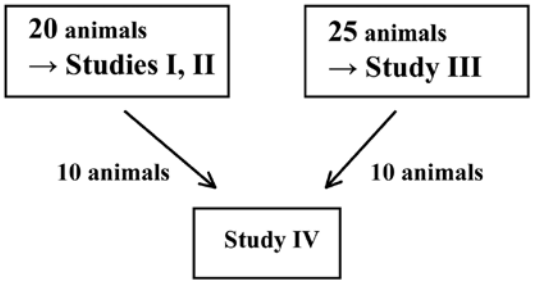

Figure 1. Experimental animals used in the studies. Twenty animals were used in studies I-II, and twenty five in the study III. Study IV included twenty animals from the studies I and III (10+10).

2. Anesthesia

General anesthesia was induced by giving ketamine hydrochloride 500 mg intramuscularly. Two mL (60 mg/mL) of pentobarbital sodium, three mL (2mg/mL) of pancuronium bromide, and 0.1 mg of fentanyl citrate were given intravenously. The pigs were intubated and ventilated with a Siemens Servo Ventilator 900 C (Siemens-Elema AB, Solna, Sweden) under continuous ECG-monitoring (S/5™ Anesthesia Monitor, Datex-Ohmeda, Helsinki, Finland). General anesthesia was maintained with continuous pancuronium promide (0.25 mg/kg/h), pentobarbital (5 mg/kg/h), and fentanyl (0.015 mg/kg/h) infusions.

3. Operative technique

The left femoral artery and vein were catheterized. Heparin (500 IU/kg) was given intravenously immediately after the vein was catheterized. Arterial blood pressure was continuously monitored and the femoral vein was used for intravenous anesthesia and blood sampling. The right femoral vein was catheterized for infusion of study drug or placebo. A small midline laparotomy was performed and an intestinal air-automated 14F tonometry catheter (Tonometrics™ Catheter, Datex-Ohmeda, Helsinki, Finland) was inserted into the lumen of ileum 50 cm proximal from the ileocecal junction and secured with a 5-0 polypropylene purse-string suture. A midline sternotomy was performed. After sternotomy, the pulmonary artery was catheterized with a pediatric 5F Swan-Ganz catheter (Baxter Health Corporation, Santa Ana, CA, USA) via the superior caval vein and the catheter was connected to a cardiac computer (S/5™ Anesthesia Monitor, Datex-Ohmeda, Helsinki, Finland). Venous drainage was provided by a cannula in the right atrium and an aortic perfusion catheter was placed in the aorta. The left ventricle was catheterized through the apex of the heart for pressure measurements. After the initial dose of heparin the activated coagulation time (ACT) was measured before CPB and at 30 minutes intervals until 105 minutes post-CPB. An additional dose (100–200 IU/kg) of heparin was given during perfusion whenever ACT was shorter than 400 seconds. Protamine was not given.

A Gambro 2-roller pump (Gambro, Lund, Sweden) and a D 705 miniflow pediatric hollow fiber oxygenator (Dideco, Mirandola, Italy) were used in all experiments. The CPB circuit was primed with 1000 mL Ringersteril™ and 5000 IU of heparin. Hemodynamic, biochemical, and intestinal tonometric baseline values were determined before normothermic perfusion (2 L/min) was started. The oxygenator was heated with a heat exchanger l (Heater™, Amer Group LTD, Tekamer, Helsinki, Finland), and temperature was kept at 37°C. After the initiation of perfusion, the aorta was clamped and the heart was arrested with 4°C (15 mL/kg) crystalloid cardioplegia (Plegisol™ , Abbot Laboratories, North Chicago, USA) which was delivered via the aortic root cannula. Myocardial temperature was continuously monitored and a pericardial insulation pad was used during the cross-clamping time. Cardioplegia (2 mL/kg) was added every 15 min and if ventricular fibrillation occurred. Arterial acid-base balance was monitored during the operation at 30 min intervals (ABL™ System 615, Radiometer Medical A/S, Copenhagen, Denmark).

Aortic clamping was released after one hour. After clamp release, ventilation was begun at 7 min and the heart was supported by continuing CPB for 15 min. If ventricular fibrillation was present after 4 min, defibrillation was used repeatedly with 1 min intervals until a hemodynamically effective sinus rhythm was achieved. Vasoactive medication was not used.

Blood from mediastinal and pleural spaces was collected and returned into the CPB system. Blood from abdomen was collected into a separate reservoir. Bleeding was measured from the mediastinal and pleural spaces and from the laparotomy wound.

Experimental animals were sacrificed during anesthesia by exsanguination after a surveillance period of 105 min after cessation of CPB.

4. Measurements

4.1. Hemodynamics

Heart rate (HR), ECG, arterial blood pressure, central venous pressure (CVP), and pulmonary artery pressure (PAP) were monitored continuously. CVP was standardized by given an infusion of Ringersteril™ i.v. before each measurement of the hemodynamic profile. Cardiac output (CO), HR, pulmonary arterial diastolic pressure (PAPD), pulmonary capillary wedge pressure (PCWP), systolic and mean arterial pressure (SAP, MAP), left ventricular pressure (LVP), and systemic vascular resistance (SVR) were determined before perfusion and after weaning from CPB at 15, 30, 60, 90, and 120 min after clamp release. CO was measured in triplicate using a thermodilution method. SVR was calculated according to standard formulas.

4.2. Blood samples

The volume of catheters used for sample collection was 1 mL. After discarding 3–5 mL of blood, nine parts of venous blood were collected in one part of 0.109M trisodium citrate at various time points in order to measure thrombin antithrombin complexes (TAT) (study I,III,IV), AT activity and cardiac biomarker troponin T (TnT)(study III). The samples were immediately transferred to the laboratory and plasma separated by centrifugation for 15 min at 2000 g at room temperature. The separated plasma was stored at -70°C until assayed. Measurements for blood gas and activated clotting time (ACT) were performed from fresh arterial whole blood.

Plasma levels of TAT were measured from the samples drawn before CPB, just before aortic declamping, and at 5, 10, 30, and 120 min after aortic declamping using a commercial kit (Enzygnost TAT micro, Dade Behring, Liederbach, Germany). AT activity was measured from the samples taken before sternotomy, before CPB, just before aortic declamping, 30, and 120 min after aortic declamping. A chromogenic assay (Berichrom Antithrombin/Antithrombina III, Dade Behring, Liederbach, Germany) was used on an automated coagulometer (BCS;Dade Behring, Liederbach, Germany). The level of TnT was determined with electrochemiluminescence immunoassay (Elecsys TroponinTSTAT®, Roche Diagnostics GmbH, Mannheim, Germany) from the samples taken before CPB and 120 minutes after the aortic declamping.

ACT was measured from blood samples obtained from the femoral artery before CPB, during CPB, just before aortic declamping, 30, 60, 90, and 120 min after aortic declamping using a two-channel Automated Coagulation Timer (Medtronic Blood Management, Parker, CO, USA). Arterial blood gas measurements were obtained

simultaneously with ACT using automatic analyzer (ABL700 Series analyzer, Radiometer Medical A/S, Brønshøj, Denmark).

The assays for TAT, AT, ACT, and TnT were all accepted as validated for human use by respective manufacturers. Since separate validation for porcine plasma was not carried out, the absolute values of each measurement need to be considered semiquantitative and only valid for intergroup comparisons.

4.3. Tonometry and blood gases

To evaluate local microcirculatory perfusion in the intestinal mucosa tonometric measurements were obtained. An air-automated tonometry catheter inserted into the ileum included a semi-permeable silicone balloon at the distal end of the catheter. Four mL of room air was pumped into the silicon balloon. CO_2 freely equilibrated between the intestinal mucosa, intestinal lumen, and the balloon. The proximal end of the catheter was connected to a tonometry module (S/5™ Tonometry Module, Datex-Ohmeda, Helsinki, Finland). The system drew a gas sample from the balloon and intestinal pCO_2 ($piCO_2$) was automatically measured every 10 min. Intramucosal pH (pHi) was automatically calculated by the monitor from $paCO_2$ and pHa values obtained from arterial gas analysis, entered by the user, and $piCO_2$ measured. Tonometric and arterial acid-base values were obtained before perfusion (0), after 30 min of perfusion, just before aortic declamping, and at 30, 60, 90, and 120 min after aortic clamp release. To further evaluate local microcirculatory perfusion in intestinal mucosa the gaps between pHa and pHi (pH(a-i)) and $paCO_2$ and $piCO_2$ (pCO2(i-a)) were calculated.

4.4. Myeloperoxidase activity

Myocardial biopsies for cardiac MPO activity were taken from the apex of the left ventricle and intestinal biopsies for intestinal wall MPO activity were taken from ileum proximal to tonometry before CPB, just before aortic declamping and during reperfusion at 5, 10, and 120 minutes after aortic declamping. The samples were immediately frozen in liquid nitrogen and stored at – 70ºC until assayed for MPO activity using a modification of the spectrophotometric method described by Suzuki et al. (1983). The method is based on the production of a blue chromogen in the MPO catalysed oxidation of 3,3',5,5'-tetramethylbenzidine by H_20_2.

4.5. Histology

A larger sample of the left ventricle (including endocardium, myocardium and epicardium), lung, and full thickness sample of ileum was taken at 120 min after aortic declamping before the animal was sacrificed or when the animal died during the experiment. Cardiac and lung samples were fixed in formalin, embedded in paraffin,

cut into 2–3 μm sections and prepared according to the routine histochemical methods prior to hematoxylin-eosin staining. Small bowel samples were fixed in formalin, embedded in paraffin, cut into 2–3 μm sections and stained with Periodic acid Schiff. The samples were taken for histological evaluation of an inflammatory reaction and presence of microvessel thrombosis. Histological parameters were scored from 0 to 3 according to the scoring criteria listed in table 1. The person carrying out the scoring was blinded to all data concerning the individual experiments.

4.6. Apoptosis

Tissue samples from left ventricle which were taken as mentioned above were further analyzed for the presence and extent of apoptosis using methods described below.

4.6.1. In situ detection of apoptotic cells

In situ detection of apoptotic cells in paraffin wax sections was performed as described earlier (Laine et al. 1996, Lukkarinen et al. 2003) with slight modifications. Briefly, deparaffinised sections were treated with 10 μg/mL of proteinase K (Boehringer, Mannheim, Germany) at +37ºC in 2 mmol/L $CaCl_2$ and 20 mmol/L TRIS-HCL, pH 7.4, for 30 minutes. The endogenous peroxidase activity was blocked by incubating the slides in 0.3% hydrogen peroxide in water for 10 min at room temperature. DNA 3' -end labeling was performed after 10 min incubation with terminal transferase buffer (Promega, Madison, WI, USA). The labeling mixture contained fresh terminal transferase buffer, 5 μmol/L non-radioactive digoxigenin-dideoxyUTP (dig-ddUTP, Boehringer, Mannheim, Germany), 45 μmol/L ddATP (Pharmacia Uppsala, Sweden), and 0.34 U/μL terminal transferase (Promega, Madison, WI, USA). The reaction was allowed to continue for one hour at +37ºC in a humidified chamber. After washing, the slides were incubated with blocking buffer containing 2% (w/v) blocking reagent and 0.05% (w/v) sodium azide (Boehringer, Mannheim, Germany) for 30 minutes. Antidigoxigenin antibody, conjugated to alkaline phosphatase (1:3000, Boehringer, Mannheim, Germany), in 2% (w/v) blocking buffer was added and incubated for two hours in a humidified chamber. The slides were treated with alkaline phosphatase buffer (0.1 mol/L NaCl, 0.05 mol/L MgCl, and 0.1 M TRIS-HCl, pH 9.5) for 10 minutes. Thereafter, 337 μg/mL nitroblue tetrazolium salt (Boehringer, Mannheim, Germany) and 175 μg/mL 5-bromo-4-chloro-3-inodyl-phosphate (Boehringer, Mannheim, Germany) were added in fresh alkaline phosphatase buffer, and the reaction was terminated 3 hours and 45 minutes later by 1 mmol/L EDTA and 10 mmol/L TRIS-HCl, pH 8.0. Finally, the slides were mounted with Gurr Aquamount (BDH Chemicals, Poole, England). For controls, terminal transferase, dig-ddUTP, or antidigoxigenin antibodies were omitted from reaction. The assay was additionally standardized using sections treated with DNase I (1 U/mL for 30 min at +37°C) to induce the formation of DNA strand breaks (positive control of apoptosis).

Apoptotic cells were counted in tissue sections stained with the antidigoxigenin antibody. A distinct color reaction within the cells was regarded to represent apoptotic DNA fragmentation. The results were expressed as proportion of TUNEL-positive cardiomyocyte nuclei of the total number of cardiomyocyte nuclei, which was counted in the corresponding DNase I-treated positive control section. Cardiomyocyte origin of the apoptotic cells was identified by the presence of myofilaments surrounding the nucleus. The in situ detection of free DNA 3´ ends is a well-established method in the detection of apoptotic cellular changes and has been validated by simultaneous electrophoretic DNA analysis in pancreatic tissue (Laine et al. 1996).

4.6.2. Cleaved caspase-3 immunohistochemistry

To further confirm the apoptotic activity in the cardiac myocytes, we investigated the activation of caspase 3 with antibodies recognizing the cleaved and active form of the enzyme (Cell Signaling Technology, Beverly, MA, USA) as previously described (Saraste et al. 2003). Antibody was detected with Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's instructions.

5. Study setting

5.1. R-hirudin and hemodynamics (study I)

Twenty pigs undergoing 60 min aortic clamping and 75 min normothermic perfusion were blindly randomized to receive an iv-bolus of recombinant hirudin (r-hirudin) lepirudin (Refludan™, Aventis Behring, Marburg, Germany) (10 mg, 0.4 mg/kg) (n=10) or placebo (20 mL NaCl) (n=10) 15 minutes before aortic clamp was released and then continued with a 135 min iv infusion of r-hirudin (3.75 mg, 0.15 mg/kg) or placebo. Thrombin-antithrombin complexes (TAT), and activated clotting times (ACT), and several hemodynamic parameters were measured before CPB, after weaning from CPB, at 30, 60, 90, and 120 minutes after aortic declamping. Intramucosal pH and pCO_2 were measured from the luminal surface of ileum simultaneously with arterial gas analysis at 30 min intervals.

5.2. R-hirudin and early functional recovery of myocardium (study II)

The study groups and setting was the same as in study I. In addition to evaluation of early hemodynamic parameters and blood samples, myocardial biopsies were taken before CPB, just before aortic declamping, during reperfusion, and after 120 min of reperfusion to quantitate leukocyte infiltration (myeloperoxidase activity, MPO). A larger sample of myocardium was taken after 120 min of reperfusion for histological evaluation and detection of apoptosis with caspase 3 and Tunel-method.

5.3. Antithrombin in myocardial and lung I/R injury (study III)

Twenty pigs undergoing 60 min aortic clamping and 75 min normothermic perfusion were randomized in a blinded setting to receive an iv-bolus of AT (250 IU/kg) (AT group, n=10) or placebo (n=10) 15 min before aortic declamping. An additional group of 5 animals received 500 IU/kg of AT in an open label setting (AT+). Thrombin-antithrombin complexes (TAT), activated clotting times (ACT), AT and myeloperoxidase (MPO) activities, troponin T, and several hemodynamic parameters were measured before CPB and after weaning from CPB up to 120 minutes after aortic declamping. Myocardial and lung biopsies were taken after 120 min of reperfusion for histological evaluation.

5.4. Intestinal post CPB inflammatory response and mucosal perfusion (study IV)

Study IV included twenty placebo animals from studies I and III. Animals underwent 60 min of aortic clamping and 75 min of normothermic perfusion. Intestinal biopsies for MPO activity were taken before CPB, just before aortic declamping and during reperfusion at 5, 10, and 120 minutes after aortic declamping. A larger sample of ileum was taken for histological evaluation after 120 minutes of reperfusion. Ileal pCO_2, and intramucosal pH were determined and arterial gases analyzed at 30 min intervals. Additionally, several hemodynamic parameters and blood thrombin-antithrombin complexes (TAT) were analyzed. Based on ileal myeloperoxidase activity (MPO) the animals were divided into two groups (CPB induced increase in MPO ("MPO+") vs. no such increase ("MPO-")) for comparison of the parameters of gut mucosal perfusion and histological injury. All subsequent analyses were carried out by comparing the MPO+ and MPO- groups to each other.

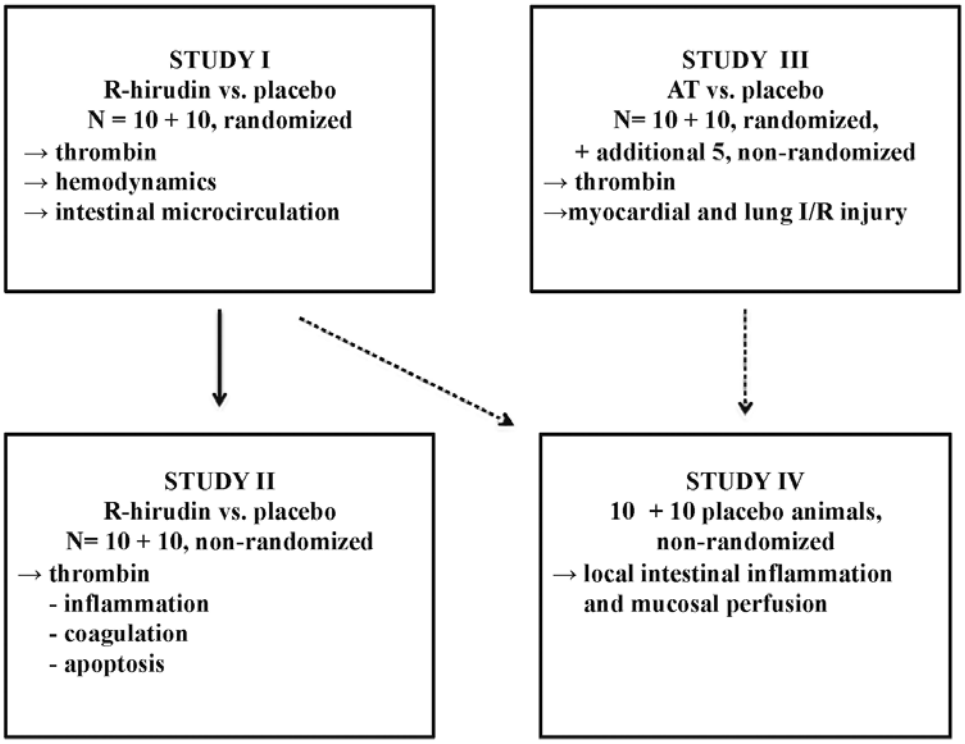

Figure 2. Study setting. Studies I and III were randomized and blinded studies, except five additional animals were included in non-randomized and open fashion in study III. Study II included twenty animals from study I. Study IV included twenty placebo animals from studies I and III.

6. Statistical analysis

Differences between the two experimental groups in continuous parameters were statistically evaluated using the repeated measure analysis of covariance model, which included the baseline value as a covariant (study I, II), the repeated measures analysis of variance with SPSS for Windows software (versions 14.01 and 11.5; SPSS Inc., Chicago, Illinois, USA) (study III, IV) and Student´s t-test (Microsoft Excel) (study I- IV). Baseline values between the two experimental groups were compared with two-tailed Student's t-test (study I, II). Intergroup differences in the uncontinuous histological scoring data were calculated using Mann Whitney U-test. The mortalities during the experiment in the two groups were compared using the Fisher's exact test (study II). A p- value < 0.05 was considered statistically significant. Data are presented as mean ± standard deviation (study I-IV).

RESULTS

1. Survival

Out of 45 animals 2 were lost and excluded from the studies at the beginning of the experiment because of a rupture of a cannulation sites during the operation (study I, III). Two animals died during reperfusion at 30 minutes after aortic declamping and two during reperfusion at 90 min after aortic declamping because of a hemodynamic collapse (study I). Altogether 39 animals survived until the scheduled end of the experiment. The difference in mortalities between the study groups was non-significant (study I). There were no differences between the groups in the time (study I; 6.8 ± 2.6 min vs. 6.2 ± 3.0 min, $p = .66$, study III; 6.0 ± 3.2 min vs. 6.0 ± 3.2 min, $p = 1.0$) needed to achieve regular hemodynamically effective rhythm nor in the number of defibrillations needed.

2. Effect on anticoagulation

ACT was well over 400 seconds after initiation of and during the CPB with the planned heparin protocol (study I, III). Both r-hirudin and AT prolonged ACT significantly. 15 minutes after the bolus of r-hirudin or AT ACT was significantly longer in these groups compared to the placebo groups (r-hirudin; 834 ± 231 sec vs. 515 ± 260 sec, $p = .008$, AT; 827 ± 240 sec vs. 440 ± 182 sec, respectively, $p < 0.001$) (study I, III). ACT increased in the AT+ group to the same level than in the AT group (study III).

AT activity rose dose dependently after the bolus of AT and remained elevated throughout the experiment (study III).

3. Effect on thrombin generation

Plasma levels of TAT were measured to determine thrombin formation. TAT levels increased in all groups during CPB (study I, III). R-hirudin abolished the pronounced reperfusion associated increase in TAT levels observed in the placebo group (study I). Instead, in the AT group, in contrast to the placebo group, TAT level gradually increased during reperfusion and there was a pronounced increase in TAT levels from 30 to 120 min after aortic declamping (study III). The increase in TAT-levels during reperfusion was more pronounced in AT+ than in AT or placebo groups (study III).

4. Myocardial troponin T release

Troponin T increased from preperfusion situation to 120 min after aortic declamping significantly in all groups but no intergroup differences were observed (placebo 0.23 ± 0.14 μg/L, AT 0.21 ± 0.15 μg/L, AT+ 0.34 ± 0.21 μg/L, $p < 0.05$, respectively) (study III).

5. Hemodynamics

5.1. Recovery of the myocardial function

HR (per min) increased gradually from 136 ± 27 in the placebo group and from 136 ± 16 in the r-hirudin group to 153 ± 22 and 156 ± 26, respectively. HR increased similarly also in the placebo and in the AT groups (study III). Ischemia during aortic declamping and CPB induced a significant loss of CO, SV, and LVP (study I, III). Importantly, in the animals receiving r-hirudin CO and SV showed recovery towards the preperfusion situation while in the placebo group no such trend was observed. The difference between the r-hirudin and placebo groups reached statistical significance at 60 min and 90 min after aortic declamping. At 15 min after aortic declamping, SV was 68 and 58% of the preischemia level in the r-hirudin and placebo groups, respectively ($p > 0.2$ for intergroup difference). In those animals receiving r-hirudin, SV increased from this nadir by 26 ± 42% at 60 min after aortic declamping while the corresponding change was -10 ± 18% in the placebo group ($p = 0.042$) (study I). In the group receiving supplementary AT (250 IU/kg) CO and SV showed only a trend of recovery during the first 15 min after CPB towards the preperfusion situation while in the placebo group no such trend was observed (study III). Animals receiving a 500 IU/kg dose of AT showed a significant improvement of CO and SV during the first 15 minutes after CPB when compared to placebo group. Even with the high dose of AT, CO and SV decreased progressively and similarly to the placebo group thereafter.

During reperfusion, LVP gradually increased back to the pre-ischemia level in the r-hirudin group (pre-ischemia 131 ± 20 mmHg, 120 min post-ischemia 133 ± 28 mmHg), while only a partial recovery in LVP in the placebo group was observed (131 ± 13 mmHg, 117 ± 21 mmHg, respectively) (study I). The difference between the groups did not reach statistical significance. LVP in the AT group showed a transient recovery during early reperfusion but was gradually dropped to 86 ± 27 mmHg at 120 minutes of reperfusion (study III). The corresponding LVP in the placebo group was 106 ± 37 mmHg ($p > 0.05$ for intergroup comparison).

5.2. Systemic hemodynamics

SVR increased markedly in the placebo groups during reperfusion (study I, III). This reperfusion induced increase in SVR was effectively abolished by r-hirudin (study I) but not by AT (study III). The difference between the r-hirudin and placebo groups reached statistical significance at 60 min and 90 min after aortic declamping (study I). There were transient drops in SAP and MAP after ischemia and CPB compared to values before CPB. R-hirudin receiving animals were able to regenerate SAP back to the pre-ischemia level. Instead, in AT receiving animals, only a transient recovery in SAP and MAP was seen (study III). However, no significant intergroup differences were seen (study I, III). PCWP was not affected by the ischemia and CPB (study I,III).

5.3. Pulmonary hemodynamics

PAPD and PVR were increased slightly during the CPB but a more pronounced increase was seen during reperfusion the period after CPB (study I, III). PVR was recorded only from part of the animals (study I, III). R-hirudin did not have a significant effect on PAPD but PVR was significantly higher in the placebo group at 60 min after aortic declamping (990 ± 337 dyne*sec/cm^5, n = 4 vs. 526 ± 191 dyne*sec/cm^5, n = 8, p = .01). AT significantly attenuated the reperfusion induced increase in PAPD but did not have significant effect on PVR (study III). The difference between the AT and placebo groups reached statistical significance at 90 min after aortic declamping (AT 22 ± 5 mmHg vs. placebo 28 ± 8 mmHg, p = 0.04) (study III). Instead, 500 IU/kg of AT substantially attenuated the reperfusion induced increase in PVR. In addition, 500 IU/kg AT potentiated the lowering effect of 250 IU/kg AT on the reperfusion induced increase in PAPD (study III).

6. Myocardial myeloperoxidase activity

MPO activity determined to measure tissue infiltration of activated neutrophils showed a slight but transient ischemia induced increase (study II, III) and an increase between 10 and 120 min of reperfusion (study II). The ischemia induced increase in MPO was not seen in the AT group (study III). The increase during later phase of reperfusion was more marked in the placebo group than in the r-hirudin group. However, statistically significant differences between the groups were not observed at any timepoint (study II, III).

7. Histology

7.1. Myocardium

All myocardial biopsies showed signs of I/R injury-related histological alterations but there were no significant differences between the study groups (study II, III). The most prominent changes were myocyte degeneration, interstitial edema and hemorrhage as well as pericardial inflammation, edema and hemorrhage. A trend of r-hirudin associated decrease in myocardial inflammatory cell infiltration was seen. No inflammatory cells were observed in vascular walls in r-hirudin group while vasculitis was present in two animals in the placebo group (study II). Instead, in the AT group, endothelial activation and vasculitis were more pronounced than in the placebo group (study III). Obliterated or thrombosed vessels were not observed in cardiac samples (study II, III).

7.2. Lung

In lung biopsies I/R injury-related histological alterations were less pronounced than in myocardial biopsies (study III). The most prominent change was alveolar hemorrhage which was significantly less severe in the AT group than in the placebo group (10.22 ± 0.44. vs. 25 ± 0.75, $p = 0.002$). Other notable changes were endothelial activation and interstitial hemorrhage but these changes did not reach statistical significance. Vascular lesions were surprisingly modest and no obliterated or thrombosed vessels were observed in lung samples (study III).

8. Apopotosis (study II)

Apoptotic DNA fragmentation was detected significantly less frequently in the r-hirudin group than in the placebo group ($0.05 \pm 0.06\%$ vs. $0.13 \pm 0.07\%$, $p = 0.042$). Caspase 3 positive myocytes were detected mainly focally concentrated at the borderzones of the injured areas. No statistical difference was seen in caspase 3 activity between the two groups.

9. Intestinal mucosal perfusion

Tonometric measurements showed that mucosal perfusion was not altered or only mildly deteriorated during CPB (study I, III, IV). Instead, during reperfusion between 30 and 120 minutes after the aortic clamp release a progressive mucosal hypoperfusion developed in all groups as evidenced by significantly increased $piCO_2$

and decreased pHi values (study I, II, IV). The increase in $piCO_2$ and the decrease in pHi was more profound in the placebo group than in the r-hirudin group but the intergroup difference was not statistically significant (study I). A progressive local microcirculatory hypoperfusion was evidenced also in gaps between pHa and pHi (pH(a-i)) and $paCO_2$ and $piCO_2$ (pCO_2(i-a)) (study I, III, IV). Progressive intestinal hypercapnia and acidosis developed between 30 and 120 min after the clamp release in the placebo groups (study I, III). Remarkably, no such local increments in acidosis or hypercapnia were observed in the r-hirudin group (study I). The rise in p(i-a)CO_2 from 30 to 120 minutes after clamp release was 5.82 ± 6.65 kPa and 0.79 ± 1.7 kPa in the placebo and r-hirudin groups, respectively ($p = 0.044$). Corresponding values for pH(a-i) were 0.21 ± 0.21 vs. 0.018 ± 0.08 ($p = 0.024$) (study I). In the AT group, no such positive effects were observed (study III).

10. Intestinal post CPB inflammatory response and mucosal perfusion

The difference between the baseline MPO value and the MPO value at the end of the experiment was taken as a measurement of inflammatory response. This definition allowed division of the animals to eight animals with an increase in MPO activity (called MPO responders or MPO+ group in the following) and to nine animals without a MPO increase (MPO-non responders or the MPO- group). The validity of this group division was tested by comparing histological injury between the two groups. MPO+ and MPO- groups proved to differ clearly and rather specifically regarding the scoring of histological injury. In both groups, intestinal biopsies showed signs of I/R injury-related histological alterations. The most prominent changes were moderate inflammation throughout the intestinal wall, extensive edema, and lymph vessel dilatation. No obliterated or thrombosed vessels were seen despite the signs of endothelial activation. The difference between MPO+ and MPO- groups was that MPO+ animals showed significantly more myocyte degeneration, endothelial activation, and vasculitis. All subsequent analysis were carried out by comparing MPO+ and MPO– groups to each other.

In the MPO- group, but not in the MPO+ group, a progressive mucosal hypoperfusion between 30 and 120 minutes after the clamp release was significant as evidenced by $piCO_2$, pHi, and corresponding gap values. However, statistically significant intergroup differences were not observed.

MPO+ and MPO- groups did not show significant differences in hemodynamic parameters (HR, CO, SV, SAP, MAP, SV, LVP, PAPD, PCWP). SVR increased during the experiment in both groups but after the CPB increase was significant ($p= 0.044$) only in the MPO+ group. However, no significant intergroup difference was observed.

ACT was well over 400 seconds after initiation of CPB and during CPB in both

groups. Plasma levels of TAT increased in MPO+ and MPO- groups significantly from pre-CPB value 2.8 ± 1.5 and 2.6 ± 1.0 to 30.1 ± 21.3 and 41.6 ± 29.2, respectively ($p < 0.05$) during CPB and aortic clamping (Fig 4). There was no difference in TAT levels between the two MPO groups during CPB and reperfusion

11. Blood gases

Progressive decrease in arterial pH was observed in all groups (study I, III). Arterial oxygenation was efficient as pO_2 increased during CPB but returned and remained at near pre-CPB values after CPB in all groups (study I, III). No statistically significant differences between the study groups were observed.

12. Bleeding

Hemoglobin decreased in all groups because of hemodilution during CPB (study I, III). The only group showing a more pronounced drop was the r-hirudin group (study I). Hemoglobin was significantly lower in the r-hirudin group compared to the placebo group at 60, 90, and 120 minutes after aortic declamping (study I). Enhanced bleeding was seen in the r-hirudin group (1753 ± 671 mL r-hirudin vs. 850 ± 376 mL placebo, $p = 0.007$). Blood collected from mediastinal and pleural spaces, which was returned to CPB circuit, was 500 ± 236 mL in the placebo group and 1200 ± 615 mL in the r-hirudin group ($p = 0.011$). However, only blood collected from abdominal cavity into a separate reservoir which was not returned to the animal, presented net blood loss. This was not significantly different between the two groups (530 ± 296 mL vs. 325 ± 223 mL, $p = 0.126$, r-hirudin vs. placebo, respectively) (study I). AT (250 IU/kg) did not cause significantly enhanced bleeding (study I). Instead, 500 IU/kg of AT caused significantly enhanced bleeding from mediastinal and pleural spaces compared to the placebo group (610 ± 195 mL vs. 291 ± 130 mL, respectively, $p < 0.001$) (study III). Bleeding from the abdominal cavity was not significantly different between the AT+ and placebo group (546 ± 255 mL vs. 304 ± 373 mL, respectively, $p = 0.18$).

Parameter	Hirudin	AT 250 IU/kg	AT 500 IU/kg
ACT	↑	↑	↑
TAT	↓	—	—
CO (mL/min	↑	⇡	↑*
SV (mL)	↑	⇡	↑*
HR (beats/min)	—	—	—
MAP (mmHg)	—	—	—
SVR	↓	—	—
PADP (mmHg)		⇣	↓
PVR		—	↓
Hb (g/L)	↓	—	
pHi	⇡	—	—
p(i-a)CO_2 (kPa)	⇣	—	—
pH(a-i)	⇣	—	—

Table 1. Summary of the central results. Overall, AT showed part of the positive results seen in r-hirudin animals, suggesting similar but less efficient effect through thrombin inhibition. Dark arrow indicates significant effect, dotted arrow a trend, and a line no effect when compared to placebo. * Of note, the effect of AT (250 and 500 IU/kg) on cardiac output (CO) and stroke volume during the first 15 minutes after CPB.

DISCUSSION

1. Methodology

This animal model was established to resemble clinical cardiac surgery and to produce clinically relevant data on CPB related adverse effects and I/R injury in myocardium, lung, and intestine. The constant hemodynamic findings in the model include loss of approximately 50% of SV and CO during ischemia with no subsequent recovery, transient increase of SVR, and increases in PVR and PADP during reperfusion. These findings are qualitatively in good accordance with various other experimental models of I/R injury (Carteaux et al. 1999, Chai et al. 1999, Eising et al. 2000, Salvatierra et al. 2001, Schreiber et al. 2006, Vähäsilta et al. 2001) and with experience from clinical CPB surgery (Cohen et al. 1999, Guru et al. 2006, De Hert et al. 1996, Kloner et al. 1994, Riedel 1999).

Thrombin is an enzyme with pleiotrophic effects on cells including platelets, leukocytes, endothelial cells, and cardiomyocytes as well as on several circulating coagulation factors. On the other hand, the complex interacting cellular and enzymatic mechanisms of ischemia-reperfusion injury makes it most difficult to study potential pathophysiological significance of thrombin per se during clinical ischemia-reperfusion injury. Therefore, we chose to study r-hirudin, the most thrombin-specific clinical anticoagulant and AT, which is physiological non-specific inhibitor of thrombin possessing also anti-inflammatory actions independently of its effects on coagulation. Thus, we feel the current approach to be a reasonable effort to better understand the potential pathophysiological significance of thrombin activity after CPB.

To address the question of potential significance of inflammation in the I/R injury, it was mandatory to have as efficient heparin anticoagulation as possible. To this end, the current experimental setting proved to be successful. On histological evaluation, no occlusive clots in myocardial or pulmonary tissue could be observed in control animals. In contrast, in the absence of thrombosis, other histological features of I/R injury were present in corresponding tissue specimens. Thus, the current model was feasible to evaluate potential effects of r-hirudin and AT on inflammation under heparinization that is compatible with the clinical use of heparin in cardiac surgery.

For the hypothesis whether the inflammatory response in the gut wall would associate with the mucosal perfusion, the extent of the inflammatory injury as well as the extent of coagulopathic (i.e. thrombosis or bleeding) tissue injuries are important as the latter would result in a model associated confounding effect. In the current model all intestinal biopsies showed signs of I/R injury-related histological alterations. The most prominent changes were moderate inflammation throughout the intestinal wall, extensive oedema, and lymph vessel dilatation. Another notable change was endothelial activation. In accordance with the steady heparinization, vascular lesions

remained rare and no obliterated or thrombosed vessels were observed. Further, hemorrhage in the intestinal wall remained sparse. Thus, methodologically the model and experimental conditions were considered suitable to address the question of association of a non-extreme and individually variable tissue inflammatory reaction and mucosal perfusion without significant confounding effects from thrombosis or bleeding.

2. The effects of r-hirudin on CPB-induced I/R injury

Generation of thrombin increased after initiation of CPB, after release of aortic clamp and during reperfusion in accordance with previous studies (Boisclair et al 1993a, Boisclair et al. 1993b, Brister et al. 1993, Eisses et al. 2004, Fernandez et al. 2006, Raivio et al. 2006). In the current study, the effect of direct thrombin inhibition on coagulation throughout drug infusion was evidenced by highly prolonged ACT values in the animals receiving r-hirudin. Instead, a significant effect on thrombin generation developed during reperfusion when the animals in the placebo group showed rapid escalation of thrombin generation but the animals receiving r-hirudin did not.

During CPB, the surgical wound is the primary source of thrombin formation (Edmunds and Coleman 2006, Hattori et al. 2005, Philippou et al. 2000, Sturk-Maquelin et al. 2003) but the source of the systemic escalation of thrombin generation during reperfusion is not known. The reperfusion induced systemic burst of thrombin generation can be explained only partly by local tissue factor expression in reperfused post-ischemic myocardium, as transcoronary markers of thrombin generation are modest (Kalweit et al. 2005). Increased serum levels of TAT have been observed after total intestinal ischemia and reperfusion in experimental settings (Schoots et al. 2004, Tsuboi et.al 2007). However, possible contributions of the splanchnic or pulmonary vascular beds to the reperfusion induced thrombin generation after CPB are not known.

Ischemia-reperfusion injury to the heart after global ischemia caused depressed myocardial function and impaired myocardial recovery during reperfusion. Inhibition of thrombin has been shown to have beneficial effects in experimental studies of myocardial I/R injury (Erlich et al. 2000, Mirabet et al. 2005, Strande et al. 2007) but the effects of direct thrombin inhibition on myocardial ischemia-reperfusion injury after cardioplegic arrest during CPB have not been studied. This study tested whether early functional recovery of post- ischemic myocardium could be improved by inhibiting reperfusion induced thrombin by r-hirudin. As a result, we found that r-hirudin receiving animals could regenerate approximately one half of the ischemia induced loss of CO during reperfusion while practically no such myocardial functional recovery was observed in the placebo group. At 60 min after

clamp release, the animals receiving r-hirudin showed 86 % of the preperfusion SV while the corresponding average figure for control animals was 49 %. The effect seemed rather specific to myocardium as no differences in heart rate, blood pressure or vascular resistance were observed between the study groups during the immediate post-ischemia period. Thus, the current data from the in vivo experimental setting offer logical support to the previous in vitro demonstration that thrombin may directly inhibit myocyte contractile function (Hird et al. 1995).

Thrombin is a pleiotrophic enzyme contributing to regulation of coagulation, inflammation, and apoptosis (Ahmad et al. 2000, Brass 2003, Choi et al. 2003, Donovan et al. 1997, Esmon 2005, Lane et al. 2005, Mann et al. 2003, Turgeon et al. 1998). Therefore, potential effects of thrombin inhibition on procoagulatory, proinflammatory, and proapoptotic features of myocardial I/R injury were explored. At histological evaluation, no occlusive clots in the myocardial microvasculature could be observed in the control animals. In contrast to the lack of thrombosis, other histological features of I/R injury were abundantly present in myocardial specimens from control animals. A significant role for thrombin in myocardial I/R injury by a proinflammatory mechanism independent of activation of coagulation and thrombus formation has been suggested (Erlich et al. 2000). In the current study, ischemia-reperfusion injury-related inflammatory changes including interstitial edema and inflammatory cell accumulation tended to be less prominent in the r-hirudin group. While the current experimental groups were too small to exclude or confirm modest anti-inflammatory effects of thrombin inhibition, the logical trends in several inflammation related parameters support the previously suggested proinflammatory role for thrombin in the I/R injury (Erlich et al. 2000).

Cardiomyocyte apoptosis is known to be present in myocardium during reperfusion after cardioplegia-induced arrest (Fischer et al. 2003, Freude et al.2000, Malmberg et al. 2006, Schmitt et al. 2002, Vähäsilta et al. 2005, Yeh et al. 2003) and may be involved in myocardial dysfunction after cardiac arrest (Anselmi et al. 2004) but the direct evidence is sparse (Schmitt et al. 2002). The present study demonstrated that inhibition of thrombin improved post-ischemic cardiac recovery and was simultaneously associated with attenuated apoptosis. The number of cardiomyocytes with DNA-fragmentation (TUNEL positivity) was significantly lower in animals receiving r-hirudin when compared with the control animals. The fact that we found caspase-3 positivity not to be affected by r-hirudin treatment may be associated with possible inhibition of an apoptotic process downstream of caspase activation (Jäättelä et al. 1998) or it may be related to the timing of myocardial biopsies in relation to the timescale of apoptotic cascade (Suzuki et al. 2001). Even a small number of myocytes affected by apoptosis may have significant impact on cardiac contractility, because single cell death may impinge upon the force generating ability of neighbouring cells depressing more severely overall muscle performance (Cheng et al. 1995). Thus, in the current setting the functional improvement in r-hirudin receiving animals might, at least partly, be attributed to inhibition of the proaptotic

effect of thrombin. However, our data do not allow establishing a direct causal relationship between thrombin inhibition, inhibition of apoptosis and improved post-ischemic cardiac performance.

One of the hemodynamic consequences of CPB may be a progressive increase in SVR. This results from complex humoral responses to surgery, CBP, and hypothermia (Carteaux et al. 1999, Kam et al. 1996). In the current study, infusion of r-hirudin prevented the increase in SVR after CPB. This effect could theoretically either improve or worsen the peripheral circulation depending on the compensatory capacity of the heart. Instead of possible systemic hypotension, myocardium was able to generate better SV and CO and so compensate the peripheral, potentially hypotension promoting effects of r-hirudin. Thus, a net effect most probably was enhanced flow in various microcirculatory vascular beds.

CPB results in reduced intestinal intramucosal blood flow, mucosal acidosis and dysfunction (Doguet et al. 2004, Ohri et al. 1994a, Ohri et al. 1994b, Ohri et al. 1997, Stamler et al. 1998, Tao et al. 1995, Tofukuji et al. 2000). In the current study, intestinal tonometry showed progressive intestinal hypoperfusion during reperfusion after CPB. However, because of several confounding factors, quantitating intestinal perfusion should rather be based on the gap between arterial and intestinal pCO_2 than on the intramucosal pH alone (Heino et al. 1998, Kolkman et al. 2000, Knichwitz et al. 1998, Vincent and Creteur 1998). To this end, the gap between the intestinal and arterial pCO_2 indicated severe splanchnic hypoperfusion in both groups. One possible explanation is bleeding seen in both groups, resulting in a decrease in hemoglobin and relative hypovolemia, which has been shown to induce splanchnic vasoconstriction and redistribution of the peripheral blood flow (Edouard et al. 1994). While r-hirudin evidently did not protect the animals from intestinal hypoperfusion, significant beneficial effect could still be demonstrated when compared with the control animals. The increasing differences between pH and pCO_2 gaps during 30–120 min of reperfusion indicates that microcapillary perfusion significantly deteriorated in the placebo group while in the r-hirudin group this phenomenon was not observed. This effect was probably mostly due to an enhanced cardiac output seen in the r-hirudin group. In addition, since low hemoglobin reduces oxygen transport and there was a hemoglobin difference in favor of the control group, the significance of CO may further increase.

3. The effects of antithrombin on CPB-induced I/R injury

Prolonged ACT values after AT supplementation indicated a clear AT induced anticoagulant effect during already strong heparin anticoagulation. However, the supplementary AT did not prevent thrombin generation during CPB.

AT did not significantly improve post ischemic myocardial recovery but it

should be noted that within the intervention group, AT was associated with small but significant recovery of SV and CO in a dose dependent manner. However, this positive effect was not carried over to the later phases of reperfusion.

Supplementation of AT (250 IU/kg) has alleviated I/R injury in the liver (Okano et al. 1995), kidney (Mizutani et al. 2003), lung (Salvatierra et al. 2001), and intestine (Özden et al. 1999). This protection has been largely attributed to the anti-inflammatory effects of AT. Scarce and controversial data of AT effects in myocardial I/R injury are available. In an isolated rat heart model physiological AT levels had no influence on myocardial I/R injury but high AT levels actually worsened tissue injury (Margreiter et al. 2003). In the current study, AT did not show anti-inflammatory effects on I/R injury in the myocardium at the level of histology by light microscopy. Neither did AT alleviate post ischemic TnT release from myocardium. The transient increase in MPO levels seen in the placebo group during the first five minutes of reperfusion was not seen in the AT group. This might suggest that AT interfered with early reperfusion induced neutrophil recruitment into the post-ischemic myocardial vascular bed. However, the possibility of more subtle anti-inflammatory effects of AT remain to be investigated.

AT did not show any influence on systemic vascular resistance. Instead, AT attenuated significantly reperfusion induced increase in pulmonary pressure in a dose dependent manner and also attenuated reperfusion induced increase in PVR when a higher dose (500 IU/kg) was given suggesting that the AT effect was preferential to the pulmonary vascular bed. Pulmonary hypertension including increased pulmonary artery pressure (PAP) and PVR are well documented phenomena in experimental studies (Carteaux et al. 1999, Chai et al. 1999, Salvatierra et al. 2001). CPB and I/R- induced perturbations in endothelial function have been suggested to contribute to reversible pulmonary hypertension during post CPB period (Carteaux et al. 1999, Fortier et al. 2004, Wan et al. 1997b, Wessel et al. 1993). In an experimental lung transplantation model in dogs, AT supplementation reduced the local inflammatory response and reduced pulmonary vascular resistance (Salvatierra et al. 2001). However, the influence of AT on CPB- and surgery-related pulmonary I/R injury has not been tested before.

In the current study, inflammatory cell exudates were not observed in either the pulmonary interstitium or in the alveolar space, a significant difference from the post lung transplant situation (Salvatierra et al. 2001). Endothelial swelling and proliferation were also present but not affected by AT. However, while AT evidently reduced functional adverse effects of lung injury after CPB, the underlying mechanisms remain unclear.

Intestinal tonometry showed progressive intramucosal acidosis and hypercapnia during and after CPB indicating reduced microvascular blood flow to the intestinal mucosa. This may be due, at least in part, to continuously decreasing CO during reperfusion. CPB and a systemic inflammatory state is associated with mesenterial endothelial injury and dysfunction with abnormal vascular reactivity, which may

contribute to the regulation of intestinal microcirculation (Doguet et al. 2004, Tokufuji et al. 2000). Previous studies suggesting that AT attenuates the local inflammatory reaction and protects from intestinal I/R injury (Tsuboi et al. 2007, Özden et al. 1999) were not supported by the data from the current CPB setting as both SVR and the compromised intestinal tissue perfusion remained unaffected by AT.

4. R-hirudin versus antithrombin

Overall, our results suggest that r-hirudin is more effective than AT in reducing the adverse effects of myocardial I/R injury during CPB. Dosing of r-hirudin was chosen according to the standard clinical recommendations in the treatment of patients with heparin induced thrombocytopenia. R-hirudin directly inhibits the active site pocket and fibrinogen binding site of free and clot bound thrombin (Weitz et al. 1990, Weitz et al. 1998). R-hirudin, in relative difference to heparin, primarily inhibits thrombin activity instead of thrombin generation (Eichinger et al. 1995, Zoldehyi et al. 1994). However, thrombin activates several clotting factors and amplifies its own formation. Thus, blocking thrombin activity by r-hirudin might secondarily inhibit further thrombin formation (Pernerstorfer et al. 2000). While the more extensive hemodilution in the r-hirudin group may have contributed to the observed difference in TAT-concentration, the data suggests that r-hirudin may be an effective inhibitor of reperfusion induced thrombin formation in addition to being a direct inhibitor of preformed thrombin.

Antithrombin (AT) is a major physiological anticoagulant inhibiting thrombin and other proteases of the coagulation (Roemisch et al. 2002, Rosenberg 1989). The AT dose of 250 IU/kg was selected because it has been used efficiently in several experimental animal studies (Mizutani et al. 2003, Okano et al. 1995, Salvatierra et al. 2001, Özden et al. 1999) and has been used safely in clinical cardiac surgery (Levy et al. 2002).

Prolonged ACT values after AT supplementation indicated that human AT was an active anticoagulant in porcine plasma. Increased TAT levels in the AT receiving animals may indicate that newly generated thrombin was bound to and inhibited by AT. Thus, one could conclude that less free thrombin is present in those animals receiving AT resulting in reduced thrombin activity. However, supplementary AT showed no protective effects on myocardial I/R injury suggesting that AT did not effectively inhibit thrombin activity. On the other hand, the anticoagulant effect of AT measured by ACT does not show the direct inhibitory effect of thrombin as AT also significantly influences the activity of several other clotting factors in coagulation. In addition, the data do not allow the evaluation of whether the apparently higher TAT levels in the AT group represented paradoxically increased rate of thrombin generation or, probably more likely, an artifact induced by different levels of circulating AT in

the experimental groups. One must also bear in mind that the interactions of a human AT with a porcine thrombin are not known.

The major issue is potential interference of the pharmacological heparin with the physiological function of AT. The main mechanisms suggested for AT's anti-inflammatory, non-anticoagulant, effects is the release of prostacyclin (PGI_2) mediated by AT interaction with endothelial cell surface glycosaminoglycans (GAG) (Mizutani et al. 2003, Salvatierra et al. 2001, Uchiba et al. 1995, Uchiba et al. 1998). Simultaneous infusion of heparin and AT could, while enhancing AT-heparin complex formation in plasma, paradoxically reduce AT-GAG coupling on endothelial surfaces resulting ultimately in reduced instead of enhanced AT functionality (Pulletz et al. 2000, Roemisch et al. 2002, Uchiba et al. 1995).

5. Intestinal post CPB inflammatory response and mucosal perfusion

The main result of this study (IV) was the lack of association between progressive hypoperfusion of the intestinal mucosal during reperfusion after CPB and inflammatory response measured with MPO and histology, two indicators of net neutrophil activation and tissue sequestration.

Only a few studies defining correlates of the CPB related systemic inflammatory response with the local inflammatory response of the gut wall exist (Doguet et al. 2004, Tokufuji et al. 20005). We are unaware of any studies analyzing specifically the relationship between the local gut wall inflammatory response with the functional mucosal injury. Therefore, our study is valuable in simulating more closely the clinical situation and still determining some central features of the local inflammatory response.

I/R injury of the intestine is a complex and multifactorial pathophysiologial process (Mallick et al. 2004, Schoenberg and Beger 1993). The hypothesis that activated neutrophils play a central role in intestinal I/R injury is supported by several studies (Cooper et al. 2004, Granger 1988, Grisham et al. 1986, Hayward and Lefer 1998, Hernandez et al. 1987, Oktar et al. 2002, Schoenberg et al. 1991, Sun et al. 1999, Özden et al. 1999). The current experimental conditions resulted only in modest changes in MPO activity during the experiment which is in significant contrast to commonly up to 5-fold increase seen in models of complete arterial ischemia followed by reperfusion (Grisham et al. 1986, Hayward and Lefer 1998, Oktar et al. 2002, Sun et al. 1999, Özden et al. 1999). In a comparison between MPO+ and MPO- groups we found that increased MPO activity was associated with less pronounced hypoperfusion of intestinal mucosa and increased SVR. This finding is novel and somewhat confounding. Under conditions of compromised CO, stable systemic blood pressure and central venous pressure, one could expect an increase in SVR

to reduce the perfusion in respective vascular beds. However, the fact that the SVR increase was more pronounced in the MPO+ group with better mucosal oxygenation during reperfusion suggests that mucosal oxygenation during reperfusion after CPB may be affected by other factors than the oxygen content of the blood and total flow through the splanchnic microcirculation. In addition, activated neutrophils may be one of the factors regulating vascular resistance after CPB, as has been earlier shown with other experimental models (Kamler et al. 1997, Ostrovsky et al. 1997).

6. Clinical implications

R-hirudin has proven to be a safe and effective anticoagulant in patients undergoing CPB (Greinacher and Lubenow 2001, Riess et al 1997). However, based on difficulties in monitoring, rather long half-life, possible enhanced bleeding tendency, and the lack of specific antidote (Greinacher and Lubenow 2001) no recommendation for human use of r-hirudin combined with heparin can be derived from the current study. The current positive hemodynamic effects of r-hirudin should be taken as demonstration of potential of improved control of thrombin using the most direct and specific thrombin inhibitor clinically available rather than a practical solution for the problem. Further studies should be conducted to test whether other thrombin modulators might have the same beneficial effects with less bleeding.

CONCLUSIONS

1. R-hirudin may be an effective inhibitor of reperfusion induced thrombin formation in addition to being a direct inhibitor of preformed thrombin. Infusion of thrombin inhibitor r-hirudin during reperfusion was associated with attenuated post-ischemic left ventricular dysfunction and decreased vascular resistance, which may potentially improve oxygen delivery to reperfused vascular beds.
2. Improved recovery of myocardium during early post-ischemic period was associated with significantly less cardiomyocyte apoptosis and with a trend in anti-inflammatory effects. Thus, inhibition of reperfusion induced thrombin may offer beneficial effects by mechanisms other than direct anticoagulant effects.
3. Supplementary AT, in doses with significant anticoagulant effect, did not alleviate myocardial I/R injury in terms of functional recovery, histological inflammatory changes or post-ischemic troponin T release. Instead, AT attenuated reperfusion induced increase in pulmonary pressure after CPB.
4. A local inflammatory response in the gut wall was not associated with perturbed mucosal perfusion after CPB. Thus, mechanisms of low-flow reperfusion injury during CPB can differ from the mechanisms of the total ischemia reperfusion injury.

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