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# Anti-Inflammatory Treatment Prevents Local and Systemic Effects of Skeletal Muscle Ischemia/Reperfusion Injury

PhD Thesis submitted by

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***To my family***

*Courage is the beginning of an act,*

*but luck controls the end.*

*- Democritus –*

## Preface

Ischemia/reperfusion injury (IRI) of lower extremities may result from thrombotic occlusion, arterial embolism, trauma or surgical intervention requiring tourniquet application with prolonged ischemia and subsequent restoration of circulation. In severe cases skeletal muscle IRI is associated with considerable pathophysiological alterations resulting in high morbidity rates. Consequently, the demand for therapeutic approaches attenuating or even preventing skeletal muscle IRI increases.

The aim of my PhD was to analyze the efficacy of various therapeutic approaches for reduction of skeletal muscle reperfusion injury by using a rat model of lower extremity IRI and provide a better understanding of the underlying mechanisms During the first series of experiments, all rats were subjected to 24 h reperfusion, which is inevitably associated with severity grade 2, since the rats are awake during reperfusion. It was my personal concern to refine the model and reduce potential pain and discomfort by shortening the reperfusion time, but still have a good model to analyze the effect of drug candidates on IRI and thereby avoiding unnecessarily high severity grades in future experiments.

This thesis includes 5 manuscripts, where I am the first author. The introduction of this thesis will be submitted as an invited review article for the journal *Biochemical Pharmacology* and runs under the title “Ischemia/reperfusion injury: Effect of simultaneous inhibition of plasma cascade systems versus specific complement inhibition” It gives an overview of IRI and focuses on the role of the plasma cascade systems in reperfusion injury. Furthermore, this thesis contains 4 original articles, which are accepted (Paper I), submitted (Paper III and IV) or in submission (Paper II). Each article is introduced by a cover page, which gives a brief overview of the scientific contents. This thesis ends with a conclusion, acknowledgements and curriculum vitae.

At the end of this preface, I want to express my gratitude to my supervisor Prof. Dr. Robert Rieben, who gave me the chance to learn in his lab and always supported me with the necessary motivation for the success. Furthermore, I would like to wholeheartedly thank my husband for his daily support, his encouraging words in hard times and simply for his love.

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## Abstract

Ischemia/reperfusion injury (IRI) of lower extremities is of crucial clinical importance, at present, no effective treatment or preventive measure is available for this clinical entity. Peripheral reperfusion injury does not only result from thrombotic occlusion, arterial embolism, trauma or transplantation, but is also a significant problem in prolonged and complicated surgical interventions requiring tourniquet application. IRI causes significant local damage of skeletal muscle tissue and, in severe cases, also remote organ damage, particularly to the lung, which may lead to multiorgan dysfunction.

This thesis investigates in detail the underlying mechanisms of skeletal muscle IRI and associated distant organ damage by analyzing the impact of the plasma cascade systems including the complement, the coagulation as well as the kinin systems. In order to study lower extremity IRI a reality-driven rat hind limb model was initially established and refined later in accordance with the 3R principles (Reduction, Replacement, Refinement).

The main objective of this thesis was to find a suitable therapeutic approach to attenuate or even prevent skeletal muscle IRI while, at the same time, clarifying the underlying mechanisms. Using the multifunctional protease inhibitor C1 esterase inhibitor, which acts on all the plasma cascade systems, led to a significant reduction of skeletal muscle IRI as well as distant organ damage, whereas the potent complement-specific inhibitor APT070 did not. Treatment with dextran sulfate (DXS), acting on complement and coagulation did not result in reduction of IRI either, although in previous studies of myocardial infarction a marked reduction of injury was shown.

In summary, the present thesis emphasizes the involvement of the plasma cascades in skeletal muscle IRI and highlights the importance of the kinin system.

# 1. Introduction

Ischemia is the clinical condition of an impaired circulation, which results in an undersupply of oxygen and nutrients in the affected tissue. Ischemia can be induced through thrombosis or embolism, but also during surgery via tourniquet application and transplantation. The absence of blood and oxygen causes time-dependent molecular as well as structural changes. In general, all organs are susceptible to ischemia, however susceptibility to the ischemic insult differs between organs. Brain tissue can cope with the ischemic burden for only a few minutes, whereas muscle tissue is able to withstand ischemia for 60 to 90 minutes without showing irreversible damage. Restoration of blood flow, termed as reperfusion, is the only effective treatment to prevent irreversible damage and necrosis of the ischemic tissue. Paradoxically, reperfusion activates a complex inflammatory response, which may finally lead to ischemia/reperfusion injury (IRI). Thus, after prolonged ischemia also reperfusion of the affected tissue can result in irreversible cell damage or necrosis as well as microvascular and endothelial injury depending on the time and severity of ischemia, for review see [1, 2].

Already in 1960, Jennings et al. described the deleterious effect of reperfusion of ischemic tissue in a canine model of myocardial IRI.[3] As compared to the induction of permanent ischemia, the authors reported an acceleration of cellular necrosis after induction of transient ischemia and subsequent reperfusion.[3] Typically, myocardial ischemia and subsequent reperfusion manifests in arrhythmias, microvascular dysfunction, myocardial stunning as well as myocyte death.[2] As seen in transplantation, IRI of the lung is characterized by non-specific alveolar damage, edema formation as well as hypoxemia. The clinical spectrum of lung IRI may range from mild hypoxemia to acute respiratory distress syndrome.[4, 5] As compared to other organs, the brain is particularly susceptible to ischemia, as it suffers irreversible neuronal damage after only 5 minutes of complete ischemia, which can be attributed to its high metabolic rate.[6, 7] For brain ischemia, as occurring in stroke, induction of reperfusion through thrombolysis or thrombectomy is only beneficial, if it is executed before the process of infarction has been completed.[8, 9] However, induction of reperfusion by using thrombolytic agents, such as urokinase seems to be very critical, as patients may suffer cerebral reperfusion injury manifesting in intracranial hemorrhage as well as fatal cerebral edema formation.[10] Renal IRI typically occurs in the setting of transplantation, which is of particular importance.[11] Renal IRI can develop to renal failure, which is characterized by an abrupt loss of the renal function to excrete wastes, concentrate urine, conserve electrolytes or maintain fluid balance and results in high mortality rates.[12] The cortical - medullary region is the most susceptible region to tubular injury, inflammation and vascular alterations.[13]

As compared to brain, heart, lung and kidney, the liver has a clear advantage based on its unique dual blood supply coming from the hepatic artery and the portal vein and therefore arterial impairments can be counterbalanced. However, serious alterations in blood supply are found in clinical settings of portal vein obstruction, transplantation, surgical intervention or trauma, which cause high morbidity and mortality.[14] Reperfusion of the hepatic ischemic tissue leads to an increase of liver enzyme, biliary strictures and in severe cases to dysfunction or failure. Of particular importance is the deleterious effect of liver IRI on other organs, which occurs secondary to liver injury.[15]

However, not only organs can be crucially affected from ischemia, but also muscle tissue, which may occur through tourniquet application during surgical intervention, trauma or thrombosis and lead to considerable pathophysiological alteration. Characteristic clinical entities of skeletal muscle IRI are edema formation, loss of muscle viability, as well as necrosis in the affected muscle tissue.[16] In severe cases, rhabdomyolysis can result in systemic complications, such as distant organ damage due to myoglobin release, cardiac arrhythmias, compartment syndrome as well as disseminated intravascular coagulopathy.[17]

Reperfusion of ischemic tissue initiates a complex inflammatory response without any involvement of pathogenic triggers, known as sterile inflammation, whereby endogenous molecules may act as alarmins or danger-associated molecular patterns (DAMPs).[18, 19] The immune response is stimulated through self-antigens, which are functional components of intact cells, but become stimulators of innate immunity when released through necrosis or secreted from injured cells.[19, 20] In 1996 Weiser et al. described a novel mechanism for reperfusion injury that involves antibody deposition and activation of complement leading to an acute inflammatory response.[21] One decade later, Zhang and Carroll et al. introduced the concept of innate autoimmunity, which is based on the finding that naturally circulating antibodies recognize self-antigens and elicit an acute inflammatory response involving the complement system.[22, 23] However, the last years of extensive research in reperfusion injury has shown that also other plasma cascade systems, including the coagulation as well as the kinin systems are of major importance. Clinical entities, like extremity IRI, myocardial infarction, stroke and also organ transplantation are associated with high morbidity and mortality rates, but an effective treatment is still missing. Therefore, therapies targeting the plasma cascade systems seem to be promising for attenuation or even prevention of IRI. The following overview is not intended to be exhaustive, but rather aims to give a brief overview of the mechanisms of IRI and mainly focuses on the role of the plasma cascade systems in reperfusion injury.

## 2. Ischemia/reperfusion-induced cellular and vascular changes

### *2.1.* Hypoxia-related cellular changes and ROS formation

During ischemia a temporary deprivation of oxygen occurs, termed as hypoxia, which affects all cellular functions, including cellular metabolism, enzymatic activity, mitochondrial function and results in cytoskeletal restructuring and modification of membrane transport. In a homeostatic condition large quantities of adenosine triphosphate (ATP) are generated by the mitochondrial oxidative metabolism to ensure energy-dependent processes, such as muscle contraction. During ischemia, the lack of oxygen leads to an impairment of oxygen-dependent processes, finally resulting in decreased ATP levels, activation of anaerobic glycolysis and related intracellular production of lactic acid as well as a drop of pH and a cytoplasmic excess of hydrogen ions. The excess of hydrogen ions activates the $Na^+/H^+$ pump, which exchanges $H^+$ ions for $Na^+$ ions. Under normal conditions a $Na^+/K^+$ ATPase is activated, however the lack of oxygen and ATP impairs a proper function, resulting in a loss of the $Na^+/K^+$ gradient. Thus, a cytosolic excess of $Na^+$ builds up, which in turn initiates the exchange of $Na^+$ for $Ca^{2+}$ via the $Na^+/ Ca^{2+}$ antiporter leading to a massive and rapid overload of cytosolic and mitochondrial $Ca^{2+}$. Additionally, the impaired production of ATP results in raised adenosine monophosphate (AMP) via adenylate kinase, which leads to the generation of xanthine, for review see Kharbanda et al. and Solaini et al.[24, 25]

Induction of blood flow restores oxygenation of the ischemic tissue and paradoxically deteriorates the ischemic condition. Through reoxygenation xanthine is processed by xanthine oxidase to superoxide, which can be further processed to other reactive oxygen species (ROS), primarily hydrogen peroxide, which are early mediators of IRI.[26] ROS is also generated as a byproduct of NADPH oxidases or uncoupled endothelial nitric oxide synthases and might be used as a determinant of endothelial function after IRI.[27, 28]

ROS formation cannot be exclusively attributed to the reperfusion period, as it was shown that mitochondrial ROS can also be generated during ischemia.[29] ROS are highly reactive and their formation causes oxidation of proteins, membrane phospholipids or nucleic acids. However, low ROS generation occurs also in homeostatic cellular conditions during metabolic processes and plays key roles in cell signaling, immune defense, antibacterial action and vascular tone regulation.[30-34] In order to uphold these functions, ROS formation needs to be strictly controlled. Therefore, a complex network of antioxidants is constantly acting to maintain redox homeostasis.[35]

## *2.2.* Protective transcriptional changes in ischemia/reperfusion injury

Oxidative stress generated through the hypoxic insult during ischemia activates cellular adaptation strategies. In order to withstand oxidative damage of proteins and lipids, cytoprotective defense mechanisms, including transcriptional changes, are initiated.[36] Therefore, a transcriptional up-regulation of specific cytoprotective genes, which are responsible for buffering cellular antioxidant capacity, is initiated. Cytoprotective gene products are responsible for detoxification of damaging electrophilic by-products of oxidant stress and include, amongst others, glutathione S-transferases, aldehyde dehydrogenases as well as quinone oxidoreductases.[37] A major role is attributed to the transcription factor NF-E2-related factor-2 (Nrf2), which is translocated into the nucleus under conditions of oxidative stress and coordinates up-regulation of cytoprotective genes.[38, 39]

The process of reprogramming during ischemia comprises modulation of metabolic mitochondrial activity through e.g. oxygenases, such as prolyl hydroxylases (PHs) and the transcription factor hypoxia-inducible factor (HIF), which is the main transcription factor at low oxygen levels. The deprivation of oxygen leads to the activation of HIFs, which initiate the transcription of genes contributing to the adaptation to the ischemic condition.[40] HIFs consist of two protein subunits, the constitutively expressed HIF1β, which binds to DNA at hypoxia response elements and HIF1α. HIF1α expression alters depending on oxygen concentration, and activation of transcription only occurs when HIF1α and HIF1β are bound together at hypoxia response elements.[41-43] In a homeostatic condition hydroxylation of the HIF1α subunit through PHs renders it susceptible to proteasomal degradation via ubiquitylation.[44, 45] Another transcription factor involved in mediating hypoxia-induced transcriptional changes is nuclear factor-kB (NF-kB).[46, 47] In a quiescent state NF-kB is held inactive by its inhibitor I-κB and is located in the cytoplasm.[48, 49] Deprivation of oxygen leads to translocation of the released NF-kB to the nucleus, binding to DNA segments and induction of target gene expression.[46, 50, 51] The HIF1α pathway is directly modulated by NF-κB.[52-54] Despite these highly regulated adaptions in order to withstand oxidative damage, increased ROS formation may overwhelm the capacity of antioxidant defense mechanisms and finally result in oxidative stress in the course of ischemia and following reperfusion.

## *2.3.* Cell death programs in ischemia/reperfusion injury

Various stimuli initiating cell death programs, such as apoptosis, necrosis or necroptosis are generated during ischemia and following reperfusion. However, apoptosis does not only occur in pathological conditions, but has also key roles in normal development and homeostasis.[55] Apoptosis, a distinct form of programmed cell death, is typically characterized by cell shrinkage, active membrane blebbing, chromatin condensation as well as fragmentation into vesicles.[56] Caspases, certain cysteine proteases, can initiate these morphological and biochemical changes through an extrinsic or intrinsic pathway. The extrinsic pathway can be initiated through the activation of death receptors of the TNF receptor family (TNFαR), such as Fas (tumor necrosis factor receptor superfamily member 6), which results in the recruitment of caspase-8 and the cleavage of its major target caspase-3.[57] Caspase-8 can alternatively cleave a pro-apoptotic Bcl-2 family protein, called BID.[58] The intrinsic pathway involves activation of caspase-9 via cytochrome c, released due to the permeabilization of the outer mitochondrial membrane, which results from cellular stress.[59, 60] Apoptotic mechanisms ensure a phagocytic removal without any leakage of the intracellular content. In contrast to apoptosis, necrosis proceeds without the action of caspases, with the exception of pyroptosis, which is a form of necrosis where a distinct subset of caspases is involved.[61]

So far necrosis has been commonly accepted to be a non-programmed, unregulated form of cell death, which is initiated as a consequence of overcoming stress. Necrosis is a rapid process with early membrane failure and cell swelling. The release of cellular debris finally results in the initiation of an inflammatory response.[62, 63] Necrosis can also proceed by regulated mechanisms and is then termed as necroptosis.[64] This recently discovered type of cell death has a crucial role in tissue injury, but also in the regulation of the immune system. Necroptosis involves receptor-interacting protein kinase 1 (RIPK1), which forms complexes with RIPK3 to mediate programmed cell death.[65-68] In contrast to earlier findings, where an allocation of apoptosis to the period of reperfusion was hypothesized,[69] it is now believed, that both apoptosis and necrosis can occur during ischemia as well as reperfusion and both contribute to IRI, even though not equally.[70, 71]

## *2.4.* Vascular barrier changes in ischemia/reperfusion injury

Impairment of the vascular barrier can cause changes in endothelial permeability, leading to edema and increased interstitial pressure. In skeletal muscle, this may result in compartment syndrome and altered tissue perfusion.[72] In a homeostatic situation the inner lining of blood vessels, the endothelium, maintains an anti-coagulatory and anti-inflammatory environment (Figure 1).[73] This is, amongst others, upheld by the protective layer of the glycocalyx, which is a negatively charged and tight mesh consisting of proteoglycans, such as syndecans, glypicans or perlecans, glycosaminoglycans including heparan sulfate, chondroitin sulfate, dermatan sulfate, keratan sulfate, associated plasma proteins and hyaluronan.[74] The glycocalyx shields the endothelium and its adhesion molecules on the luminal side and thus prevents the interaction of inflammatory cells with the endothelium.[75] Furthermore, many anti-inflammatory or anti-coagulatory proteins contain a heparin-binding domain, which leads to the interaction with glycosaminoglycans like heparan sulfate. The concentration of these proteins on the glycocalyx ensures the maintenance of the quiescent state. Heparin-binding domain containing proteins include, amongst others, the multifunctional acute phase protein C1 esterase inhibitor, anti-thrombin III, thrombomodulin, tissue factor pathway inhibitor and also the anti-oxidant extracellular superoxide dismutase.[76-80] However, during ischemia the glycocalyx may be partially lost, termed as shedding.[81, 82] In 2007, Rehm et al. showed for the first time that cardiopulmonary ischemia in surgery of the ascending aorta with cardiopulmonary bypass and also infrarenal ischemia in surgery for infrarenal aortic aneurysm in humans causes the shedding of syndecan-1 and also heparan sulfate.[83] Shedding renders the anti-inflammatory and anti-coagulatory state to a pro-inflammatory and pro-coagulatory one and facilitates interactions of leukocytes with the endothelium,[75, 84] leading to enhanced sensitivity of the endothelial cells to activation by cytokines.[85] Changes in the integrity of the glycocalyx may finally result in vascular leakage and increased permeability as well as in the loss of vasculoprotective properties.[73, 86-88]

Not only the glycocalyx has an important role in maintaining the vascular barrier, but also endothelial junctions (EJs), which include tight junctions, adherens junctions and gap junctions. Additionally, EJs are crucial for preserving tissue integrity, intercellular communication and controlling the entry of leukocytes. EJs transfer intracellular signals, which regulate contact-induced inhibition of cell growth, apoptosis, gene expression and new formation of vessels.[89-91] In pathologic conditions the integrity of the endothelial cell layer can be severely affected, resulting in disruption of cellular junction or even in the detachment of cells from the vascular wall, leading to vascular damage and altered permeability.[72]

In particular, vascular endothelial (VE)-cadherin is an important component of endothelial adherens junctions and has a crucial role in controlling vascular permeability,[92] since modifications in its expression may alter the vascular barrier function. Recently, we showed in a rat model of lower extremity IRI, increased VE-cadherin expression along with edema formation in lung tissue, which may be attributed to activated repair mechanisms.[93] Modifications of VE-cadherin and following increased permeability may result from induction of tyrosine phosphorylation through histamine, vascular endothelial growth factor, platelet-activating factor and TNFα, or from VE-cadherin cleavage via enzymatic proteolysis.[94-98] In 2012, Orsenigo et al. demonstrated that phosphorylation of VE-cadherin in veins is induced via hemodynamic forces.[99] Phosphorylated VE-cadherin is internalized and ubiquitinated in response to permeability-increasing agents such as bradykinin and histamine leading to a quick and reversible opening of endothelial cell junctions and plasma leakage.[99]

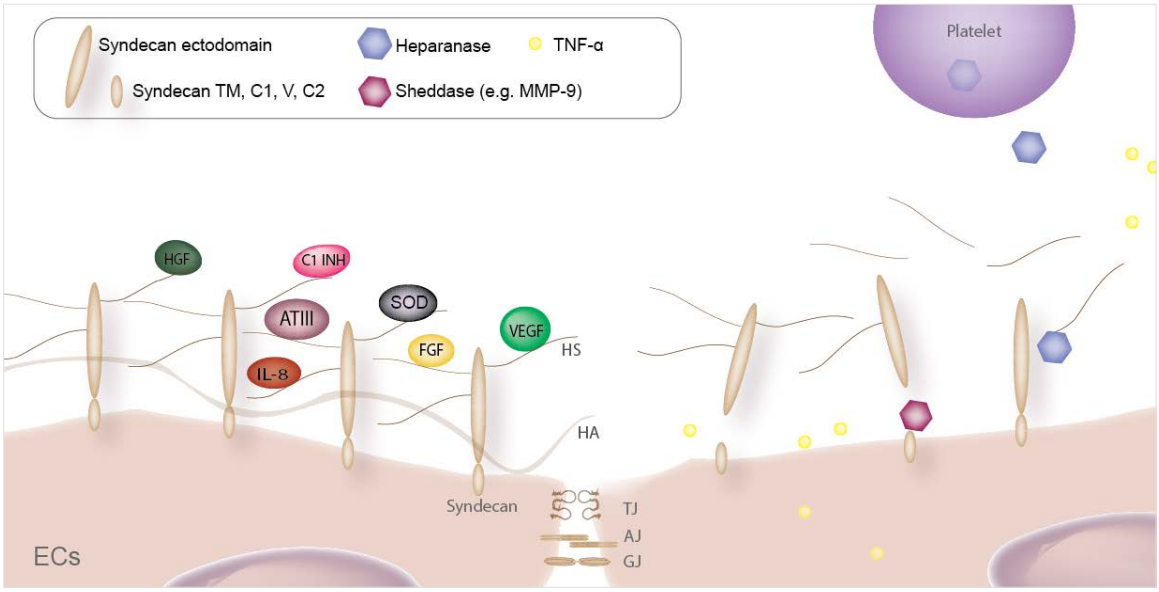


**Figure 1. Shedding of the glycocalyx**. In a homeostatic condition, the intact glycocalyx binds a myriad of proteins (mainly via HS), which in total maintain an anti-inflammatory and anti-coagulatory state (left cell). However, during an ischemic event this state is rendered into a pro-inflammatory and pro-coagulatory one, since shedding of proteoglycans (syndecans), and HS occurs (right cell). HS can be cleaved from syndecans via the endoglycosidase heparanase, which is secreted by activated platelets.[100] The ectodomain of syndecans can be cleaved from its intracellular domain via different mechanisms. TNF-α, secreted by endothelial cells or by neutrophils can cause changes in the glycocalyx either dependent or independent of leukocyte adhesion, as it up-regulates endothelial-derived metalloproteases and may cause endothelial release of superoxide, both resulting in shedding.[101] MMPs are known sheddases of syndecans.[102] AJ, adherens junction; ATIII, antithrombin III; C1, conserved domain 1; C1 INH, C1 esterase inhibitor; C2, conserved domain 2; ECs, endothelial cells; FGF, fibroblast growth factor; GJ, gap junction; HA, hyaluronic acid; HGF, hepatocyte growth factor; HS, heparan sulfate; IL-8, interleukin-8; MMP-9, matrix metalloprotease-9; SOD, superoxide dismutase; TJ, tight junction; TM, transmembrane domain; TNF-α, tumor necrosis factor-alpha; V, variable domain; VEGF, vascular endothelial growth factor. This illustration is not drawn to scale.

## *2.5.* Inflammatory cell trafficking

Ischemic tissue injury is associated with an inflammatory process, which is characterized by rapid activation of resident cells and production of pro-inflammatory mediators (IL-1β, IL-6, IL-8, and TNFα) as well as infiltration of various types of inflammatory cells including neutrophils, different subtypes of lymphocytes, monocyte/macrophages, platelets, mast cells and other cells into the affected tissue.[103-106] These cellular events collectively contribute to IRI.

Endothelial cells can be activated by an ischemic insult, causing increased vascular permeability as well as enhanced expression of adhesion molecules. It was shown that elevated plasma concentrations of soluble E-selectin and P-selectin indicate activation of endothelia cells in acute ischemic stroke.[107] Upon restoration of blood flow inflammatory cells adhere to primed endothelial cells. Endothelial adhesion molecules, such as E- and P-selectin recognize fucosylated carbohydrate structures, whereas leukocytes express the equivalent ligands P-selectin glycoprotein ligand-1, E-selectin ligand-1 as well as hyaluronan receptor CD44 and also L- selectin on the surface.[108] Binding of endothelial selectins enable leukocytes to roll along the vessel wall. Lymphocyte function–associated antigen (LFA)-1, and Mac-1 initiate the slow rolling on the endothelium before transendothelial or paraendothelial migration across postcapillary venules into the surrounding tissue by following a gradient of chemotactic factors.[108-110] LFA-1 and its endothelial ligand intercellular adhesion molecule (ICAM)-1 play an essential role in the process of migration of neutrophils. It was shown that ICAM-1 and selectins act through interdependent pathways to regulate rolling, adhesion and migration.[109, 111, 112] Firm adhesion is mainly triggered by complete activation of leukocyte integrins such as LFA-1 or VLA-4, which enables them to tightly bind endothelial ligands including ICAM-1 and VCAM-1, respectively.[113, 114]

The anaphylatoxins C5a and C3a play crucial roles in the modulation of the immune response during IRI by inducing chemotaxis and activation of neutrophils via C3a- or C5a receptors.[115, 116] Activation of neutrophils and migration into the inflamed tissue is followed by subsequent release of ROS and other tissue destructive mediators such as neutrophil elastase, cathepsin G as well as matrix metalloproteinase.[117-120] Next to neutrophils, also dendritic cells, macrophages, platelets and mast cells are involved in IRI.[121] Following ischemia, monocytes start to migrate into the harmed tissue and develop into dendritic cells or macrophages, depending on the specific tissue microenvironment. There, dendritic cells function as antigen-presenting cells for lymphocytes and orchestrate the inflammatory response.

Dendritic cells express receptors for endogenous danger signals, microbial structures as well as cytokines and chemokines and play a critical role in IRI.[122, 123] Platelet accumulation in post-ischemic microvessels depends on leukocyte adhesion and leukocyte-platelet aggregate adhesion causes a prolonged increase in microvessel permeability.[124] In 2006, Xu et el. demonstrated, that activation of platelets occurs early during reperfusion and their activation depends on the duration of ischemia and is proportional to the extent of myocardial injury.[125] Activated platelets play an important role in the process of IRI, as shown in a mouse model of myocardial infarction.[125] Mast cells are multifunctional, inflammatory as well as pro-fibrotic mediators and are well known for their contributions to allergic reactions but it was also reported, that they play an important role in IRI.[126] In 1998, Frangogiannis and co-workers showed that cardiac mast cells degranulate after myocardial ischemia and release preformed mediators, such as histamine and TNFα.[127] The authors suggest that mast cell-derived TNFα may be a crucial factor in upregulating IL-6 in infiltrating leukocytes and initiate the cytokine cascade responsible for myocyte ICAM-1 induction and subsequent neutrophil-induced injury.[127]

The contribution of the innate immune system to IRI is well established.[128, 129] Emerging data suggest an important role for lymphocytes, in particular T cells, but also B cells in IRI.[130] In 2003, Burne-Taney et al. demonstrated for the first time the pathogenic role for B cells in acute renal IRI.[131] In 2007, Hurn et al. showed that severe combined immuno-deficient (SCID) mice lacking T and B cells had reduced lesion size and inflammation in a model of stroke.[132] The importance of lymphocytes in IRI was also shown in other organs.[131, 133, 134] However, lymphocytes do not only contribute to the enhancement of injury responses after ischemia, but could also play beneficial roles.[135]

# 3. Endogenous danger signals

## *3.1.* Alarmins/ DAMPs

In 2005, Oppenheim introduced the term “alarmin” to differentiate endogenous inflammatory stimuli from PAMPs, which are pathogen-derived inflammatory stimuli.[136, 137] Alarmins or danger/damage associated molecular patterns (DAMPs) are the endogenous analogues to PAMPs. Constitutively available alarmins can be released upon tissue damage and thus activate the immune system. Their uncontrolled release can contribute to the excessive inflammatory responses seen in IRI.[138] Many endogenous inflammatory stimuli are identified, which are able to activate the innate immune system, including defensins, cathelicidin, eosinophil-derived neurotoxin (EDN) as well as high-mobility group protein B1 (HMGB1), S100 proteins and heat shock proteins.[137, 138] One of the best known alarmins is HMGB1, which is a nuclear constituent loosely bound to chromatin, activating dendritic cells as well as enhancing humoral and cellular antigen-specific immune responses.[139, 140] Inhibition of HMGB1 has shown beneficial effects in IRI.[141] Additionally, it was demonstrated that HMGB1 levels may be used as indicator of inflammation and may be a novel target for controlling inflammation during cardiopulmonary bypass.[142] Alarmins are recognized by toll like receptors (TLRs) of inflammatory cells of the innate immune system, such as immature dendritic cells DCs. They mature upon recognition and start to express MHC molecules as well as produce pro-inflammatory cytokines, in particular IL-12 and thus induce an inflammatory response. TLRs including TLR2, TLR4 and TLR9 seem to mediate effects of various alarmins.[143] In 2010, Wu and colleagues presented that endogenous HMGB1 promotes kidney damage after IRI, possibly through the TLR4 pathway.[144] Very recently, Ding et al. showed that the HMGB1–TLR4 axis plays a pathogenic role in triggering cardiomyocyte apoptosis during myocardial IRI.[145] An important role of HMGB1–TLR4 interaction was also identified in acute lung injury following liver IRI.[146] In 2011, Huang et al. could demonstrate that histones function as alarmins via recognition by TLR9 in hepatic IRI.[147] In general, this inflammatory reaction proceeds without the presence of microbial triggers and is therefore termed as sterile inflammation.[18] The initiation of sterile inflammation via the innate immune system does not necessarily require the rupture of cells and release of alarmins (Figure 2).

## *3.2.* Microparticles

Microparticles are phospholipid microvesicles, which are shed from the plasma membrane of activated or dying eukaryotic cells.[148] The release of microparticles and alarmins can proceed simultaneously, as microparticles can be filled with molecules having alarmin activity. Some features of microparticles may result from their content of alarmins. Microparticles demonstrate pro-inflammatory and pro-thrombotic activities after their release,[149] which can be induced by endogenous danger signals that are expressed following IRI.[1] In blood most frequently found microparticles derive from platelets, whereas microparticles from erythrocytes, granulocytes, monocytes, lymphocytes, and endothelial cells can also be found, but less frequently.[150-152] Microparticles resemble their cell of origin as they express the same antigens, contain the same cell surface proteins, cytoplasmic contents, and nuclear components.[153] Recently, it was shown that platelet activation in myocardial IRI is associated with increased generation of microparticles, which were platelet-, monocyte- as well as endothelial cell-derived.[154] Interactions between platelets, monocytes and endothelial cells play an important role in the pathogenesis of myocardial IRI.[154] Furthermore, it was demonstrated that circulating endothelial- as well as platelet-derived microparticles correlate to the size of myocardium at risk in patients with myocardial infarction.[155] Increased levels of platelet-derived microparticles were also reported for patient suffering from critical limb ischemia.[156] Microparticles seem to be more than just biomarkers of disease and should be considered as circulating bioactive effectors of immunity.[157]

### *3.3. Neo-epitopes and natural antibodies*

The hypoxic condition during ischemia mediates structural alterations of proteins, leading to the formation of neo-epitopes. Based on cellular reorganization these proteins are mobilized to the cell surface and get accessible for natural antibodies, which are produced without a previous infection (Figure 2). The involvement of natural antibodies in inflammatory responses is known for decades.[158] Natural antibodies are mainly produced by $CD5^+$ B-1 cells.[159, 160] In 1996, Weiser et al. highlighted the importance of natural antibodies in IRI by using RAG-$1^{-/-}$ mice (deficient in mature lymphocytes), which underwent skeletal muscle IRI.[21] The authors showed, that RAG-$1^{-/-}$ mice were protected from IRI and reconstitution of normal serum restored skeletal muscle reperfusion injury, indicating that IRI is mediated by preexisting natural antibodies.[21, 161] The specific self-reactive IgM antibody clone (CM-22) was shown to initiate intestinal and also skeletal muscle IRI.[162, 163] Nonmuscle myosin heavy chain type II A and C (NMHC II) was identified as target self-antigen in skeletal muscle and intestinal IRI, which is exposed on hypoxic cells and rather not released as a result of cell death.[23] Interestingly, reconstitution of immunoglobulin-deficient mice with normal human IgM restored injury in a model of intestinal IRI, indicating that human natural IgM recognizes similar ischemia specific self-antigens as murine natural IgM does.[164] Furthermore, in a model of intestinal IRI it was demonstrated that the identified neo-epitope of NMHC II is conserved between mice and rats by using the synthetic peptide mimetope N2 with the amino acid sequence of the hinge region of nonmuscle myosin heavy chain II. [165] The N2 peptide also prevents specific IgM binding to ischemic antigens in the heart, resulting in a significant reduction in myocardial IRI.[166] Another identified neo-epitope in models of intestinal and cerebral IRI is annexin IV, recognized by the IgM clone B4.[167, 168]. Moreover, oxidized lipids, generated through phospholipid peroxidation, have strong pro-inflammatory properties and may act as self-antigens and also initiate innate immune responses.[169, 170]

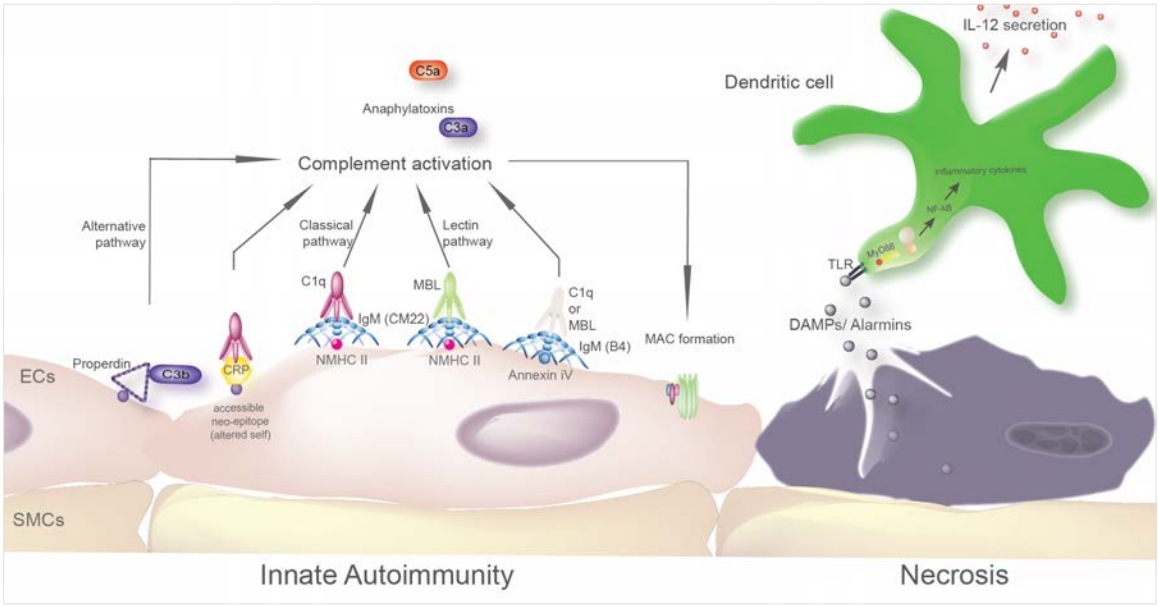


**Figure 2. Endogenous danger signals trigger an inflammatory response.** The ischemic condition leads to the formation of neo-epitopes of proteins, such as NMHC II and annexin IV and their mobilization to the cell surface. Accessible neo-epitopes can be recognized by certain immunoglobulin clones and initiate the complement cascade via different pathways, finally leading to the formation of the MAC. Altered self can also be recognized by CRP and properdin, resulting in the activation of the complement system. Prolonged ischemia and reperfusion may lead to a necrotic rupture of cells, whereby intracellular components are released, functioning as DAMPs or alarmins, and can be recognized by dendritic cells via TLRs. Activation of the transcription factor NF-kB results in the release of pro-inflammatory cytokines, in particular IL-12. CRP, C-reactive protein; DAMPs, danger/damage associated molecular patterns; ECs, endothelial cells; IgM, immunoglobulin M; IL-12, interleukin 12; MAC, membrane attack complex; NF-kB, nuclear factor-kB; NMHC II, non-muscle myosin heavy chain type II; TLR, toll-like receptor. This illustration is not drawn to scale.

## 4. Systemic inflammatory response syndrome and multiple organ dysfunction

Local excessive activation of the immune system following prolonged ischemia and subsequent reperfusion can extend to the systemic inflammatory response syndrome (SIRS) and may result in distant organ damage or even in the multiple organ dysfunction syndrome (MODS).[171, 172] Characteristically, SIRS manifests in local and systemic production as well as release of various mediators, including pro-inflammatory cytokines, components of the plasma cascade systems, acute phase proteins, and accumulation of inflammatory cells at the site of tissue damage.[173] Large quantities of pro-inflammatory cytokines, which are generated during the period of reperfusion are excessively released into circulation, acting as mediators of SIRS. Released cytokines may include TNF, interleukin (IL)-6, IL-1b, IL-8, monocyte chemotactic protein-1 (MCP-1), transforming growth factor-b1 (TGF-b1), and RANTES (Regulated on Activation, Normal T cell Expressed and Secreted).[174] Recently, it was shown that IL-19 mediates tissue damage and targets several major organs in acute renal IRI.[175] Inflammatory cells, such as neutrophils are activated and recruited by pro-inflammatory cytokines, which leads to the release of proteases and ROS. MODS is of essential clinical importance in lower extremity, intestinal and also renal IRI.[4, 176-182] In severe cases, reperfusion of the ischemic tissue mediates a complex inflammatory response, involving the activation of the complement, coagulation as well as kinin system and may finally cause cardiopulmonary failure as well as renal and hepatic dysfunction.[183] Lung tissue is the most susceptible tissue affected in MODS, ranging from mild dysfunction to severe respiratory failure and is followed by cardiovascular failure, renal, neurological and hepatic dysfunction.[180]

# 5. Role of plasma cascade systems in ischemia/reperfusion injury

## *5.1.* The complement system

The complement system was discovered more than one century ago and since that time it is increasingly growing in importance. The complement system is a highly regulated network and consists of more than 30 fluid-phase and membrane-bound proteins mainly but not exclusively synthesized in the liver. Complement activation aims to opsonize antigens resulting in enhanced phagocytosis of pathogens, initiate cellular lysis via formation of the membrane attack complex (MAC) as well as attracts inflammatory cells through generation of anaphylatoxins. However, activation of the complement system may also occur in the absence of pathogenic triggers (sterile inflammation) in the course of tissue injury, contributing to the pathophysiology of IRI. Under healthy conditions, the complement system is tightly regulated via host cell protective proteins to maintain homeostasis. Host cell protective proteins include the decay-accelerating factor (CD55), which is a potent inhibitor of the C3 convertase of the classical, lectin and alternative pathway,[184] the membrane cofactor protein (MCP, CD46), [185] complement receptor 1 (CR1, CD35), [186, 187] complement receptor 2, factor H,[188] C1 esterase inhibitor (C1 INH), C4 binding protein, CD59 as well as S protein, which is a plasma glycoprotein expressed by endothelial cells.[189]

The complement system consists of 3 pathways, the classical pathway, the lectin pathway and the alternative pathway. The pathways differ in the molecules involved in the initiation sequence, but converge at the level of C3 activation. The classical pathway is activated by binding of the C1 complex, which comprises C1q, C1r and C1s. C1q can bind to the Fc portion of antibody-antigen complexes as well as to C-reactive protein (CRP) in complex with antigens.[190] As mentioned above, antibodies cannot only recognize non-self structures but also altered self-structures (e.g. neo-epitopes), leading to the initiation of an inflammatory response. Binding of the C1 complex results in the cleavage of the downstream complement component 4 (C4) and C2 via C1s, leading to the formation of the C3 convertase C4bC2a.

Similar to the classical pathway, natural antibodies can activate complement via the lectin pathway by binding of mannose-binding lectin (MBL) to antibody-antigen complexes.[191] Furthermore, the lectin pathway can be activated by recognition of polymannose carbohydrate structures via MBL or ficolin, which are in complex with MBL-associated serine proteases (MASPs).[192, 193] Activation of the lectin pathway results in autoactivation of MASP2 and subsequent cleavage of C4 to C4a and C4b as well as C2 to C2b and C2a.

The alternative pathway is initiated when C3b, constantly generated in low levels (tick-over), binds to the certain carbohydrates, lipids as well as proteins. Binding of C3b leads to the recruitment of factor B, which is cleaved by factor D and thus enables the formation of the alternative pathway convertase C3bBb and is stabilized by properdin.[194] Recently, a novel role for properdin has emerged. Properdin is not only the stabilizer of the alternative pathway C3 convertase, but is also a pattern recognition molecule and can recognize dangerous non-self-structures as well altered self-structures, including necrotic, apoptotic or malignant cells.[195, 196] Binding of properdin to certain structures directly leads to the binding of C3b and results in the activation of the complement system.[195, 196] The C3 convertases C4bC2a as well as C3bBb generate more C3b, which amplifies the complement system and paves the way for MAC formation. The C5 convertase is formed by the C3 convertases via complex formation with C3b. C3bBb3b and C4bC2aC3b cleave C5 to C5a and C5b. The MAC comprises C5b, C6, C7, C8 as well as multiple molecules of C9, finally resulting in pore formation and lysis of the cell. The split products C3a as well as C5a are of special importance, as they function as chemoattractants for phagocytic cells. Furthermore, they fulfill a role as vasodilators, are able to induce contraction of smooth muscles and trigger the release of histamine from mast cells.[197, 198]

### *5.1.1. Role of complement in ischemia/reperfusion injury*

In 1971, Hill and Ward were the first to describe the involvement of the complement system in IRI by using a rat model of myocardial IRI.[199] Since that time more than 40 years have passed and the complement system is widely accepted and has become an integral part of the underlying mechanisms of IRI. The contribution of complement to tissue injury in IRI was shown in various organs and tissue types, ranging from brain,[200, 201] heart,[202, 203] lung,[204] liver,[205] and kidney,[206] to gut,[207] and skeletal muscle[21]. The importance of individual components of the complement system in IRI was demonstrated by using either genetically modified animal models, which are deficient in certain complement components or via administration of specific synthetic proteins, pooled plasma proteins or monoclonal antibodies to inhibit complement activation. The prominence of the complement component C1 in contribution to IRI was analyzed in various models of IRI. Interestingly, none of the studies could demonstrate, that C1 is crucial for the manifestation of tissue injury. In 2006, Mocco et al. showed that C1q deficient mice were not protected from stroke.[200] Similar results were presented for myocardial as well as gastrointestinal IRI.[207, 208]

Recently, Elvington et al. demonstrated in a murine model of stroke that C1q/MBL deficient mice were protected from cerebral injury.[201] In contrast to C1 deficient mice, deficiency in MBL clearly demonstrated its crucial contribution to IRI.[201] It was shown that MBL deficient mice display reduced myocardial, cerebral, renal as well as skeletal muscle IRI.[208-212] MBL deficient mice demonstrated reduced gastrointestinal IRI, but not a reduction in distant lung damage.[207] In 2010, Cervera et al. presented that genetically-defined MBL deficiency facilitates anti-inflammatory responses after acute stroke that results in long lasting beneficial effects on post-stroke functional recover in humans.[213] The significant contribution of the lectin pathway to IRI was also shown in MASP deficient mice, as they were protected from myocardial and also intestinal IRI.[214] MASP-1 and MASP-3 deficient mice lack alternative complement pathway activation based on a non-existing cleavage of factor D, which remains in its proenzyme form.[215] Therefore, MASP-1 and MASP-3 seem to be involved in activation of both the lectin and alternative pathways.[215, 216] In a study of gastrointestinal IRI C2/factor B deficient mice were protected from local injury and also from distant lung injury, whereas addition of C2 restored injury, demonstrating that gastrointestinal IRI is mediated via the lectin and/or classical pathway.[207] C4 deficient mice are protected from intestinal and also skeletal muscle IRI, further highlighting the importance of the lectin and/or classical pathway.[21, 217] Also complement component C3 deficient mice showed reduced tissue injury following cerebral as well as skeletal muscle ischemia and following restoration of blood flow. [21, 200]

Knockout of factor H, a specific regulator of the alternative complement pathway, does not affect infarct volume in stroke, which may indicate that fH has no role in the regulation of brain ischemic damage.[218] Knockout of the alternative pathway component factor B revealed protection from tissue damage in brain and also kidney, but not intestinal IRI.[201, 219, 220]

Mice deficient in C5 or C6 displayed no reduction of cerebral injury in models of stroke.[200, 201] Knockout of the MAC inhibitory protein CD59 demonstrated aggravated injury in models of liver IRI and also stroke.[221, 222] A double deficiency in CD55/CD59 greatly exacerbated renal IRI revealing CD55 and CD59 act synergistically to inhibit complement-mediated renal IRI.[223] Very recently, Miwa et al. showed an elegant study analyzing renal IRI by using decay-accelerating factor/CD59 double knockout mice, which were mated with mice deficient in various complement components or receptors and demonstrated that deletion of C3, factor B, properdin, C3aR, or C5aR significantly ameliorated renal IRI, whereas deficiency of C4, or MBL had no effect.[224] Furthermore, the authors reported that treatment of DAF/CD59 knockout mice with either anti-C5 or anti-properdin monoclonal antibody ameliorated IRI and finally concluded that complement is activated via the alternative pathway during the early phase of reperfusion, and both anaphylatoxin-mediated inflammation and the membrane attack complex contribute to tissue injury.[224] In C5a receptor or C3a receptor deficient mice, it was shown that renal IRI was significantly reduced.[225] All in all, knockout of individual complement components revealed that activation pathways in IRI highly depend on the affected organ.

### *5.1.2. Complement-specific therapeutics in ischemia/reperfusion injury*

The development and use of complement inhibitors in animal models of IRI is the first step in reaching the long-term objective the translation from “bench to bedside”. In that regard many studies were conducted, demonstrating promising results. One good but rather indirect example is the use of the synthetic peptide mimetope N2 with the amino acid sequence of the hinge region of nonmuscle myosin heavy chain II. Several rodent studies showed that the treatment with the peptide N2, inhibiting the binding of natural antibodies specific for the N2-neo-epitope and preventing complement activation, reduces skeletal muscle, mesenteric and also myocardial IRI.[23, 165, 166]

Another very promising approach is the use of soluble complement receptor 1 (sCR1), which is a highly effective complement inhibitor. It is a modified fragment of the complement receptor 1 (CR1), has binding sites for C3b and also C4b and inactivates C3/C5 convertases. The sCR1 construct APT070 (Mirococept) consists of the first 3 consensus domains of the human CR1 and a membrane-targeted synthetic peptide, which mediates the binding to phospholipids on the cell surface and protects the cell against complement activation.[226, 227] Beneficial effects of APT070 were shown in our lab in an in vivo study of myocardial infarction by using a closed-chest pig model, but also in other studies.[202, 228, 229] Interestingly, neuroprotective effects of the CR1 short consensus repeats were shown in rat models of cerebral IRI.[230, 231]

Cobra venom factor (CVF), a structural and functional analog of complement C3 isolated from cobra venom, reduces myocardial IRI.[232] Mice treated with the recombinant humanized CVF (HC3-1496) are protected from myocardial IRI with resultant preservation of cardiac function.[233] Beneficial effects were also found for cerebral IRI, where CVF significantly reduced post-ischemic cerebral infarct volume and atrophy in adult and neonatal rats.[234] In 2012, Elvington et al. used the alternative pathway inhibitor CR2-fH, which targets sites of complement activation via CR2-mediated recognition of C3 opsonins, and demonstrated protective effects in a mouse model of stroke.[201] Furthermore, neurological improvement and stroke volume reduction were achieved by C3a-receptor antagonist treatment.[200]

Treatment with the endogenous mannose-binding lectin (MBL)/ficolin-associated protein-1 (MAP-1) demonstrated reduced myocardial IRI by inhibition of the lectin pathway via displacement of MASP-1, MASP-2, and MASP-3 from the MBL complex.[235] Attenuation of IRI was also obtained by using C5a receptor antagonists, which was shown in models of stroke, renal, intestinal, skeletal muscle as well as hepatic IRI.[236-239] In contrast to the above shown findings, some animal studies demonstrated that complement inhibition did not reduce IRI, as shown in models of renal and skeletal muscle IRI as well as acute thromboembolic stroke.[93, 240, 241]

So far not many complement inhibitory drug candidates were successful and promising enough to be applied in human clinical trials. Particularly worth mentioning is pexelizumab (Alexion Pharmaceuticals, Inc.), a recombinant humanized single-chain antibody fragment that prevents cleavage of C5a, has shown a reduction in mortality when applied in coronary bypass graft surgery and myocardial infarction.[242-245] However, it was reported that pexelizumab failed to improve end points of death, shock, or heart failure of patients in the APEX trial, the largest randomized trial of patients undergoing percutaneous coronary intervention for acute ST-elevation myocardial infarction.[246, 247] Furthermore, unsatisfying results were achieved from the COMMA (The COMplement inhibition in Myocardial infarction treated with Angioplasty) trial, where 960 patients with myocardial infarction were treated with pexelizumab. Primary objective was reduction of infarct size and secondary objective included a 90-day composite clinical outcome (death, new or worsening congestive heart failure, cardiogenic shock, or stroke). The primary objective was not reached, as no differences were found between pexelizumab and placebo treated patients.[248] Based on these results pexelizumab is no longer on the market, although mortality was reduced in on-pump coronary artery bypass grafting.[249, 250] Eculizumab (Soliris, Alexion Pharmaceuticals, Inc.), a fully humanized monoclonal antibody selectively inhibiting terminal complement activation by binding with high affinity to the C5 fragment, is currently used in clinical studies to investigate its efficacy and safety in ABO incompatible transplantation, in the prevention of acute and chronic rejection either with a living or a deceased donor kidney as well as in the prevention of delayed graft function.[251]

The sCR1 TP10 was applied in a clinical trial (multicenter, randomized, double-blind, placebo-controlled trial, including 59 patients) in IRI following lung transplantation and demonstrated a decreased duration of mechanical ventilation as compared to a placebo group.[204] Furthermore, TP10 was used in a clinical trial (randomized multicenter, prospective, placebo-controlled, double-blind study including 564 high-risk patients) of cardiac surgery on cardiopulmonary bypass demonstrating no improvement in primary endpoints of the study, whereas mortality and ischemic damage was reduced.[252]

## *5.2.* The coagulation system

Whenever bleeding occurs the coagulation system plays a crucial role in protecting the body from blood loss, which is achieved through the formation of thrombi. However, the coagulation system can also be initiated in nonbleeding situation, such as sterile inflammation. Traditionally, it can be activated via the intrinsic or extrinsic pathway of coagulation.[253, 254] Activation via the extrinsic pathway of coagulation occurs through tissue factor, which is exposed at the site of injury and is followed by the formation of factor VII-Tissue factor (TF) complexes.[255, 256] Subsequently, factor Xa is activated, which, together with factor Va, cleaves prothrombin to thrombin.[257] After cleavage of fibrinogen by thrombin, fibrin monomers polymerize to form stable fibrin clots. Activation of the intrinsic pathway of coagulation via factor XII was attributed to the contact of blood with negatively charged surfaces (contact activation) in *in vitr*o experiments.[258] Following activation, factor XIIa cleaves factor XI, which finally results in the formation of fibrin clots.

As patients deficient in factor XII display no bleeding tendency the traditional "waterfall" model of coagulation was reconsidered. The current version of the coagulation cascade suggests that the TF pathway consists of the factor VIIa/TF complex as well as the factor Xa/Va (prothrombinase) complex. In contrast, the intrinsic pathway, an alternative route to generate factor Xa, consist of factor XI(a), the factor IXa/VIIIa complex, and the factor Xa/Va complex, but does not include factor XII. Both pathways converge at the level of the factor Xa/Va (prothrombinase) complex.[259-261]

In 2005, Renne et al. demonstrated that the deficiency of factor XII and factor XI are not associated with abnormal hemostasis in mice, but impair formation of occlusive thrombi in arterial injury models, which indicates that these pathways, which are not essential for hemostasis participate in arterial thrombosis.[262, 263]

An important mechanism for degradation of fibrin into soluble fibrin degradation products is the fibrinolytic system. In a homeostatic condition fibrinolysis is kept quiescent. It is tightly controlled by fibrinolysis inhibitors, including plasminogen activator inhibitor (PAI) -1 and -2. It was shown that the fibrinolytic pathway can be initiated via direct plasminogen activation through tissue plasminogen activator, urokinase plasminogen activator, kallikrein (KK) or factor XII, resulting in the generation of plasmin.[264, 265] Fibrin molecules are cross-linked by factor XIIIa. Degradation of polymerized fibrin leads to generation of fibrin degradation products. Characteristically, degradation by plasmin releases fragments known as D-dimers, which indicates the breakdown of a cross-linked fibrin clot. In contrast, the presence of fibrinogen degradation products can occur from the breakdown of fibrinogen, fibrin monomers, or cross-linked fibrin.[266-268]

### *5.2.1. Role of the coagulation system in ischemia/reperfusion injury*

As compared to the complement system not many studies were conducted using knockout models of individual coagulation factors to analyze the impact on the development of IRI. Nevertheless, the importance of the coagulation system in the development of IRI was shown in models of myocardial infarction as well as stroke.[269, 270] Very recently, it was demonstrated that a complete lack of fibrinogen may be detrimental, whereas a partial reduction of fibrinogen, like in heterozygous mice, can improve renal function and overall outcome in a murine model of renal IRI.[271] Furthermore, in a murine model of myocardial IRI it was shown that deficiency in protease-activated receptor 2 (PAR2, also known as coagulation factor II (thrombin) receptor-like 1), which is activated by proteases including factor VIIa as well as factor Xa, reduces myocardial infarction and heart remodeling after IRI.[272] In murine models of factor XII or factor XI deficiency it was shown that mice are protected from ischemic brain injury.[273, 274] Moreover, factor VII deficiency does not protect from ischemic stroke as shown in a recent case report.[275] Factor VII deficiency does not correspond to any protection from thrombosis.[275] TF deficiency or lack of PAR-1 was reported to be protective in a murine model of renal IRI.[276]

### *5.2.2. Coagulation-specific therapeutics in ischemia/reperfusion injury*

Inhibition of TF by using a specific monoclonal antibody has shown promising results in a rabbit model of myocardial infarction.[270] Similar results were obtained by functional inhibition of thrombin via application of hirudin.[270] Furthermore, the authors found no effect on infarct size by defibrinogenating rabbits with ancrod, a defibrinogenating agent by cleaving the A-chains of fibrinogen, suggesting a requirement of thrombin generation but not fibrin deposition in myocardial IRI.[270, 277] In a model of intestinal IRI, treatment of human TF knock-in transgenic mice with a human monoclonal anti-TF antibody showed an attenuation of intestinal IRI induced severe distant lung damage.[278] The use of monoclonal anti-TF antibodies has demonstrated beneficial results also in a model of stroke.[279] Furthermore, it was demonstrated that inhibition of factor XII-mediated activation of factor XI, by using an antibody directed against the apple 2 domain of factor XI, provides protection against acute ischemic stroke in mice, while leaving other factor XI and factor XII functions intact.[280] Inhibition of factor XII was beneficial in models of stroke by using the factor XII inhibitor PCK (Phe-Pro-Arg-chloromethylketone) or recombinant infestin-4 fused to human albumin (rHA-infestin-4) and also silent brain ischemia when mice were treated with rHA-infestin-4.[274, 281, 282] Inhibition of factor VIIa in a mouse model of myocardial IRI was reported to attenuate injury, which was associated with reduced inflammation, indicated by reduced neutrophil influx as well as reduced expression of pro-inflammatory genes and proteins in the left ventricle.[283, 284] Also anti-coagulants including heparin or hirudin showed promising results in the reduction of IRI.[276, 285-287]

Anticoagulant therapy is the standard of care in patients presenting venous thrombosis, acute coronary syndrome and in cardiopulmonary bypass for heart surgery. [288-290] Recent clinical studies (randomized, double-blind, dose-finding multicenter trial in patients with acute ischemic stroke) have shown that the application of low molecular weight heparin certoparin in stroke appears to be controversial, since the functional outcome was not improved.[291, 292]

Rivaroxaban, the first orally bioavailable direct factor Xa inhibitor, showed beneficial effects in acute coronary syndrome, reducing the risk of the composite end point of death from cardiovascular causes, myocardial infarction, or stroke.[293-296] Other anti-coagulants applied e.g. for the prevention of thrombosis after hip or knee replacement, or prevention of stroke include dabigatran and warfarin and are reviewed elsewhere.[297-300]

Already in 1978, Godfrey and Salaman demonstrated promising effects of aprotinin in renal IRI.[301] Aprotinin is a potent inhibitor of plasmin, trypsin, chymotrypsin, kallikrein, thrombin and activated protein C via forming reversible enzyme-inhibitor complexes, for review see Mahdy et al.[302] Protective effects of aprotinin were also presented in other animal models of IRI including myocardial, lung and also skeletal muscle IRI.[303-305] Despite the demonstrated positive effects of aprotinin, its application may be associated with undesired effects. It was reported that the use of aprotinin in cardiac surgery was associated with increased risk of myocardial infarction or heart failure as well as stroke or encephalopathy, which was followed by suspension of aprotinins license.[306] However, the beneficial effects of aprotinin outweigh its risks in appropriately managed patients undergoing isolated CABG surgery and it is recommended to the EU that the suspension of the license for aprotinin in this context may be lifted.[307]

## *5.3.* The kinin system

The discovery of components of the kinin system occurred in the first half of the last century.[308, 309] Our current knowledge of the kinin system indicates pivotal roles in the regulation of blood pressure, inflammatory reaction as well as modulation of vascular permeability and vasodilation. Factor XII has a crucial role in the kinin system. Activation of factor XII occurs by binding to negatively charged surfaces including glass, metals and certain polymers as well as collagen, cholesterol sulfate, sulfatides, acid phospholipids, fatty acids, several charged carbohydrates, mast cell released heparin and misfolded proteins (contact activation).[310, 311] Factor XIIa leads to the activation of prekallikrein to form kallikrein and the latter cleaves high molecular weight kininogen (HK), leading to the formation of bradykinin, which mediates an increase in vascular permeability.[312] In plasma under normal conditions, about 70% of prekallikrein is bound to HK as a noncovalently linked complex, which does not result in cleavage of HK.[313] A reason why HK is not cleaved in normal conditions might be the regulation by C1 INH. When bound to HK, prekallikrein expresses an active site, which is inhibited by C1 INH.[314] After formation of active bradykinin, binding to its b2 receptor on the surface of endothelial cells occurs, whereas des-arg-9-bradykinin acts on b1 receptors, both causing vasodilation and increased vascular permeability.[315] In contrast to b2 receptors, b1 receptors are not constitutively expressed but may be induced by pro-inflammatory cytokines.[315, 316]

### *5.3.1. Role of the kinin system in ischemia/reperfusion injury*

The role of the kinin system in IRI is well established, especially in ischemic stroke.[317] In 2011, Revenko et al. reported that selective depletion of plasma prekallikrein via antisense oligo- nucleotide technology displayed beneficial effects in the prevention of thromboembolic diseases, which highlights the role of prekallikrein in thrombus formation.[318] Deficiency in tissue kallikrein, a major kinin-releasing enzyme present in arteries, was shown to aggravate cardiac remodeling and to increase mortality rate in a murine model of myocardial infarction, indicating tissue kallikrein has a protective role in heart failure.[319, 320] In contrast, HK deficient mice are protected from thrombosis after artificial vessel wall injury and lack the pro-inflammatory mediator bradykinin.[321] It was also shown that HK deficient mice developed smaller brain infarctions and less severe neurological deficits without an increase in infarct-associated hemorrhage. [321]

Several studies focused on the role of bradykinin receptor b1 or b2 in IRI. It was shown that mice deficient in both bradykinin receptors are extremely vulnerable to renal IRI.[322] B2 receptor gene knockout in a murine model of stroke demonstrated an increase in mortality rate, neurological deficit scores as well as infarct size in comparison to wild-type mice, indicating that the b2 receptor promotes survival and protects against brain injury.[323] In 2009, Austinat et al. presented, that b1 receptor knockout mice developed significantly smaller brain infarctions and less neurological deficits compared to wild-type mice, whereas b2 receptor deficiency did not result in neuroprotection or reduction of cerebral edema formation.[324] Similar results were shown in a model of myocardial infarction, where b1 receptor knockout mice demonstrated significantly reduced infarct size as compared to wild-type animals.[325] Also in intestinal IRI it was shown that b1 receptor deficient mice display protective anti-inflammatory and anti-lethality effects.[326]

### *5.3.2. Kinin system–specific therapeutics in ischemia/reperfusion injury*

Souza et al. demonstrated, that when b1 receptor deficient mice were pretreated with HOE 140, a well-known antagonist of b2 receptors, protective anti-inflammatory and anti-lethality effects were reversed, as b2 activation also leads to vasodilatation and protection against reperfusion injury in intestinal IRI.[326] Furthermore, it was reported that treatment with the b1 receptor inhibitor R-715 (Biomatik Corporation) significantly reduced brain edema in a mouse model of stroke.[324] Similar results were shown in a murine model of myocardial infarction, where treatment with the b1 receptor inhibitor (des-Arg9, Leu8)-bradykinin significantly reduced infarct size, but not the b2 receptor inhibitor HOE 140.[325] These results are confirmed by a study in renal IRI, where b1 receptor antagonism (R-954) showed beneficial results, but not treatment with the b2 receptor antagonist HOE 140.[327] As bradykinin b1 receptors are inducible proteins that are rarely present under physiological conditions they might be a promising pharmacological target for various disease including IRI.[328]

In a rat model of myocardial IRI gene transfer of kallistatin, a tissue kallikrein binding protein, demonstrated reduced myocardial infarct size.[329] Beneficial effects in IRI were identified for DX-88, a selective recombinant inhibitor of human plasma kallikrein, which reduced brain edema as well as the number of degenerating cells in a murine model of cerebral IRI.[330]

So far not many clinical trials targeting the kinin system in IRI exist. Recently, a clinical trial (randomized double-blind, cross-over study) using the bradykinin b2 receptor antagonist HOE-140 in healthy male volunteers, subjected to forearm IRI could not confirm a major role for endogenous bradykinin, acting via the b2 bradykinin receptor, in the mechanism of IRI (ClinicalTrials.gov Identifier: NCT00965120).[331]

None of the currently known potent and selective peptide and non-peptide agonists of b2 bradykinin receptor have been selected for a clinical assessment in cardiovascular indications. One major challenge of this approach is the still unanswered question of whether there is a sufficient safe therapeutic window between potential cardioprotective and pro-inflammatory effects following b2 bradykinin receptor agonism.[316, 332]

## *5.4.* Interaction of plasma cascade systems

In the past years more and more interactions between the individual plasma cascade systems have been identified. Differentiating between single complement pathways or even between the plasma cascade systems becomes increasingly difficult. When it comes to multifaceted interactions the MASPs have an important role. It was demonstrated that MASP1 has thrombin-like activity cleaving and activating fibrinogen and factor XIII although less efficiently than thrombin.[333] Furthermore, MASP1 is able to cleave high molecular weight kininogen (HK) resulting in bradykinin production. MASP2 is also able to cleave HK but not leading to bradykinin release.[334] It is well known that MASP2 cleaves the complement components C2 as well as C4, moreover MASP2 has factor Xa-like activity and is capable of cleaving prothrombin, generating thrombin, which finally results in the formation of cross-linked fibrin.[335] Interestingly, MASPs do also have a dual role within the complement system, as MASP1 and 3 were identified to activate the alternative complement pathway through cleaving pro-factor D into mature factor D.[336]

Also other complement components were presented to interact with the coagulation- and kinin system, respectively. C5a was shown to induce tissue factor activity, C5b-9 catalyzes prothrombin cleavage to thrombin and the gC1q receptor binds factor XII as well as HK.[337-340] Nevertheless, also components of the coagulation system are capable of activating other plasma cascade systems, as shown for thrombin, factors IXa, Xa and XIa, which cleave C3 and also C5 resulting in generation of biologically active anaphylatoxins.[341, 342] Factor XIIf, a fragment resulting from further cleavage of factor XIIa by kallikrein or plasmin, is involved in the complement system by activating the classical pathway via C1r of the C1 component.[343] Plasmin interacts with the complement system by cleaving C3 and also C5.[344] Moreover, kallikrein can replace factor D for the activation of the alternative pathway C3 convertase and cleaves factor B.[345] These multifaceted interactions highlight the complexity of the inflammatory response in IRI.

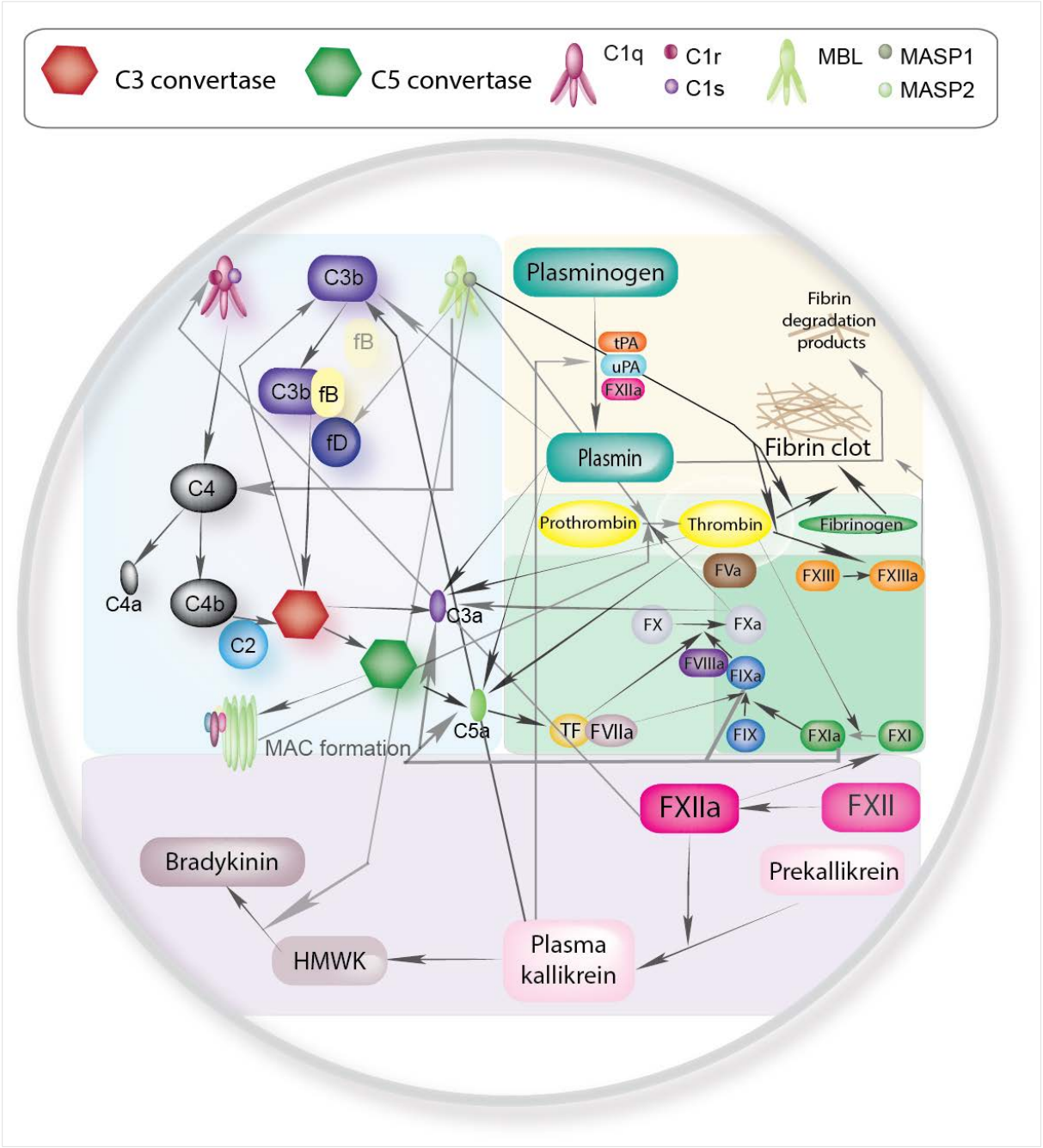


**Figure 3. Interaction of plasma cascade systems.** The plasma cascade systems, including the complement, the coagulation as well as the kinin systems are highly interactive. A detailed description can be found in section 5.4. fB, factor B; F, factor; fD, factor D; HMWK, high molecular weight kininogen; MAC, membrane attack complex; MASPs, mannose binding lectin associated proteases; MBL, mannose binding lectin; tPA, tissue plasminogen activator; uPA, urokinase plasminogen activator. This illustration is not drawn to scale.

### *5.4.1. Simultaneous inhibition of plasmatic systems in ischemia/reperfusion injury*

So far only a limited number of regulators or inhibitors, which act on more than one cascade have been identified. The following section highlights the multifunctional regulators C1 esterase inhibitor (C1 INH), low molecular weight dextran sulfate (DXS) as well as antithrombin III (ATIII).

C1 INH is a member of the serpin family of proteinase inhibitors and was first described almost 60 years ago.[346, 347] As its name leads to assume, Ratnoff and Lepow identified C1 INH by its efficacy to inhibit the first component of the complement system and also until now C1 INH is the only known inhibitor of the serine proteases C1r and C1s of the classical complement pathway.[347] Several decades later C1 INH was found to have far more target proteases than originally thought. C1 INH is not only a potent inhibitor of the classical pathway, but also of the lectin pathway, by inhibiting MASP1/2 as well as the alternative pathway through inhibition of factor B binding to C3b.[192, 348] However, the biological actions of C1 INH do also affect components not belonging to the complement system, such as factor XII, which is primarily inhibited by C1 INH.[349, 350] Furthermore, it was shown that also the coagulation system components factor XIa as well as thrombin are targets of C1 INH.[351, 352] Another important role of C1 INH was found in the fibrinolytic system by regulating kallikrein, tissue plasminogen activator and also plasmin.[353-355]

C1 INH is an acute-phase protein and its serum level rises during acute inflammation through production by various cell types, including endothelial cells, fibroblasts, monocytes, macrophages or hepatocytes.[356, 357] The transcription rate of C1 INH is enhanced due to expression of pro-inflammatory cytokines, such as IFN-alpha, IFN-gamma or interleukin 6.[358, 359] Patients deficient in C1 INH suffer from the potentially life-threatening disorder hereditary angioedema (HAE), emphasizing the importance of C1 INH in the healthy organism.[360] HAE patients suffer from edema formation in the upper airways and gastrointestinal tract,[361] mediated by bradykinin, a member of the kinin system that enhances capillary permeability. As C1 INH does not only act on the complement- but also on the coagulation and the kinin systems, it represents a promising therapeutic option to treat IRI. Positive effects were shown in animal models of heart, brain, liver and skeletal muscle IRI.[93, 362-365] In 2007, Fattouch et al. presented the effects of C1 INH in a randomized double-blind study of ST segment elevation myocardial infarction in patients who underwent emergent reperfusion with coronary artery bypass grafting.[366] C1 INH significantly reduced plasma levels of C3a and C4a, serum cardiac troponin I as well as stroke volume, whereas early mortality was not different between placebo control group and C1 INH-treated patients.

Currently, some clinical trials using C1 INH are on the way, including prevention of rejection in kidney transplantation (phase I/II trial; ClinicalTrials.gov identifier: NCT01134510), acute ischemic stroke (phase I trial, ClinicalTrials.gov identifier: NCT01694381) as well as prevention of IRI during liver transplantation (phase I trial, ClinicalTrials.gov identifier: NCT01886443).

Similar to C1 INH, also ATIII is well known for its plasma cascade system-overlapping actions. As its name led suggest ATIII has a crucial role in regulating the coagulation system. ATIII inhibits the blood coagulation proteases factor IX, X, XI, XII, thrombin as well as factor VII.[367-370] Furthermore, inhibitory actions of the kinin system were identified, as ATIII regulates kallikrein and also kinin.[371] It was also demonstrated that ATIII inhibits the complement components C1s as well as MASP1 and 2 in the presence of heparin.[372, 373] Due to its multifunctional actions on the plasma cascade systems ATIII appears to be a promising therapy in IRI. Very recently, Wang et al. presented that ATIII elicits potent cardioprotective responses in a model of myocardial IRI.[374] Moreover, ATIII exerts effects against liver and also retinal IRI as well as distant lung damage in a model of hind limb IRI.[375-377] ATIII is currently analyzed in various clinical trials, including a limited number of trials investigating its effects in IRI, such as liver IRI (phase I trial, ClinicalTrials.gov identifier: NCT01886443).

The synthetic polyanion low molecular weight dextran sulfate (DXS) is known for its heparin-like anti-coagulant and complement inhibitory activity by potentiating C1 esterase inhibitor mediated inactivation of C1s as well as binding of the regulatory protein factor H.[76, 378, 379] Furthermore, it modulates biological effects of contact activation, by inhibiting intrinsic coagulation without affecting the fibrinolytic potential of FXIIa/ kallikrein.[380] It was shown that DXS can act like a “repair coat” and therefore protects the endothelium from damage.[381] In vivo work in our laboratory demonstrated that DXS significantly protects from IRI in a closed chest porcine model of acute myocardial infarction.[382] Furthermore, we have shown that DXS attenuates IR induced acute graft injury and facilitates long-term survival in a rat model of heart transplantation.[383] In 2004, Laumonier et al. reported that DXS, in combination with cyclosporine A, prevents acute vascular rejection in a hamster to rat cardiac xenotransplantation model.[384] Recently, we found that DXS treatment in a model of skeletal muscle IRI did not reduce reperfusion injury (Duehrkop et al. unpublished). However, since low molecular weight DXS belongs to the glycosaminoglycans, it might have anti-coagulatory properties bearing the risk of bleeding complications and therefore may limit its use in clinical situations.[385] There are currently no ongoing clinical trials for DXS in IRI.

# 6. Conclusion

The involvement of the plasma cascade systems in IRI is well documented. A vast number of publications demonstrated the beneficial effects of complement, coagulation or kinin systems inhibitors in various animal models of IRI. However, results obtained from animal studies may be interpreted with caution, since clinical trials have often shown rather disappointing results for specific complement, coagulation or kinin systems inhibitors in preventing or attenuating IRI. A general conclusion is not easily possible, as study design and determination of primary as well as secondary endpoints in clinical trials play a crucial role for the outcome.

It becomes more and more obvious that the plasma cascade systems are highly interactive and a clear differentiation between the systems turns out to be increasingly challenging. The traditional, separated view of the systems may need to be revised and a consideration as an overall whole instead of distinct systems may be preferable. Therefore, ideal therapeutic approaches should simultaneously target more than one plasma cascade system, like C1 INH does, but should not result in complete inhibition, since this may lead to increased risks of infection and uncontrolled bleedings, respectively.

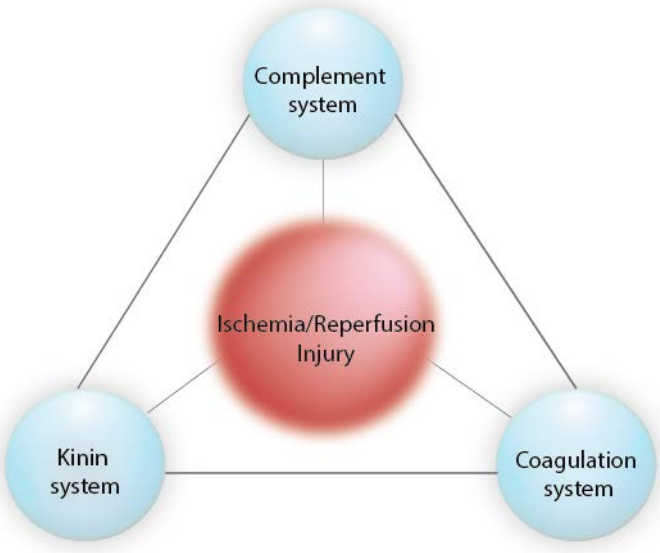


**Figure 4. Plasma cascade systems in ischemia/reperfusion injury.** Each plasma cascade system contributes to the development of IRI. Complex interactions additionally complicate the development of new therapeutic approaches. Therefore, therapeutic approaches targeting more than one system at the same time seem to be most promising for the attenuation or even prevention of IRI.

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# Paper I

## C1 Esterase Inhibitor Reduces Lower Extremity Ischemia/Reperfusion Injury and Associated Lung Damage

Claudia Duehrkop[1,2], Yara Banz[1,3], Rolf Spirig[4], Sylvia Miescher[4], Marc W. Nolte[5], Martin Spycher[4], Richard A. G. Smith[6], Steven H. Sacks[6] and Robert Rieben[1]

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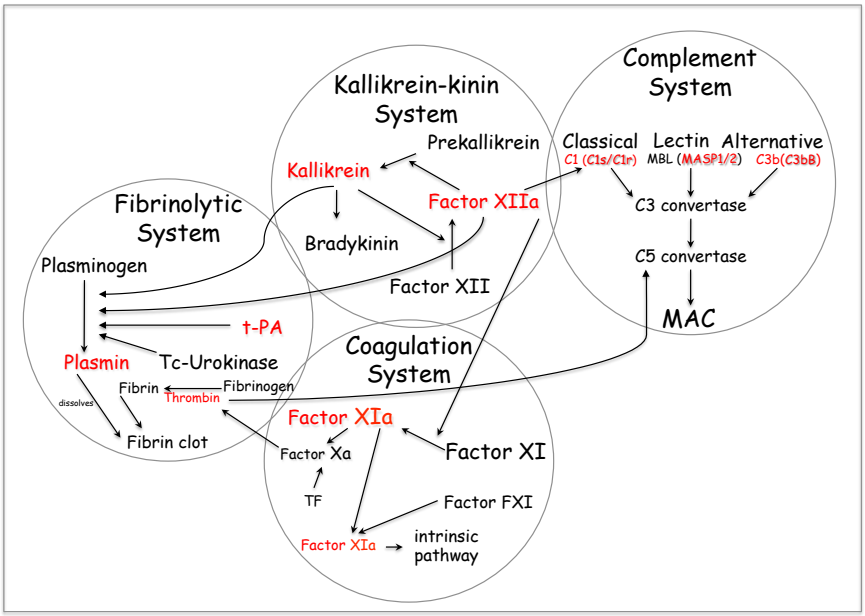
**Aim:** The aim was to investigate the effect of exogenous human plasma-derived C1 esterase inhibitor (C1 INH) and the soluble, membrane-targeted CR1 construct APT070 on tourniquet-induced ischemia/reperfusion injury (IRI) and associated distant lung damage by using a rat lower extremity IRI model.

**Conclusion:** Our data suggest that the positive effects of C1 INH may not primarily result from complement inhibition, emphasizing the importance of the kinin- and coagulation cascades in peripheral IRI, as APT070 did not reduce reperfusion injury in this model.




C1 INH is a multifunctional protein, acting on multiple inflammatory cascades relevant in IRI pathology. Proteases regulated by C1 INH are highlighted in red.

# C1 Esterase Inhibitor Reduces Lower Extremity Ischemia/Reperfusion Injury and Associated Lung Damage

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**Key words:** C1 Esterase Inhibitor, Ischemia/Reperfusion Injury, Inflammation, Complement Pathways, Contact System

## Abstract

**Background—**Ischemia/reperfusion injury of lower extremities and associated lung damage may result from thrombotic occlusion, embolism, trauma, or surgical intervention with prolonged ischemia and subsequent restoration of blood flow. This clinical entity is characterized by high morbidity and mortality. Deprivation of blood supply leads to molecular and structural changes in the affected tissue. Upon reperfusion inflammatory cascades are activated causing tissue injury. We therefore tested preoperative treatment for prevention of reperfusion injury by using C1 esterase inhibitor (C1 INH).

**Methods and Findings**—Wistar rats systemically pretreated with C1 INH (n=6), APT070 (a membrane-targeted myristoylated peptidyl construct derived from human complement receptor 1, n=4), vehicle (n=7), or NaCl (n=8) were subjected to 3h hind limb ischemia and 24h reperfusion. The femoral artery was clamped and a tourniquet placed under maintenance of a venous return. C1 INH treated rats showed significantly less edema in muscle ($P<0.001$) and lung and improved muscle viability ($P<0.001$) compared to controls and APT070. C1 INH prevented up-regulation of bradykinin receptor b1 ($P<0.05$) and VE-cadherin ($P<0.01$), reduced apoptosis ($P<0.001$) and fibrin deposition ($P<0.01$) and decreased plasma levels of pro-inflammatory cytokines, whereas deposition of complement components was not significantly reduced in the reperfused muscle.

**Conclusions—**C1 INH reduced edema formation locally in reperfused muscle as well as in lung, and improved muscle viability. C1 INH did not primarily act via inhibition of the complement system, but via the kinin and coagulation cascade. APT070 did not show beneficial effects in this model, despite potent inhibition of complement activation. Taken together, C1 INH might be a promising therapy to reduce peripheral ischemia/reperfusion injury and distant lung damage in complex and prolonged surgical interventions requiring tourniquet application.

## Introduction

Lower extremity ischemia/reperfusion injury (IRI), which may result from thrombotic occlusion, embolism, trauma or surgical intervention through tourniquet application and subsequent restoration of blood flow, is of essential clinical importance. The deprivation of blood and oxygen, termed as ischemia, leads to time-dependent molecular and structural changes of the affected tissue. Complex inflammatory cascades are subsequently activated when blood flow is restored, leading to ischemia/reperfusion injury (IRI). The hypoxic state of ischemia leads to expression of non-muscle myosin heavy chain type II or annexin IV on the cell surface, which function as neo-epitopes for natural antibodies.[1],[2] This immune complex formation already occurs prior to tourniquet release and paves the way for activation of the complement system. Natural antibodies can activate complement via C1q and the classical pathway or via the lectin pathway by binding of mannose-binding lectin (MBL) to carbohydrate structures, particularly on IgM,[3],[4] generating potent anaphylatoxins and ultimately resulting in the formation of a pore and lysis of the cell. The roles of natural antibodies and the complement system in IRI are well established, but the coagulation- and the kinin systems have been shown to be of equal importance.[5] The coagulation system plays a pivotal role in IRI in the intestine, brain, lung and heart.[6], [7], [8], [9] The fact that the complement system may be activated by thrombin, a protease of the coagulation system, highlights the complexity of the inflammatory response in IRI.[10] In a homeostatic situation, the inner lining of blood vessels, the endothelium, maintains an anti-coagulatory and anti-inflammatory environment.[11] This is, amongst others, upheld by the protective layer of the glycocalyx, a negatively charged, tight meshwork of proteoglycans, including heparan sulfate and other glycosaminoglycans and associated plasma proteins. However, during ischemia the glycocalyx may be partially lost.[12], [13] This shedding renders the anti-inflammatory and anti-coagulatory state a pro-inflammatory and pro-coagulatory one and facilitates interaction of leukocytes with the endothelium.[14]

The activation of this multifaceted network of cascades in IRI manifests itself in edema formation and muscle necrosis. IRI of the extremities is often accompanied by remote organ damage, affecting organs like the liver, lung, kidney or intestine and may lead to the development of multiple organ dysfunction syndrome.[15] In particular, remote lung damage, which results from the systemic inflammatory response, is a common issue.[16] It has been shown that the expression of pro-inflammatory cytokines is required for remote lung injury, resulting in increased vascular permeability.[17]

APT070, also known as Mirococept, is a highly effective complement inhibitor. It is a modified fragment of the complement receptor 1 (CR1) and has binding sites for C3b and also C4b.[18] APT070 consists of the first 3 consensus domains of the human CR1 and a membrane-targeted synthetic peptide, which mediates the binding to phospholipids on the cell surface and therefore protects the cell against complement activation.[19] Beneficial effects of APT070 were shown in our lab in an in vivo study of myocardial infarction by using a closed-chest pig model.[20]

C1 esterase inhibitor (C1 INH) is one of the main regulators of the complement system, as it interacts with all three pathways and additionally plays a pivotal role in the coagulation- and kinin systems.[21] Patients deficient in C1 INH suffer from the potentially life-threatening disorder hereditary angioedema (HAE), emphasizing the importance of C1 INH in the healthy organism.[22] HAE patients suffer from edema formation in the upper airways and gastrointestinal tract,[23] mediated by bradykinin, a member of the kinin system that enhances capillary permeability. As C1 INH does not only act on the complement- but also on the coagulation- and the kinin systems, it represents a promising therapeutic option to treat IRI. Positive effects were already shown in IRI of the heart, brain, liver and muscle.[24], [25], [26,27] We therefore hypothesized, that C1 INH treatment in peripheral IRI would reduce local edema formation as well as lung damage. The effect of exogenous human plasma-derived C1 INH on tourniquet-induced IRI was investigated in a rat hind limb model and the underlying mechanisms of protection were analyzed.

## Materials and Methods

### Animals and housing

All experiments were conducted in accordance with the terms of the Swiss animal protection law and were approved by the animal experimentation committee of the cantonal veterinary service (Canton of Bern, Switzerland).[28] Male Wistar rats (wild type, bred at the central animal facility, University of Bern) were kept in groups of three in a clear 1500 $cm^2$ Euro-standard Type IV S cage (Tecniplast, Buguggiate, Italy) under standard housing conditions with food and water ad libitum. Cages were individually ventilated at 20 ± 2°C and 45-65% relative humidity with a circadian rhythm of 12/12 h. During the light cycle animals were exposed to an intensity of 200 lux. For the experiments, rats weighing between 250 and 350 g were used.

### Reagents

C1 esterase inhibitor (Berinert®) as well as the vehicle (10 mg/ml glycine, 2.9 mg/ml sodium citrate, 8.5 mg/ml sodium chloride, pH 7.0) were provided by CSL Behring (CSL Behring GmbH, Marburg, Germany). APT070 was provided by King's College (London, UK) and consists of the first three short consensus repeats of human complement receptor 1. APT070 is modified with a membrane-targeting amphiphilic peptide based on the naturally occurring membrane-bound myristoyl-electrostatic switch peptide.[18] APT070 was provided in a solution of phosphate-buffered saline (PBS, pH 7.4) containing mannitol (50 mg/ml) and arginine (17.4 mg/ml).

### Experimental groups

Rats were divided into five groups. The experimental group (n=6, C1 INH group) received a dose of 50 IU/kg (50 IU/ml) of human C1 INH. Control group 1 (n=8, NaCl group) received 1 ml/kg of 0.9% sodium chloride. Control group 2 (n=7, vehicle group) received 1 ml/kg C1 INH vehicle prior to ischemia. Control group 3 (n=4, APT070 group) received 9 mg/kg (9 mg/ml) of APT070 before induction of ischemia and control group 4 (n=4, normal) underwent no intervention.

**Anesthesia and analgesia**

Anesthesia was induced with 2.5% isoflurane in oxygen in a box and later maintained by inhalation of 1.5% isoflurane on a nose mask. Analgesia was provided by 0.05 mg/kg of buprenorphine (Temgesic, Reckitt Benckiser, Switzerland AG) injected subcutaneously 30 minutes prior to surgical intervention. The total duration of anesthesia was approximately 6 h after which the rats were allowed to wake up. To provide adequate analgesia for the 24 h reperfusion period buprenorphine injection was repeated when animals were completely awake. After completion of 24 h reperfusion, rats were anesthetized again as described above and sacrificed by exsanguination during organ removal.

**Surgical procedure**

For assessment of limb perfusion the fur was completely removed from both hind limbs with an electric shaver. The rats were kept on a heating pad to maintain the body temperature at 37°C. Approximately 30 minutes after induction of anesthesia, the femoral artery and vein were exposed via a groin incision and a tourniquet (standardized weight of 450 g) was placed underneath the femoral vessels to block collateral circulation.[29] The femoral artery was then occluded for 3 h with two microvascular clamps (B1-V, S&T, Neuhausen, Switzerland). Rat hind limbs were not exsanguinated, but a comparable state was achieved by allowing venous return during the entire period of ischemia in order to prevent venous congestion and additional injury through microcirculatory impairment, which would not represent the clinical situation. After 3 h of ischemia the limb was reperfused for 24 h during which the rats were allowed to wake up with appropriate analgesia. At the end of the experiments, tissue samples of both the ischemic as well as the contralateral gastrocnemic muscles as well as the lungs were taken for subsequent analyses.

**Assessment of edema formation**

For assessment of edema formation two samples of the gastrocnemic muscle from both legs were taken and immediately weighed to obtain the wet weight. The muscle samples were then dried for 24 h at 80°C after which a constant dry weight was achieved. Subsequently, the wet / dry ratio was calculated.

**Analysis of muscle viability**

IRI severely affects muscle viability, which may ultimately result in muscle necrosis. In order to investigate the influence of C1 INH on muscle viability the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma, St. Louis, USA) assay was performed. MTT is a yellow-colored tetrazolium salt, which is converted to purple colored formazan crystals by metabolically active cells. Muscle samples from the gastrocnemic muscle were taken, washed in PBS, blotted dry and incubated in 0.1 mg MTT/ml PBS in a total volume of 3 ml at 37°C, rotating in the dark for 2 h. Thereafter, muscle samples were blotted dry and incubated in 100% isopropanol at 37°C, rotating in the dark overnight to elute the formazan crystals from the tissue for measurement of the optical density (OD). 200 µl of thus obtained supernatant was measured in a microplate (Nunc, 96 well, maxisorp, transparent, Roskilde, Denmark) with a microplate reader at 560 nm (Ref. 690 nm; Infinite M1000 spectrophotometer, Tecan, Männedorf, Switzerland). After drying the muscle samples at 80°C for 24 hours the OD per mg dry weight was calculated and compared with values of contralateral control legs.

**Histological assessment of damage**

For assessment of hemorrhage, total myocyte damage as well as infiltration of neutrophil granulocytes, tissue samples from the gastrocnemic muscle were fixed in 4% formalin for 24-72 h. Thereafter, all samples were embedded in paraffin, cut into 3 µm thick sections and stained with hematoxylin and eosin.

**Immunofluorescence analyses of tissue samples**

Immunofluorescence staining using specific antibodies was used to quantify the deposition of IgM (3020-08; Southern Biotech, AL, USA) and IgG (3030-08; Southern Biotech), C1q (A0136, Dako, Baar, Switzerland), MBL (clone 14C3 kindly provided from Prof. G. Stahl, Boston, USA), C4b/c (LSB 4228, LifeSpan BioSciences Inc., Seattle, WA, USA), C3b/c (A0062, Dako) and factor B (341272, Calbiochem, Darmstadt, Germany). Furthermore, we analyzed fibrin deposition (F0111; Dako, Baar, Switzerland), expression of heparan sulfate (HS; 370255, Amsbio, Abingdon, UK), bradykinin receptor b1 (ABR-011, Alomone Labs, Jerusalem, Israel), bradykinin receptor b2 (ABR-012, Alomone Labs) as well as VE-cadherin (sc-6458, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Tissue samples from the gastrocnemic muscle of both legs and the lung were taken, washed in PBS, blotted dry and embedded in OCT matrix (Tissue-Tek, Sakura Finetek Europe B.V., Leiden, The Netherlands) on dry ice.

The samples were immediately stored at -20°C until cryosections were cut. Sections were fixed in acetone and rehydrated in Tris-buffered saline (TBS). Primary antibodies were incubated overnight at 4°C and secondary antibodies were incubated for 1 h at room temperature (RT). Subsequently, slides were mounted and coverslipped. Pictures were taken with a fluorescent microscope (Leica DMI 4000B, Leica Microsystems Schweiz AG, Heerbrugg, Switzerland) and analyzed using Image J (National Institutes of Health, Bethesda, MD, USA) and GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA, USA.). Endothelial expression of VE-cadherin as well as bradykinin receptor b1 and b2 was analyzed in lung tissue. For this analysis, the inner lining of the vessels was selected by hand, the surface area calculated and the intensity of immunofluorescence measured. Area under the curve values were obtained and divided by the surface area to achieve a final value in intensity per square pixel.

**Assessment of apoptosis using TUNEL**

For assessment of apoptosis in muscle and lung tissue a TdT-mediated dUTP nick end labeling (TUNEL) assay (in situ Cell Death Detection Kit, TMR red, Roche, Mannheim, Germany) was used. In brief, cryosections of muscle and lung tissue were fixed in acetone for 5 minutes at RT, washed and permeabilized with 0.1% Triton-X-100 on ice. Sections were incubated with TUNEL reaction mixture for 1 h at 37°C in the dark. After a washing step sections were mounted, coverslipped and analyzed with a fluorescent microscope.

**Analysis of infiltration of myeloperoxidase positive cells in lung tissue**

For quantitative analysis of infiltration of myeloperoxidase (MPO) positive cells in lung tissue, embedded and frozen tissue was cut into 5 µm thick sections, fixed in acetone and hydrated in TBS. Tissue sections were stained with an antibody for MPO (A0398, Dako) as well as DAPI (4',6-diamidino-2-phenylindole) to stain nuclei. Primary antibody was incubated overnight at 4°C and the secondary antibody (C2306, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) as well as DAPI were incubated for 1 h at RT. MPO positive cells were counted and divided by total number of cells.

**Cytokine / chemokine / growth factor analysis using multiplex array**

A multiplex immunoassay consisting of magnetic beads conjugated with a capture antibody specific for a target protein was used to detect an array of cytokines, chemokines, and growth factors (Bio-Plex Pro Rat Cytokine Group I panel, Bio-Rad, Hercules, CA, USA). The assay was performed according to the manufacturer's instructions. Briefly, plasma was diluted 1:3 and incubated with antibody-coupled magnetic beads. A washing step was followed by incubation with biotinylated detection antibody.

After streptavidin-phycoerythrin incubation cytokine / chemokine / growth factor concentrations were measured. Recombinant proteins were used to establish standard curves. Analyte concentrations were calculated using the Bio-Plex Manager Software.

**Statistical analysis**

Data are expressed as mean ± standard deviation (SD). Statistical significance was determined by one-way analysis of variance with Dunnett's post-test against NaCl control, using GraphPad Prism 5 software. P values of $< 0.05$ were considered statistically significant. Determination of n-numbers per group was performed without formal power analysis, based on preliminary experiments with C1 INH.

## Results

### Effect of C1 INH treatment on edema formation and muscle viability as well as histological assessment of muscle damage

Edema formation in tissue samples was analyzed as wet / dry ratio. Edema was indicated by an increase in wet / dry ratio. C1 INH treatment (ratio 4.6 ± 0.18, Figure 1B right) led to a significant ($P < 0.001$) reduction of fluid accumulation in the gastrocnemic muscle in comparison to NaCl control (5.6 ± 0.71, Figure 1B left). When rats were treated with the complement inhibitor APT070 (5.5 ± 0.79) no attenuation of edema was found (Figure 1A). Furthermore, C1 INH treated rats also showed a significant ($P < 0.001$) reduction in lung edema (4.7 ± 0.11) as compared to NaCl controls (5.1 ± 0.15), whereas APT070 treatment (5.0 ± 0.10) did not lead to reduction of edema formation (Figure 1C). Analysis of muscle viability using the MTT assay showed that C1 INH treatment (viability 93 ± 15.3%) led to a significant increase ($P < 0.001$) of viability in comparison to NaCl control (63 ± 9.4%). Again, this was not the case for APT070 treatment (69 ± 2.0%) (Figure 1D**)**. Histologically, hemorrhage, edema formation and myocyte destruction were apparent in NaCl-, vehicle- and APT070 treated rats, whereas C1 INH treated rats showed only minimal tissue damage (Figure 1E-H).

### Deposition of IgM and IgG in reperfused muscle as well as in lung tissue

Immunofluorescence stainings were performed to investigate the impact of C1 INH treatment on antibody deposition in muscle and lung tissue. As compared to normal rats no IgG deposits (Figure 2A) or IgM deposits (Figure 2G) were found in contralateral legs. In the reperfused legs high antibody deposition was found for NaCl and vehicle control groups, whereas C1 INH ($P < 0.01$) as well as APT070 ($P < 0.001$) treatment significantly reduced antibody deposition in comparison to NaCl. Representative immunofluorescence images showed an intense staining for IgG (Figure 2B) and IgM (Figure 2H) in reperfused muscles of NaCl treated rats. For C1 INH treated rats reduced deposition was detected (IgG, Figure 2C; IgM, Figure 2I). In lung tissue no differences between groups could be detected (Figure 2D-F and J-L).

**Assessment of deposition of C3b/c and factor B in muscle and lung tissue**

Deposition of factor C3, a central component of the complement system, was analyzed using immunofluorescence staining for the C3b/c. High deposition of C3b/c was found in the contralateral as well as in the reperfused muscle tissue and the lung of NaCl as well as vehicle treated rats. C3b/c deposition was significantly reduced by APT070 treatment in both legs ($P<0.01$) and in the lung ($P<0.001$), and by C1 INH in the contralateral leg only ($P<0.01$), but not in the reperfused leg or the lung (Figure 3A-F). Complement factor B, which is specific for alternative pathway activation, was highly deposited in the reperfused and in the contralateral leg as well as in lung of the NaCl control and vehicle groups. C1 INH ($P<0.01$) as well as APT070 ($P<0.001$) treatment significantly reduced deposition of factor B in the contralateral leg but not in the lung and reperfused leg (Figure 3G-L).

**Assessment of deposition of C1q, MBL and C4b/c in muscle tissue**

To assess deposition of classical- and lectin-pathway specific complement components, stainings for C1q (classical pathway, Figure 4A-F), MBL (lectin pathway, Figure 4G-I) and C4b/c (classical and lectin pathways, Figure 4J-L) were performed. An increased C1q deposition was found for NaCl controls in the gastrocnemic muscle of both legs compared to normal rats (Figure 4A). Enhanced C1q deposition was significantly reduced in the reperfused leg by APT070 but not by C1 INH treatment, whereas no significant differences between groups were detected in the contralateral leg. No inter-group differences were found for deposition of MBL (Figure 4G) as well as C4b/c (Figure 4J) in both reperfused and contralateral legs.

**Impact of C1 INH treatment on fibrin deposition as well as heparan sulfate expression in muscle and lung tissue**

To analyze the involvement of the coagulation system in peripheral IRI and distant lung damage, muscle as well as lung tissue was stained for fibrin deposition (Figure 5A-F). Fibrin deposits were found in the reperfused muscle in the NaCl, vehicle and APT070 treated groups and were significantly reduced by C1 INH ($P < 0.05$) (Figure 5A-C). C1 INH also reduced fibrin deposition in the lung tissue as compared to NaCl control ($P < 0.01$) (Figure 5D-F). The glycocalyx component heparan sulfate (HS) was detected by immunofluorescence staining (Figure 5G-L). Reduced HS expression was found in the contralateral and reperfused muscle of NaCl controls. C1 INH-treated rats showed significantly preserved expression of HS in tissue of the contralateral muscle as compared to the NaCl control group ($P < 0.01$). However, in the reperfused muscle no differences could be detected between the C1 INH and NaCl groups (Figure 5G-I).

APT070 treated rats showed significantly preserved expression of HS in the reperfused muscle in comparison to NaCl treated rats ($P < 0.05$). No inter-group differences were found for HS expression in lung tissue (Figure 5J-L).

**Effect of C1 INH treatment on apoptosis in muscle and lung tissue**

Apoptosis was measured using the TUNEL assay. The ratio of TUNEL-positive cells to total cell number was calculated. Whereas in the contralateral muscle no apoptotic cells were detected, cells in the reperfused muscle of the NaCl (0.78 ± 0.24), vehicle (0.96 ± 0.06) and APT070 (0.76 ± 0.29) treated groups showed a high degree of apoptosis. C1 INH (0.08 ± 0.18), but not APT070, treatment led to a significant reduction of apoptotic cells ($P < 0.001$) in the reperfused muscle (Figure 6A-G). Similar results were found for lung tissue, where C1 INH treated rats (0.24 ± 0.34) also showed significantly less apoptosis as compared to the NaCl (0.93 ± 0.05), vehicle (0.79 ± 0.18) and APT070 (0.90 ± 0.10) (Figure 6H-N).

**Effect of C1 INH treatment on expression of bradykinin receptor b1 as well as b2 in lung tissue**

Lung sections were stained for bradykinin receptor b1 (Figure 7 A-D) as well as b2 (Figure 7E-H). Specificity of bradykinin receptor staining was verified by competitive inhibition with the respective b1- or b2-peptides (data not shown). In contrast to APT070, C1 INH inhibited up-regulation of bradykinin receptor b1 in lung tissue as compared to control groups ($P < 0.05$ vs. NaCl). No inter-group differences were found for receptor bradykinin receptor b2 expression.

**Analysis of infiltration of myeloperoxidase positive cells and expression of VE-cadherin in lung tissue**

Infiltration of pro-inflammatory cells such as neutrophil granulocytes was assessed by immunofluorescence staining for myeloperoxidase. No significant differences between NaCl controls and the other treatment groups could be observed. However, C1 INH treated rats showed a trend for reduction of MPO-positive cells (Figure 8A, B). Analysis of the expression of VE-cadherin, a protein important for the endothelial barrier function, in lung tissue showed an up-regulation in the NaCl control group as compared to normal rats. Up-regulation of VE-cadherin was prevented in C1 INH treated rats ($P < 0.01$) (Figure 8C-F).

**Analysis of plasma levels of cytokines, chemokines and growth factors after 24 h reperfusion**

Multiplex suspension array technology was used to quantify levels of different cytokines, chemokines and growth factors in EDTA-plasma taken after 24 h of reperfusion. Analysis revealed that C1 INH treatment significantly reduced levels of Interleukins (IL) IL-1α, IL-7, IL-17 and IL-18 as well as IFN-γ, MIP-1α (macrophage inflammatory protein, CCL3), MIP-3α (CCL20) and TNF-α ($P < 0.05$). EPO (erythropoietin), CXCL1, RANTES (regulated and normal T cell expressed and secreted, CCl5), VEGF (vascular endothelial growth factor), IL-4, IL-5, IL-10, MCP-1 (monocyte chemotactic protein 1) and M-CSF (macrophage colony-stimulating factor) were not affected. Data are expressed as means ± SD. IL-1beta, IL-2, IL-6, IL12p70, IL-13, G-CSF, GM-CSF were below detection level and are not listed (Table 1).

## Discussion

The present study aimed to investigate the effects of C1 INH treatment on peripheral IRI and related remote lung damage. Originally, the application of C1 INH was described in the potentially life-threatening disease hereditary angioedema (HAE).[30] Lower extremity IRI is associated with edema formation in the affected tissue, which is multifactorial and results amongst others from increased vascular permeability.[31] It is also known that limb ischemia may cause distant lung damage, including pulmonary pathology with fibrin-rich microthrombus formation, vascular congestion and pulmonary edema.[32] In the present study we show that C1 INH protected from peripheral IRI by reduction of skeletal muscle edema and maintenance of muscle cell viability. In addition, lung edema formation was prevented by C1 INH treatment. Edema formation in muscle as well as lung tissue required reperfusion. Rats subjected to ischemia only did not show gastrocnemic muscle or lung edema (histologically assessed, data not shown), suggesting that local, humoral or cellular components within the reperfused limb were responsible for mediating distant lung damage.[15]

In order to investigate the mechanisms of edema reduction and improvement of muscle viability, we analyzed the involvement of the complement, coagulation and kinin systems since all three systems play important roles in IRI pathophysiology.[9,20,33] First, the effect of C1 INH on binding of natural IgG as well as IgM antibodies was determined by immunofluorescence. Indeed, as compared to normal control rats, no significant increase of antibody deposition was found in contralateral muscle tissue as well as in lung. In reperfused muscle we detected high deposition of IgG as well as IgM in NaCl and vehicle treated groups, which was significantly reduced by treatment with C1 INH and APT070. Both are inhibitors of the complement system, but their evident direct effect on natural antibody binding has not been described so far.

In order to assess which complement pathways were mainly affected by C1 INH treatment, deposition of C3b/c (all pathways), factor B (alternative pathway), MBL (lectin pathway), C1q (classical pathway) as well as C4b/c (classical and lectin pathway) were investigated. Previous studies which analyzed the effect of C1 INH treatment on peripheral IRI did not investigate deposition of complement components at all or only as hemolytic C3 and C4 titers.[34] In our study, we showed that deposition of complement components C4b/c and MBL was not increased by peripheral IRI. However, an increased binding of C1q, C3b/c and factor B was found in the contralateral as well as in the reperfused leg, but was not significantly reduced by C1 INH treatment in the reperfused leg. Also in lung tissue C1 INH showed no significant effects on C3b/c and factor B deposition.

In contrast, the specific complement inhibitor APT070 significantly reduced deposition of C1q and C3b/c in the reperfused and contralateral legs as well as C3b/c in the lung, while not preventing edema formation or increasing tissue viability. This finding was unexpected as beneficial effects of APT070 were shown earlier for remote and systemic injury following intestinal ischemia and reperfusion in rats and myocardial reperfusion injury in pigs.[35],[20] That APT070 indeed prevented complement activation was also confirmed in vitro by CH50 test as well as cell ELISA and cytotoxicity assay with porcine cells and human serum (data not shown). Based on the above mentioned data we conclude that the beneficial effects of C1 INH treatment were not primarily due to inhibition of the complement system.

Systemic circulation of activated complement components has been shown in models of IRI and deposition of such components on the endothelium of distant organs and tissues may therefore play a role.[36] Another possibility would be that locally produced bradykinin may lead to distant edema formation in the lung once reperfusion starts. In our study, C1 INH treatment led to reduced fibrin deposition in muscle as well as lung tissue. This finding is in line with C1 INH being the main inhibitor of coagulation factors XIa as well as XIIa.[21] Also in a mouse model of stroke it was recently demonstrated that C1 INH treatment reduced intracerebral fibrin formation.[37] An important mechanism for degradation of fibrin into soluble fibrin degradation products is the fibrinolytic system. It was reported that the fibrinolytic pathway can be initiated via direct plasminogen activation through tissue plasminogen activator (tPA), kallikrein (KK) or factor XII, which results in the generation of plasmin.[38,39] However, as C1 INH inhibits plasminogen activators like FXII, KK as well as to a lesser extent tPA and plasmin itself, increased fibrinolysis will probably not be the main reason for reduced fibrin deposition.[40,41] Rather, C1 INH dependent inhibition of the activation of the coagulation system may be responsible for the observed significant reduction of fibrin deposits.

Furthermore, as a marker of the integrity of the glycocalyx, we analyzed expression of HS in muscle as well as lung tissue. In muscle, shedding of HS was detected as decreased expression in the NaCl and the vehicle group in the reperfused as well as the contralateral leg, but we did not detect any effect of peripheral IRI on HS expression in the lung. All treatment groups, including vehicle, showed intermediate HS expression patterns between NaCl controls (low) and normal rat tissue [42]. Among these, statistical significance for preservation of HS expression was reached for C1 INH in the contralateral and for APT070 in the reperfused leg.

Next to activation of the fibrinolytic system, factor XIIa initiates the intrinsic pathway of coagulation via FXI activation and also the kinin cascade by cleaving plasma prekallikrein, leading to the formation of bradykinin. Active bradykinin binds to its b2 receptor on the surface of endothelial cells, whereas des-arg-9-bradykinin acts on b1 receptors, both causing vasodilation and increased vascular permeability.[43] In contrast to b2 receptors, b1 receptors are not constitutively expressed but are induced by pro-inflammatory cytokines. We found normal levels of the constitutively expressed receptor b2 in all groups. However, compared with normal rats, bradykinin b1 receptor expression was increased in NaCl controls and this up-regulation was prevented by treatment with C1 INH but not by APT070. This finding corresponds with the reduced edema formation in lung tissue found in C1 INH but not in APT070 treated or control rats and with an earlier report showing that blocking of the b1 receptor, but not b2 receptor, diminished brain edema formation in mice.[33] Similar results were also shown for lung as well as intestinal IRI by using bradykinin receptor antagonists to prevent or attenuate IRI.[44,45] However, reduced expression of b1 receptors could also be attributed to a reduction of pro-inflammatory cytokine levels via C1 INH, as bradykinin receptor expression can be induced through pro-inflammatory cytokine release.

Pulmonary damage secondary to local IRI can result from embolism but also from circulatory distribution of inflammatory mediators locally produced in the affected tissue.[46] There is no evidence, that exclusively bradykinin is responsible for edema formation in lung in the present model. It was reported that locally produced humoral mediators can cause leukocyte accumulation in lung tissue, which results in pulmonary damage by clogging of the capillaries and release of lysosomal enzymes by leukocytes.[15,47] Furthermore, an important role in edema formation is attributed to leukotriene B4 and other inflammatory mediators, like serotonin or histamine. [48,49]

Two studies reported that C1 INH, via expression of sialyl Lewisx tetrasaccharides and binding to E- and P-selectins, prevents adhesion and migration of leukocytes to the endothelium in vitro as well as in vivo.[50,51] We analyzed infiltration of MPO-positive cells in lung tissue and indeed a trend, albeit not significant, was found for a reduction of MPO positive cells by C1 INH.

We also analyzed the expression of VE-cadherin, which is a component of adherens junctions of endothelial cells and contributes to their barrier function.[52] In vessels of lung tissue we found elevated levels of VE-cadherin in NaCl, vehicle as well as APT070 treated rats, whereas C1 INH treated rats showed VE-cadherin expression levels similar to normal rats. Currently, not much is known about the mechanisms by which VE-cadherin-mediated cell-cell junctions are regulated.

It could be speculated that in the present study the increase of VE-cadherin expression could be due to repair mechanisms, whereas C1 INH maintains endothelial cell integrity and avoids activation of these mechanisms.[53] However, a more detailed analysis of the mechanism of VE-cadherin regulation in IRI would be necessary to support this hypothesis, which is beyond the scope of the present study.

In IRI apoptosis plays an important pathophysiological role and is an event of reperfusion, as it requires energy and is associated with cell shrinkage and phagocytosis without loss of membrane integrity.[54] In our study, C1 INH treated rats showed significantly less apoptosis as compared to the NaCl control group. These data confirm earlier reports describing that C1 INH improves the outcome of myocardial IRI via anti-apoptotic activity independent of its serine protease inhibitory activity by normalization of ratio of the Bcl-2/Bax expression.[55] Furthermore, it was shown that C1 INH reduced infarction size in a mouse model of myocardial infarction via inhibition of leukocyte transmigration into the ischemic tissue, which is also not mediated through its protease activity.[56]

The systemic inflammatory response, which is initiated in IRI is characterized by the release of pro-inflammatory cytokines, like TNF-α.[57] Our results demonstrated that C1 INH treatment led to significantly reduced levels of several pro-inflammatory cytokines. In a model of myocardial IRI it was shown that IL-17A plays a pathogenic role by inducing cardiomyocyte apoptosis and neutrophil infiltration.[58] We found reduced plasma levels of IL-17A in C1 INH treated rats, which fits with the observed reduction of apoptosis in muscle and lung tissue by C1 INH treatment. Also MIP-1α plays an important role in mediating an acute inflammatory response – another chemokine that was significantly reduced in C1 INH treated rats in our study.[59]

In 2004, Inderbitzin and colleagues presented a study of transgenic mice overexpressing human C1 INH (plasma levels of 1-2 mg/ml), which were used for a lower torso IRI model. They found that muscle as well as lung tissue was protected from endothelial cell damage by measuring the amount of extravasation of 125I-labelled albumin, reflecting a direct functional measurement of endothelial integrity.[53] We showed here for the first time in non-transgenic animals that C1 INH at a low, clinically applicable dose of 50 IU/kg significantly reduced peripheral IRI in muscle and, in particular, that also lung injury was significantly reduced.

In conclusion, C1 INH is a multifaceted protein, which acts on multiple inflammatory cascades relevant in IRI pathology. Via inhibition of kallikrein, FXIa, FXIIa as well as the complement system, it regulates IRI associated inflammatory and thrombotic processes. Our data support the regulatory effect of C1 INH on the coagulation- and the kinin system in IRI. A very potent inhibitory effect of human C1 INH on edema formation and apoptosis in skeletal muscle as well as in lung was observed. In addition, the up-regulation of bradykinin receptor b1 was prevented by C1 INH. These results may be a hint that C1 INH plays an important role in inhibition of the kinin system in this animal model of hind limb IRI. Furthermore, C1 INH also prevented fibrin deposition. Analysis of the effect of C1 INH on the complement cascades revealed that C1 INH reduced peripheral IRI not primarily by inhibition of the complement system. This conclusion is supported by APT070 data, which showed a significant reduction of C1q and C3b/c in the reperfused leg, but did not reduce edema formation in muscle and lung tissue. Furthermore, C1 INH reduced plasma levels of IFN-γ, IL-1α, IL-7, IL-17A, IL-18, MIP-1α, MIP-3α and TNF-α. All in all, C1 INH may provide a promising therapy to reduce peripheral IRI as well as distant lung injury in complicated and prolonged surgical interventions requiring tourniquet application.

## Acknowledgements

The gift of anti-MBL antibody by Prof. Gregory Stahl, Harvard Institutes of Medicine, USA, is kindly acknowledged. We are grateful to Dr. Regula von Allmen (University Hospital, Bern) for expert surgical advice. Further, we wish to thank Julie Denoyelle and Anjan Bongoni (Department of Clinical Research, University of Bern) for technical assistance as well as Dr. Boris Leuenberger and his team at the Institute of Pathology for preparation of histological slides. This study was performed with the support of the Microscopy Imaging Center (MIC), University of Bern.

**Table 1. Plasma levels of cytokines, chemokines and growth factors (in pg/ml) at baseline and after 24 h of reperfusion**

| Marker† | Baseline | NaCl | C1 INH |
|---|---|---|---|
| EPO | 278.9 ± 183.2 | 729.0 ± 427.7 | 346.9 ± 353.1 |
| CXCL1 | 93.0 ± 14.5 | 127.4 ± 77.0 | 78.3 ± 68.8 |
| IFN-γ | 57.6 ± 34.9 | 120.3 ± 100.2 | 19.1 ± 11.4* |
| IL-1α | 9.0 ± 4.9** | 83.1 ± 44.9 | 16.9 ± 10.3* |
| IL-4 | 22.6 ± 11.4 | 57.5 ± 39.3 | 8.3 ± 4.2 |
| IL-5 | 96.5 ± 11.2 | 172.7 ± 73.6 | 108.3 ± 23.7 |
| IL-7 | 69.1 ± 22.9** | 240.9 ± 98.3 | 67.2 ± 42.0** |
| IL-10 | 306.6 ± 51.3 | 776.3 ± 508.0 | 279.1 ± 94.2 |
| IL-17A | 7.0 ± 1.5** | 27.1 ± 14.0 | 8.3 ± 3.4** |
| IL-18 | 1103.0 ± 720.6** | 4155.0 ± 1390.0 | 1115.0 ± 580.4** |
| MCP-1 | 425.0 ± 58.0* | 1693.0 ± 982.4 | 1905.0 ± 638.3 |
| MIP-1α | 1097.0 ± 968.0* | 4629.0 ± 3045.0 | 1203.0 ± 762.0* |
| MIP-3α | 10.0 ± 8.6** | 48.0 ± 20.2 | 12.0 ± 9.5** |
| RANTES | 164.0 ± 89.1 | 310.2 ± 309.7 | 389.0 ± 442.7 |
| TNF-α | 19.1 ± 6.1 | 40.4 ± 24.9 | 9.1 ± 7.5* |
| VEGF | 12.0 ± 4.9 | 16.0 ± 6.0 | 11.4 ± 2.0 |
| M-CSF | 293.6 ± 50.8 | 381.2 ± 117.1 | 459.1 ± 79.2 |

EPO indicates Erythropoietin; CXCL1, Chemokine (C-X-C motif) Ligand 1; IFN-gamma, Interferon-gamma; IL, Interleukin; MCP-1, Monocyte chemotactic protein-1; MIP, Macrophage inflammatory protein; RANTES, Regulated and normal T cell expressed and secreted; TNF-α, Tumor necrosis factor-α; VEGF, Vascular endothelial growth factor; M-CSF, Macrophage colony-stimulating factor. Values are mean ± SD. P<0.05*; P<0.01** by ANOVA with Dunnett's post test vs. NaCl. Multiplex analysis of the shown markers was performed using a standard rat 24-plex panel from Bio-Rad. †IL-1β, IL-2, IL-6, IL12p70, IL-13, G-CSF, GM-CSF were below detection level and are not listed.

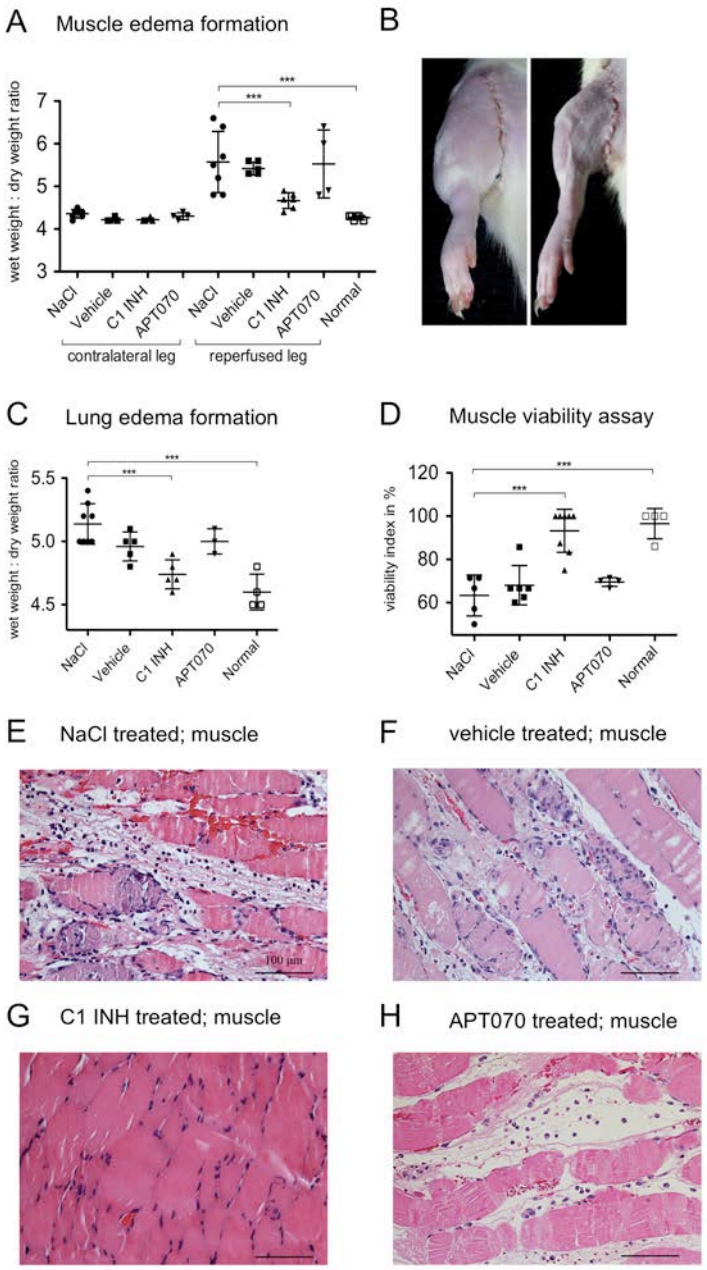


**Figure 1. Effect of C1 INH on edema formation, muscle viability and histological assessment of muscle damage.** (A) Analysis of edema in the gastrocnemic muscle of both the contralateral- and reperfused legs. NaCl treated rats were compared with C1 INH, APT070 as well as vehicle treated and normal rats. C1 INH treatment reduced muscle wet weight / dry ratio for C1 INH as compared to NaCl controls. (B) Representative images of edema formation after 24 h reperfusion for treatment with NaCl (left) and C1 INH (right). (C) Edema formation in the lung. C1 INH treatment led to a reduced lung wet / dry weight ratio as compared to NaCl controls. (D) Viability of the gastrocnemic muscle as assessed by MTT. C1 INH treatment improved muscle viability as compared with NaCl controls. (**E**-**H**) Hematoxylin / eosin stained histological samples of reperfused gastrocnemic muscle. Representative images are shown for NaCl (**E**) and vehicle (**F**) controls as well as C1 INH (**G**) and APT070 (**H**) treatment. One-way ANOVA followed by Dunnett's post hoc test for significance vs. NaCl controls was used. Error bars indicate mean ± SD. *P≤0.05; **P<0.01; ***P<0.001.

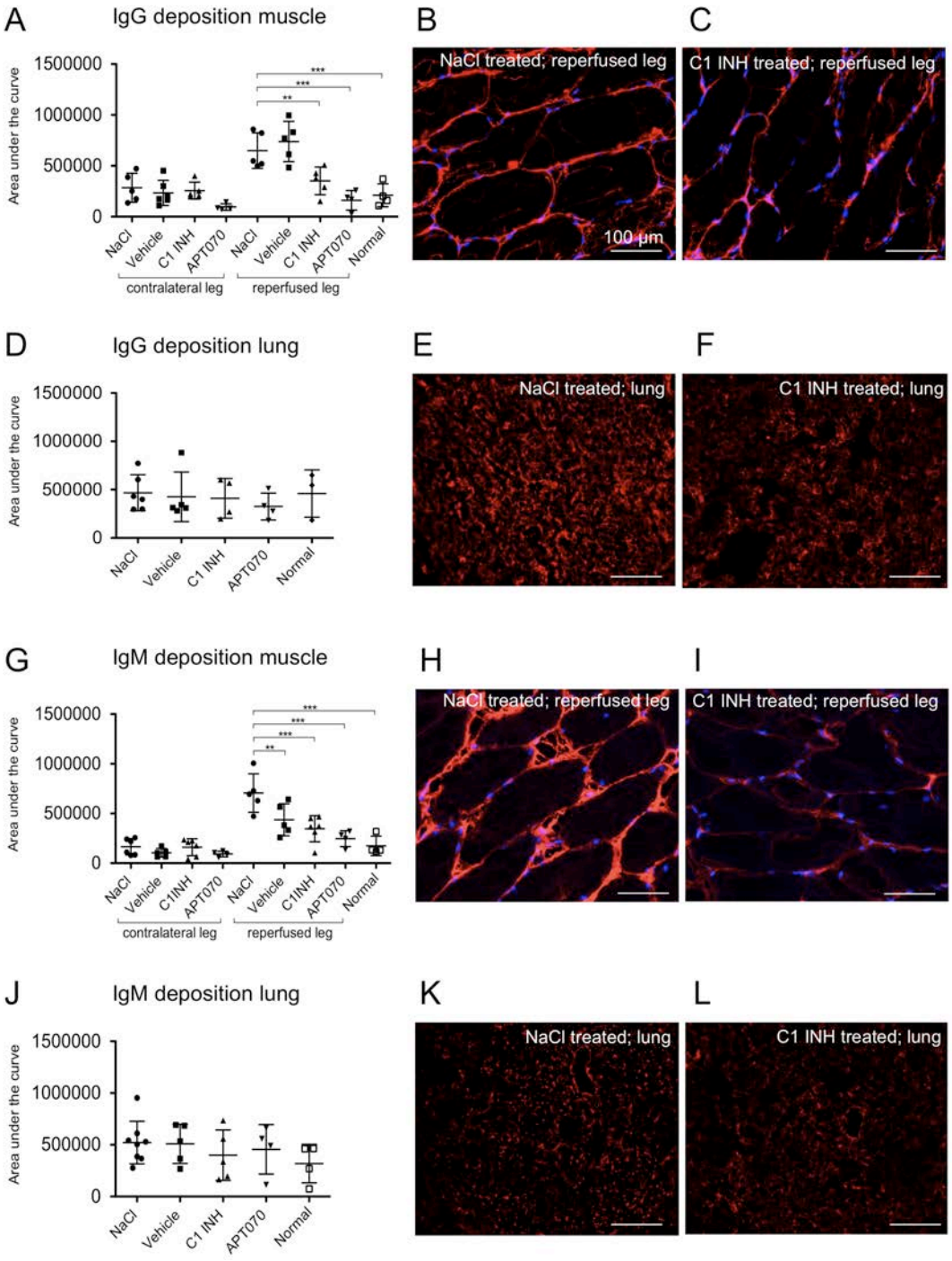


**Figure 2. Analysis of deposition of IgM and IgG in muscle as well as in lung tissue.** (A, D, G and J) Quantitative analysis of immunofluorescence stainings. (A) Detection of IgG in muscle and (**D**) in lung tissue. (G) Detection of IgM in muscle and (**J**) in lung tissue. (B and C) Representative immunofluorescence images of IgG deposition in muscle and (E and F) in lung tissue of either a NaCl or C1 INH treated rat. (H and I) Representative immunofluorescence images of IgM deposition in muscle and (K and L) in lung tissue of either a NaCl or C1 INH treated rat. IgM as well as IgG detectable in the red channel (CY3), counterstaining with DAPI (blue channel). One-way ANOVA followed by Dunnett's post hoc test for significance vs. NaCl controls was used. Error bars indicate mean ± SD. *P<0.05; **P<0.01; ***P<0.001.

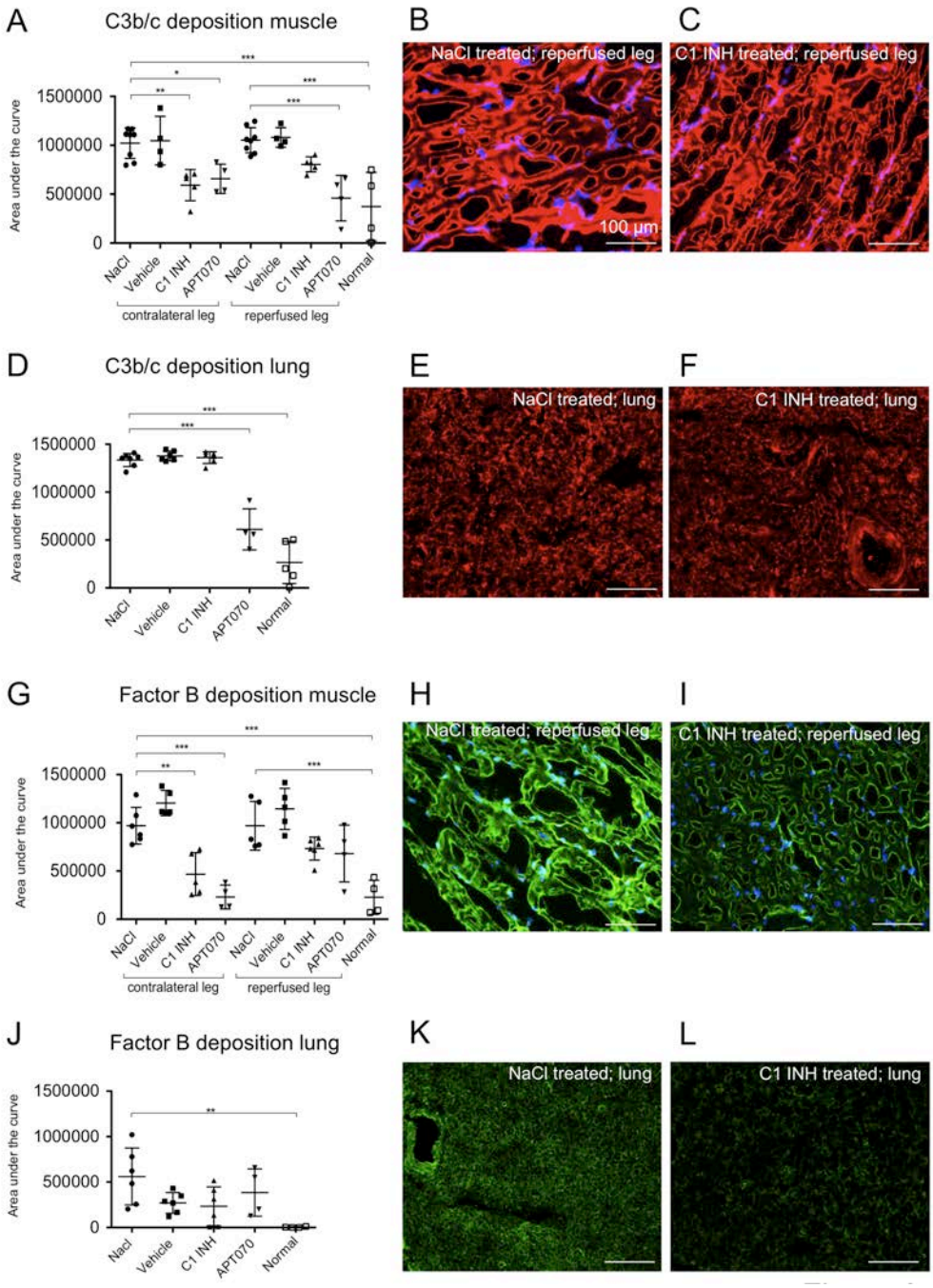


**Figure 3. Deposition of C3b/c and factor B in muscle and lung tissue.** (A, D, G and J) Quantification data of C3b/c and factor B deposition in muscle and lung tissue, respectively. (B and C) Representative immunofluorescence images of C3b/c deposition in muscle tissue. (**E** and **F**) Representative immunofluorescence images of C3b/c deposition in lung tissue. Counterstaining with DAPI (blue channel, only shown for muscle tissue), C3b/c visible in the red channel (CY3). (**H** and **I**) Representative immunofluorescence images of factor B deposition in muscle tissue. (**K** and **L**) Representative immunofluorescence images of factor B deposition in lung tissue. Counterstaining with DAPI (blue channel), factor B visible in the green channel (Alexa 488). One-way ANOVA followed by Dunnett's post hoc test for significance vs. NaCl controls was used. Error bars indicate mean ± SD. *P<0.05; **P<0.01; ***P<0.001.

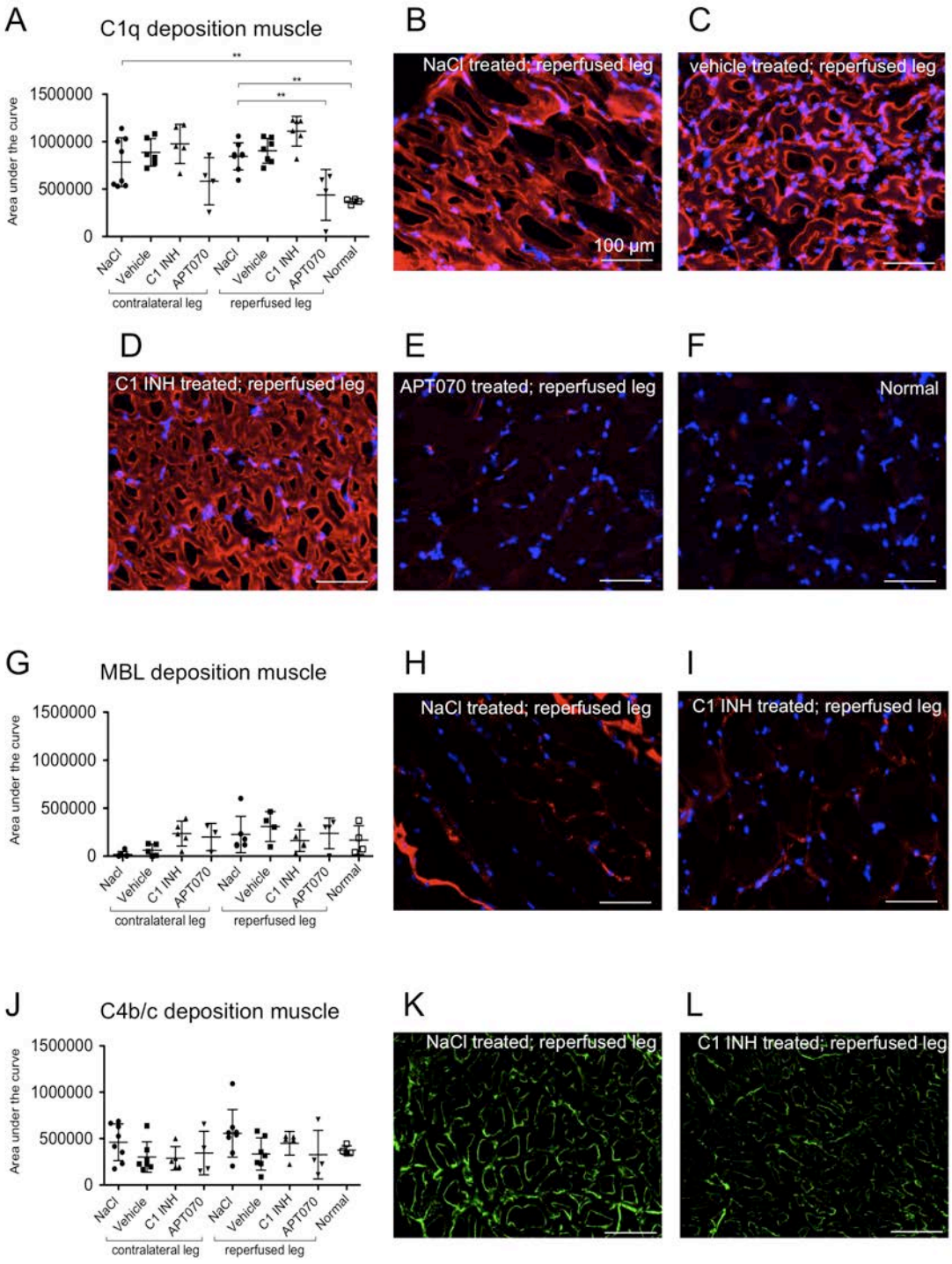


**Figure 4. Deposition of C1q, MBL and C4b/c in muscle tissue.** (A, G and J) Quantification data of C1q, MBL and C4b/c deposition in muscle tissue. (B-F) Representative immunofluorescence images of C1q deposition depending on treatment. (H and I) Representative immunofluorescence images of MBL deposition in muscle tissue. Counterstaining with DAPI (blue channel), C1q or MBL visible in the red channel (CY3). (K and L) Representative immunofluorescence images of C4b/c in muscle tissue, C4b/c visible in the green channel (Alexa 488). One-way ANOVA followed by Dunnett's post hoc test for significance vs. NaCl controls was used. Error bars indicate mean ± SD. *P<0.05; **P<0.01; ***P<0.001.

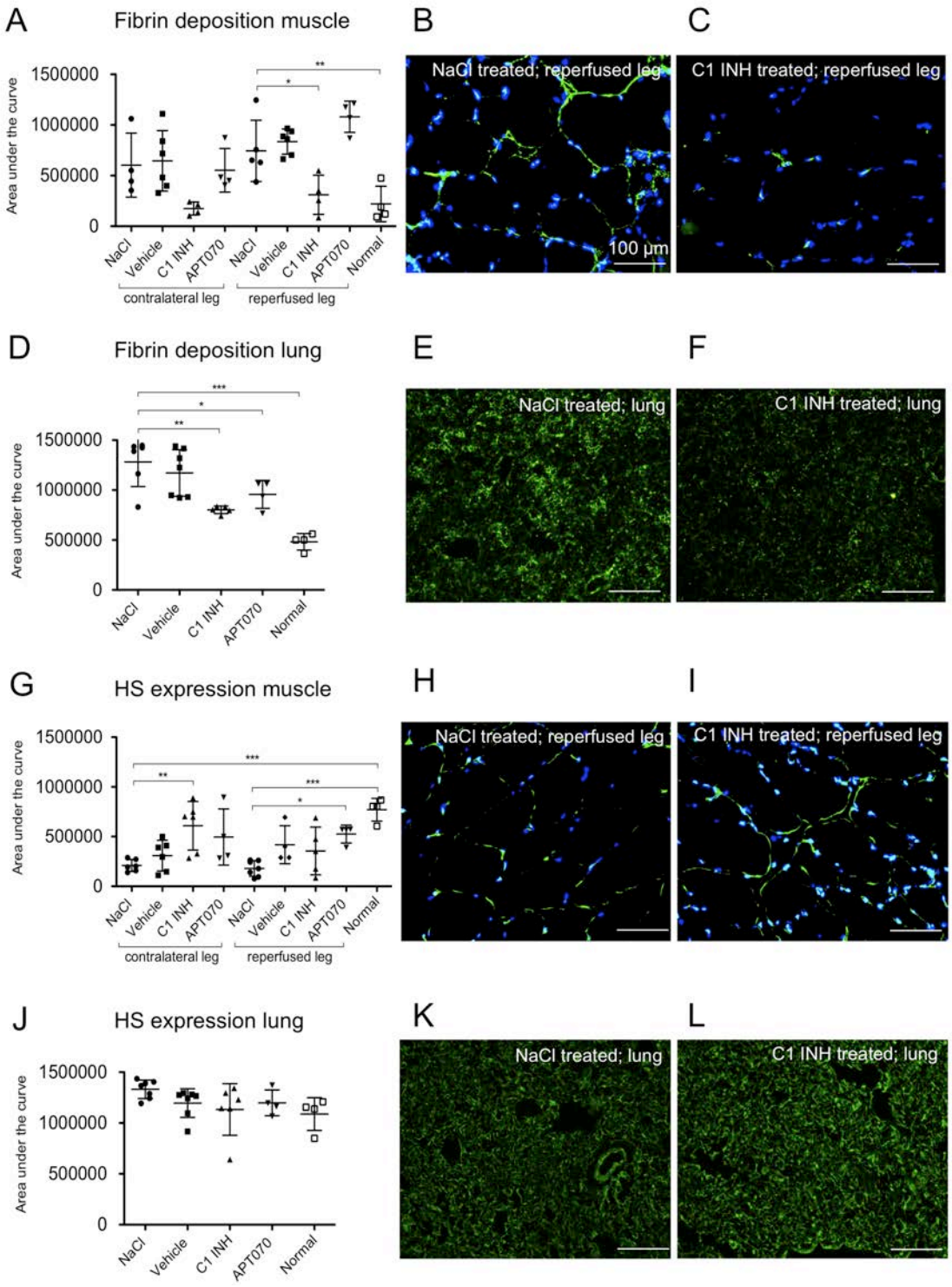


**Figure 5. Fibrin deposition as well as heparan sulfate (HS) expression in muscle and lung tissue.** (A, D, G and J) Quantification data from immunofluorescence stainings. (B and C) Representative immunofluorescence images for fibrin deposition. Counterstaining with DAPI (blue channel), fibrin visible in the green channel (FITC). E and F, Representative immunofluorescence images for fibrin deposition in lung tissue. (H and **I**; **K** and **L**) Representative immunofluorescence images of HS expression in muscle and lung, respectively. HS visible in the green channel (FITC). One-way ANOVA followed by Dunnett's post hoc test for significance vs. NaCl controls was used. Error bars indicate mean ± SD. *P<0.05; **P<0.01; ***P<0.001.

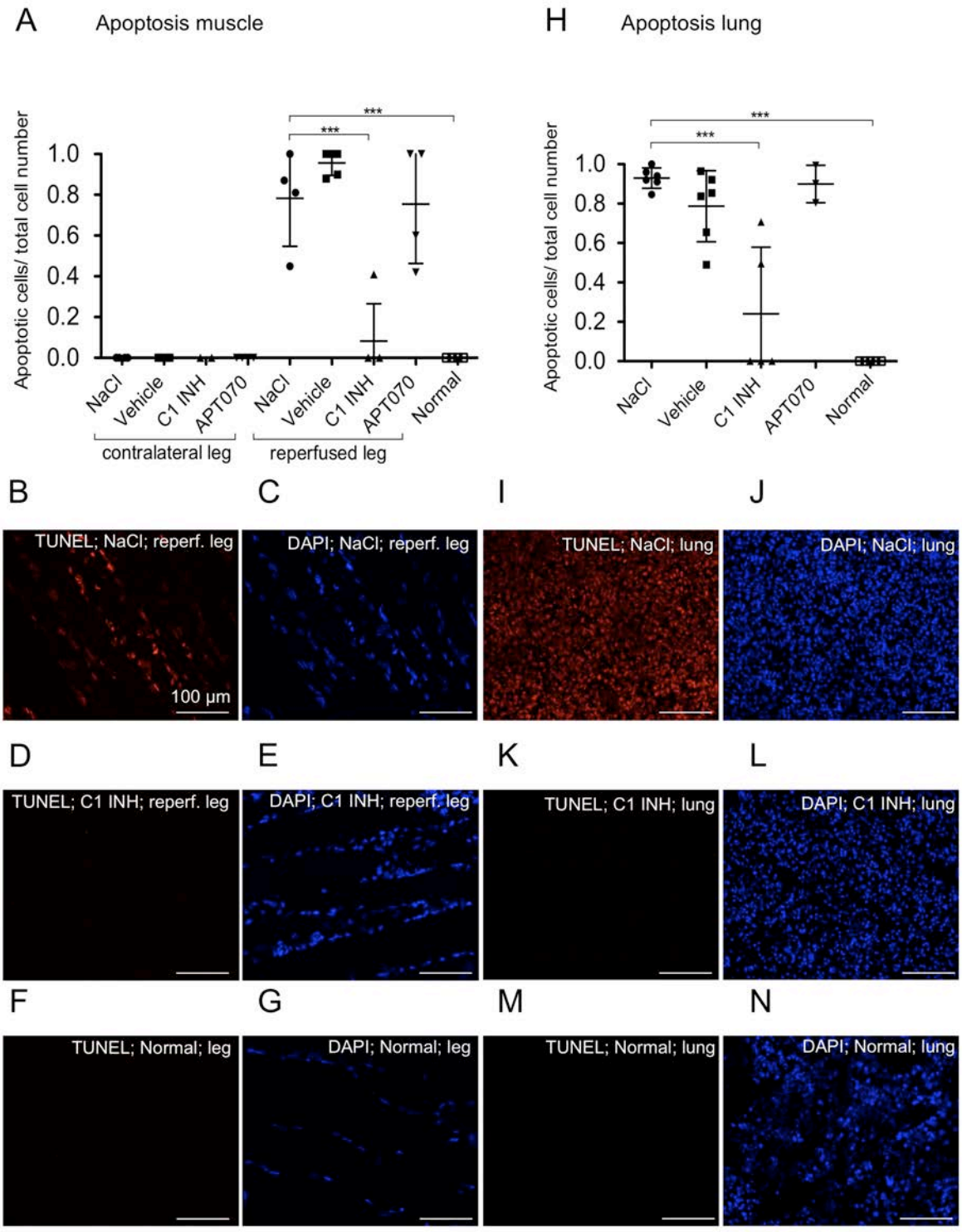


**Figure 6. Frequency of apoptotic cells in muscle and lung tissue.** (A and H) Quantitative analysis of TUNEL staining in muscle and lung tissue, respectively. (**B-G**) and (**I-N**) Representative immunofluorescence images of TUNEL staining of reperfused muscle and lung, respectively. TUNEL-positive cells are shown in red (**B, D, F, I, K, M**), corresponding DAPI staining of all nuclei in blue (**C, E, G, J, L, N**). One-way ANOVA followed by Dunnett's post hoc test for significance vs. NaCl controls was used. Error bars indicate mean ± SD. *P<0.05; **P<0.01; ***P<0.001.

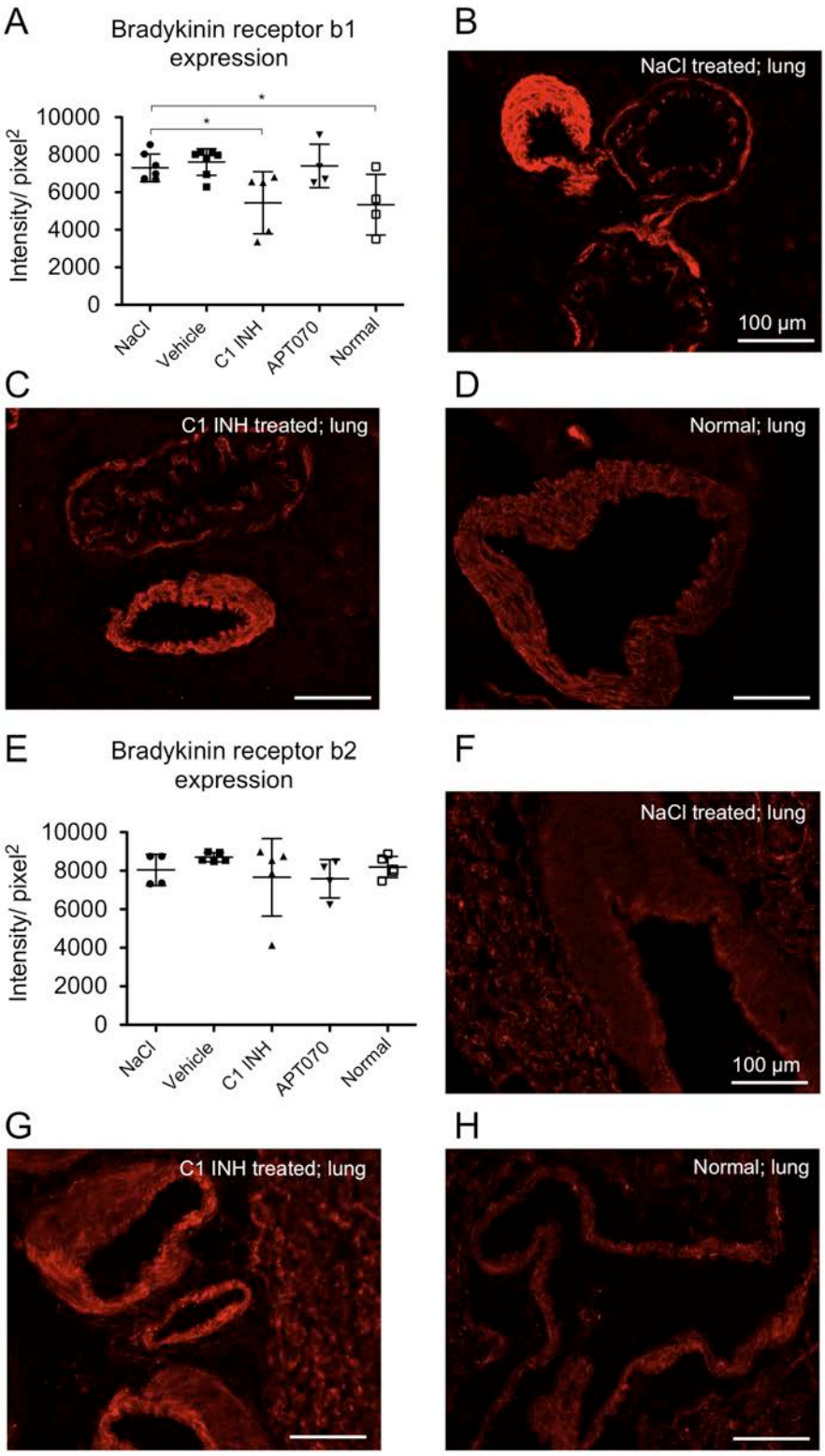


**Figure 7. Endothelial expression of bradykinin receptor b1 as well as b2 in lung tissue.** (A and E) Quantification data from immunofluorescence stainings. (B-D) Representative immunofluorescence images of bradykinin receptor b1 as well as (**F**-**H**) bradykinin receptor b2 staining in vessels of lung tissue. One-way ANOVA followed by Dunnett's post hoc test for significance vs. NaCl controls was used. Error bars indicate mean ± SD. *P<0.05; **P<0.01; ***P<0.001.

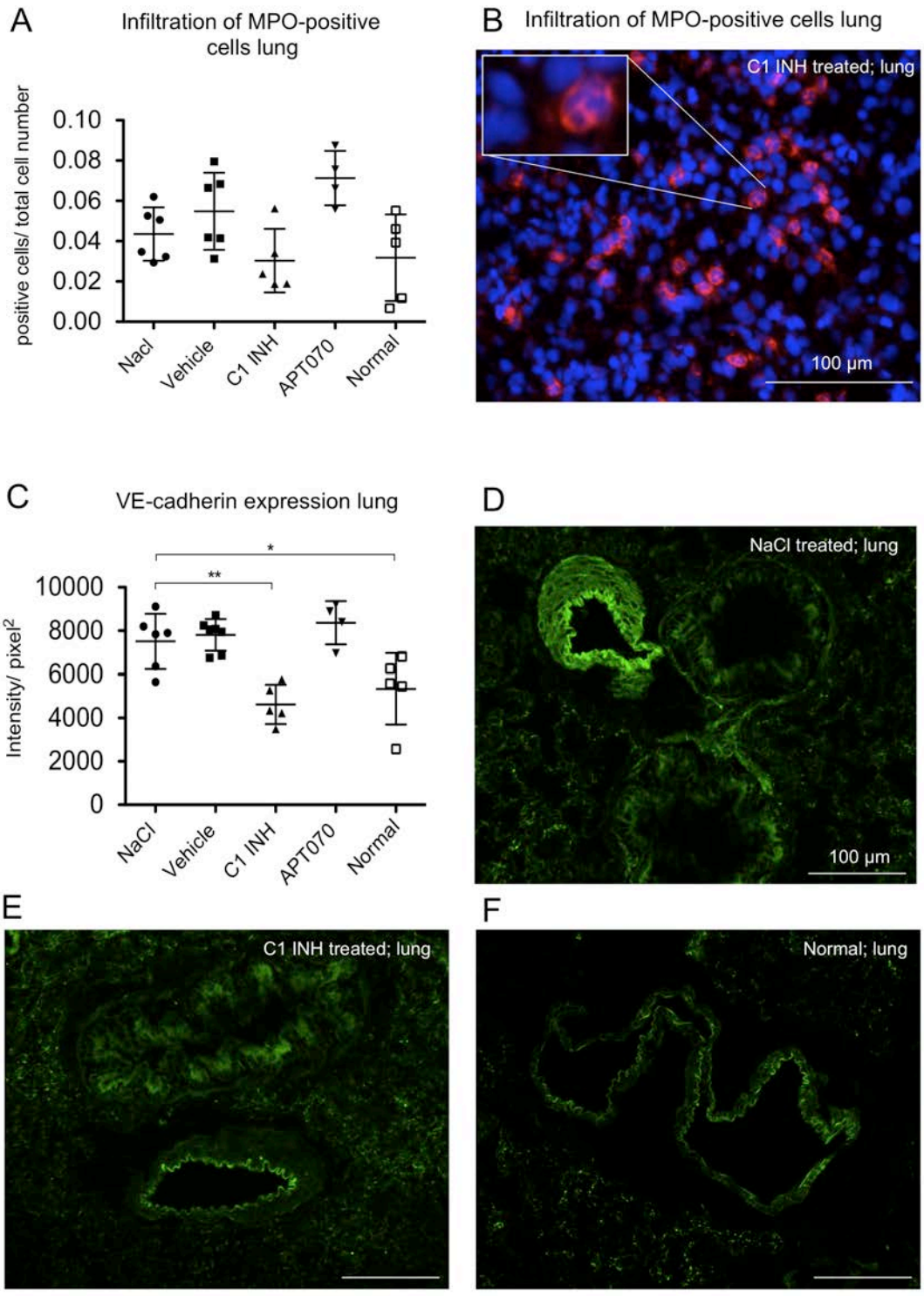


**Figure 8. Infiltration of myeloperoxidase positive cells in lung tissue as well as VE-cadherin expression.** (A) Quantitative evaluation and (**B**) representative immunofluorescence image of MPO expression in lung tissue. The blue channel shows DAPI staining, the red channel (CY3) shows MPO positive cells. (C) Quantification data from immunofluorescence stainings of VE-cadherin. (D–F) Representative images of VE-cadherin staining. One-way ANOVA followed by Dunnett's post hoc test for significance vs. NaCl controls was used. Error bars indicate mean ± SD. *P<0.05; **P<0.01; ***P<0.001.

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# Paper II

## C1 esterase inhibitor reduces distant organ damage caused by tourniquet-induced lower extremity ischemia/reperfusion injury

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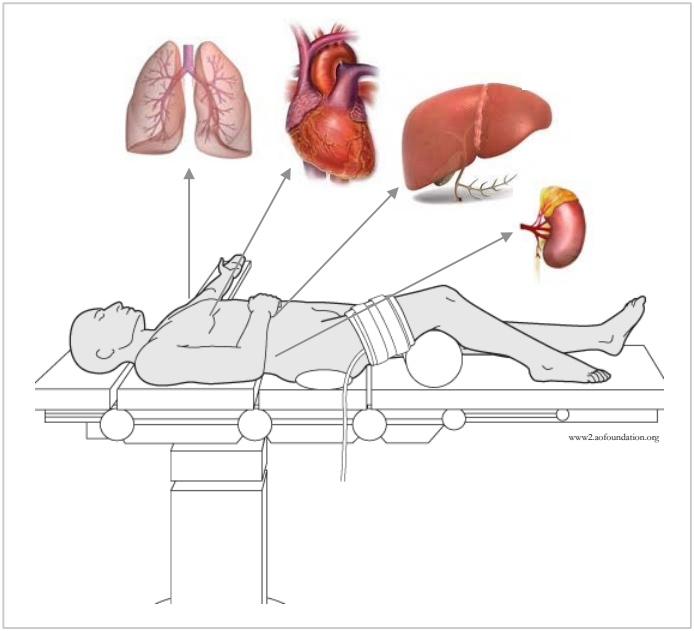
**Status:** in submission for publication to ***Critical Care Medicine***

**Aim:** The aim of this study was to investigate the effect of C1 esterase inhibitor on distant organ damage caused by tourniquet-induced ischemia/reperfusion injury of the lower extremity.

**Conclusion:** Individual organs differ in terms of susceptibility to systemic effects of lower extremity IRI. Furthermore, C1 INH showed beneficial effects in all of the organs and may therefore be a promising drug candidate to reduce distant organ damage induced by lower extremity IRI




Adapted from www.aofoundation.org

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***Key Words***: C1 esterase inhibitor; ischemia/reperfusion injury; distant organ damage; lower extremity; tourniquet

## Abstract

**Objective:** Distant organ damage is of clinical importance in lower extremity ischemia/reperfusion injury. Organ damage results from activation of the complement, coagulation and kinin systems caused by the original insult. Aim of the study was to analyze the effect of C1 esterase inhibitor (C1 INH) on distant organ damage caused by reperfusion injury of the lower extremity.

**Design:** Rats were subjected to 3h hind limb ischemia and 24h reperfusion. C1 INH, vehicle, the complement inhibitor APT070, or NaCl were injected intravenously 5min before ischemia. Deposition of IgM, complement factors C1q, C3b/c and fB, fibrin, as well as expression of tissue factor, heparan sulfate and bradykinin receptors b1 and b2 were analyzed in lung, liver, heart and kidney.

**Subjects:** 29 male Wistar rats weighing 250-350 g.

**Measurements and main results:** Histology revealed no or only minimal tissue damage in heart, liver and kidney, whereas edema was detected in lung. Expression of tissue factor, bradykinin receptor b2 and heparan sulfate were not affected in these distant organs ($P>0.05$). In contrast, significantly increased deposition or expression was found of IgM (liver), C1q (liver), C3b/c (lung, kidney), factor B (lung, liver, heart), fibrin (lung, liver, heart), and bradykinin receptor b1 (lung, heart). C1 INH significantly reduced deposition of C3b/c ($P<0.05$) in kidney, factor B ($P<0.05$) in liver, and fibrin in lung ($P<0.01$), and prevented up-regulation of bradykinin receptor b1 in lung ($P<0.05$) and heart ($P<0.001$).

**Conclusions:** Distant organs were affected to different degrees by reperfusion injury of the lower extremity. We conclude that individual organs differ in terms of susceptibility to systemic effects of lower extremity reperfusion injury. C1 INH showed beneficial effects in all organs and may therefore reduce distant organ damage induced by reperfusion injury of an extremity.

## Introduction

Distant organ damage is of essential clinical importance in lower extremity ischemia/reperfusion injury (IRI) which may result from thrombotic occlusion, embolism, trauma or surgical intervention through tourniquet application and subsequent restoration of blood flow.(1) Reperfusion of an ischemic extremity initiates a complex inflammatory response, involving activation of the complement, coagulation, kinin as well as the fibrinolytic system, which may, in severe cases, result in cardiopulmonary failure as well as renal and hepatic dysfunction.(2) However, the lung is the most vulnerable distant organ affected.(3) The observed damage may range from mild dysfunction to severe respiratory failure and is often followed by cardiovascular failure, renal, neurological and hepatic dysfunction.(4)

Locally, the ischemic insult results in formation of neo-epitopes and subsequent binding of naturally occurring antibodies, mainly during the reperfusion phase.(5) In 1996, Weiser et al. showed that IRI is mediated by natural antibodies and activation of the complement system, which was confirmed by Carroll and co-workers, and termed “innate autoimmunity”.(6, 7) The three complement pathways are triggered by different activators and differ in the molecules involved in the initiation sequence, but converge at the C3 activation step. The complement component C3 is a key molecule involved in innate immunity and its activation is crucial for the effector functions of the complement system, for review see Ricklin et al..(8) Excessive local complement activation following prolonged ischemia and subsequent reperfusion may in its full-blown form manifest as systemic inflammatory response syndrome (SIRS) and thus contributing to multiple organ dysfunction.(9, 10) The complement system and the other plasma cascade systems form a highly interactive network, which makes a strict separation of these individual systems impossible. Activation of the complement system can result in the initiation of the coagulation system and vice versa. In 1997, Ikeda et al. demonstrated that the anaphylatoxin C5a can induce tissue factor (TF) expression on human umbilical vein endothelial cells (HUVEC).(11) In 2006, Huber-Lang et al. presented a new complement activation pathway, where thrombin acts as a potent C5 convertase to generate C5a.(12) The coagulation factor XII (FXIIa) has a key role in the plasma cascade systems, as it can activate the classical complement pathway via C1q, but also triggers the coagulation system via activation of factor XI and the fibrinolytic system through plasminogen activation.(13-15) In the kinin system, FXIIa cleaves prekallikrein to form kallikrein finally leading to the formation of bradykinin, which mediates increased vascular permeability.(16) Another crucial determinant of vascular permeability is the endothelial glycocalyx, which is a carbohydrate-rich layer located between the fluid phase of the blood and the endothelium.(17)

It forms a complex network consisting of proteoglycans, glycosaminoglycans, such as heparan sulfate, as well as soluble molecules, and upholds anti-coagulant as well as anti-inflammatory conditions in the healthy vasculature.(18) However, a partial loss of the glycocalyx, termed as shedding, decreases vascular barrier function and renders the endothelium pro-inflammatory and pro-coagulant. In 2007, Rehm et al. showed for the first time in humans that ischemia resulting from major vascular surgery causes shedding of the glycocalyx.(19)

The heavily glycosylated C1 esterase inhibitor (C1 INH) is a multi-functional acute-phase protein, which plays key roles in regulating C1r, C1s and MASP1/2 of the complement system, FXIIa, activated factor XI (FXIa) and thrombin of the coagulation system as well as plasma kallikrein of the kinin system, for review see Davis et al.(20) Recently, we showed beneficial effects of human C1 INH in a rat model of lower extremity IRI. There we found a reduction of skeletal muscle IRI as well as associated distant lung damage.(21) Also in earlier studies it was demonstrated, that C1 INH reduces or even protects from IRI of the brain, heart, liver or gut.(22-25)

Distant organ damage following lower extremity IRI is a relevant clinical problem with increased morbidity and mortality rates.(26) An effective therapy to either prevent or reduce distant organ damage is currently still missing. The aim of the present study was therefore to determine the effect and mechanisms of C1 INH on distant organ damage (heart, liver, lung and kidney) caused by tourniquet-induced IRI of the lower extremity by using a rat model. We hypothesized, that C1 INH treatment regulates deposition or expression of complement, coagulation as well as kinin system components in the affected remote organs and may therefore reduce distant organ damage.

## Material and methods

All animal experiments were conducted in compliance with the Guide for the Care and Use of Laboratory Animals and Swiss national guidelines.(27) The study was approved by the animal ethics committee (permission number 71/10) of the cantonal veterinary service (Cantone of Bern, Switzerland).

### Animals

Male Wistar rats (wild type, bred at the central animal facility, University of Bern) were kept in clear 1500 $cm^2$ Euro-standard Type IV S cages under standard housing conditions with food and water ad libitum. Cages were individually ventilated at 20 ± 2°C and 45-65% relative humidity with a circadian rhythm of 12/12 h. For the experiments, rats weighing between 250 and 350 g were used.

### Reagents

Human plasma-derived C1 INH (Berinert) and the vehicle (10 mg/ml glycine, 2.9 mg/ml sodium citrate, 8.5 mg/ml sodium chloride, pH 7.0) were provided by CSL Behring. APT070, a highly effective complement inhibitor, which has binding sites for C3b and also C4b, was provided by King's College (London, UK).(28) APT070 consists of the first 3 consensus domains of the human CR1 and a membrane-targeted synthetic peptide, which mediates the binding to phospholipids on the cell surface and therefore protects the cell against complement activation.(29) APT070 was formulated in a solution of phosphate-buffered saline (PBS, pH 7.4) containing mannitol (50 mg/ml) and arginine (17.4 mg/ml).

### Experimental groups

Rats were divided into five groups. The treatment group (n=6, C1 INH group) received a dose of 50 IU/kg (conc. 50 IU/ml) of C1 INH. Control group 1 (n=8, NaCl group) received 1 ml/kg of 0.9% sodium chloride. Control group 2 (n=7, vehicle group) received 1 ml/kg C1 INH vehicle prior to ischemia. Control group 3 (n=4, APT070 group) received 9 mg/kg (9 mg/ml) of APT070 before induction of ischemia and control group 4 (n=4, normal) underwent no intervention.

**Anesthesia and analgesia**

Anesthesia was induced with 2.5% isoflurane in oxygen in a box and maintained by inhalation of 1.5% isoflurane on a nose mask. Analgesia was provided by 0.05 mg/kg of buprenorphine injected subcutaneously 30 minutes prior to surgery. Total duration of anesthesia was 6 h after which the rats were allowed to wake up. To provide adequate analgesia for the 24 h reperfusion period buprenorphine injection was repeated when animals were completely awake. After completion of 24 h reperfusion, the rats were anesthetized again as described above and sacrificed by exsanguination during organ harvest.

**Surgery**

Rats were kept on a heating pad to maintain body temperature at 37°C. The femoral artery and vein were exposed via groin incision and a tourniquet (standardized weight of 450 g) placed underneath the femoral vessels to block collateral circulation.(30) The femoral artery was then occluded for 3 h with two microvascular clamps. Venous return was maintained during the entire period of ischemia to prevent congestion and additional injury through microcirculatory impairment. Ischemia was followed by 24 h reperfusion during which the rats were allowed to wake up with appropriate analgesia. At the end of the experiments, standardized tissue samples from lung, liver, kidney and heart were taken.

**Histological assessment of damage**

For assessment of hemorrhage, total damage as well as infiltration of neutrophils, organ samples were fixed in 4% formalin for 24-72 h. Thereafter, all samples were embedded in paraffin, cut into 3 µm thick sections and stained with hematoxylin and eosin.

**Immunofluorescence analyses of tissue samples**

Immunofluorescence staining was used to quantify deposition of IgM (3020-08; Southern Biotech), C1q (A0136, Dako), C3b/c (A0062, Dako) and factor B (341272, Calbiochem). Furthermore, we analyzed fibrin deposition (F0111; Dako), expression of tissue factor (sc23596, Santa Cruz), heparan sulfate (HS; 370255, Amsbio), bradykinin receptor b1 (ABR-011, Alomone Labs) and bradykinin receptor b2 (ABR-012, Alomone Labs). Tissue samples were washed in PBS, blotted dry and embedded in OCT matrix (Tissue-Tek) on dry ice. Cryosections were fixed in acetone and rehydrated in Tris-buffered saline (TBS).

Primary antibodies were incubated overnight at 4°C and secondary antibodies were incubated for 1 h at room temperature (RT). Pictures were taken with a Nikon camera attached to a fluorescent microscope (Leica DMI 4000B) using oil immersion objectives and analyzed using Image J GraphPad Prism 5 software. For the analysis of endothelial expression of tissue factor as well as bradykinin receptor b1 and b2, the inner lining of the vessels was selected by hand, the surface area calculated and the intensity of immunofluorescence measured. Area under the curve values were obtained and divided by the surface area to achieve a final value in intensity per square pixel.(21)

**Statistical analysis**

Data are expressed as mean ± standard deviation (SD). Statistical significance was determined by one-way analysis of variance with Dunnett's post-test against NaCl control, using GraphPad Prism 5 software. P values of $< 0.05$ were considered statistically significant. Determination of n-numbers per group was performed without formal power analysis, based on previous experiments with C1 INH.(21)

## Results

### Effect of lower extremity ischemia/reperfusion injury on histopathology of distant organs

Tissue sections of heart, liver, kidney and lung were stained with hematoxylin and eosin. Light microscopic assessment of distant organ damage was unremarkable for heart and liver in all groups. In particular no necrosis and no inflammatory infiltrate was noted in any of these organs. Also, no relevant tissue edema was found in heart and liver. Minimal tubular swelling was detected in the kidneys of the NaCl and vehicle treated animals but not in the naïve and drug treated rats. Assessment of lung tissue revealed a primarily interstitial infiltration of neutrophil granulocytes, accompanied by interstitial edema, alveolar fluid accumulation and some extravasation of red blood cells in the NaCl, Vehicle and APT070 treated rats after 24 h of reperfusion of the lower extremity, whereas morphology of lung tissue of C1 INH treated rats was comparable to normal rats (Figure 1).

### Deposition of IgM and complement components in heart, lung, liver and kidney caused by ischemia/reperfusion injury of the lower extremity

Analysis of IgM deposition in heart, kidney and lung tissue after 3 h ischemia followed by 24 h reperfusion of the lower extremity revealed no significant differences to normal rats, In contrast, IgM deposition in liver tissue of NaCl treated rats was significantly higher as compared to normal rats (Figure 2, Table 1). Similar results were detected for C1q deposition, as NaCl control rats showed increased C1q deposition in liver tissue compared to normal rats ($P<0.001$), whereas lung, kidney and heart tissue demonstrated no inter-group differences. C1q deposition was significantly reduced in APT070 treated rats ($P<0.001$), but not in C1 INH treated rats (Table 1).

No inter-group differences were detected for C3b/c in heart and liver tissue, whereas in lung ($P<0.001$) and kidney ($P<0.05$) tissue NaCl control animals demonstrated significantly increased C3b/c deposition as compared to normal rats. In lung tissue APT070, but not C1 INH, significantly reduced C3b/c deposition ($P<0.001$). In contrast, in kidney tissue, C3b/c deposition was significantly reduced ($P<0.05$) in C1 INH-, but not in APT070 treated rats (Figure 3, Table 1).

After 24 h reperfusion of the lower extremity factor B (fB) deposition was significantly increased in NaCl controls as compared to normal rats in lung ($P<0.01$), liver ($P<0.01$) and heart ($P<0.05$), but not in kidney. In liver tissue, C1 INH ($P<0.05$) as well as APT070 ($P<0.001$) led to significantly reduced fB deposition. In lung and heart tissue, neither C1 INH nor APT070 could reduce fB deposition (Table 1).

**Effect of C1 INH on expression of tissue factor and fibrin deposition in heart, lung, liver and kidney**

Analysis of the expression of TF in the inner lining of the vessels, the endothelium, in heart, kidney and lung tissue revealed no significant differences as compared to normal rats (Figure 4, Table 2). However, analysis of fibrin deposition demonstrated significantly increased fibrin deposition of NaCl control rats in lung ($P<0.001$), liver ($P<0.05$) and heart ($P<0.05$), but not in kidney tissue as compared to normal rats. In lung tissue both, C1 INH ($P<0.01$) and APT070 ($P<0.05$) significantly reduced fibrin deposition, but this was not the case in liver and heart (Figure 5, Table 2).

**Effect of C1 INH on kinin system components bradykinin receptor b1 and b2 as well as heparan sulfate in heart, lung, liver and kidney**

Expression of the inducible bradykinin receptor b1 was analyzed in the inner lining of blood vessels. Kidney as well as liver tissue presented no inter-group differences. However, in lung ($P<0.05$) as well as heart ($P<0.001$) tissue NaCl treated rats demonstrated significantly increased expression of the bradykinin receptor b1 in comparison to normal rats. In both, lung ($P<0.05$) and heart ($P<0.001$) tissue C1 INH treatment prevented up-regulation of the b1 receptor, which was not the case after APT070 treatment (Figure 6, Table 3).

Investigation of the constitutively expressed bradykinin receptor b2 revealed no up-regulation of expression in lung, heart, liver and kidney tissue as compared to normal rats (Table 3). Furthermore, we analyzed the expression of the glycosaminoglycan heparan sulfate (HS) in lung, heart, liver and kidney after 24 h reperfusion of the lower extremity. Also for HS expression we detected no significant up-regulation as compared to normal rats (Table 3).

## Discussion

The aim of this project was to analyze the involvement of the complement, coagulation and kinin systems in distant organ damage associated with lower extremity IRI. Furthermore, investigation of the effect of C1 INH on components of the complement, coagulation and kinin systems was carried out.

Histopathological assessment revealed essentially no distant tissue damage in heart and liver, and only minimal tubular swelling was observed in the kidneys. From these findings we conclude that the systemic injury induced in our experimental setting was not severe enough to induce structural alterations visible by light microscopy in these organs. This is in line with an earlier study in which similar results were obtained.(2) In contrast, analysis of lung tissue demonstrated a primarily interstitial infiltration of neutrophils as well as interstitial edema and alveolar fluid accumulation after 3 h ischemia and 24 h reperfusion of the lower extremity. These results confirm earlier findings, identifying the lung as the most vulnerable organ in lower extremity IRI-induced distant organ damage as well as multiple organ dysfunction syndrome.(31, 32) This may be explained by locally produced pro-inflammatory components, which are distributed via the blood circulation and reach the lung first and therefore render it the most vulnerable organ for remote IRI.(31) Furthermore, activation of neutrophils and subsequent accumulation in lung tissue is an early step in the development of lung injury and may also play an important role.(33) C1 INH treated rats demonstrated improved structural integrity of the lung which has also been shown in earlier studies in a sheep model of lung transplantation.(34)

In a next step deposition of IgM was investigated, as it was demonstrated before, that IgM binding to the affected tissue occurs due to the ischemic insult in mice.(5, 35-37) An increase of IgM deposition in lung, heart and kidney tissue was not detected as compared to normal rats. In liver tissue, IgM is significantly increased in the NaCl group as compared to normal rats, It has been demonstrated, that neo-epitopes, such as non-muscle myosin heavy chain type II, are formed and get accessible due to the hypoxic insult during ischemia, which results in the binding of natural antibodies and the activation of the classical or lectin complement pathway during the reperfusion period.(5, 38, 39) Analysis of deposition of the classical pathway component C1q in individual organs demonstrated an increase only in liver tissue, whereas lung, heart and kidney showed no increased C1q deposition as compared to normal rats. Increased C1q deposition found in liver tissue may correlate with the increased IgM deposition detected in liver tissue, which may indicate classical complement activation in the liver (Table 1), C1q deposition might also be attributed to the presence of apoptotic cells or C-reactive protein, to both of which binding of C1q was described earlier.(40, 41)

C1q deposition in liver was significantly reduced by APT070, but not C1 INH. Similar results were found for C3b/c deposition in lung tissue, where APT070 significantly reduced deposition, but not C1 INH. However, in kidney tissue C3b/c was significantly reduced by C1 INH, but not APT070. Furthermore, increased factor B deposition in liver tissue was significantly reduced by C1 INH and also APT070 (Table 1). Effective complement inhibition by APT070 as well as C1 INH was extensively shown, as both are potent complement inhibitors.(42-45)

In order to investigate the involvement of the coagulation system in distant organ damage, TF expression as well as fibrin deposition was analyzed (Table 2). The coagulation system can be activated via the intrinsic pathway or the extrinsic pathway with an increased expression of TF on endothelial cells, induced by pro-inflammatory cytokines or C-reactive protein.(46) Our results demonstrated that TF expression was unchanged in the analyzed organs as compared to normal rats. These results suggest that the extrinsic pathway is not activated in distant organs and the coagulation system may thus be initiated via the intrinsic pathway or via the complement system.(47-49) Fibrin deposits were detected in lung, liver and heart, but not in kidney. Only in the lung C1 INH as well as APT070 significantly prevented fibrin deposition, which is in line with the inhibitory action of C1 INH on FXIIa, FXIa and thrombin leading to fibrin formation, whereas the beneficial effects of APT070 could be indirect via complement inhibition i.e. prevention of C5b-9 induced prothrombinase activation.(50, 51)

Furthermore, we analyzed the expression of the kinin system components bradykinin receptor b1 (BRB1) and b2 (BRB2) as well as the glycosaminoglycan heparan sulfate (HS) (Table 3). Under normal physiological conditions the BRB1 is only minimally expressed, whereas an inflammatory condition rapidly induces its expression.(52) Binding of bradykinin to the constitutively expressed BRB2 as well as the inducible BRB1 both lead to vasodilation and vascular leakage, which contributes to the pathophysiology of IRI.(53) An up-regulation of BRB1 was found in lung and heart tissue of NaCl treated rats, but not in liver and kidney. However, C1 INH-, but not APT070-treatment, prevented up-regulation of BRB1 in lung and also in heart tissue. It is known, that the up-regulation of BRB1 occurs under the control of specific cytokines released in conditions of trauma or stress, including interleukin (IL)-1β and tumor necrosis factor (TNF)-α.(54) When analyzing the expression of BRB2 as well as HS, we found no inter-group differences. From these findings we conclude that BRB2 as well as HS expression in lung, liver, heart and kidney is not affected in our model of lower extremity IRI. In contrast, in skeletal muscle significantly less HS was detected in the contralateral and also reperfused leg.(21)

## Conclusion

The aim of this study was to analyze the effect of lower extremity IRI on lung, liver, heart and kidney. Furthermore, we wanted to investigate the efficacy of C1 INH in regulating selected parameters of the complement, coagulation as well as kinin system and thereby preventing distant organ damage. Our results show that IgM deposition as well as TF, BRB2 and HS expression was not affected in distant organs by lower extremity IRI, except IgM in liver. However, we detected significant alterations of C1q, C3b/c, factor B and fibrin deposition as well as an up-regulation of BRB1 expression. C1 INH treatment demonstrated beneficial effects in reduction of C3b/c, factor B and fibrin deposition as well as prevention of up-regulation of BRB1. We found that individual organs were not equally affected. From these findings we conclude that the individual organs differ in terms of susceptibility to systemic effects of lower extremity IRI. Furthermore, C1 INH showed beneficial effects in all of the organs and may therefore be a promising drug candidate to reduce distant organ damage induced by lower extremity IRI.

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## Disclosure

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**Table 1 Deposition of IgM as well as complement components C1q, C3b/c and factor B**

| | NaCl (n=8) | Vehicle (n=7) | C1 INH (n=6) | APT070 (n=4) | Normal (n=4) | |
|---|---|---|---|---|---|---|
| IgM | 5.21 ± 2.06 | 5.09 ± 1.89 | 3.99 ± 2.44 | 4.56 ± 2.39 | 3.18 ± 1.86 | Lung |
| | 7.33 ± 1.76 | 6.08 ±1.95 | 5.08 ± 2.93 | 7.44 ± 5.07 | 2.35 ± 1.46* | Liver |
| | 3.19 ± 1.13 | 3.18 ± 1.15 | 1.92 ± 0.56 | 3.33 ± 1.01 | 1.90 ± 0.48 | Heart |
| | 1.84 ± 0.76 | 2.28 ± 0.61 | 2.49 ± 1.33 | 3.05 ± 0.51 | 2.42 ± 0.92 | Kidney |
| C1q | 12.30 ± 2.03 | 14.06 ± 0.51 | 12.38 ± 1.71 | 10.86 ± 1.88 | 8.38 ± 3.76 | Lung |
| | 12.58 ± 1.75 | 12.49 ± 1.34 | 12.91 ± 1.38 | 5.09 ± 2.88*** | 3.84 ± 2.73*** | Liver |
| | 6.13 ± 3.66 | 4.56 ± 2.38 | 4.64 ± 2.19 | 3.73 ± 2.54 | 3.48 ± 3.40 | Heart |
| | 8.45 ± 3.63 | 7.82 ± 2.70 | 5.98 ± 1.74 | 8.51 ± 2.25 | 6.60 ± 3.29 | Kidney |
| C3b/c | 13.35 ± 0.68 | 13.77 ± 0.50 | 13.61 ± 0.61 | 6.11 ± 2.14*** | 2.65 ± 2.20*** | Lung |
| | 3.08 ± 0.49 | 2.95 ± 0.46 | 3.06 ± 0.24 | 2.52 ± 0.66 | 2.66 ± 0.85 | Liver |
| | 3.05 ± 0.91 | 3.21 ± 0.32 | 2.88 ± 0.39 | 2.96 ± 0.69 | 2.59 ± 0.46 | Heart |
| | 1.69 ± 0.41 | 1.39 ± 0.55 | 0.94 ± 0.33* | 1.55 ± 0.08 | 0.89 ± 0.34* | Kidney |
| factor B | 5.61 ± 3.13 | 2.71 ± 1.14 | 2.33 ± 2.14 | 3.84 ± 2.60 | 0.04 ± 0.05** | Lung |
| | 6.87 ± 1.63 | 4.18 ± 2.48 | 2.62 ± 0.64* | 0.95 ± 0.34*** | 0.87 ± 0.78** | Liver |
| | 5.60 ± 2.00 | 5.22 ± 2.09 | 3.47 ± 1.92 | 4.54 ± 2.05 | 2.07 ± 1.27* | Heart |
| | 7.71 ± 3.28 | 10.05 ± 3.09 | 9.18 ± 3.69 | 7.15 ± 3.30 | 6.89 ± 2.59 | Kidney |

Rats were subjected to 3 h unilateral hind limb ischemia, which was followed by 24 h reperfusion. Organs were analyzed for deposition of IgM and complement components by immunofluorescence stainings. Shown data are fluorescence intensities (area under the curve of histograms) divided by 100'000 and are expressed as mean ± standard deviation (SD). Statistical significance was determined by one-way analysis of variance with Dunnett's post-test against NaCl control. P values of < 0.05 were considered statistically significant. *P<0.05; **P<0.01; ***P<0.001.

**Table 2 Expression of coagulation system component tissue factor as well as deposition of fibrin**

| | NaCl (n=8) | Vehicle (n=7) | C1 INH (n=6) | APT070 (n=4) | Normal (n=4) | |
|---|---|---|---|---|---|---|
| TF | 7.35 ± 1.59 | 5.98 ± 1.54 | 5.28 ± 3.43 | 9.04 ± 0.17 | 6.57 ± 1.96 | Lung |
| | 7.30 ± 1.32 | 6.85 ± 1.25 | 7.23 ± 1.05 | 6.71 ± 1.33 | 6.46 ± 1.07 | Heart |
| | 8.84 ± 0.37 | 7.73 ± 1.41 | 8.62 ± 0.39 | 7.59 ± 0.56 | 6.92 ± 3.07 | Kidney |
| Fibrin | 12.82 ± 2.45 | 11.72 ± 2.33 | 8.02 ± 0.38** | 9.56 ± 1.40* | 4.81 ± 0.83*** | Lung |
| | 2.46 ± 0.33 | 2.04 ± 0.73 | 1.39 ± 0.57 | 3.63 ± 1.43 | 1.04 ± 0.66* | Liver |
| | 2.11 ± 0.96 | 1.99 ± 1.02 | 2.22 ± 1.08 | 3.00 ± 1.90 | 0.39 ± 0.07* | Heart |
| | 0.90 ± 0.30 | 1.31 ± 0.23 | 1.34 ± 0.48 | 1.45 ± 0.88 | 0.68 ± 0.17 | Kidney |

Organs were analyzed for expression of tissue factor (TF) as well as deposition of fibrin by immunofluorescence stainings. Shown data for TF are intensities per pixel$^2$ divided by 1000 (TF expression in blood vessels) and fibrin data are fluorescence intensities (area under the curve of histograms) divided by 100'000, all expressed as mean ± standard deviation (SD). Statistical significance was determined by one-way analysis of variance with Dunnett's post-test against NaCl control. P values of < 0.05 were considered statistically significant. *P<0.05; **P<0.01; ***P<0.001.

**Table 3 Expression of kinin system components bradykinin receptor b1 (BRB1), bradykinin receptor b2 (BRB2) as well as heparan sulfate (HS)**

| | NaCl (n=8) | Vehicle (n=7) | C1 INH (n=6) | APT070 (n=4) | Normal (n=4) | NaCl (n=8) |
|---|---|---|---|---|---|---|
| BRB1 | 7.30 ± 0.74 | 7.61 ± 0.71 | 5.44 ± 1.65* | 7.40 ± 1.16 | 6.34 ± 1.31* | Lung |
| | 8.91 ± 0.46 | 8.65 ± 0.78 | 8.14 ± 0.79 | 8.38 ± 1.13 | 8.52 ± 0.72 | Liver |
| | 7.30 ± 0.67 | 8.15 ± 0.70 | 3.98 ± 0.53*** | 7.99 ± 0.56 | 4.53 ± 0.80*** | Heart |
| | 6.81 ± 0.93 | 6.13 ± 1.43 | 5.69 ± 1.85 | 8.59 ± 0.86 | 5.69 ± 0.95 | Kidney |
| BRB2 | 8.04 ± 0.81 | 8.70 ± 0.24 | 7.66 ± 2.01 | 7.58 ± 0.99 | 8.20 ± 0.55 | Lung |
| | 8.58 ± 0.39 | 7.99 ± 1.25 | 8.24 ± 2.61 | 5.67 ± 2.14 | 6.73 ± 1.98 | Liver |
| | 8.32 ± 1.25 | 7.48 ± 1.62 | 8.18 ± 0.75 | 7.63 ± 0.82 | 7.81 ± 1.26 | Heart |
| | 8.22 ± 1.15 | 7.73 ± 0.91 | 7.60 ± 1.13 | 7.22 ± 1.35 | 7.23 ± 1.24 | Kidney |
| HS | 13.31 ± 0.91 | 11.96 ± 1.40 | 11.33 ± 2.54 | 11.99 ± 1.27 | 10.88 ± 1.61 | Lung |
| | 3.38 ± 0.19 | 3.49 ± 0.07 | 3.53 ± 0.07 | 3.98 ± 0.00 | 3.25 ± 042 | Liver |
| | 3.14 ± 0.47 | 3.15 ± 0.21 | 2.83 ± 0.91 | 2.87 ± 0.85 | 2.71 ± 0.61 | Heart |
| | 3.56 ± 0.04 | 3.37 ± 0.47 | 3.17 ± 0.65 | 3.10 ± 0.64 | 3.57 ± 0.04 | Kidney |

Organs were analyzed for expression of BR1, BRB2 as well as HS by immunofluorescence stainings. Shown data for BRB1 and BRB2 are intensities per pixel2 divided by 1000 (expression in blood vessels) and HS data are fluorescence intensities (area under the curve of histograms) divided by 100'000, all expressed as mean ± standard deviation (SD). Statistical significance was determined by one-way analysis of variance with Dunnett's post-test against NaCl control. P values of < 0.05 were considered statistically significant. *P<0.05; **P<0.01; ***P<0.001.

**Table 4 Summary of the effect of hind limb IRI and C1 INH treatment on different parameters in remote organs**

| | Lung | Liver | Heart | Kidney |
|---|---|---|---|---|
| IgM | → | ↑ | → | → |
| C1q | → | ↑ | → | → |
| C3b/c | ↑ | → | → | X ↑ |
| factor B | ↑ | X ↑ | ↑ | → |
| TF | → | → | → | → |
| Fibrin | X ↑ | ↑ | ↑ | → |
| BRB1 | X ↑ | → | X ↑ | → |
| BRB2 | → | → | → | → |
| HS | → | → | → | → |

Organs were analyzed for deposition and expression of selected markers by immunofluorescence stainings. "→" no deposition or increase of expression in NaCl controls as compared to normal rats. "↑" deposition or increase of expression in NaCl controls compared to normal rats. "X" significant reduction of deposition or prevention of up-regulation by C1 INH treatment.

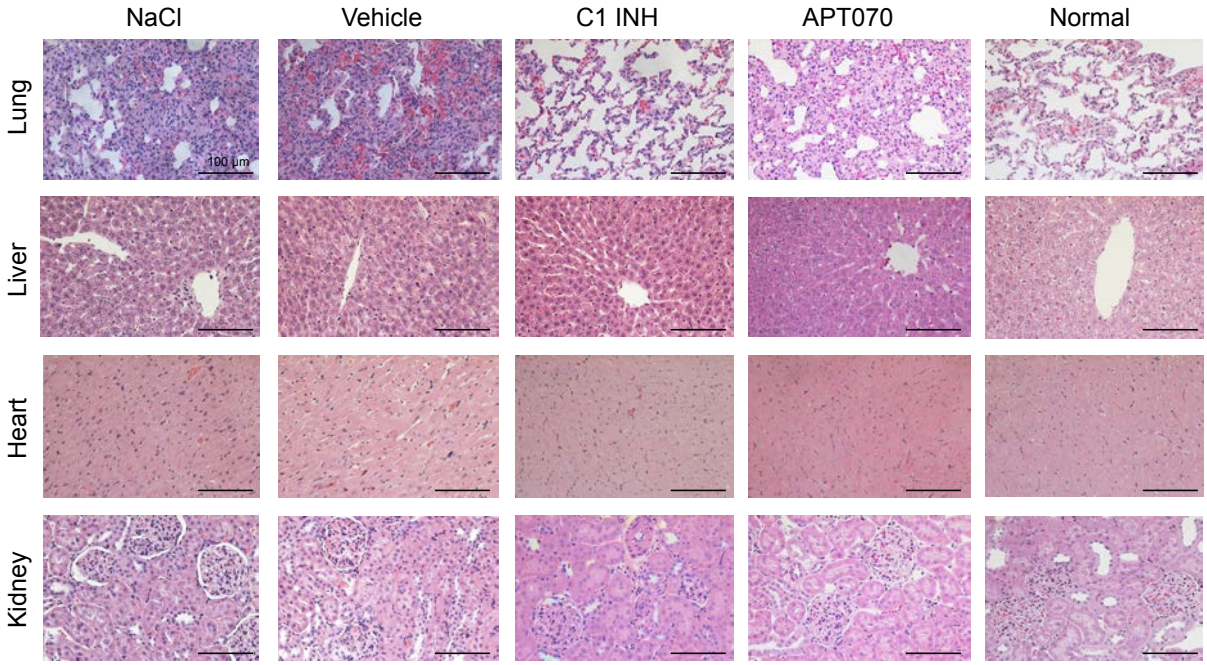


**Figure 1 Histological assessment of distant organ damage.** Histopathological analysis of heart, liver, kidney and lung tissue sections. Light microscopic assessment of distant organ damage was unremarkable for heart and liver with minimal tubular swelling in the kidneys of the NaCl and vehicle treated animals. Assessment of lung tissue revealed a primarily interstitial infiltration of neutrophil granulocytes as well as interstitial edema, alveolar fluid accumulation and some extravasation of red blood cells in the NaCl, Vehicle and APT070 treated rats. These changes however were less pronounced in the APT070 group as compared to the two control groups.

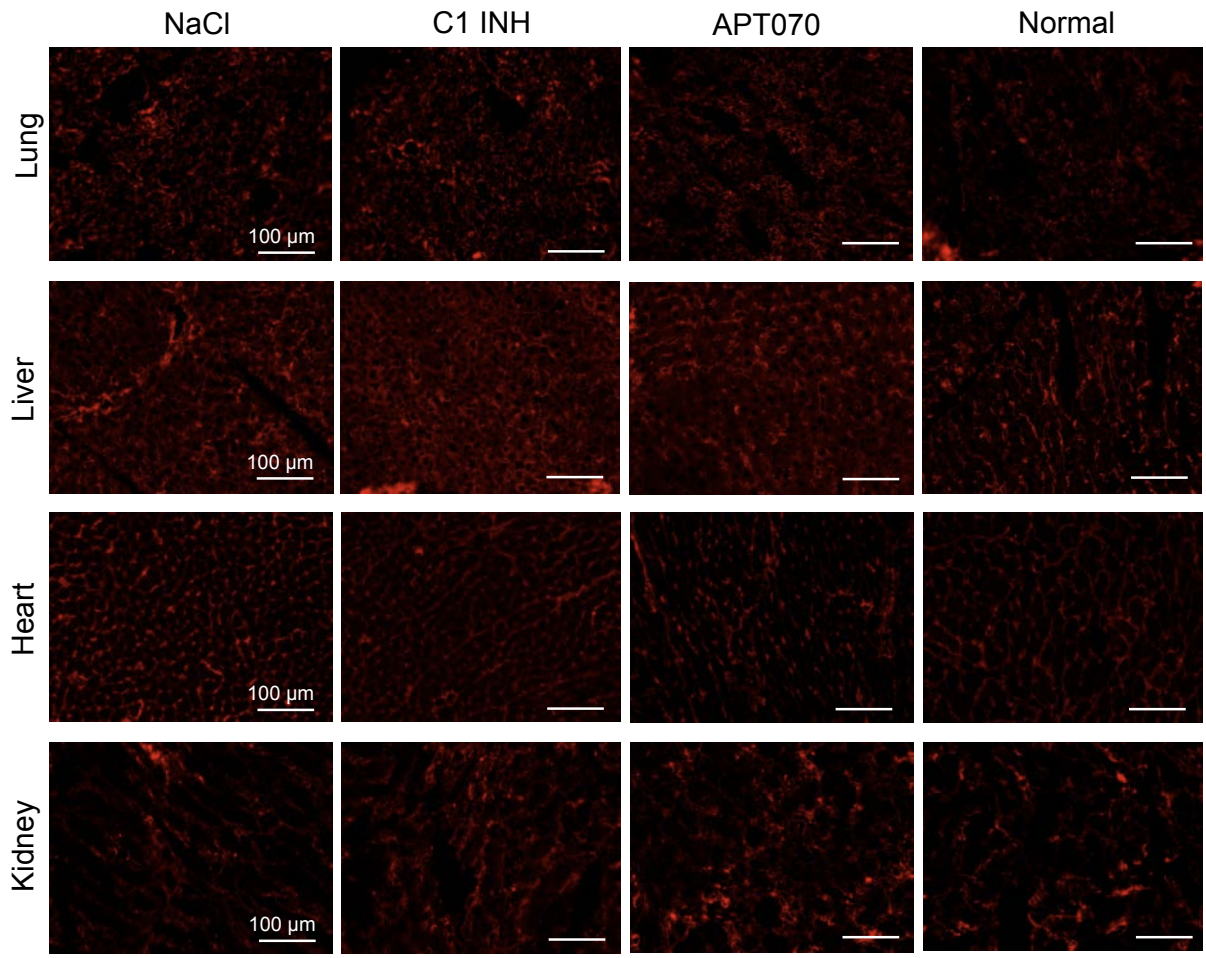


**Figure 2 Analysis of IgM deposition in distant organs.** Analysis of IgM deposition by using immunofluorescence stainings. No statistical differences were found for IgM deposition in heart, kidney and lung, whereas in liver NaCl treated rats displayed a significant increase of IgM deposition as compared to normal rats. IgM detected in the red channel (CY3).

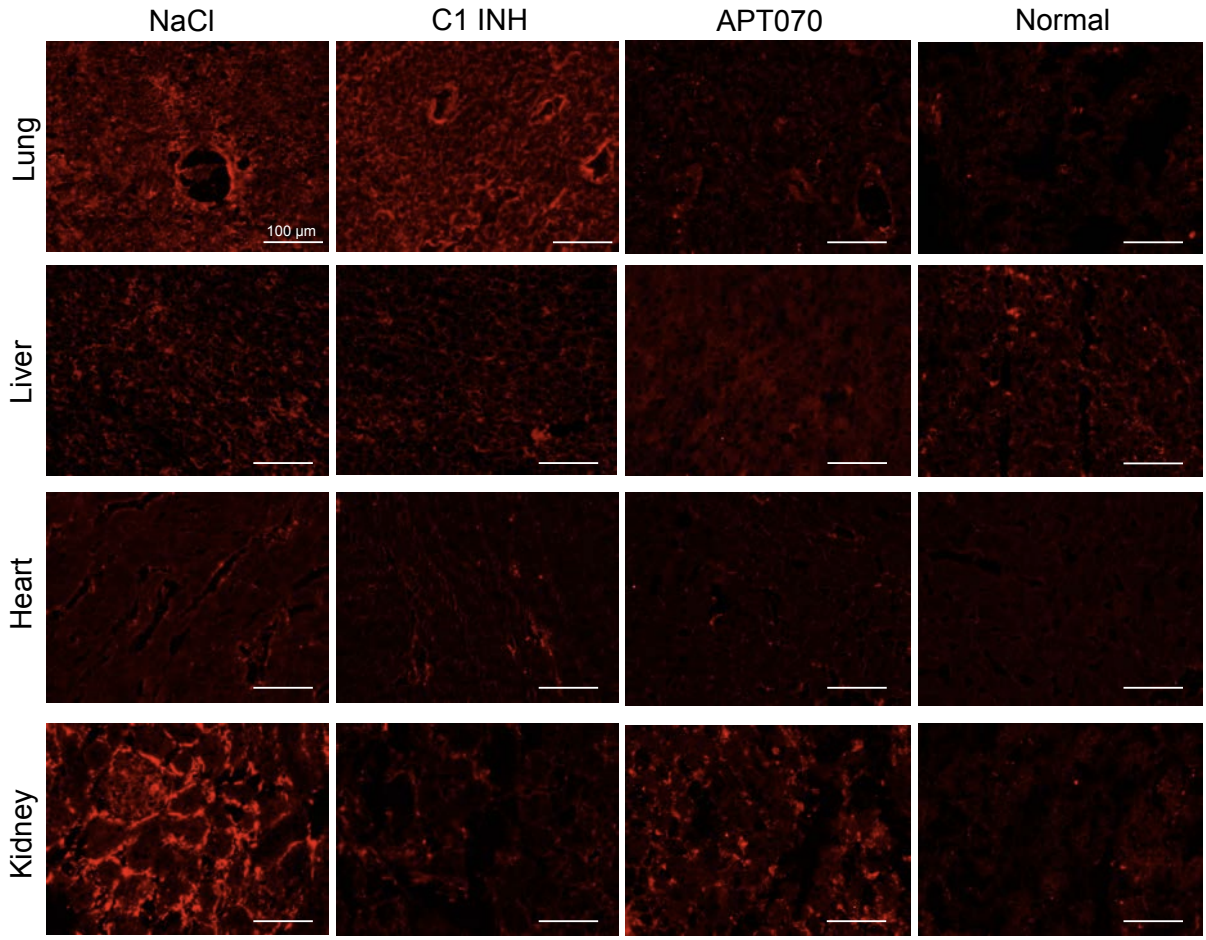


**Figure 3 Investigation of distant organ C3b/c deposition.** Assessment of C3b/c deposition by using immunofluorescence stainings. Analysis of C3b/c (red channel) in lung tissue revealed significantly increased C3b/c deposition in NaCl and C1 INH treated rats as compared to normal rats. C3b/c deposition was prevented in APT070 treated rats. Also in kidney tissue increased C3b/c deposition was found in NaCl treated rats, which was significantly reduced by C1 INH treatment, but not APT070.

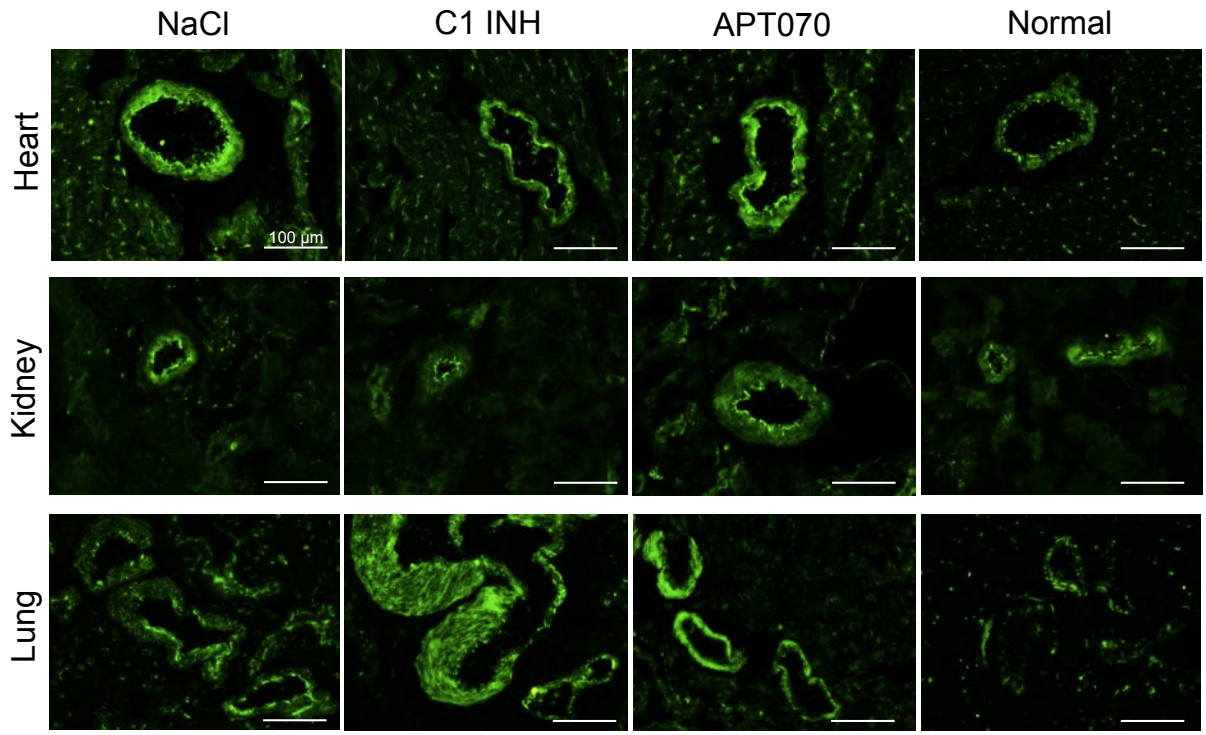


**Figure 4 Assessment of tissue factor (TF) expression in heart, kidney and lung.** Expression of TF was analyzed by using immunofluorescence stainings. No statistical differences were found for tissue factor expression in heart, kidney and lung. TF detected in the green channel (Alexa 488).

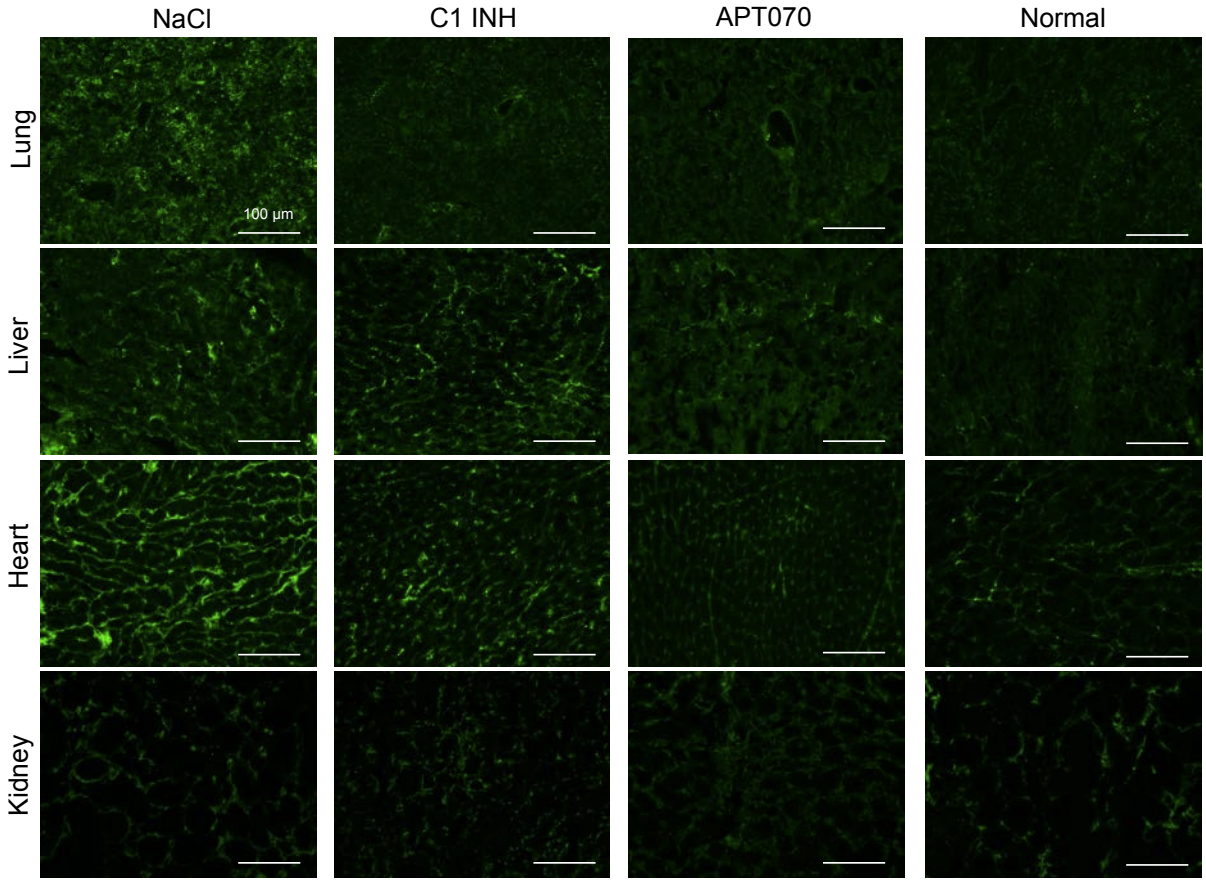


**Figure 5 Analysis of fibrin deposition in distant organs.** Analysis of fibrin deposition was performed via immunofluorescence stainings. Assessment of fibrin deposition in lung tissue revealed increased deposition in NaCl treated rats, which was significantly reduced by C1 INH treatment. In liver and heart tissue significantly increased fibrin was found for NaCl treated rats, but no significant reduction was detected for C1 INH treated rats. No fibrin deposition was detected in kidney as compared to normal rats. Fibrin detected in the green channel (Alexa 488).

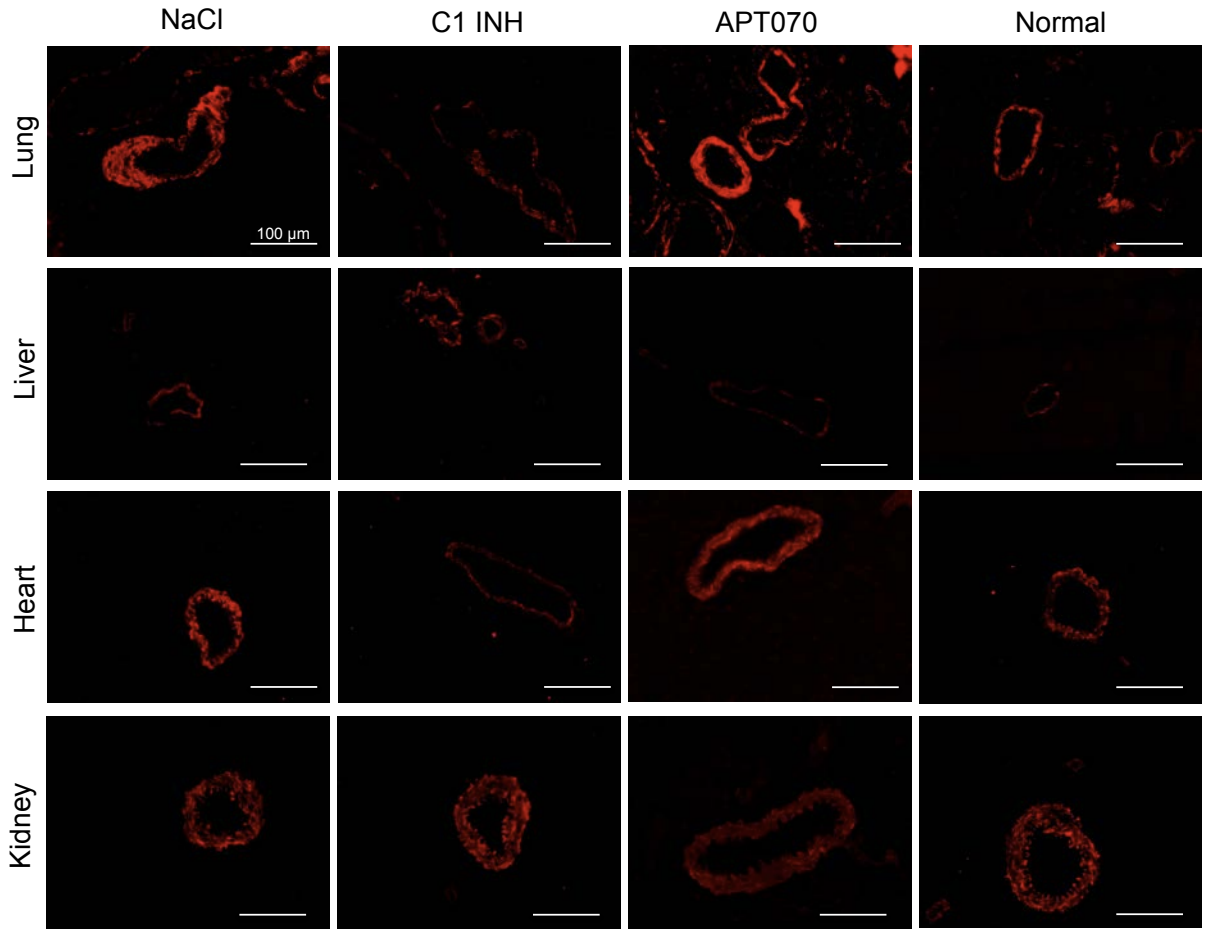


**Figure 6 Investigation of bradykinin receptor b1 expression in distant organs.** Analysis of bradykinin receptor b1 expression in lung and heart tissue revealed an up-regulation of b1 expression in NaCl treated rats, which was prevented by C1 INH treatment. For kidney and liver no increase in bradykinin receptor b1 expression was detected as compared to normal rats. Bradykinin receptor b1 detected in the red channel (CY3).

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# Paper III

## Refinement of tourniquet-induced peripheral ischemia/reperfusion injury in rats: Comparison of 2h vs. 24h reperfusion

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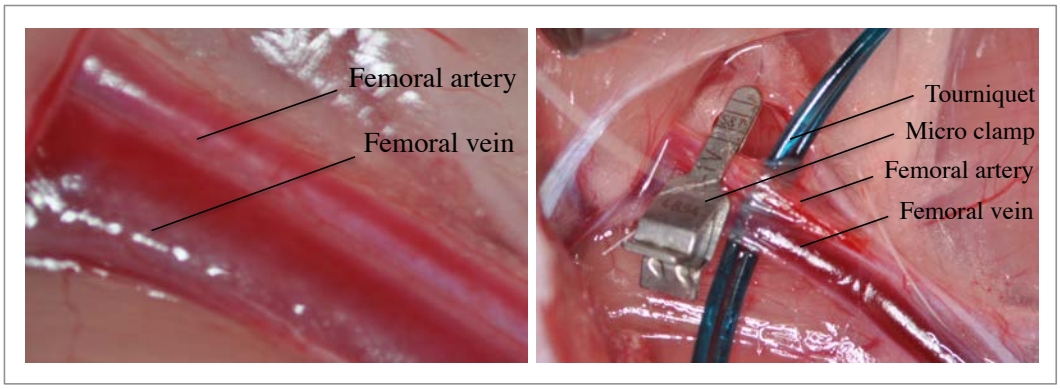
**Status:** under review in ***Laboratory Animals*** (submitted 17.05.2013)

**Aim:** The aim was to establish an acute hind limb IRI model with shortened reperfusion times in consideration of 3R principles (Reduction, Refinement & Replacement) which would minimize the potential pain and suffering of animals, but still represent a good model to analyze the effect of drug candidates on IRI.

**Conclusion:** An acute model with 2 h reperfusion time is sufficient to assess basic parameters of IRI in the rat hind limb setting, whereas for in-depth analysis of mechanisms of actions, muscle viability and distant organ damage, rats need to be subjected to 24 h reperfusion.



**Refinement of tourniquet-induced peripheral ischemia/reperfusion injury in rats: Comparison of 2h vs. 24h reperfusion**

**Short title:** Refinement of an IRI rat model

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## Abstract

Prolonged ischemia of skeletal muscle tissue, followed by reperfusion, leads to ischemia/reperfusion injury (IRI), which is a feared local and systemic inflammatory reaction. With respect to 3R, we wanted to determine which parameters for assessment of IRI require a reperfusion time of 24 h and for which 2 h reperfusion are sufficient. Rats were subjected to 3 h of hind limb ischemia and 2 h or 24 h reperfusion. Human plasma derived C1 inhibitor was used as a drug to prevent reperfusion injury. For 2 h reperfusion the rats stayed under anesthesia throughout (severity grade 1), whereas for 24 h they were awake under analgesia during reperfusion, grade 2. The femoral artery was clamped and a tourniquet placed, under maintenance of venous return. C1 INH was systemically administered 5 minutes before induction of ischemia. Local muscle edema formation and deposition of IgG and IgM showed no differences between 2 h and 24 h ($P>0.05$), whereas lung edema was only observed after 24 h. Muscle viability was significantly lower after 24 h vs. 2 h reperfusion ($P<0.05$). Increased plasma CK-MM and PDGF-bb could be detected after 2 h, but not after 24 h reperfusion. In contrast, deposition of C3b/c and fibrin in muscle was only detected after 24 h ($P<0.001$). In conclusion, for a first screening of drug candidates to reduce IRI, 2 h reperfusion are sufficient and reduce the severity of the animal experiment. 24 h reperfusion are only needed for in-depth analysis of mechanisms of IRI, including lung damage.

**Keywords:** Refinement, Ischemia/Reperfusion Injury, C1 inhibitor, Tourniquet, Rat

Lower extremity ischemia may occur due to embolic/ thrombotic or traumatic vascular occlusion, or alternatively due to tourniquet application during surgical intervention. Reperfusion of the ischemic extremity is crucial for tissue survival. However, due to reperfusion also complex inflammatory cascades are activated, which aggravate ischemic tissue damage and lead to a local as well as systemic inflammatory reaction. In this phenomenon, called ischemia/reperfusion injury (IRI), key roles are attributed to the complement-, the coagulation- and the kinin system.[1,2,3] Locally, lower extremity IRI is manifested in muscle edema formation, rigidity, loss of muscle viability, apoptosis and necrosis. In addition, local injury is often accompanied by distant organ damage, affecting organs like lung, heart, kidney as well as liver and may result in multiple organ dysfunction syndrome.[4,5] Especially acute lung injury is a consequence of lower extremity IRI and is characterized by increased microvascular permeability and pulmonary edema.[6]

Lower extremity IRI has been extensively studied in different species like pigs, dogs or rabbits.[7,8,9] Although 75 % of all experimental animals are rodents a comparison is often impossible. Screening the literature reveals a variety of methods with respect to the induction of ischemia, which is done by placement of an elastic rubber band, a tourniquet, on the proximal part of the limb, and/or clamping the femoral or iliac artery, or even the infrarenal aorta.[10,11, 12] However, not only the technique of ischemia induction varies, but also the time periods of ischemia and reperfusion show strong heterogeneity. In acute models rats are subjected to periods of ischemia ranging from 2 h to 4 h, whereas reperfusion times range from 1 h to 24 h.[11,13,14]

The aim of the present study was to establish an acute hind limb IRI model with shortened reperfusion times in consideration of 3R principles,[15] which would minimize the potential pain and suffering of animals, but still represent a good model to analyze the effect of drug candidates on IRI. To demonstrate the efficacy of the present rat model we used C1 esterase inhibitor (C1 INH) as a model drug candidate, which is a crucial regulator of plasmatic cascades activated in the process of inflammation. We hypothesized that for a first screening of drug candidates, 2 h of reperfusion would be sufficient, whereas for detailed analysis of mechanisms as well as distant lung injury 24 h reperfusion are required. Furthermore, we wanted to compare the effect of exogenous human plasma-derived C1 INH on tourniquet-induced limb IRI after 2 h and 24 h reperfusion in a rat model.

## Animals

All animal experiments were conducted in compliance with the Guide for the Care and Use of Laboratory Animals and Swiss national guidelines and were approved by the animal ethics committee (permission number 71/10) of the cantonal veterinary service (Canton of Bern, Switzerland).[16] Male Wistar rats (wild type, bred at the central animal facility, University of Bern, Switzerland, body weight 250-350 g) were kept together at three in a clear 1500 $cm^2$ Euro-standard Type IV S cage (Tecniplast, Buguggiate, Italy) with overall dimensions of 48 x 37.5 x 21 cm in order to enable social interactions and welfare. However, after surgical interventions rats were kept single for 24 h reperfusion to prevent mutual injury. Rats were housed under standard conditions with food (maintenance diet, Provimi Kliba, Kaiseraugst, Switzerland) as well as water *ad libitum* and poplar wood as bedding material (Lignocel, Provimi Kliba). For cage enrichment rats were provided with paper tissue, pieces of wood and tubes. Cages were individually ventilated at 20 ± 2°C and 45-65% relative humidity with a circadian rhythm of 12/12 h. During the light cycle animals were exposed to an intensity of 200 lux.

## Materials and Methods

### Reagents

C1 esterase inhibitor (Berinert®) was provided by CSL Behring (CSL Behring GmbH, Marburg, Germany).

### Experimental groups

Rats were divided into 5 groups. The experimental groups of 2 h reperfusion (n=7) and 24 h reperfusion (n=6) received 50 IU/kg (50 IU/ml) of exogenous human C1 INH. The control groups, which were subjected to either 2 h (n=7) or 24 h (n=7) of reperfusion received no C1 INH. In addition, tissue and plasma samples of normal, healthy rats (n=4) were included in the study for comparison. Timelines of the experimental procedures are given in Figure 1a.

## Anesthesia and analgesia

Induction of anesthesia was performed with 2.5% isoflurane in oxygen in an anesthetic induction chamber and later maintained by inhalation of 1.5% isoflurane on a nose mask. Arterial oxygen saturation, heart rate, breath rate, rectal temperature, pulse distention, and breath distention were continuously monitored using the mouse ox plus system (Starr Life Sciences, Oakmont, PA, USA). Thirty minutes prior to surgical intervention, 0.05 mg/kg of buprenorphine (Temgesic, Reckitt Benckiser, Switzerland AG, 1 mg/ml) was injected subcutaneously to the anesthetized rats to provide analgesia. The total duration of anesthesia was approximately 6 h after which the rats of the 24 h reperfusion groups were allowed to wake up, whereas rats which underwent only 2 h reperfusion remained under anesthesia until euthanasia by exsanguination (Figure 1a). When the animals of the 24 h reperfusion groups were completely awake, buprenorphine injection was repeated to provide adequate analgesia. The condition of rats was continuously monitored for the first 5 h of reperfusion and then after 19 h and 24 h. The administration of analgesia was strictly controlled to avoid pica behavior. After completion of 24 h reperfusion, rats were anesthetized again as described above and sacrificed by exsanguination during organ removal.

## Surgery

Rats had an acclimatization period of at least 6 days prior surgical intervention. The fur was completely removed from both hind limbs with an electric shaver for assessment of limb perfusion by Laser Doppler. To maintain the body temperature at 37°C rats were kept on a heating pad (T/pump professional system, Gaymar Industries, Inc., NY, USA). For induction of unilateral hind limb ischemia, approximately 30 minutes after induction of anesthesia, the femoral artery and vein were exposed via a groin incision and a tourniquet (standardized weight of 450 g) was placed underneath the femoral vessels to block collateral circulation.11 The femoral artery was occluded for 3 h with two microvascular clamps (B1-V, S&T, Neuhausen, Switzerland) (Figure 1b). Rat hind limbs were not exsanguinated, but a comparable state was achieved by allowing venous return during the entire period of ischemia in order to prevent venous congestion and additional injury through microcirculatory impairment, which would not represent the clinical situation. After 3 h of ischemia the limb was reperfused for either 2 h or 24 h. At the end of the experiments, tissue samples of both the ischemic and contralateral gastrocnemic muscles as well as the lungs were taken for subsequent analyses.

## Assessment of edema formation

Edema formation was assessed by using wet weight to dry weight ratio (wet / dry ratio). Two samples of the gastrocnemic muscle from both legs as well as the left lobe of the lung were taken and immediately weighed to obtain the wet weight. Muscle and lung samples were then dried for 24 h at 80°C after which a constant dry weight was achieved. Subsequently, the wet / dry ratio was calculated.

## Analysis of muscle viability

IRI severely affects muscle viability, which may ultimately result in muscle necrosis. For analysis of muscle viability the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma, St. Louis, USA) assay was performed. Muscle samples from the gastrocnemic muscle were taken, washed in PBS, blotted dry and incubated in 0.1 mg MTT/ ml PBS in a total volume of 3 ml at 37°C, rotating in the dark for 2 h. Thereafter, muscle samples were blotted dry and incubated in 100% isopropanol at 37°C, rotating in the dark over night to elute the formazan crystals from the tissue for measurement of the optical density (OD). 200 µl of thus obtained supernatant was measured in a microplate (Nunc, 96 well, maxisorp, transparent, Roskilde, Denmark) with a microplate reader at 560 nm (Ref. 690 nm; Infinite M1000 spectrophotometer, Tecan, Männedorf, Switzerland). After drying the muscle samples at 80°C for 24 h the OD per mg dry weight was calculated and compared with values of contralateral legs.

## Histological assessment of damage

Hemorrhage, total myocyte damage as well as infiltration of neutrophil granulocytes were assessed in tissue samples from the gastrocnemic muscle. Tissue samples were fixed in 4% formaldehyde in PBS for 24-72 h, embedded in paraffin, cut into 3 µm thick sections and stained with hematoxylin and eosin.

## Analysis of creatine kinase-MM level

For analysis of creatine kinase-MM (CK-MM) level a solid phase enzyme-linked immunosorbent assay (ELISA, Life Diagnostics, West Chester, USA) was performed. According to manufacturer's instruction, standards and EDTA-plasma samples, taken after 2 h or 24 h reperfusion, were incubated in microtiter wells for 45 minutes at room temperature. Subsequently, wells were washed, horseradish peroxidase conjugated detection antibody was added and incubated for 45 minutes. After washing, TMB reagent was added, incubated for 20 minutes and stopped by adding stop solution. Finally, optical density was spectrophotometrically measured at 450 nm. For analysis GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA, USA.) was used.

## Analysis of PDGF-bb levels

An in-house developed immunoassay consisting of carboxylated non-magnetic beads (for PDGF-bb, region: 38, Cat. No: 171506038, Bio-Rad, Hercules, CA, USA) conjugated with specific capture (rabbit anti PDGF-bb, 500-47, PeproTech, Hamburg, Germany) and detection antibodies (biotinylated rabbit anti PDGF-bb, 500-P47Bt, PeproTech) was used to detect PDGF-bb. Briefly, EDTA-plasma was diluted 1:3 in cytokine assay buffer (Bio-Rad) and incubated with antibody-coupled magnetic beads. A washing step was followed by incubation with biotinylated detection antibody. After streptavidin-phycoerythrin (Qiagen, Hilden, Germany) incubation PDGF-bb concentration was measured. Recombinant protein (rat PDGF-bb, 520-BB, R&D Systems, Minneapolis, MN, USA) was used to establish a standard curve. PDGF-bb concentrations were calculated using the Bio-Plex Manager 6.0 Software (Bio-Rad).

## Immunofluorescence analyses of tissue samples

Immunofluorescence staining using specific antibodies was used to quantify the deposition of IgM (3020-08; Southern Biotech, AL, USA) and IgG (3030-08; Southern Biotech), C1q (A0136, Dako, Baar, Switzerland), C4b/c (LSB 4228, LifeSpan BioSciences Inc., Seattle, WA, USA), C3b/c (A0062, Dako), factor B (341272, Calbiochem, Darmstadt, Germany) and fibrin (F0111; Dako, Baar, Switzerland). Tissue samples from the gastrocnemic muscle of both legs were taken and washed in PBS, blotted dry and embedded in OCT matrix (Tissue tek, Sakura, USA) on dry ice. The samples were immediately stored at -20°C until cryosections were cut. Sections were fixed in acetone and rehydrated in tris-buffered saline (TBS).

Primary antibodies were incubated over night at 4°C and secondary antibodies were incubated for 1h at room temperature (RT). Subsequently, slides were mounted and covered. Pictures were taken with a fluorescent microscope (Leica DMI 4000B, Leica Microsystems Schweiz AG, Heerbrugg, Switzerland) and analyzed using Image J (National Institutes of Health, Bethesda, MD, USA) and GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA, USA.).

## Statistical analysis

Data are expressed as means ± standard deviation (SD). One-way ANOVA followed by Bonferroni's post hoc test for significance was achieved by using the GraphPad Prism 5 software. A 95% confidence interval was established and *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001 were considered statistically significant. Determination of n-numbers per group was performed without formal power analysis, based on preliminary experiments with C1 INH in our laboratory.

## Results

### Edema formation in muscle and lung as well as muscle viability

Edema formation in muscle and lung tissue was assessed by wet / dry ratio. Non-treated animals showed clear edema formation in reperfused gastrocnemic muscle tissue after 2 h (wet / dry ratio = 5.4 ± 0.4, ratio for normal control animals = 4.3 ± 0.05, $P < 0.0001$) as well as after 24 h reperfusion (5.6 ± 0.7), whereas C1 INH treated rats presented significantly less edema after 2 h (4.6 ± 0.2, $P < 0.001$ vs. non-treated) and 24 h (4.7 ± 0.2, $P < 0.0001$) reperfusion (Figure 2a-e). As compared with normal rats (4.6 ± 0.1) no lung edema was detected after 2 h reperfusion for non-treated (4.7 ± 0.2) as well as for C1 INH treated rats (4.7 ± 0.1). However, after 24 h reperfusion significant edema formation was found for non-treated rats (5.1 ± 0.2), which was significantly reduced ($P < 0.001$) in C1 INH treated rats (4.7 ± 0.1, Figure 2f). Muscle viability was analyzed by using the MTT assay. Results are given as viability index in % of the contralateral control leg, which was set to 100%. As compared to normal rats (96.5 ± 7.0 %) muscle viability was not significantly decreased after 2 h (80.5 ± 21.1 % for non-treated and 88.1 ± 7.3 % for C1 INH treated rats). In contrast, after 24 h reperfusion, C1 INH treatment (96.2 ± 12.9 %) significantly improved viability compared to non-treated rats (63.3 ± 9.5 %, Figure 2g). Analysis of correlation between the muscle viability index in % and wet / dry ratio revealed a significant ($P < 0.001$) negative correlation (Pearson coefficient = -0.53, Figure 2h).

### Histopathology and measurement of CK-MM and PDGF-bb plasma levels

H&E stained muscle sections were assessed for overall muscle damage. C1 INH treated rats showed only minimal damage after 2 h as well as 24 h reperfusion, whereas non-treated rats presented strong edema formation, myocyte destruction and infiltration of neutrophil granulocytes (Figure 3a-d). For analysis of CK-MM and PDGF-bb plasma levels ELISA and singleplex bead-based assay were performed, respectively. After 2h of reperfusion C1 INH treated animals (1122 ± 468 ng/ml) showed a significantly decreased level ($P < 0.01$) of CK-MM as compared to non-treated rats (1989 ± 485 ng/ml), whereas PDGF-bb levels were not significantly decreased by C1 INH treatment (2468 ± 390 pg/ml) in comparison to non-treated rats (2219 ± 566 pg/ml). However, after 24 h reperfusion CK-MM as well as PDGF-bb levels were significantly decreased and back to baseline (Figure 3e,f).

## Antibody deposition in muscle tissue

Deposition of IgG and IgM in gastrocnemic muscle tissue was assessed by immunofluorescence staining. As compared to normal rats only minimal antibody deposition was detected in contralateral legs after 2 h and 24 h reperfusion, respectively, whereas in the reperfused leg IgG deposition was increased at both time points ($P < 0.0001$). Treatment with C1 INH led to a significant decrease of IgG deposition after 2 h ($P < 0.0001$) as well as after 24 h ($P < 0.05$) reperfusion as compared to non-treated rats (Figure 4a). Analogous results were found for IgM deposition, for which C1 INH treated rats also showed significantly less than non-treated rats after 2 h ($P < 0.05$) and 24 h ($P < 0.0001$) reperfusion (Figure 4f).

## Analysis of C3b/c, factor B and fibrin deposition in muscle tissue

Analysis of C3b/c deposition revealed no significant differences between all groups after 2 h reperfusion. However, after 24 h reperfusion non-treated rats presented C3b/c deposition in contralateral as well as reperfused legs, which was significantly reduced ($P < 0.001$) in C1 INH treated rats (Figure 5a). In contrast to C3b/c deposition, factor B deposition was found in the contralateral and reperfused legs after 2 h and 24 h reperfusion and was significantly reduced in C1 INH treated rats ($P < 0.01$). However, after 24 h reperfusion C1 INH could not significantly reduce factor B deposition in reperfused legs (Figure 5f). Furthermore, we analyzed the effect of C1 INH on fibrin deposition after 2 h and 24 h reperfusion and found no fibrin after 2 h reperfusion as compared to normal rats. After 24 h reperfusion non-treated rats demonstrated fibrin deposition in contralateral and reperfused legs, which was significantly reduced ($P < 0.05$) in C1 INH treated rats (Figure 5k).

## Discussion

In order to refine rat IRI models and to enable comparability between different studies we wanted to determine parameters for which it is necessary to subject rats to 24 h reperfusion and for which 2 h reperfusion are sufficient to reduce potential pain and distress. To assess differences between early (2 h) and late reperfusion (24 h) in a model of acute limb IRI, we selected 8 parameters, including muscle edema formation, muscle viability, distant lung edema formation, plasma CK-MM level, plasma PDGF-bb level, antibody-, complement components C3b/c and factor B- as well as fibrin deposition, and analyzed the effect of the drug candidate C1 INH. The muscle edema test was used for a first screening of the efficacy of C1 INH to reduce the accumulation of body fluids in muscle tissue. Edema formation was detected after 2 h as well as after 24 h reperfusion and was significantly reduced by C1 INH treatment. C1 INH is a potent regulator of the kinin system, which is a key mediator in edema response.[17] C1 INH interacts with kallikrein and FXIIa and may therefore prevent edema formation.[18, 19] Another key parameter in the present study was the assessment of distant lung injury, by analyzing lung edema formation. Lung edema formation caused by limb IRI is a severe clinical problem, which should be also considered in animal experiments.[20] Investigation of lung injury by analysis of lung edema revealed that after 2 h of reperfusion no detectable lung edema was present, whereas after 24 h reperfusion significant edema formation occurred, which was reduced by C1 INH treatment. MTT assay was used for assessment of muscle viability. Analysis of the effect of C1 INH treatment on muscle viability showed significant improvement after 24 h reperfusion as compared to non-treated groups, whereas after 2 h reperfusion viability was not severely affected. Furthermore, we measured CK-MM in plasma samples, as it plays a significant role in energy homeostasis of cells with high energy requirements, such as myocytes and its level is routinely used as an indicator of skeletal muscle injury.[21] Under normal conditions CK-MM is located in the cytosol and mitochondria of tissue and leaks into the blood due to muscle cell rupture, cell damage and in disease.[22] High CK-MM levels in plasma were detected after 2 h reperfusion and could be significantly reduced by C1 INH treatment. However, after 24 h reperfusion only very low levels of CK-MM were measured, as CK-MM is a marker of early detection of muscle injury and has a serum half-life of only about 1h in rats.[23] Additionally, we were looking at the plasma level of PDGF-bb, which is known to have angiogenic effects, regulates the tonus of blood vessels and is involved in wound healing.[24, 25] At low levels PDGF-bb is expressed in endothelium, platelets and macrophages, which increases following mechanical injury and atherosclerosis.[26]

As compared to normal values, we found elevated plasma levels after 2 h, but not after 24 h reperfusion. Similar results were shown in a study of tourniquet-induced IRI in hand surgery, where PDGF-bb was significantly increased compared to normal values as early as after 10 minutes reperfusion.[27]

Our data showed that C1 INH treatment did not reduce plasma PDGF-bb level after 2 h reperfusion. However, we cannot conclude to which source the increased plasma PDGF-bb level can be attributed. Furthermore, we analyzed deposition of antibodies, complement fragments, as well as fibrin, which provide reliable and quantifiable data to reveal the underlying mechanisms of actions of drug candidates in IRI. Analysis of antibody as well as complement deposition was selected, as the hypoxic state of ischemia leads to an alteration of proteins, such as non-muscle myosin heavy chain type II or annexin IV, which function as neo-epitopes for natural antibodies.[28,29] We found deposition of IgM and IgG in the reperfused leg after 2 h and 24 h reperfusion, which was significantly reduced by C1 INH treatment. In the contralateral legs only minimal antibody deposition was detected, suggesting that neo-epitopes are only locally exposed and IRI did not cause systemic formation of neo-epitopes. Furthermore, we investigated the key component of the complement system C3b/c. After 24 h reperfusion C3b/c was highly deposited in the contralateral and reperfused legs of non-treated rats and could be significantly reduced by C1 INH treatment, whereas after 2 h reperfusion only low amounts of C3b/c deposits were detected. Analysis of the complement component factor B revealed high deposition after 2 h and 24 h reperfusion in the contralateral and reperfused muscle, which was significantly reduced by C1 INH treatment. After 24 h reperfusion no significant reduction of factor B by C1 INH was detected in the reperfused leg. It is known, that C1 INH down regulates the function of the alternative pathway, including C3b/c as well as factor B, which fits with our data.[30]

Reperfusion following ischemia mediates the activation of the coagulation system. We analyzed the role of the coagulation system by measuring fibrin deposition in the gastrocnemic muscle. After 2 h reperfusion fibrin deposition in the reperfused leg was not detected, but this was the case after 24 h reperfusion. C1 INH treatment significantly reduced fibrin deposition as compared to non-treated rats, which is in line with earlier findings, where it was shown that C1 INH plays a crucial role in the coagulation system, as it is the main inhibitor of factor XIa and also modulates thrombin activity.[31, 32]

The results of this study indicate that for a first screening of drug candidates, analysis of muscle edema, plasma CK-MM level and PDGF-bb as well as antibody and complement deposition after 2 h reperfusion are sufficient and therefore recommended. In contrast, analysis of muscle viability, distant lung damage as well as underlying mechanisms by investigating antibody or fibrin deposition, a prolonged reperfusion period of 24 h is necessary (table 1). We could show that C1 INH treatment reduces muscle- and lung edema, maintains muscle viability and reduces plasma CK-MM level. Furthermore, it prevents from antibody-, C3b/c, factor B and fibrin deposition. In contrast to Thaveau et al., who report that the contralateral leg may be used as a control in the experimental setting of unilateral hind limb ischemia, we found C3b/c-, factor B and fibrin deposits also in the contralateral muscle.[33] Whether or not the contralateral leg may be used as a control in unilateral hind limb ischemia should therefore be carefully considered based on the parameters to be assessed. In summary, this study demonstrates that an acute model with 2 h reperfusion time is sufficient to assess basic parameters of IRI in the rat hind limb setting and may therefore be suitable for a first screening of drug candidates. However, for in-depth analysis of mechanisms of actions, muscle viability and distant organ damage, rats need to be subjected to 24 h reperfusion.

## Acknowledgments

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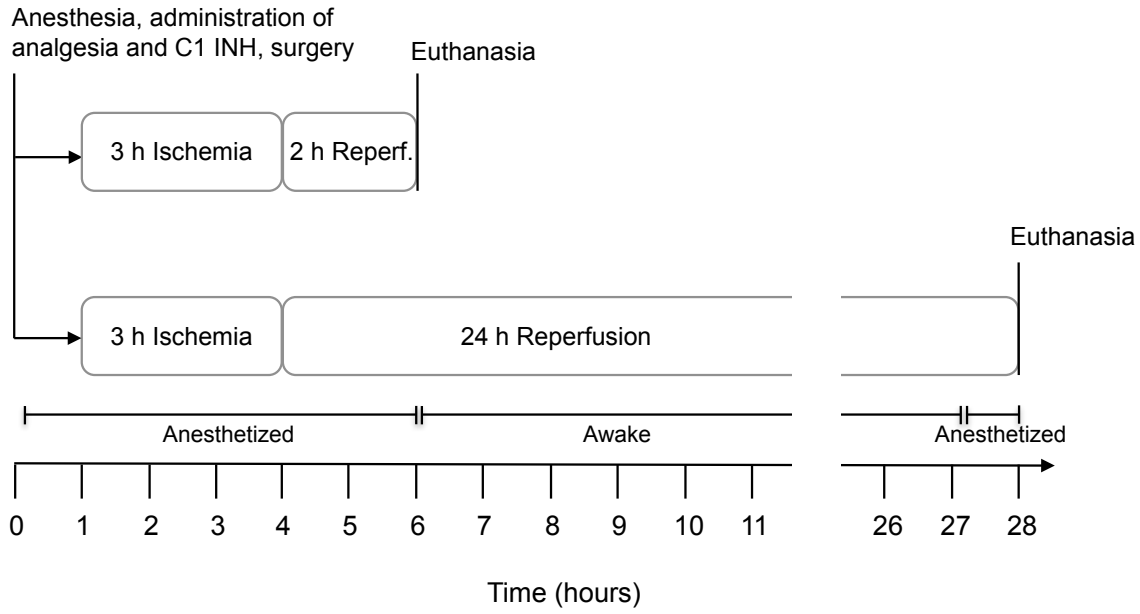
## Disclosure

Robert Rieben received grant support and Claudia Dührkop was paid in part from CSL Behring AG.

(a)




(b)

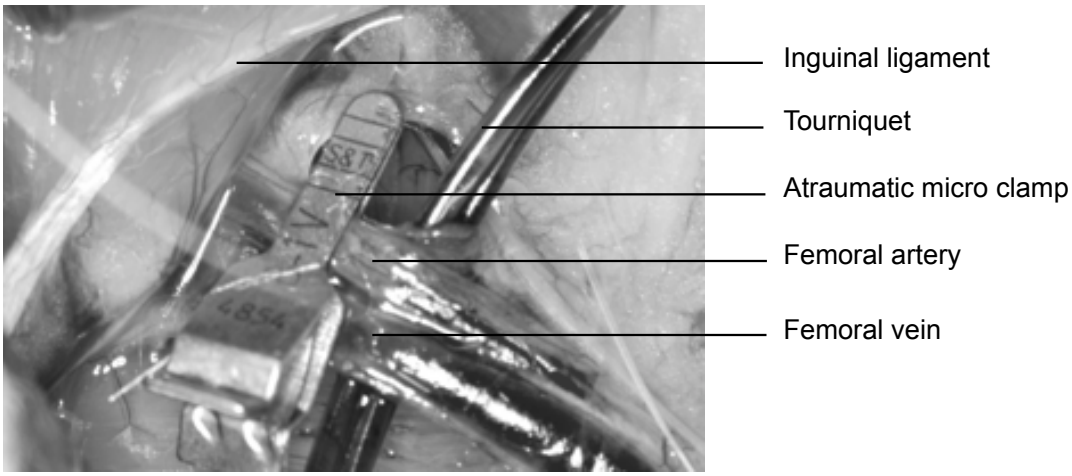



**Figure 1** Experimental design and surgical procedure. **(a)** Male Wistar rats were subjected to 3 h of ischemia, followed by either 2 h or 24 h of reperfusion. During 2 h of reperfusion rats were kept under anesthesia, whereas for 24h reperfusion rats were awake. **(b)** For induction of ischemia, the femoral artery was occluded by using an atraumatic micro clamp. Additionally, a tourniquet was placed underneath the femoral vessels under maintenance of venous return. (For details see materials and methods section)

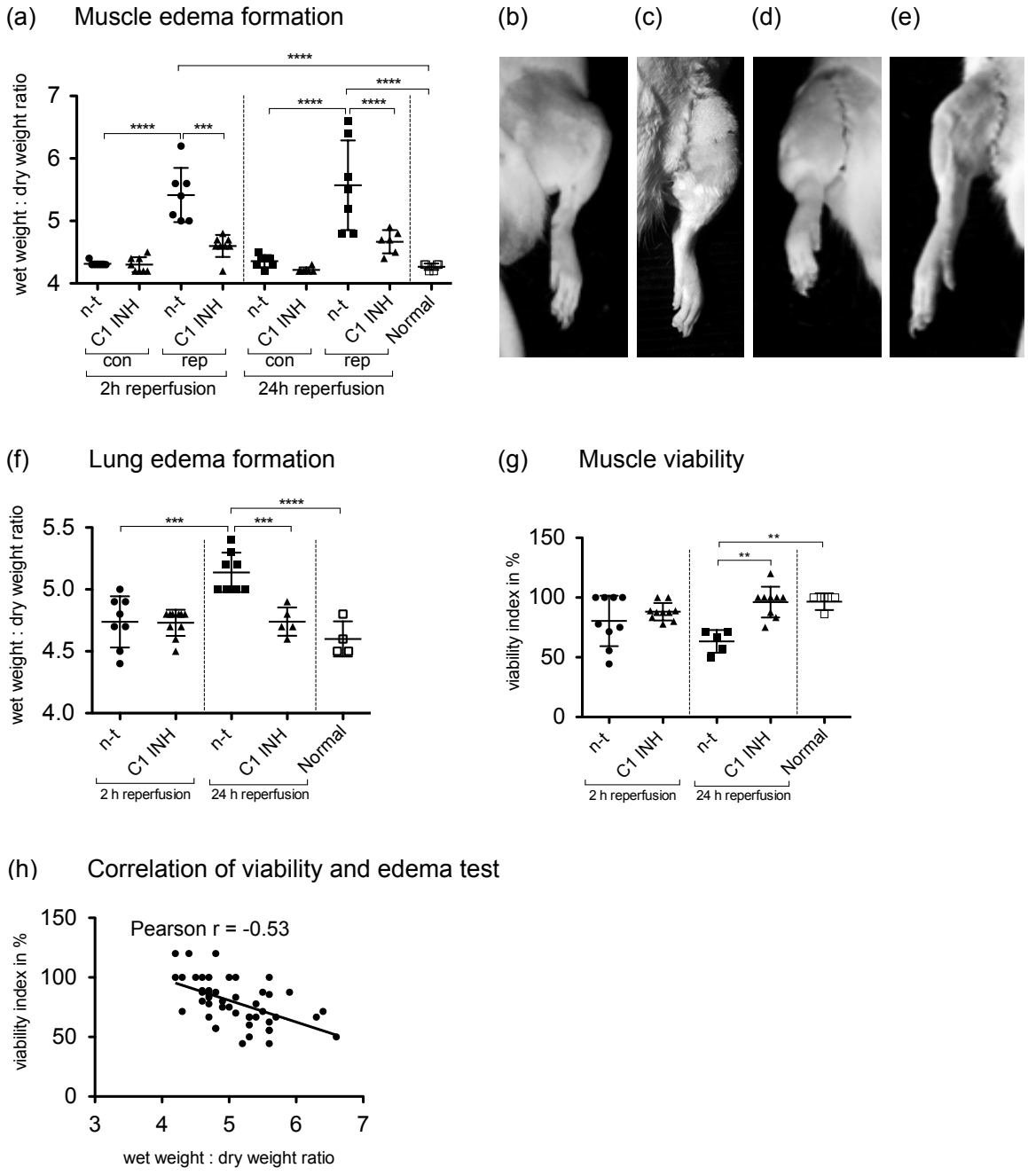



**Figure 2** Effect of C1 INH on edema formation in muscle and lung as well as muscle viability after 2 h and 24 h reperfusion**. (a)** Muscle edema formation was analyzed in contralateral (con) and reperfused (rep) legs. Edema is indicated by an increase in wet to dry ratio. **(b-e)** Representative images of baseline and edema formation after 2 h and 24 h reperfusion. **(b)** baseline. **(c)** non-treated (n-t) rat after 2 h reperfusion. **(d)** non-treated (n-t) rat after 24 h reperfusion. **(e)** C1 INH treated rat after 24 h reperfusion. **(f)** Lung edema formation was analyzed after 2 h and 24 h by using wet / dry ratio. **(g)** Assessment of viability of gastrocnemic muscle after 2 h and 24 h by MTT assay. **(h)** Analysis of correlation between muscle viability and muscle edema formation. One-way ANOVA followed by Bonferroni's post hoc test for significance was used. Error bars indicate mean ± SD. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

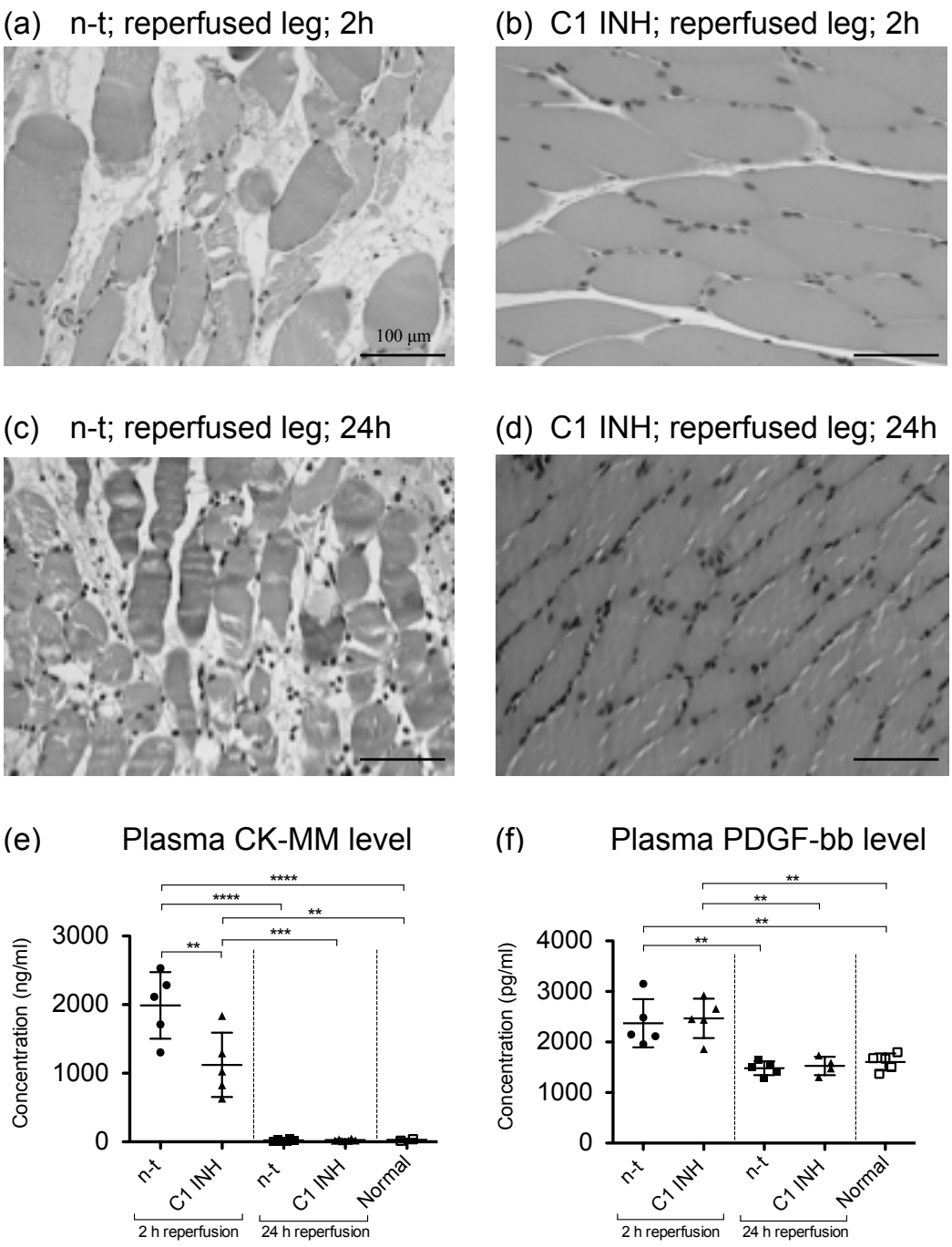



**Figure 3** Histopathological assessment of muscle damage as well as plasma levels of CK-MM and PDGF-bb. **(a-d)** Representative images of gastrocnemic muscles from non-treated as well as C1 INH treated rats, which were subjected to either 2 h or 24 h reperfusion. **(e,f)** Analysis of CK-MM and PDGF-bb plasma levels after 2 h and 24 h reperfusion. One-way ANOVA followed by Bonferroni's post hoc test for significance was used. Error bars indicate mean ± SD. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

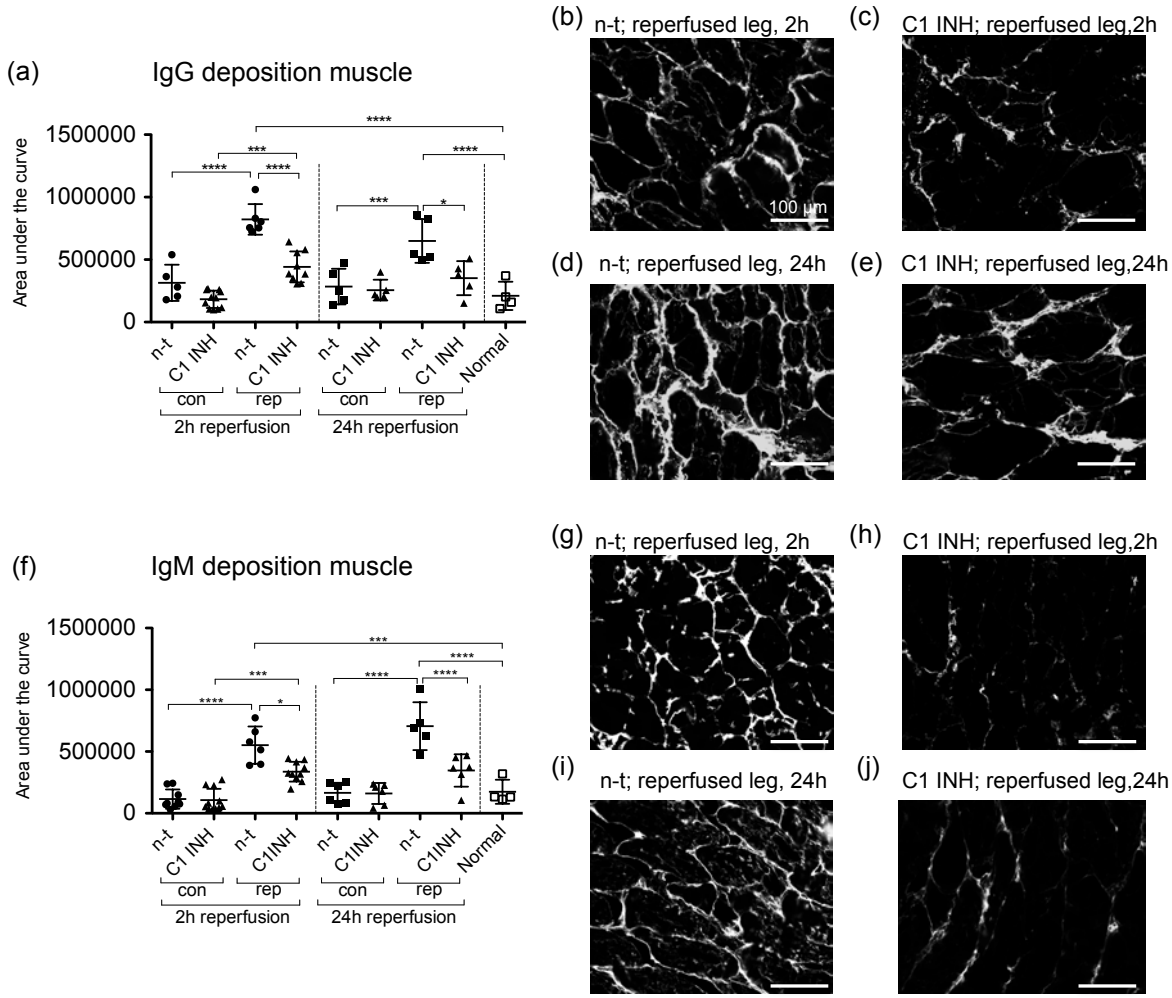



**Figure 4** Effect of C1 INH on IgG and IgM deposition in gastrocnemic muscle after 2h and 24h reperfusion. **(a,f)** Quantitative analysis of immunofluorescence stainings. **(b-e)** and **(g-j)** show representative images of IgG and IgM deposition, respectively. One-way ANOVA followed by Bonferroni's post hoc test for significance was used. Error bars indicate mean ± SD. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

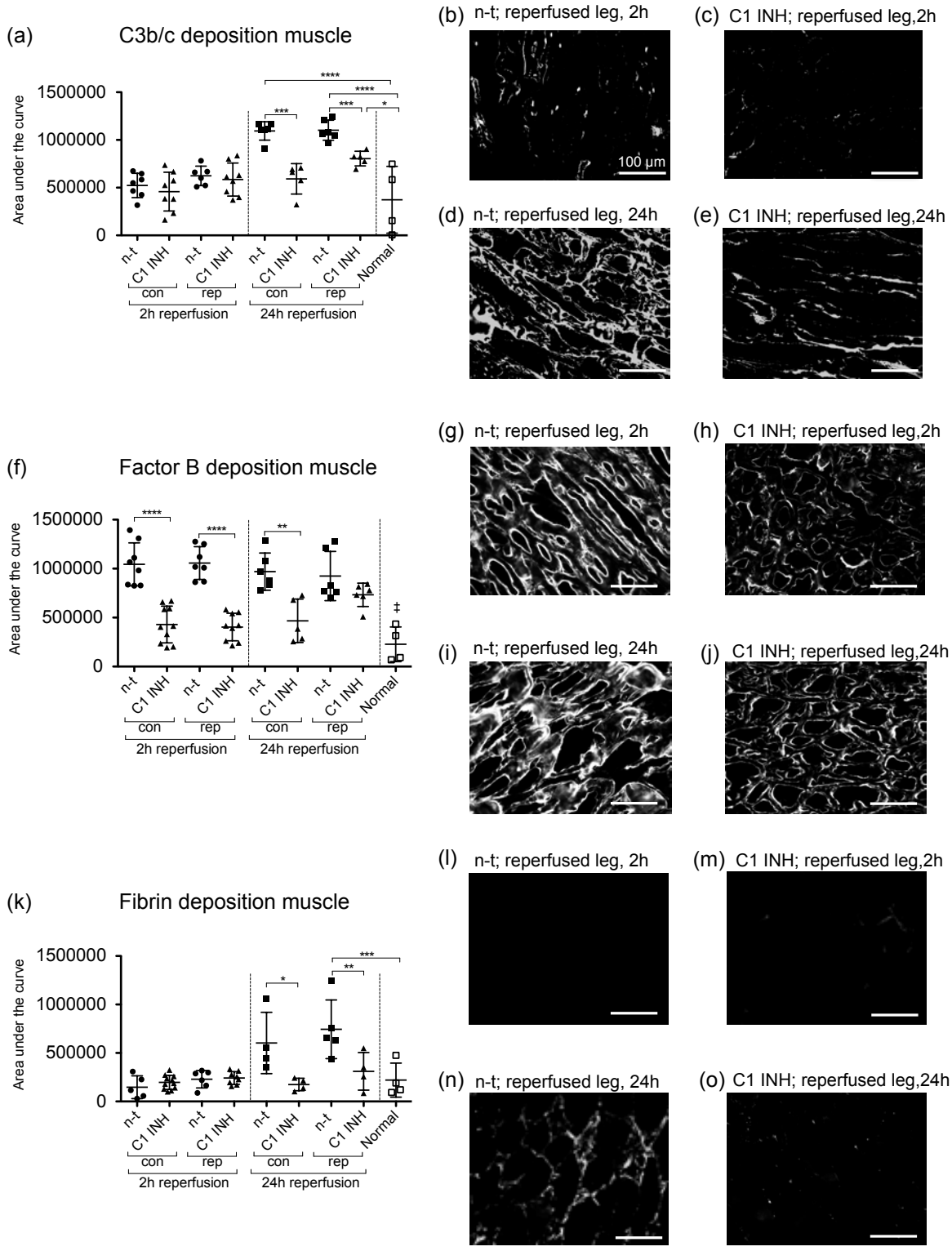



**Figure 5** Assessment of C3b/c, factor B and fibrin deposition after 2h and 24h reperfusion. **(a, f, k)** Quantitative analysis of immunofluorescence stainings. **(b-e)**, **(g-j)** and **(l-o)** show representative images of C3b/c, factor B and fibrin deposition, respectively. ‡ All non-treated groups are significantly different from normal rats. One-way ANOVA followed by Bonferroni's post hoc test for significance was used. Error bars indicate mean ± SD. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

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# Paper IV

## Use of dextran sulfate in tourniquet-induced skeletal muscle reperfusion injury

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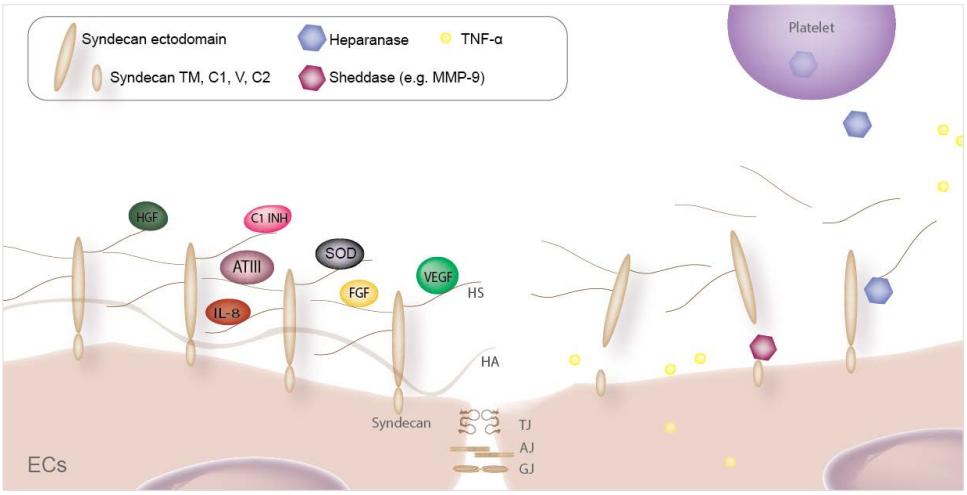
**Status:** under review in ***Journal of Surgical Research*** (submitted 28.07.2013)

**Aim:** The aim was to investigate the effect of dextran sulfate (DXS) on tourniquet-induced limb IRI and distant lung damage after 2 h and 24 h reperfusion, respectively.

**Conclusion:** In contrast to cardiac reperfusion injury, the complement system does not seem to play a major role in reperfusion injury of skeletal muscle. Rather, the kallikrein/kinin cascade may be of importance. These findings contribute to the understanding of lower extremity ischemia/reperfusion injury as well as highlight the different mechanisms on which IRI bases in skeletal muscle and heart.




In a homeostatic state, the endothelium maintains an anti-coagulatory and anti-inflammatory environment, which is uphold by a protective layer, the glycocalyx. During ischemia the glycocalyx may be partially shed, which turns the anti-inflammatory and anti-coagulatory into a pro-inflammatory and pro-coagulatory state. DXS can act like a “repair coat” and therefore may protect the endothelium from damage.

# Use of dextran sulfate in tourniquet-induced skeletal muscle reperfusion injury

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Running title: Dextran sulfate in skeletal muscle reperfusion injury

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**Keywords**: Tourniquet; Hind Limb; Ischemia/Reperfusion Injury; Dextran sulfate

## Abstract

**Background**: Establishment of a blood-free environment is a prerequisite in reconstructive surgery of extremities, which is achieved by tourniquet application. Prolonged tourniquet application is associated with ischemia/reperfusion injury (IRI) and considerable pathophysiological alterations.

**Material and methods:** Wistar rats were subjected to 3h of hind limb ischemia and 2h or 24h reperfusion. To induce ischemia the femoral artery was clamped and a tourniquet placed under maintenance of the venous return. DXS was systemically injected 10min before reperfusion. Tissue samples were analyzed for deposition of antibodies, complement C3b/c, fibrin, expression of VE-cadherin and bradykinin receptors b1 and b2.

**Results:** Antibody deposition in the reperfused leg was reduced by DXS after 2h ($P<0.001$ for IgG/IgM) and 24h ($P<0.001$, IgM), and C3b/c deposition was reduced in muscle and lung ($P<0.001$). DXS reduced fibrin deposits in the contralateral leg after 24h reperfusion. However, DXS did not reduce edema in muscle and lung ($P>0.05$), nor improve muscle viability ($P>0.05$). Expression of bradykinin receptor b1 and VE-cadherin were increased in lung after 24h reperfusion in DXS- and non-treated rats. Bradykinin receptor b2 was not affected by IRI.

**Conclusion:** In contrast to studies in myocardial infarction, DXS did not reduce IRI in this model. Neither edema formation nor viability of the reperfused muscle was improved by DXS, whereas deposition of complement and coagulation components was significantly reduced. Our data therefore suggest that IRI of skeletal muscle may not primarily be caused by complement or coagulation, but that the kinin system may play an important role.

## Introduction

Establishment of a blood-free environment is a prerequisite in reconstructive- and orthopedic surgery of extremities, which is routinely achieved by tourniquet application.(1) While short-term tourniquet application for less than 30 minutes shows no effect on post-operative pain and return to activities,(2) prolonged application can be associated with ischemia/reperfusion injury (IRI) and considerable pathophysiological alterations, which may affect the outcome of surgery.(3, 4) Characteristic clinical entities of IRI are the formation of edema, loss of muscle viability, as well as necrosis in the affected extremity.(5, 6)

In a homeostatic condition the vascular endothelium is coated by the glycocalyx, a negatively charged network of proteoglycans, glycosaminoglycans, and plasma proteins, which upholds an anti-inflammatory as well as anti-coagulatory condition.(7) A shedding of the glycocalyx, as occurring during ischemia and reperfusion, results in a loss of protection and changes the anti-inflammatory as well as anti-coagulatory endothelial surface into a pro-inflammatory and pro-coagulatory one, fostering also vascular permeability.(8, 9) Events which arise due to reperfusion and which significantly contribute to IRI, are the activation of plasmatic cascade systems, including the complement, coagulation, kinin and the fibrinolytic systems (for review see Eltzschig and Eckle).(10) The complement system can be activated via three different pathways, which finally results in the formation of the membrane attack complex, leading to lysis of the affected cell.(11) In 2006, Zhang et al. identified non-muscle myosin heavy chain type II (NMHC II) as a neo-epitope for natural antibodies.(12) Due to the hypoxic state during ischemia NMHC II is mobilized to the cell surface and via binding of natural antibodies activation of the complement system may occur.(13) The coagulation system can be activated via the intrinsic pathway through factor XII by contact activation or via the extrinsic pathway, where tissue factor – factor VIIa complexes are formed, which may finally results in intravascular clotting, vessel occlusion or thrombotic pathology.(14, 15) Edema formation may results from activation of the kinin system and therefore binding of bradykinin and des-Arg9-bradykinin to bradykinin receptors, leading to vasodilation and increased vascular permeability, see ref. (16) for review. Bradykinin formation can be initiated via two different pathways, contact activation or activation via the tissue pathway.(17) Activated FXIIa converts prekallikrein to kallikrein, which leads to the formation of bradykinin. In the tissue pathway the conversion of prekallikrein to tissue kallikrein is an enzymatic intracellular process, which results in bradykinin formation. However, also other inflammatory mediators, such as leukotriene B4 or histamine were shown to contribute to edema formation.(18, 19)

Furthermore, FXII as well as kallikrein or tissue plasminogen activator initiate the fibrinolytic pathway by activation of plasminogen, which leads to the generation of plasmin and the degradation of fibrin clots.(20, 21)

The synthetic polyanion low molecular weight dextran sulfate (DXS) is known for its heparin-like anti-coagulant and complement inhibitory activity by potentiating C1 esterase inhibitor mediated inactivation of Cls as well as binding of the regulatory factor H.(22-24) Furthermore, it modulates biological effects of contact activation, by inhibiting intrinsic coagulation without affecting the fibrinolytic potential of FXIIa/ kallikrein.(25) It was shown that DXS can act like a “repair coat” and therefore protects the endothelium from damage.(26) In vivo work in our laboratory demonstrated that DXS significantly protects from IRI in a closed chest porcine model of acute myocardial infarction.(27) Furthermore, we have shown that DXS attenuates IR induced acute graft injury and facilitates long-term survival in a rat model of heart transplantation.(28) Finally, in 2004, Laumonier et al. reported that DXS, in combination with cyclosporine A, prevents acute vascular rejection in a hamster to rat cardiac xenotransplantation model.(29)

The aim of the current project was to investigate the effect of the DXS on peripheral muscle IRI by using a rat hind limb model as well as to analyze the underlying mechanisms. We hypothesized, if the complement and coagulation systems are primarily responsible for IRI in the presented rat model, DXS treatment would maintain muscle viability, reduce local edema formation as well as distant lung damage.

## Material & Methods

### Animals and housing

All animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals as well as the Swiss animal protection law and were approved by the animal experimentation committee of the cantonal veterinary service (Canton of Bern, Switzerland). Before experimentation male Wistar rats weighing between 250 and 350 g (wild type, bred at the central animal facility, University of Bern) were kept in groups of three in clear 1500 $cm^2$ Euro-standard Type IV S cages (Tecniplast, Buguggiate, Italy) under standard housing conditions with food and water ad libitum. After surgical intervention, animals, which were subjected to 24 h reperfusion, were kept separated. Cages were individually ventilated at 20 ± 2°C and 45-65% relative humidity.

### Reagents

Low molecular weight dextran sulfate (DXS, MW 5000) buffered with citric acid in NaCl 0.9 % was provided by TikoMed AB (Viken, Sweden).

### Experimental groups

Rats were divided into 5 groups. The experimental groups of 2 h reperfusion (n=8) and 24 h reperfusion (n=6) received 20 mg/kg of DXS 10 minutes prior to induction of reperfusion. Control groups subjected to either 2 h (n=7) or 24 h (n=6) of reperfusion received no DXS (non-treated, n-t). Tissue samples of normal, healthy rats (n=4) were included for comparison.

### Anesthesia and analgesia

2.5% isoflurane in oxygen was used for anesthesia induction in a special box and later maintained by inhalation of 1.5% isoflurane on a nose mask. Analgesia was provided by subcutaneous injection of 0.05 mg/kg of buprenorphine (Temgesic, Reckitt Benckiser, Switzerland AG) 30 minutes prior surgical intervention. The total duration of anesthesia was approximately 6 h after which the 24 h reperfusion rats were allowed to wake up and the 2 h reperfusion rats were euthanized. For the 24 h reperfusion period buprenorphine injection was repeated when animals were completely awake. Rats were anesthetized again as described above and sacrificed by exsanguination during organ removal after completion of 24 h reperfusion.

## Surgical procedure

In order to maintain body temperature at 37°C rats were kept on a heating pad. Approximately 30 minutes after induction of anesthesia, the femoral artery and vein were exposed via a groin incision, and a tourniquet (standardized weight of 450 g) was placed underneath the femoral vessels to block collateral circulation. For occlusion of the femoral artery two microvascular clamps (B1-V, S&T, Neuhausen, Switzerland) were used. Rat hind limbs were not exsanguinated, but a comparable state was achieved by allowing venous return during the entire period of ischemia in order to prevent venous congestion and additional injury through microcirculatory impairment, which would not represent the clinical situation. 3 h of ischemia was followed by 2 h reperfusion under anesthesia or 24 h reperfusion during which the rats were allowed to wake up with appropriate analgesia. At the end of all experiments, tissue samples of both the ischemic as well as the contralateral gastrocnemic muscles and the lungs were taken for further analyses.

## Assessment of edema formation

Formation of edema was analyzed by taking two samples of the gastrocnemic muscle from both legs and additionally lung, which were immediately weighed to obtain the wet weight. Tissue samples were then dried for 24 h at 80°C after which a constant dry weight was achieved and the wet weight to dry weight ratio was calculated.

## Analysis of muscle viability

For investigation of the effect of DXS on muscle viability the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma, St. Louis, USA) assay was performed. MTT is a yellow-colored tetrazolium salt, which is converted to purple colored formazan crystals by metabolically active cells. Samples from the gastrocnemic muscles were taken, washed in PBS, blotted dry and incubated in 0.1 mg MTT/ml PBS in a total volume of 3 ml at 37°C, rotating in dark for 2 h. To elute the formazan crystals from the tissue, muscle samples were blotted dry and incubated in 100 % isopropanol at 37°C, rotating overnight in the dark. 200 µl of thus obtained supernatant was measured in a microplate (Nunc, 96 well, maxisorp, transparent, Roskilde, Denmark) at 560 nm (Ref. 690 nm; Infinite M1000 microplate reader, Tecan, Männedorf, Switzerland). After drying the muscle samples at 80°C for 24 h the OD per mg dry weight was calculated and compared with values of contralateral control legs and normal rats.

## Histological assessment of damage

Infiltration of neutrophil granulocytes, hemorrhage as well as total myocyte damage was histologically assessed. For this purpose, tissue samples of the gastrocnemic muscles were fixed in 4% formalin for 24-72 h, embedded in paraffin, cut into 3 µm thick sections and stained with hematoxylin and eosin.

## Immunofluorescence analyses of muscle and lung samples

Immunofluorescence stainings were performed to quantify the deposition of IgM (used antibody: catalog no. 3020-08; Southern Biotech, AL, USA) and IgG (3030-08; Southern Biotech) as well as C3b/c (A0062, Dako). Furthermore, fibrin deposition (F0111; Dako, Baar, Switzerland), expression of bradykinin receptor b1 (ABR-011, Alomone Labs, Jerusalem, Israel), bradykinin receptor b2 (ABR-012, Alomone Labs) as well as VE-cadherin (sc-6458, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) were analyzed. Samples from the gastrocnemic muscle of both legs and lung were taken after 2 h or 24 h reperfusion, washed in PBS, blotted dry and embedded in OCT matrix (Tissue tek, Sakura, USA) on dry ice. The samples were immediately stored at -20°C until cryosections were cut. Sections were fixed in acetone and rehydrated in tris-buffered saline (TBS). Primary antibodies were incubated overnight at 4°C and secondary antibodies for 1 h at room temperature (RT). Subsequently, slides were mounted and coverslipped. Pictures were taken with a fluorescent microscope (Leica DMI 4000B, Leica Microsystems Schweiz AG, Heerbrugg, Switzerland) and analyzed using Image J (National Institutes of Health, Bethesda, MD, USA) and GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA, USA.). Endothelial expression of VE-cadherin as well as bradykinin receptor b1 and b2 was analyzed in lung tissue. For this analysis, the inner lining of the vessels was selected by hand, the surface area calculated and the intensity of immunofluorescence measured. Area under the curve values were obtained and divided by the surface area to achieve a final value in intensity per square pixel.

## Sandwich-ELISA

An enzyme-linked immunosorbent assay (ELISA) was used to measure the inhibitory effect of DXS on IgG, IgM, C1q, C4b/c and C3b/c binding to a polystyrene microplate (NUNC maxisorp) coated with synthetic Gal alpha 1,3 Gal disaccharide conjugated to polyacrylamide (PAA-Bdi, Lectinity Inc., Moscow, Russia).

The hydrophilic PAA-Bdi polymer acts as surrogate for the B-disaccharide, which is the most important porcine xenoantigen,(30) and efficiently binds antibodies as well as complement components from human serum.(31) In brief, 10 µg/ml PAA-Bdi in carbonate buffer (pH 9.6) was immobilized to the surface of a 96 well plate at 4°C, overnight. After blocking the coated plate with 1% BSA in PBS to prevent non-specific binding, normal human serum (NHS, 1:30), containing different concentrations of DXS, was incubated on the PAA-Bdi coated plate for 90 minutes at 37°C. Primary antibodies IgM (goat anti-human IgM FITC, Sigma, F-5384), IgG (gt-a-hu IgG FITC, Sigma, F-5512), C1q (rb-a-hu C1q, Dako A0136), C4b/c (rb-a- C4b/c FITC, Dako F0169) or C3b/c (rb-a- C3b/c FITC, Dako F0201) were then incubated on the plate. A washing step with PBS containing 0.05 % Tween 20 followed, as well as an incubation step with an alkaline phosphatase-conjugated antibody. Finally, a solution of diethanolamine buffer and the substrate 4-NPP was added and the OD measured at 405/ 490 nm. For analysis, GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA, USA) was used.

## Hemolytic assay (CH50)

A standard CH50 test, as described earlier (26, 32), was used to assess the complement-inhibitory function of DXS. In brief, $10^9$ sheep erythrocytes (BioMérieux, Geneva, Switzerland) per mL $VBS^{++}$ were coated with rabbit anti-sheep antibodies (amboceptor) for sensitization. In order to obtain a 50 % lysis of the sensitized erythrocytes NHS was diluted 1:100. When exposed to complement components in NHS, the antibody-loaded sheep erythrocytes ($10^8$ cells/mL in $DGVB^{++)}$ activate the classical complement pathway, and are therefore lysed. The degree of lysis was then calculated from the amount of free hemoglobin. DXS was added at different concentrations to the diluted NHS and its effect on complement-mediated hemolysis was measured spectrophotometrically at 412 nm. For analysis, GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA, USA.) was used.

## Statistical analysis

All data are expressed as mean ± standard deviation (SD). One-way analysis of variance with Dunnett's post-test against control (no DXS) was used for in vitro experiments, as every concentration of DXS should be compared with the control. Bonferroni's post test (GraphPad Prism 5 software) was used for all other experiments to determine statistical significance between groups. P values of $< 0.05$ were considered statistically significant. Determination of n-numbers per group was performed without formal power analysis, based on preliminary experiments with DXS(26).

## Results

### Edema formation, muscle viability and histopathology

Analysis of muscle edema formation in gastrocnemic muscles after 2 h and 24 h reperfusion revealed a significant increase in non-treated (2 h: ratio 5.4 ± 0.43, $P < 0.001$; 24 h: ratio 5.6 ± 0.72, $P < 0.0001$) and also in DXS treated (2 h: ratio 5.1 ± 0.45, $P < 0.05$, 2 h; 24 h: ratio 5.5 ± 0.77, $P < 0.001$) rats as compared to normal ones (ratio 4.3 ± 0.05). Normal rats showed no edema formation (Figure 1A). An increase of wet/dry ratio in lung samples was not found for non-treated (ratio 4.7 ± 0.20) and DXS treated rats (ratio 4.8 ± 0.12) after 2 h reperfusion. However, after 24 h reperfusion wet/dry ratios significantly increased in non-treated rats (ratio 5.1 ± 0.16, $P < 0.001$) and also in DXS treated rats (ratio 5.3 ± 0.23, $P < 0.0001$) in comparison to normal rats (ratio 4.6 ± 0.14, Figure 1B). Viability of the reperfused muscle was not significantly impaired after 2 h reperfusion (non-treated 57 ± 49 mOD/mg; DXS 76 ± 46 mOD/mg) as compared to normal rats (69 ± 62 mOD/mg). In contrast, as compared to normal rats, muscle viability was significantly decreased after 24 h reperfusion in non-treated rats (46 ± 1 mOD/mg, $P < 0.05$), but not in DXS-treated animals (53 ± 14 mOD/mg, Figure 1C). Histopathological assessment of reperfused muscle after 2 h and 24 h revealed infiltration of neutrophil granulocytes, edema formation and myocyte destruction in non-treated and DXS treated rats (Figure 1 D-H).

### Deposition of immunoglobulin G and M

Antibody deposition was analyzed in gastrocnemic muscle and lung tissue by immunofluorescence. Contralateral muscle tissue showed no IgG deposition after 2 h and 24 h reperfusion. In contrast, significant IgG deposition was found in reperfused muscle tissue after 2 h ($P < 0.001$) and 24 h ($P < 0.05$) reperfusion. However, in DXS treated rats significantly less IgG deposits were detected after 2 h ($P < 0.0001$) but not after 24 h reperfusion (Figure 2A). Analysis of lung tissue revealed no inter-group differences in IgG deposition as compared to normal rats (Figure 2B).

Also IgM deposits were not detected in contralateral legs as compared to normal rats after 2 h and 24 h reperfusion. Similar to IgG results, also IgM deposition was significantly increased in reperfused legs of non-treated rats after 2 h ($P < 0.001$) and 24 h ($P < 0.001$) reperfusion. In contrast to IgG, IgM deposition was significantly decreased in DXS treated rats after 2 h ($P < 0.001$) and 24 h ($P < 0.001$) reperfusion (Figure 2C). In lung tissue no significant increase of IgM deposition was detected as compared to normal rats (Figure 2D).

## Deposition of complement components C1q and C3b/c in tissue

C1q as well as C3b/c deposition in gastrocnemic muscle and lung tissue was analyzed by immunofluorescence. C1q deposition was detected after 2 h reperfusion in the contralateral leg ($P < 0.01$) and in the reperfused leg ($P < 0.001$) as compared to normal rats. DXS treated rats demonstrated significantly reduced C1q deposition in both legs. After 24 h reperfusion increased C1q deposition was also found in both legs (contralateral leg, $P < 0.05$; reperfused leg, $P < 0.01$). A trend for reduction by DXS was visible in the contralateral leg, however not significantly (Figure 3A). Also in lung tissue C1q deposition was increased as compared to normal rats, which was also not reduced by DXS treatment (Figure 3B). As compared to normal rats no C3b/c deposition was detected in contralateral and reperfused tissue after 2 h reperfusion. However, after 24 h reperfusion non-treated rats presented significant C3b/c deposition in both, contralateral ($P < 0.001$) and reperfused legs ($P < 0.001$) as compared to normal rats, whereas DXS treatment significantly reduced C3b/c deposition in contralateral ($P < 0.001$) and reperfused ($P < 0.05$) legs (Figure 3C**).** After 24 h reperfusion also in lung tissue of non- ($P < 0.001$) and DXS ($P < 0.001$) treated rats significant C3b/c deposition was detected as compared to normal rats. However, DXS treated rats presented significantly less C3b/c deposits as compared to non-treated rats ($P < 0.01$, Figure 3D).

## Deposition of fibrin

Fibrin deposition was significantly increased after 24 h reperfusion in non-treated rats and was detected in both, contralateral ($P < 0.05$) and reperfused legs ($P < 0.01$). DXS treatment significantly reduced fibrin deposition only in contralateral ($P < 0.01$) but not reperfused legs (Figure 4A). Also in lung tissue significant fibrin deposition was detected after 24 h reperfusion in non-treated ($P < 0.001$) as compared to normal rats. However, DXS treatment significantly ($P < 0.001$) reduced fibrin deposition in the lungs (Figure 4B).

## Expression of bradykinin receptors b1 and b2 as well as VE-cadherin

Analysis of the endothelial expression of kinin system components bradykinin receptor b1 and b2 in lung tissue after 24 h reperfusion revealed a significant increase of b1 expression in non-treated ($P < 0.05$) and also DXS treated rats ($P < 0.05$) as compared to normal animals (Figure 5A). The constitutively expressed b2 receptor showed no inter-group differences and no increase of expression as compared to normal rats (Figure 5B). Furthermore, analysis of VE-cadherin demonstrated increased expression in non-treated rats ($P < 0.05$), which was not reduced by DXS treatment (Figure 5C).

### Assessment of the inhibitory effect of DXS by using sandwich-ELISA

An ELISA was used to assess whether DXS is able to inhibit the deposition of the complement components C1q, C4b/c and C3b/c on PAA-Bdi coat – as to be expected for a complement inhibitor – or whether also the binding of IgM and IgG antibodies to the coat antigen would be blocked. The results demonstrated no significant inhibition of IgM, IgG and also C1q in this setting. Furthermore, DXS demonstrated a dose-dependent, significant reduction of the deposition of C3b/c ($P < 0.001$) and C4b/c ($P < 0.001$) with IC50 values of 32 µg /mL and 55 µg /mL, respectively (Figure 6).

### Hemolytic assay (CH50)

A standard CH50 test as shown in Figure 7 revealed a dose-dependently decreased serum classical complement activity when DXS was used. The concentrations at which 50% inhibition of the activity was reached (IC50) were 30 µg /mL for the CH50 test.

## Discussion

In reconstructive surgery sometimes long operation times are required, which are frequently associated with IRI. The latter is highly dependent on the time of ischemia and may therefore range from mild edema formation to severe necrosis of the affected tissue. However, the consequences of IRI are not locally restricted and in severe cases also distant organs can be affected.(33) A frequent observation is respiratory dysfunction in patients suffering from lower extremity IRI.(34)

In the present study, analysis of muscle and lung edema formation after 3 h ischemia and 2 h or 24 h reperfusion revealed significant edema formation in both non-treated and also DXS treated rats. Muscle viability of reperfused legs in non-treated rats was not affected after 2 h, but significantly so after 24 h reperfusion. Assessment of histopathology in reperfused gastrocnemic muscles demonstrated edema formation, infiltration of granulocytes and damage of myocytes in non-treated as well as DXS-treated rats. Analysis of IgG and IgM deposition in gastrocnemic muscles after 2 h and 24 h reperfusion revealed significantly increased deposition in non-treated rats, which was reduced by DXS treatment. Furthermore, deposition of the complement component C1q was significantly increased after 2 h and also after 24 h reperfusion in contralateral as well as ischemic legs of non-treated rats. After 2 h reperfusion DXS significantly reduced C1q deposition in both legs, whereas this was not the case after 24 h reperfusion. Also in lung tissue DXS did not reduce C1q deposition. Deposition of the complement component C3b/c in non-treated and also DXS treated rats was not significantly different to normal animals after 2 h reperfusion. However, after 24 h reperfusion C3b/c was deposited in contralateral and reperfused legs of non-treated rats and was significantly reduced by DXS treatment. Similar results were found for lung tissue, where increased C3b/c deposition was present in non-treated rats after 24 h reperfusion and DXS treatment resulted in significantly reduced C3b/c deposition. These results are in line with earlier findings, which showed that DXS is a potent complement inhibitor (26-28, 35), and they may also explain the significant reduction of antibody deposition in gastrocnemic muscles. Indeed, activation of complement may potentiate antibody deposition due to increased tissue damage and therefore increased exposure of epitopes for natural antibodies. Complement inhibition may therefore indirectly lead to reduced antibody binding in the tissue. This assumption is supported by our in vitro experiments, in which DXS decreased serum classical complement activity in a CH 50 test as well as reduced the deposition of complement components, but not antibody binding, on a PAA-Bdi coated plate.

In the present model – in line with earlier findings – DXS treatment resulted in significantly reduced fibrin deposition in contralateral and reperfused legs as well as in lung tissue after 24 h reperfusion, whereas after 2 h of reperfusion no fibrin deposition was detected.(36) Furthermore, degradation of fibrin into soluble fibrin degradation products may occur via the fibrinolytic system.(37) It is known that the fibrinolytic pathway can be initiated via direct plasminogen activation through tissue plasminogen activator (tPA), kallikrein (KK) or factor XII, which results in the generation of plasmin.(20, 21) Thus, the reduction of fibrin deposition in our model cannot be exclusively attributed to the inhibition of the coagulation system, as DXS could also induce an increased activation of plasmin resulting in amplified fibrinolysis.

In contrast to our expectations based on data from myocardial infarction studies (27), DXS treatment did not result in a significant increase of viability or reduced edema formation. However, DXS indeed prevented activation of the complement and coagulation systems, which, even for complement alone, is sufficient to prevent IRI in the case of myocardial infarction.(38) Additional analyses of the specific mechanisms for IRI in skeletal muscle tissue were therefore conducted, including the kinin system and expression of VE cadherin. When analyzing the inducible bradykinin receptor b1 in lung tissue, up-regulation of endothelial expression was detected in non-treated and also in DXS treated as compared to normal animals. This finding fits with the increased edema formation found in lung tissue. It is known, that DXS inhibits complement and coagulation, but not the kinin system.(36)

Furthermore, analysis of expression of VE-cadherin, an endothelial specific adhesion molecule located between endothelial cells and contributing to barrier function, was conducted in lung tissue.(39) VE-cadherin expression was significantly increased in non- as well as DXS treated rats as compared to normal animals. So far not much is known about VE-cadherin-mediated cell-cell junction regulating mechanisms. It could be speculated that the increase of VE-cadherin expression was induced due to repair mechanisms on which DXS has no effect.

## Conclusion

In the present rat model of tourniquet-induced lower extremity IRI, DXS (MW 5000) was not able to significantly reduce skeletal muscle edema, distant lung edema formation as well as maintenance of muscle viability. However, DXS reduced deposition of complement and fibrin in the affected tissue as well as remotely in the lung. The reduction of fibrin deposition may be due to inhibition of the coagulation system or due to amplified fibrinolysis. In contrast, in a porcine model of myocardial IRI, DXS showed cardioprotective effects by reducing myocardial infarct size via inhibition of complement as well as reduced expression of pro-coagulant factors.(27) Although skeletal muscles and cardiac muscles are both striated muscles, they significantly differ in their cellular structure. Therefore, IRI in skeletal and cardiac muscle may base on different mechanisms, which could explain the divergent effect of DXS on skeletal muscle versus myocardial IRI. In both models DXS significantly inhibited the complement and coagulation system, albeit with different consequences. Based on our results, we conclude that the complement as well as coagulation systems are not primarily responsible for IRI in the present rat model of skeletal muscle IRI, but that the kinin system may play a role.(40)

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## Disclosure

The authors have declared no competing interests.

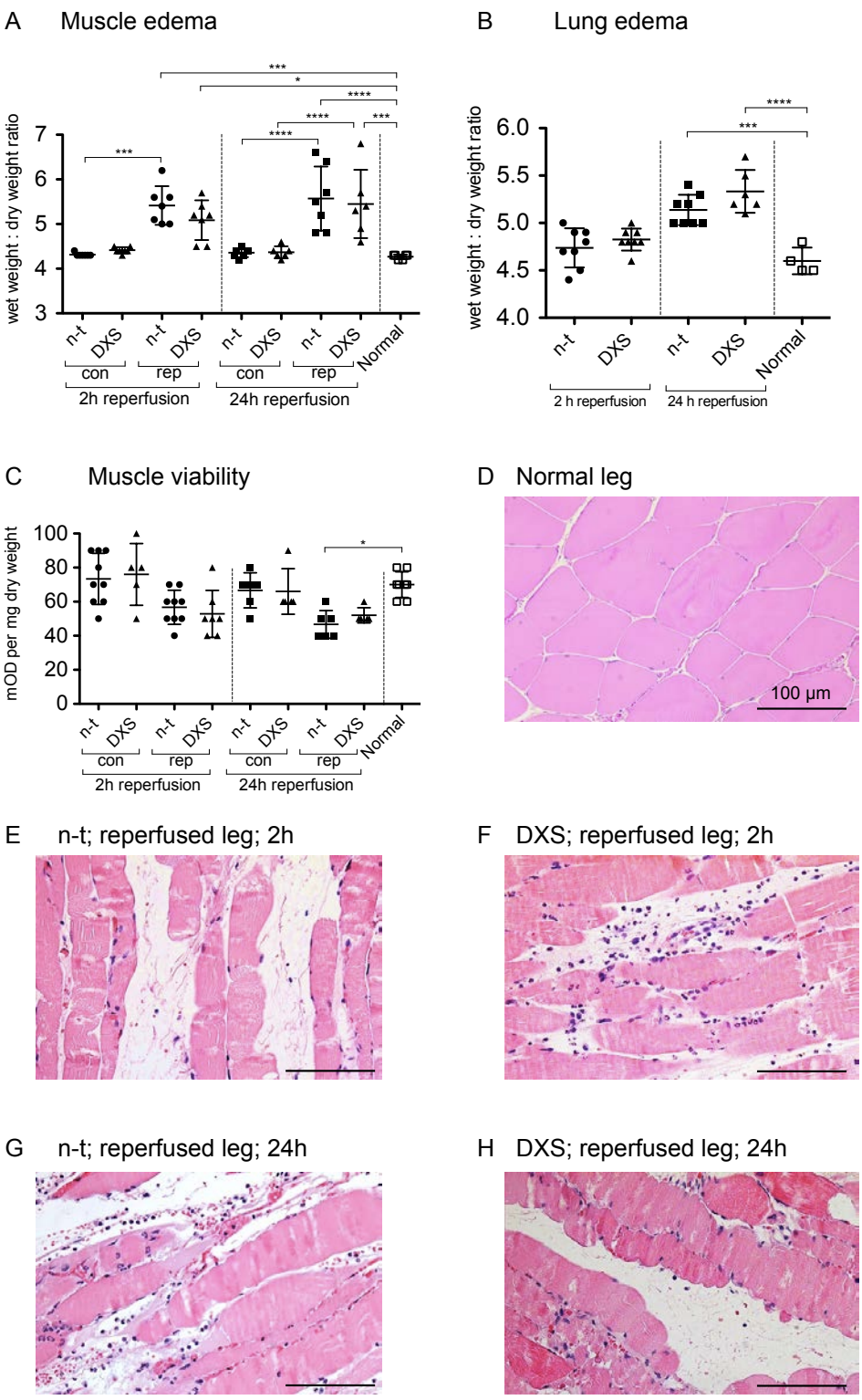


**Figure 1** Edema formation, muscle viability and histopathology. Analysis of edema formation in gastrocnemic muscle (A) and lung (B) after 2 h and 24 h reperfusion. Viability of muscle samples was analyzed using the MTT assay (C). Representative images of hematoxylin-eosin stained muscle tissue after 2 h or 24 h reperfusion for histopathological assessment (18). One-way ANOVA followed by Bonferroni's post hoc test for significance was used. Scatterplots of individual experiments are shown with indication of mean ± SD. *P<0.05; **P<0.01; ***P<0.001.

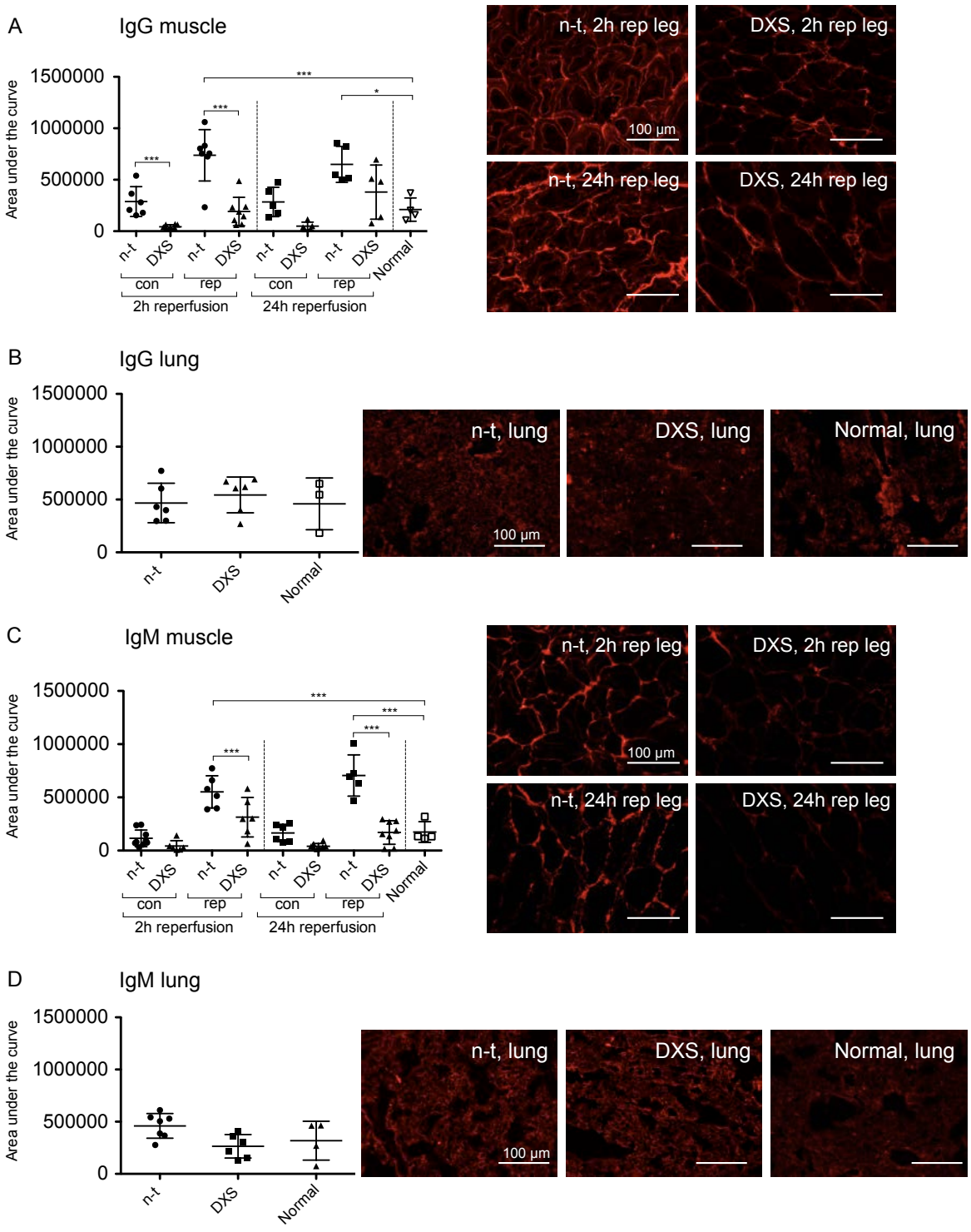


**Figure 2** Deposition of immunoglobulins G and M**.** Antibody deposition was analyzed in gastrocnemic muscle and lung tissue by immunofluorescence staining. Representative images are shown for detection of IgG in muscle (A) and lung (B) as well as for IgM in muscle (C) and lung tissue (D). One-way ANOVA followed by Bonferroni's post hoc test for significance was used. Scatterplots of individual experiments are shown with indication of mean ± SD. *P<0.05; **P<0.01; ***P<0.001.

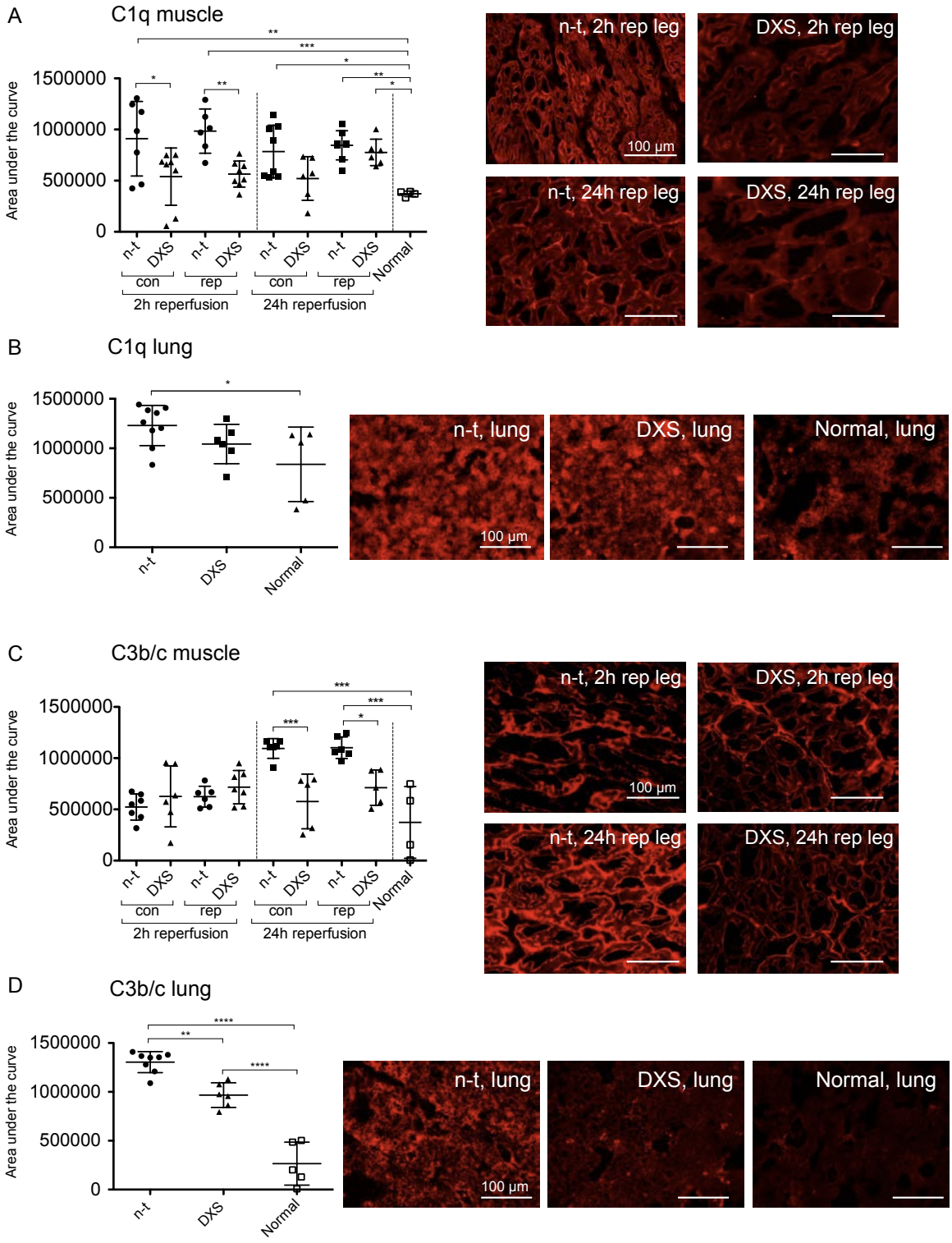


**Figure 3** Analysis of C1q and C3b/c deposition in muscle and lung. Quantitative analyses of immunofluorescence stainings and representative images are shown for detection of complement components C1q in muscle (A) and lung (B) as well as C3 deposition (C3b/c) in muscle (C) and lung (D) by CY3-labeled antibodies on cryosections. One-way ANOVA followed by Bonferroni's post hoc test for significance was used. Scatterplots of individual experiments are shown with indication of mean ± SD. *P<0.05; **P<0.01; ***P<0.001.

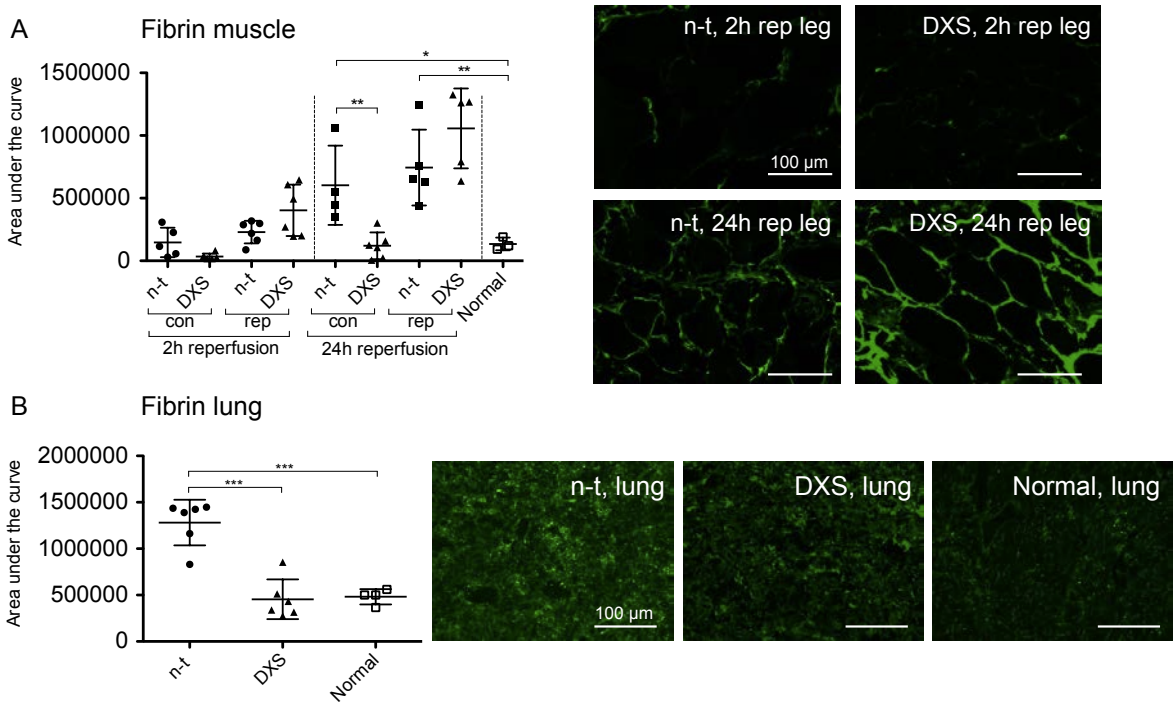


**Figure 4** Analysis of fibrin deposition in muscle and lung. Quantitative analysis of immunofluorescence stainings and representative images are shown for detection of fibrin in muscle (A) and lung (B) by a FITC-labeled antibody on cryosections. One-way ANOVA followed by Bonferroni's post hoc test for significance was used. Scatterplots of individual experiments are shown with indication of mean ± SD. *P<0.05; **P<0.01; ***P<0.001

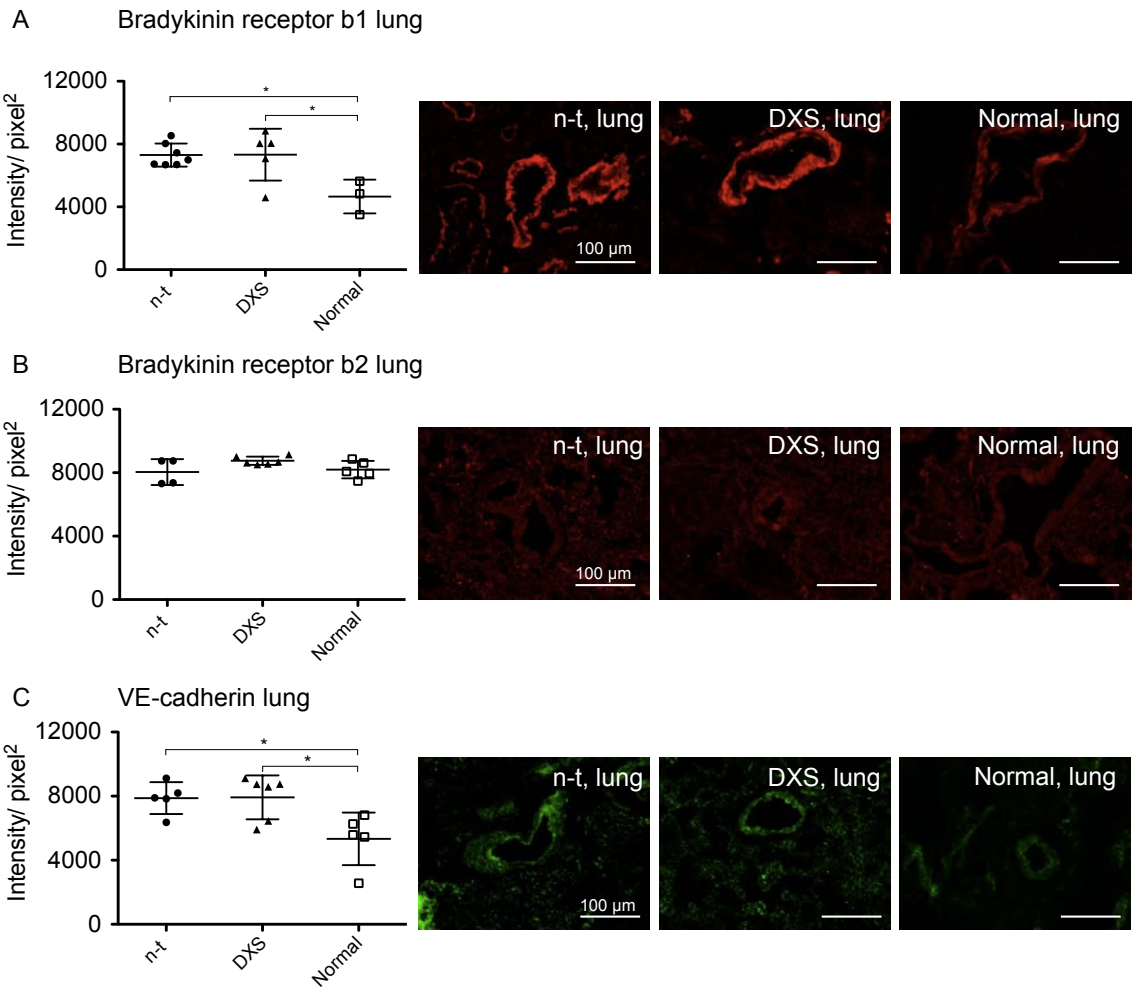


**Figure 5** Expression of bradykinin receptors b1 and b2 as well as VE-cadherin. Analysis of bradykinin receptors b1 (A) and b2 (B) as well as VE-cadherin (C) expression lung tissue after 24 h reperfusion. Quantitative analysis of immunofluorescence stainings and representative images are shown by Cy3 or Alexa 488-labeled antibodies on cryosections. One-way ANOVA followed by Bonferroni's post hoc test for significance was used. Scatterplots of individual experiments are shown with indication of mean ± SD. *P<0.05; **P<0.01; ***P<0.001.

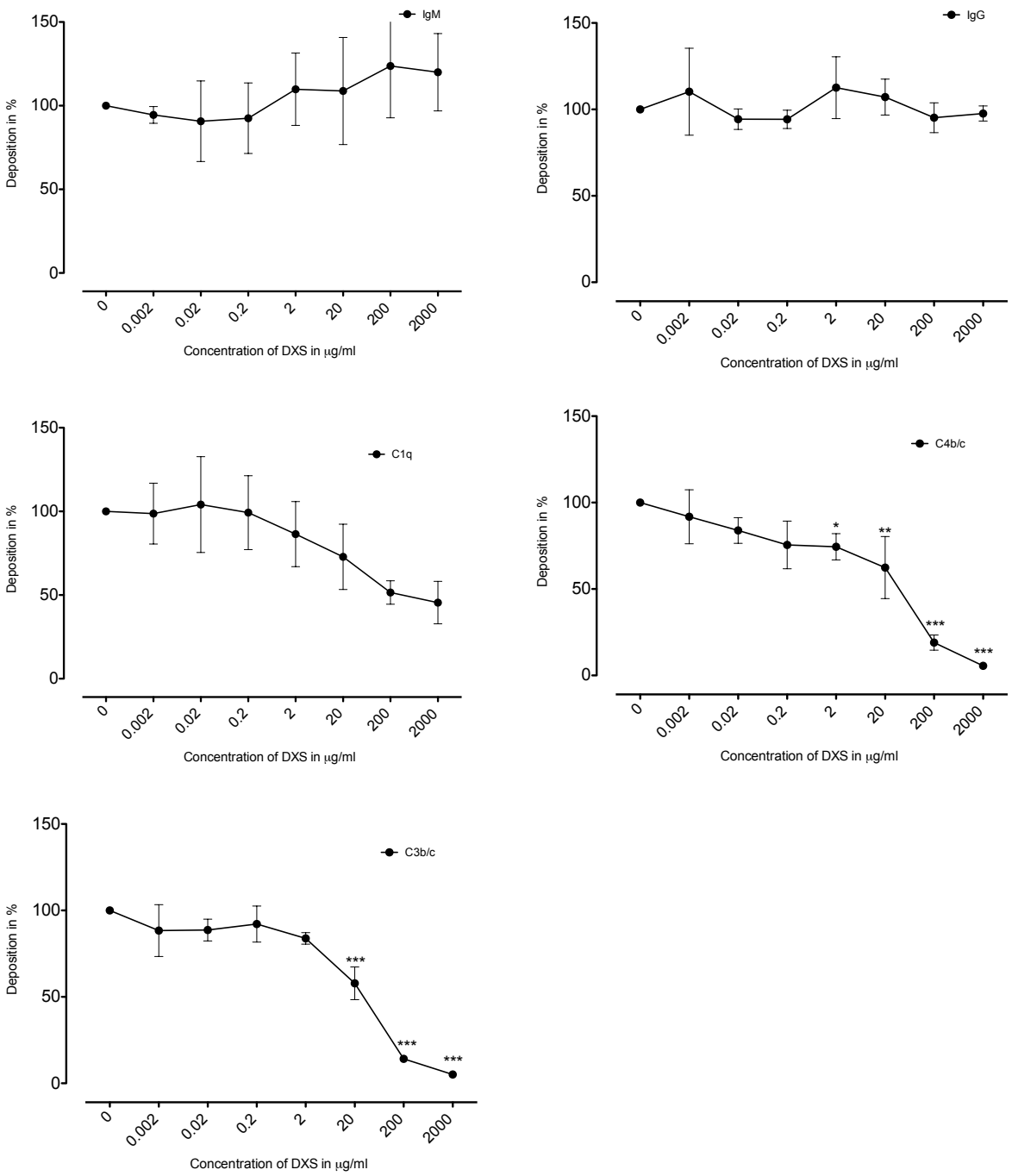


**Figure 6** In vitro assessment of the inhibitory effect of DXS on antibody binding and complement deposition on an antigen (PAA-Bdi) coated polystyrene plate. One-way ANOVA followed by Dunnett's post hoc test for significance vs. NHS only was used for comparison of the different DXS concentrations. Error bars indicate SD. *P<0.05; **P<0.01; ***P<0.001.

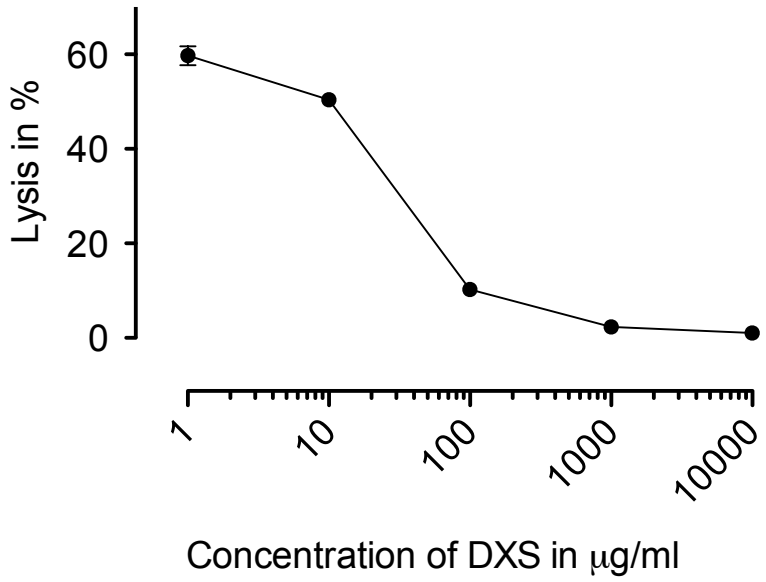


**Figure 7** Hemolytic assay for the classical (CH50) complement pathway. The CH50 test was performed to evaluate the efficacy and specificity of complement inhibition. DXS dose-dependently inhibits the classical pathway of complement activation. Error bars indicate SD.

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## Integrated Discussion and Conclusion

Apart from coronary heart disease and stroke, acute limb ischemia and subsequent reperfusion is one of the most common peripheral vascular emergencies, associated with extensive morbidity. Ischemia/reperfusion injury results in the activation of a complex inflammatory cascade, in which the complement, coagulation as well as the kinin systems play crucial roles. IRI of extremities has been analyzed before, but until now an effective treatment of this critical situation is missing.

It is well known that C1 esterase inhibitor has an important physiological role in regulating the complement, the kinin as well as the coagulation systems and may thus be a promising drug candidate to reduce IRI in skeletal muscle. C1 INH treatment led to significant reduction of edema formation and apoptosis as well as maintained muscle viability in a rat model of lower extremity IRI. In contrast, APT070, a membrane-targeted construct derived from human complement receptor 1, showed potent reduction of complement deposition, but did not result in reduction of injury *(Paper I)*. Similar results were obtained when rats were treated with low molecular weight dextran sulfate, since deposition of coagulation and also complement components was reduced, but no attenuation of injury was detected *(Paper IV)*. Interestingly, IRI in skeletal and cardiac muscle may base on different mechanisms, as in both models DXS significantly reduced deposition of complement and coagulation components, however with different outcomes *(Paper IV).* C1 INH presented also beneficial effects in regulating components of the plasma cascade systems in distant organs, affected due to tourniquet-induced lower extremity IRI *(Paper I and II)*. Furthermore, we found that individual organs are not equally susceptible to IRI *(Paper II).*

In another study, an acute hind limb IRI model with shortened reperfusion times was established in order to prevent unnecessarily high severity grades in consideration of the 3R principles. We demonstrated that 2 h reperfusion is sufficient to assess basic parameters, whereas for in-depth analysis of mechanisms of actions rats need to be subjected to 24 h reperfusion *(Paper IV).* This finding may prevent unnecessarily high severity grades in future studies.

In conclusion this thesis demonstrates that C1 INH may be a promising drug to reduce peripheral IRI and distant organ damage in complex and prolonged surgical interventions on extremities requiring tourniquet application. C1 INH is already clinically approved as therapy for hereditary angioedema, a rare disorder characterized by recurrent episodes of severe swellings. Based on the above-mentioned findings, a next step might be its clinical application in orthopedic surgery in the setting of hip- or knee replacement requiring tourniquet application.

All in all, targeting more than one plasma cascade system may be most effective, but different types of tissue may require different therapeutic treatment. As shown in paper IV, DXS significantly inhibited deposition of complement and coagulation components, but did not reduce IRI in a model of lower extremity IRI. In contrast, in myocardial IRI (Banz et al. 2005, doi:10.1093/eurheartj/ehi421) reperfusion injury was significantly attenuated by DXS. Importantly, complete inhibition of the complement and coagulation systems may result in increased risks of infection and uncontrolled bleedings, respectively. Therefore, ideal therapeutic approaches should simultaneously target more than one plasma cascade system, like C1 INH, but should not result in complete inhibition.

Inhibition of plasma cascade systems has demonstrated promising results in various animal models of IRI. However, results obtained from animal studies need to be interpreted with caution, since clinical trials have often shown rather disappointing results for specific complement-, coagulation- or kinin system inhibitors in preventing or attenuating IRI. An outlook on clinical application of C1 INH is therefore not easily possible. In addition, study design and determination of primary as well as secondary endpoints may crucially influence the results of clinical trials.

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# Curriculum Vitae

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## - Personal Details -

| | |
|---|---|
| Sex | female |
| Date of Birth | 07.10.1983 |
| Civil status | married |
| Languages | German (mother tongue), English (fluent), Swiss German (basic), Swedish (A1) |

## - Education -

| **Period** | **Institution** | **Degree** | **Field of study** |
|---|---|---|---|
| 2009 - 2013 | University of Bern<br>Switzerland | PhD | Immunology |
| 2006 - 2008 | University of Veterinary Medicine<br>Hannover, Germany | M.Sc. | Biochemistry |
| 2003 - 2006 | University of Osnabrück<br>Germany | B.Sc. | Zoology |

## - Teaching -

| | | |
|---|---|---|
| Annually | Teaching of medical students | (2 weeks-courses) |
| | Teaching of pupils in 11th grade of high school | (2 weeks-courses) |
| 2010 | Co-supervision of Master thesis | (6 month) |

# - Education & Training -

| Year | Course/ Training |
|---|---|
| 2013 | European Meeting on Complement (Jena, Germany) |
| | Basic statistics and analysis of small and large data sets |
| | Swiss experimental surgery symposium |
| 2012 | Continuing training for performance of animal experiments (SGV Meeting) |
| | Swiss experimental surgery symposium |
| | Conference of the society of cardiovascular diseases (USGG) |
| | Swiss Pharma Science Day (SSPhS) |
| | International Complement Workshop (ICW, Crete, Greece) |
| | Scientific writing (University of Bern) |
| 2011 | European Meeting on Complement (Leiden, the Netherlands) |
| | International Symposium in Defects in Innate Immunity and Inflammation (Zurich) |
| | Wolfsberg Meeting (Meeting of Swiss Ph.D. students in the field of Immunology) |
| | Continuing training for performance of animal experiments (SGV Meeting) |
| | Natural Science Day (Workshop of MSD Merck, Sharp & Dohme AG, Zurich) |
| 2010 | Confocal microscopy |
| | Vascular cell biology |
| | Book club: Abbas “Cellular and molecular immunology” |
| | Continuing training for performance of animal experiments (SGV Meeting) |
| | Course Flow Cytometry |
| 2008 | FELASA: Certificate for directing animal experiments (Category-C) |

## Poster presentations at international meetings

Claudia Dührkop-Sisewitsch, Yara Banz, Rolf Spirig, Sylvia Miescher, Marc W. Nolte, Martin Spycher, Steven H. Sacks, Richard A. G. Smith, Robert Rieben. **C1 esterase inhibitor protects against peripheral ischemia/reperfusion injury and distant lung damage.** 14th Meeting on Complement in Human Disease — Jena, Germany, 2013

Claudia Dührkop-Sisewitsch, Yara Banz, Rolf Spirig, Sylvia Miescher, Marc W. Nolte, Martin Spycher, Robert Rieben. **Effects of C1 esterase inhibitor on distant organ damage induced by ischemia/reperfusion injury of the lower extremity** 14th Meeting on Complement in Human Disease — Jena, Germany, 2013

Claudia Dührkop-Sisewitsch, Robert Rieben. **Effect of low molecular weight dextran sulfate on tourniquet-induced limb ischemia/reperfusion injury and distant lung damage.** 14th Meeting on Complement in Human Disease — Jena, Germany, 2013

Claudia Dührkop-Sisewitsch, Yara Banz, Rolf Spirig, Sylvia Miescher, Marc W. Nolte, Martin Spycher, Robert Rieben. **Exogenous C1 esterase inhibitor protects against peripheral ischemia/ reperfusion injury.** The 10th International Conference on New Trends in Immunosuppression and Immunotherapy (Immuno 2013) — Barcelona, Spain, 2013

Claudia Dührkop-Sisewitsch, Yara Banz, Rolf Spirig, Sylvia Miescher, Marc W. Nolte, Martin Spycher, Robert Rieben. **Effects of C1 esterase inhibitor on attenuation of ischemia/ reperfusion injury in a rat hind limb model.** 24th European Complement Meeting on Human Disease — Chania, Greece, 2012

## Oral presentations at national meetings

Claudia Dührkop-Sisewitsch, Yara Banz, Rolf Spirig, Sylvia Miescher, Marc W. Nolte, Martin Spycher, Robert Rieben. **Efficiency of C1 Esterase Inhibitor Treatment to Prevent Peripheral Ischemia/Reperfusion Injury.** 13. Unionstagung der Schweizerischen Gesellschaften für Gefäßkrankheiten — Bern, Switzerland, 2012

## Poster presentations at national meetings

Claudia Dührkop-Sisewitsch, Yara Banz, Rolf Spirig, Sylvia Miescher, Marc W. Nolte, Martin Spycher, Robert Rieben. **Plasma-derived C1 Esterase Inhibitor Attenuates Tourniquet-induced Ischemia/Reperfusion Injury** — AGLA and Cardiovascular Biology Meeting, Bern, Switzerland, 2013

Claudia Dührkop-Sisewitsch, Yara Banz, Robert Rieben. **A reality-driven rat hind limb model for studying skeletal muscle ischemia/ reperfusion injury: comparison of 2h versus 24h reperfusion.** Swiss Experimental Surgery Symposium — Fribourg, Switzerland, 2013

Claudia Dührkop-Sisewitsch, Yara Banz, Rolf Spirig, Sylvia Miescher, Marc W. Nolte, Martin Spycher, Robert Rieben. **Efficiency of C1 Esterase Inhibitor Treatment to Prevent Peripheral Ischemia/Reperfusion Injury.** 13. Unionstagung der Schweizerischen Gesellschaften für Gefäßkrankheiten — Bern, Switzerland, 2012

Claudia Dührkop, Robert Rieben. **A Reality-Driven Rat Hind Limb Model For Studying Skeletal Muscle Ischemia/ Reperfusion Injury** — Swiss Experimental Surgery Symposium — Geneva, Switzerland, 2012

Claudia Dührkop-Sisewitsch, Yara Banz, Rolf Spirig, Sylvia Miescher, Marc W. Nolte, Martin Spycher, Robert Rieben. **C1 Esterase Inhibitor Treatment in Skeletal Muscle Ischemia/ Reperfusion Injury. Swiss Pharma Science Day** — Bern, Switzerland, 2012

# - Awards -

| Year | Prize | Conference |
|---|---|---|
| 2012 | Prize for the 2nd best Poster | Swiss Experimental Surgery Symposium |
| 2012 | Prize for the 3rd best Poster | Swiss Pharma Science Day |

- In Vivo experiments (microsurgery)
    - Rat hind limb ischemia/reperfusion injury model
    - Establishing animal models
    - Laser Doppler Imaging (Moor LDI)
    - Edema assessment
    - Muscle Viability assay

- Cell culture (PAEC, HUVEC, PK15, CACO, MDCK)
- Immunofluorescence & Histology
- Microscopic analysis by using immunofluorescence and light microscope
- Basics in scanning electron microscopy & transmission electron microscopy
- ELISA based experiments
- Western Blot and Gel electrophoresis

  (Analysis with ChemiDoc™ XRS and The Odyssey® Infrared Imaging System)
- CH-50
- Basics in surface plasmon resonance
- Basics in Bioplex
- Basics in fluorescence activated cell sorting (FACS)
- Polymerase Chain Reaction (PCR)
- DNA purification and sequencing
- Single Nucleotide Polymorphism (SNP) genotyping
- Basics in R (language and environment for statistical computing and graphics)

# - List of Publications -

Claudia Duehrkop; Yara Banz; Rolf Spirig; Sylvia Miescher; Marc W. Nolte; Martin Spycher; Steven H. Sacks; Richard A. G. Smith; Robert Rieben. **C1 Esterase Inhibitor Reduces Lower Extremity Ischemia/Reperfusion Injury and Associated Lung Damage**
*– Original article published in PLOS one (accepted 05.07.2013)*

Claudia Duehrkop; Robert Rieben. **Refinement of tourniquet-induced peripheral ischemia/ reperfusion injury in rats: Comparison of 2h vs. 24h reperfusion**
*– Original article submitted 17.05.2013, Laboratory Animals, under review*

Claudia Duehrkop; Yara Banz; Rolf Spirig; Sylvia Miescher; Marc W. Nolte; Martin Spycher; Robert Rieben. **C1 esterase inhibitor reduces distant organ damage caused by tourniquet-induced lower extremity ischemia/reperfusion injury**
*– Original article in submission (Critical care medicine)*

Claudia Duehrkop; Julie Denoyelle; Robert Rieben. **Use of dextran sulfate in tourniquet-induced skeletal muscle reperfusion injury**
*– Original article submitted 28.07.2013, Journal of Surgical Research, under review*

Claudia Duehrkop; Robert Rieben. **Ischemia/reperfusion injury: effect of simultaneous inhibition of plasma cascade systems versus specific complement inhibition**
*–Review in submission (Biochemical Pharmacology)*

## - Published Abstracts -

Duhrkop-Sisewitsch, C.; Banz, Y.; Spirig, R.; Miescher, S.; Nolte, M. W.; Spycher, M.; Rieben, R. **Effects of C1 esterase inhibitor on attenuation of ischemia/reperfusion injury in a rat hind limb model.** Immunobiology 2012, 217(11) 1165-1166

Dührkop-Sisewitsch, C.; Banz, Y.; Spirig, R.; Miescher, S.; Nolte, M. W.; Spycher, M.; Sacks H.S.; Smith R.A.G.; Rieben, R. **C1 esterase inhibitor protects against peripheral ischemia/reperfusion injury and distant lung damage.** Molecular Immunology 2013

Dührkop-Sisewitsch, C.; Rieben, R. **Effects of C1 esterase inhibitor on distant organ damage induced by ischemia/reperfusion injury of the lower extremity.** Molecular Immunology 2013

Dührkop-Sisewitsch, C.; Rieben, R. **Effect of low molecular weight dextran sulfate on tourniquet-induced limb ischemia/reperfusion injury and distant lung damage.** Molecular Immunology 2013

## - Patent Pending -

**C1-Esterase Inhibitor for the Treatment and Prevention of Remote Ischemia-Reperfusion Injury (A216)**