Infusion of C1-inhibitor to prevent ischemia-reperfusion injury in pigs

Involvement of the contact system

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September 2016

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Trykk: Reprosentralen, Universitetet i Oslo

Acknowledgements

To my supervisors. Thank you for your full support, guidance and motivation. I would not have come this far without your help. Thank you for believing in me and giving me a chance to work you, for always being there, for helping me with every aspect of my thesis and for sharing your experience and knowledge. My deepest appreciation is particularly extended to my main supervisors: Professor Harald Thidemann Johansen and Professor Rigmor Solberg. Also my appreciation is extended to my collaboration supervisor: Scientist Erik Waage Nielsen. Thank you for providing materials for my experiments and for your guidance. You have all been an inspiration.

To the whole gang at PROTARG. Thank you for welcoming me with open arms when I first arrived. Your help and guidance concerning my laboratory work is very much appreciated. My deepest gratitude is extended to: PhD. Student Ngoc Nguyen Lunde, Senior Engineer Hilde Nilsen, and Master Student Nuriddin Abdukadir.

To Grete Hasvold and Léon Reubsaet. Thank you for helping me with measurements of BK1-5. If it were not for you guys, it would not have been possible.

To my family and friends. Thank you for your support and encouragement during this last year and particularly the last weeks of the thesis. Even though many of you do not understand what my thesis is about, your company and presence are were appreciated.

Anh Hoang Duong

Oslo, September 2016

Abstract

INTRODUCTION: Ischemia-reperfusion injury (IRI) is a clinical condition caused by thrombosis or embolisms but can also be induced during surgery or transplantation. The consequences of IRI can be a strong inflammatory response depending on duration and severity of the ischemia. Previous studies focusing on contact activation (also known as kallikrein-kinin pathway) during IRI has indicated that this system is prothrombotic by activating intrinsic pathway of coagulation and proinflammatory by producing the vasoactive peptide bradykinin. C1-inhibitor is a regulator of the contact system by its ability to inhibit the proteinases in this cascade system, primarily FXIIa and plasma kallikrein. The hypothesis explored in this work was if administration of C1-inhibitor could prevent activation of the contact system.

MATERIALS&METHODES: This study is based on materials from an animal model of IRI with a surgical procedure of an aortic cross clamp for 45 minutes followed by prolonged reperfusion. Both whole blood samples and plasma samples were collected before, during and after ischemia in three groups of pigs: Control group (received saline), C1-inhibitor group (received C1-inhibitor) and sham (did not undergo aortic cross clamp). To investigate activation of the contact system plasma kallikrein activity was measured with a chromogenic substrate (S-2302; H–D–Pro–Phe–Arg–p–nitroanilide) and a fluorescence substrate (Bz–Pro–Phe–Arg–AMC). Cleavage products of high molecular weight kininogen (HK) and formation of C1-inhibitor complexes with plasma kallikrein were analyzed by immunoblotting. The metabolite of bradykinin (1-5; BK1-5) was analyzed with LC-MS/MS.

RESULTS: Measurements of plasma kallikrein activity and the stable metabolite of bradykinin, BK (1-5), indicated contact activation during ischemia in the control group. C1-inhibitor attenuated the generation of plasma kallikrein activity. BK1-5 was found to be present in blood samples after 45 minutes of ischemia but disappeared after five hours of reperfusion. This indicates that bradykinin is generated during ischemia but not during the period of reperfusion. Measurements of HK showed no differences between the control and C1-inhibitor groups and C1-inhibitor complexes were not observed.

Abbreviations

ACE	Angiotensin converting enzyme
AMC	7-amino-4-methylcoumarin
ANOVA	Analysis of variance
AU	Arbitrary unit
B1	Bradykinin-1-receptor
B2	Bradykinin-2-receptor
Bz	Benzo
Bz-Pro-Phe-Arg	Benzo-proline-phenylalanine-arginine
C1-inhibitor	Complement 1 inhibitor
Cys	Cysteine
DDT	Dithiotreitol
dH_2O	Destilled water
DXS	Dextran sulphate
ELISA	Enzyme-linked-immunosorbent assay
F	Fluorescence
fXI(a)	Factor XI(a)
fXII(a)	Factor XII(a)
fXII(f)	Factor XII(f)
Glx	Glutamine or glutamate

HAE	Hereditary angioedema
HK	High molecular weight kininogen
IRDye	Infrared fluorescent dye
IRI	Ischemia-reperfusion injury
K	Plasma kallikrein
LDS	Lithium dodecyl sulphate
MASP	MBL-assosiated serine proteinase
MBL	Mannose-binding lectin
NIR	Near-infrared
OD	Optical density
PK	Prekallikrein
pNA	Paranitroaniline
S-2302	H–D–Pro–Phe–Arg–p–nitroanilide
SEM	Standard error of the mean
SERPING1	Serine proteinase inhibitor G1
t1/2	Half-life
α_2M	Alpha-2-macroglobulin

Table of contents

1 Introduction

1.1 Ischemia-reperfusion injury (IRI)

Ischemia is a clinical condition of an impaired circulation, and is often associated with microvascular dysfunction or endothelial injury. The result is reduction of oxygen supply (hypoxia) and nutrients to the affected tissue. Ischemia can occur through formation of thrombosis or embolisms but can also be induced during surgery or transplantation. The deficiency of oxygen and blood supply causes time-dependent molecular and structural changes in tissue. In general, all organs or tissues are susceptible to ischemia; however, the severity outcome differs between organs.

Reperfusion (restoration of blood flow) is the only effective treatment to prevent irreversible damage and necrosis of the ischemic tissue. Paradoxically, reperfusion causes complex inflammatory response which may lead to ischemia-reperfusion injury (IRI). The complex inflammatory response caused by IRI does not involve pathogenic triggers, and is known as sterile inflammation such as inflammation caused by the vasoactive peptide, bradykinin. The consequence of the inflammatory response is activation of the complement system through production of a number of inflammatory mediators (Arumugam, Shiels, Woodruff, Granger, & Taylor, 2004), and the contact system through activation of intrinsic pathway and production of proinflammatory mediator bradykinin (Wu, 2015).

Impairment of vascular barriers can cause changes in endothelial permeability, leading to edema and increased interstitial pressure. Therefore, the plasma cascade systems, such as complement system, are promising targets for attenuation of IRI. Although several studies have showed beneficial effect though inhibition of the complement system using complement inhibitors, the pivotal roles in IRI are also attributed to other cascade (Duehrkop & Rieben, 2014). C1-inhibitor has a major role in controlling the activation of these plasma cascade system mentioned earlier, and animal studies have proven that treatment with C1-inhibitor reverses the increased vascular permeability (Han, MacFarlane, Mulligan, Scafidi, & Davis, 2002; Han Lee, Pappalardo, Scafidi, & Davis Iii, 2003). In the condition of swelling attacks in hereditary angioedema patients (with deficient C1-inhibitor) administration of C1-inhibitor reduce swelling attacks (Agostoni & Cicardi, 1992).

The research focus in this thesis is whether activation of the contact system happens during IRI. C1-inhibitor is a regulator of the contact system, and the role of C1-inhibitor to attenuate IRI has been postulated for decades (Horstick, 2002). Bradykinin is an important mediator causing edema and an interesting hypothesis is if bradykinin is alone or partly responsible for the inflammation and local damage in the tissue.

1.2 Hereditary angioedema (HAE)

Hereditary angioedema (HAE) is a rare autosomal genetic disorder caused by mutation in the C1-inhibitor gene (Massimo Cugno, Zanichelli, Foieni, Caccia, & Cicardi, 2009). A major biological role of C1-inhibitor is to regulate vascular permeability, illustrated by symptoms of increased vascular permeability in patients with HAE (see below), due to incomplete or deficient C1-inhibitor activity. C1-inhibitor also suppresses inflammation.

Increased vascular permeability in HAE patients can be located subcutaneous or submucosal which include face, genitals, gastrointestinal tract, airways and extremities (Fig. 1.1). The symptoms of HAE are often associated with pain syndromes, nausea, vomiting and diarrhea depending location of the swelling. HAE differs from symptoms of angioedema caused by allergy because it is without urticaria (Kaplan, 2009).

During attacks of HAE, some plasma proteolytic cascades are activated, the most important is the contact system, and several biological active mediators are generated. Studies have shown that bradykinin is a predominant mediator of enhanced vascular permeability in HAE attacks. Bradykinin is generated by activation of the contact system resulting in increased vascular permeability by binding potently to the bradykinin receptor B2 (B2-receptor) on vascular cells and less on the bradykinin receptor B1 (B1-receptor) (Alvin E Davis, 2006). Consistent with these findings in human is studies of the homozygous C1-inhibitor knockout mice. The study reported strong evidence that bradykinin contributes substantially to the vascular permeability via the B2-receptor (Han et al., 2002).

Symptoms of HAE are often misinterpreted as an allergic reaction which lead to a wrong diagnosis of this patient group. As HAE symptoms are not mediated by histamine, treatment with antihistamines will not work on HAE patients (Massimo Cugno et al., 2009; Frank, 2008) .

1.2.1 Treatment

At present there are some drugs for the treatment of HAE, which can be divided into prophylactic and acute treatment.

Prophylactic treatment is given to reduce the frequency of attacks. There are three drugs in this group which are: (I) Danazol, an antiandrogen with the possible effect through increase of C1-inhibitor synthesis in the liver, whereas estrogen has the opposite effect. (II) Anti-fibrinolytica, which might counteract angioedema attack but the mechanism of action on liberation of bradykinin is limited. (III) C1-inhibitor isolated from plasma.

Current acute treatments are: (I) C1-inhibitor given intravenously can be used both prophylactically and acute. C1-inhibitor counteracts and reverse angioedema characteristics of the disorder (Harald Thidemann Johansen, Seip, & Nielsen, 2016). There are three C1-inhibitors on the Norwegian marked, being Berinert® and Cinryze® isolated from human plasma and Ruconest® which is recombinant C1-inhibitor. (II) Plasma kallikrein inhibitor (Kalbitor®) that is more potent to inhibit plasma kallikrein than C1-inhibitor. It inhibits the liberation of bradykinin from HK and the edema is reduced. (III) B2-antagonist Firazyr® is a synthetic decapeptide that counteracts the effects of bradykinin.

Interestingly, there are also drugs that induce bradykinin-mediated edema, including ACE-inhibitors and gliptins (Harald Tiedemann Johansen, 2016).

1.2.2 Diagnosis

The symptoms of HAE often overlap with angioedema with other etiology like allergy. Therefore, the first and main step is to establish a possible family history of such attacks. Even though HAE is considered to be a genetic disorder, still about 20% of patients do not have any family history of the disease (Agostoni & Cicardi, 1992; Tosi, 1998).

Blood tests are indicated for all patients with suspected HAE. Usually C1-inhibitor and C4 are measured because most HAE patients have reduced levels of C1-inhibitor in plasma, lower C4 and normal C1q and C3 levels. C1q, C3 and C4 are complement components that can be measured in blood. Commercial kits are available in most hospital clinical laboratories for measuring concentrations of C1-inhibitor and C4 (Weis, 2009).

The newest international consensus from 2010 in Canada has summarized an algorithm document to approach a guideline for diagnosis, therapy and management of HAE (T. Bowen et al., 2010).

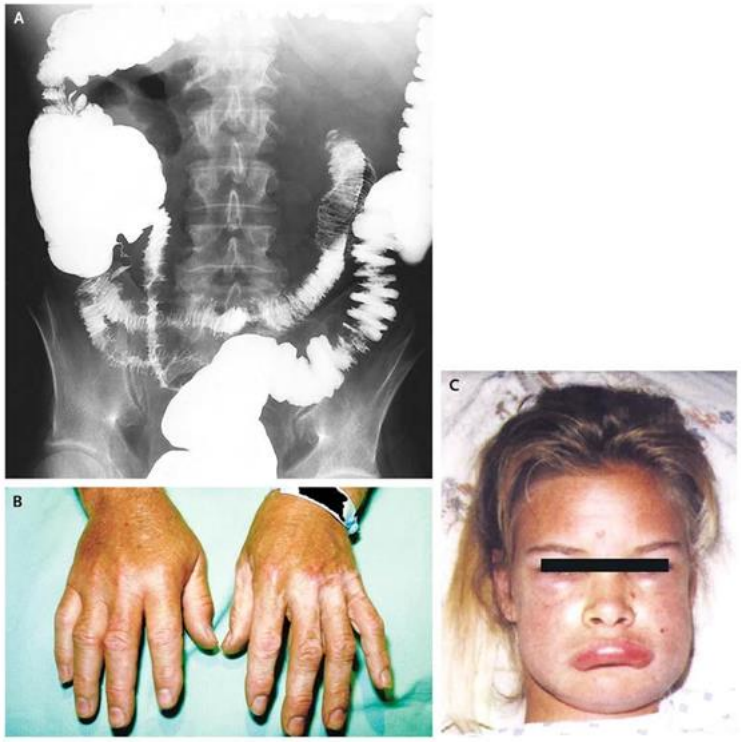

Figure 1.1. Illustrations of some symptoms in HAE patients. A. The abdomen during attack. The intestine is swollen and the wall thickened. **B.** Swollen hands which are asymmetrical. **C.** A swollen face. The figure is adapted from (Zuraw, 2008).

Undiagnosed or untreated disorder can lead to fatal consequences as obstruction of the airways (asphyxia) can cause death.

1.2.3 Pathogenesis and types of HAE (I, II and III)

As written above, HAE is an autosomal genetic disorder, meaning that one allele can be passed from a chromosome from a parent. The mutation lies in the gene coding for C1-inhibitor. There are reports of primary point mutation; missense and nonsense point mutation as well frameshift-, splice-, deletion-, insertion-, mutation (B. Bowen, Hawk, Sibunka, Hovick, & Weiler, 2001). About 300 different mutations in the C1-inhibitor gene have currently been identified in patients with HAE in the database HAEdb (http://hae.enzim.hu, (Kalmár, Hegedüs, Farkas, Nagy, & Tordai, 2005)).

There are two main types of HAE: type 1 (85 %) and type 2 (15 %) and both have mutation in C1-inhibitor gene. Type 1 is caused by zero or very low concentrations of C1-inhibitor in plasma. Type II is caused by lack of a functional C1-inhibitor but the level of C1-inhibitor in plasma is normal (Rosen, Charache, Pensky, & Donaldson, 1965).

A third type of HAE has recently been described and referred to as HAE type III. It appears to be strongly associated with exogenous estrogen as attacks and often occurs in women using birth control hormones, hormonal replacement therapy containing estrogen or during pregnancy (Binkley & Davis, 2000). A possible mechanism for the type III HAE is that a mutation in the sequence of factor XII can make factor XII "overactive". This can lead to more kallikrein production than normal (Björkqvist et al., 2015; Bork, Barnstedt, Koch, & Traupe, 2000; Dewald & Bork, 2006).

1.3 Proteinases

Proteinases, also known as proteases, proteolytic enzymes or peptidases, are specialized enzymes which catalyze the cleavage of proteins or peptides by hydrolysis. Today we know that they are extremely important in many of the signaling pathways (Turk, 2006).

The modern classification of proteinases follows the database MEROPS (MEROPS, http://merops.sanger.ac.uk). Proteinases are classified by structural similarities in the parts of the molecules responsible for their enzymatic activity. Proteinases are grouped into proteinase families and lastly into clans. The families contain related amino acid sequences and clans contain related structures (Rawlings, Barrett, & Finn, 2015).

According to the mechanism of action of proteinases, they can be classified into five major classes: aspartic, serine, cysteine, threonine and metallo proteinases. The names are reflecting the centrally important catalytic function. More than one third of all known proteinases belong to the serine proteinases (Page & Di Cera, 2008). Serine proteinases have an active site which contains the amino acid serine. C1-inhibitor, encoded by the SERPING1 gene (this name reflects the function of the inhibitor), belongs to family I4 and clan ID. Serpin inhibitors are unique as the structures not only inhibit proteinases but also destroy proteinase activity with the action of a "mouse trap" (Huntington & Carrell, 2001).

1.3.1 C1-inhibitor

C1-inhibitor is extremely important as it controls the activity of the complement system, coagulation, fibrinolytic and kinin release system (Bock et al., 1986). C1-inhibitor inhibits the macromolecular subcomponents (C1q, C1s, C1r) in the complement system, factor XIIa (fXIIa), factor XIIf (fXIIf), and plasma kallikrein in the contact system, factor XIa and thrombin in coagulation, and tPA and plasmin in the fibrinolytic system.

C1-inhibitor has two domains. The C-terminal is a serpin domain which binds and block the activity of its target proteinase like a “mouse trap”. Because of its structure, the mobile active loop “acts” like a bait for the target proteinase. The N-terminal is a non-serpin domain which gives C1-inhibitor the capacity to bind lipopolysaccharide and E-selectin, cell adhesion molecules. Because of this, C1-inhibitor influences regulation of the inflammatory reaction (Cicardi, Zingale, Zanichelli, Pappalardo, & Cicardi, 2005; Huntington & Carrell, 2001; Lomas et al., 2005). The action of C1-inhibitor and the covalent binding to target proteinase is illustrated in figure 1.3B.

C1 inhibitor is primarily synthesized in the liver and circulates in the blood. A mutation in a single amino acid substitution results in loss of the C1-inhibitory activity in HAE. When the concentration of C1-inhibitor is reduced, the generation of bradykinin occurs more easily. C1-inhibitor is the only plasma inhibitor of fXIIa and fXIIf. These factors in activated forms are involved in activating the kinin cascade pathway system by converting plasma prekallikrein (PK) to plasma kallikrein (K). High molecular weight kininogen (HK) is cleaved by plasma kallikrein and releases bradykinin, a potent vasoactive peptide that causes symptoms like swelling. The two major inhibitors that inhibit this action are C1-inhibitor and alpha-2-macroglobulin. Fig. 1.2 shows a simplified diagram of the production of kallikrein.

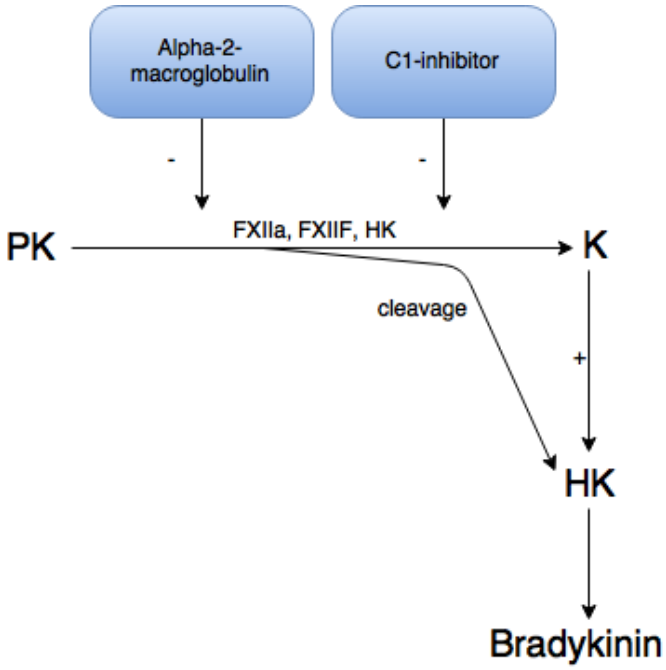

Figure 1.2. Production of bradykinin in human plasma. High molecular weight of kininogen (HK) circulates as complex with prekallikrein (PK) or factor XI (fXI). When factor XIIa (fXIIa) or factor XIIf (fXIIf) cleave PK to kallikrein (K), bradykinin is released from HK.

1.3.2 Alpha-2-macroglobulin

Alpha-2-macroglobulin (α_2M) is a unique inhibitor and is present in plasma. It is functioning as a "molecular trap" for a proteinase. There is a "bait region" in the inhibitor as for C1-inhibitor, but not of the same type (Fig. 1.3). A small exposed 25 amino acid sequence stretch which is located inside α_2M is the bait region and can be cleaved by a proteinase. This results in an immediate and distinct conformational change. The uniqueness in this is that the proteinase which is "trapped," is physically excluded from large substrates, but it is still able to cleave smaller substrates. Therefore α_2M is regarded as an inhibitor (Borth, 1992).

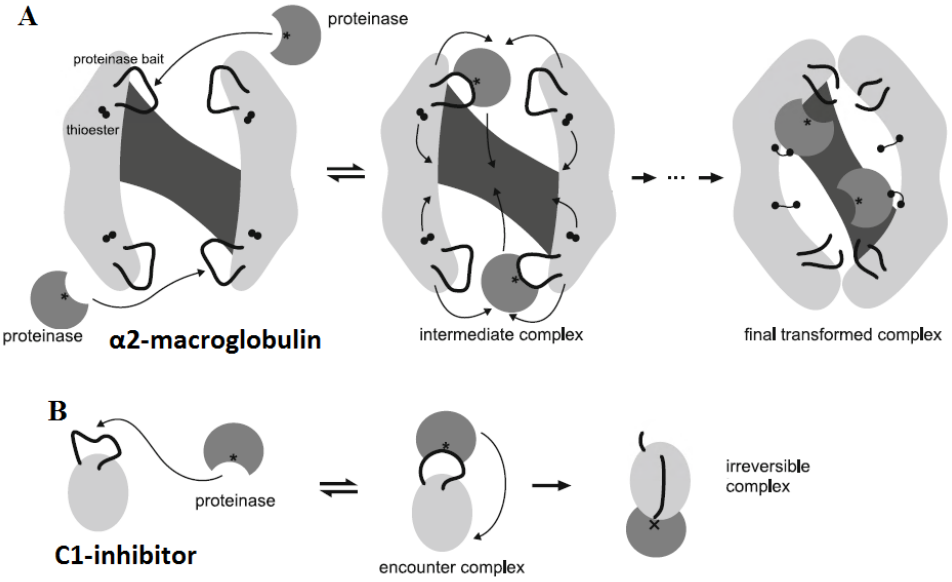

Figure 1.3. Illustrations of the different types of inhibition by α2M(A) and C1-inhibitor (B). Native α_2M **(A)** and serpin inhibitor, such as C1-inhibitor **(B)** undergo conformational change during the inhibition process and form irreversible complexes with the target. The figure is modified from (Gál, Dobó, Beinrohr, Pál, & Závodszky, 2013).

The human α2M is composed of four subunits of 180 kDa. The α_2M tetramer composed of a pair of identical subunits are joined by disulphide bridge and two such dimers are joined by non-covalent bonds to form a tetrameric molecule, that is why it is called “dimer of dimers”. Each subunit has a bait region. Despite having four subunits, α_2M has only two proteinase binding sites which are identical and independent of each other. Fig. 1.4A illustrates the action of the conformational change and trapping in α2M. The “trapping” arms represent each subunit. A receptor binding site is exposed after the conformational change.

Cleavage of the bait region by a proteinase exposes internal thiol ester bond between Cys and Glx present in the identical subunits. Follow this cleavage the thiol ester become “activated” and is susceptible to cleavage attack by nucleophiles. The receptor binding cite on α_2M trap/complex get recognized by receptors on hepatocytes in the circulation that clears α_2M trap/complex rapidly from plasma (Armstrong & Quigley, 1999; Rehman, Ahsan, & Khan, 2013).

This inhibitor is found in vertebrates (for instance humans), invertebrates, and in birds and reptile egg white (Sottrup-Jensen, 1989) and is produced in the liver of mammals (Andus et al., 1983; Armstrong & Quigley, 1999).

α_2M has the ability to inhibit virtually all proteinase, regardless of its specificity and catalytic mechanism (Rehman et al., 2013). It can trap proteinases released by granulocytes and other cells during inflammation and can also regulate extracellular proteolytic activity causing clotting and fibrinolysis. Also it can protect against invasive pathogens.

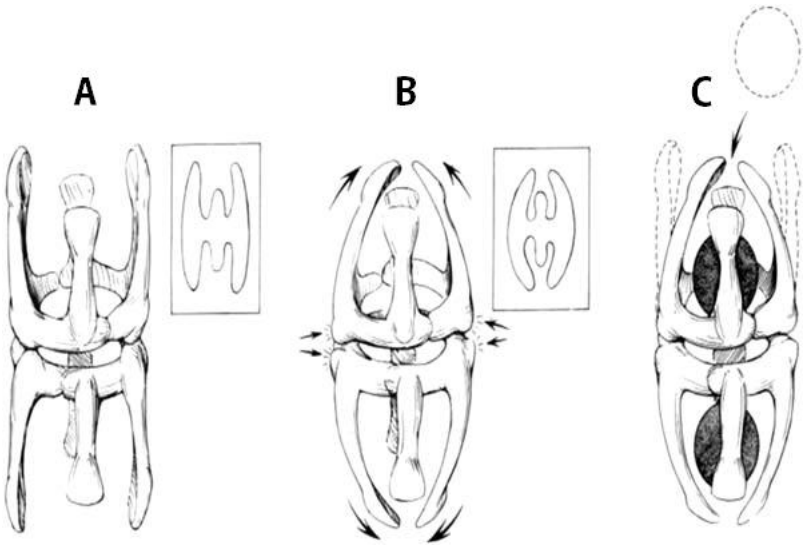

Figure 1.4. The molecular model of α2M. A. Native α2M. **B**. Cleavage of thiol ester bonds allows the trap arms to swing (arrows). **C.** A proteinase trapped by α2M. The figure is adapted from the article (Rehman et al., 2013).

1.4 Plasma contact system activation

The contact system is a proteinase enzyme cascade that is initiated by factor XII (fXII) activation on cardiovascular cells. It consists of four plasma proteins that assemble when blood contacts with negative artificial charged surfaces or activators. The four plasma proteins are: fXII (also called Hageman factor), fXI, plasma prekallikrein and a non-enzymatic cofactor, the high molecular weight kininogen (HK). Activation of the enzyme cascade results in blood coagulation, kinin formation, plasminogen activation (Maas, Oschatz, & Renné, 2011). Activation of this system can trigger blood coagulation and is responsible for generation of the proinflammatory mediator such as bradykinin. Deficiencies in contact factors are rare, and activation of contact system has been implicated in various of human disease, however the functions in humans remain a mystery.

FXII is synthesized in the liver and circulates in plasma as zymogen, proenzyme. It converts to the active form fXIIa after contact with negative charged surfaces or activators *in vitro*. Some examples of negative artificial charged surfaces are: glass, kaolin, ellagic acid, or

dextran sulphate and examples of activators are: misfolded proteins like aggregated amyloid beta peptide or fibril in collagen. Such activators of fXII can be released from damaged tissue, infection, inflammation (Maas et al., 2011). This is the main factor that contributes to crosstalk between the enzyme cascades. The two main pathways that generate bradykinin are illustrated in figure 1.5.

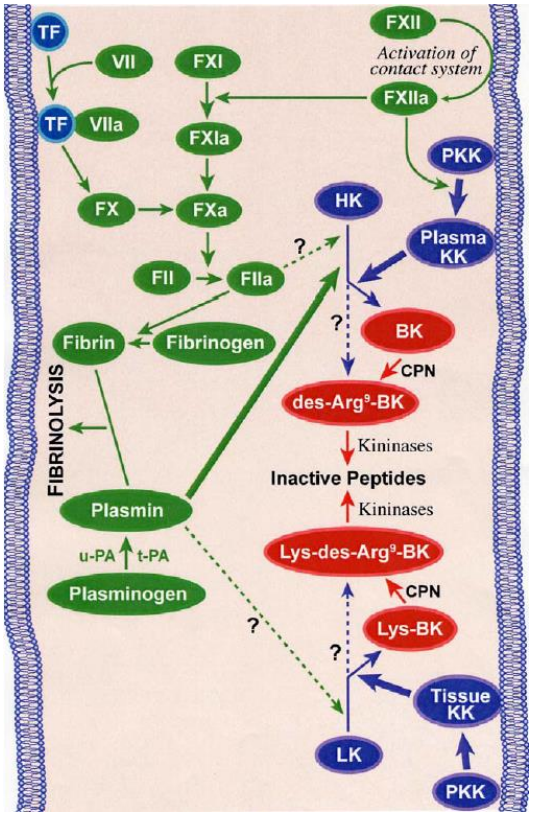

Figure 1.5. The blood cascade systems. A sophisticated network presents the contact system (kallikrein-kinin system; blue and red), both intrinsic and extrinsic coagulation cascade (green) and fibrinolysis (green). The solid lines are established pathways, while dashed lines are speculative or experimental activation pathways. The figure is adapted from (Moreau et al., 2005).

When blood makes contact with artificial surfaces the generation of fibrin is initiated by activation of fXII through what is referred to as the "intrinsic pathway". Activated forms for fXII (fXIIa) catalysis the formation of other active factors like fXIa, eventually causing the generation of fibrin.

However, defect fXII neither affects hemostasis nor causes bleeding. The first discovery of fXII was in 1955. The short version of the discovery; a blood sample of a patient named Hageman was examined to have prolonged clotting time in the tube, without the patient having symptoms of hemorrhage. Later the hematologist Oscar Ratnoff discovered that Hageman lacked an unidentified clotting factor (therefore the name Hageman factor). The patient died of pulmonary thrombosis (Caen & Wu, 2010). Later the question was raised whether defect fXII might lead to thrombosis. In epidemiologic studies defect fXII did not show to have higher overall incidence of thrombotic complications such as deep vein

thrombosis, myocardial infarction or stroke. Interestingly, in experimental studies with knock out mice defect in fXII (fXII-/-) indicated no thrombus formation, but rather protects the animals from experimental cerebral ischemia and pulmonary embolism (Renné, Schmaier, Nickel, Blombäck, & Maas, 2012).

Plasma kallikrein and fXIIa have been described as plasminogen activators, but both appears to have weak plasminogen activator effect (M. J. Gallimore, Fareid, & Stormorken, 1978; Goldsmith, Saito, & Ratnoff, 1978).

1.4.1 Bradykinin

The small peptide bradykinin can be generated from two pathways. One of the pathways is high molecular weight kininogen (HK) which is released from prekallikrein. Bradykinin is a mediator of inflammation and has been implicated in disease pathology such as asthma by inducing bronchoconstriction and increase in vascular permeability (Fuller, Dixon, Cuss, & Barnes, 1987). Increase in bradykinin in plasma has also been observed in HAE patients during attack (Shoemaker, Schurman, Donaldson, & Davis, 1994).

Bradykinin can activate two types of receptors: bradykinin-1-reseptor (B1-receptor; induced during an inflammation) or bradykinin-2-reseptor (B2-receptor; constitutively expressed in cells) (McLean, Ahluwalia, & Perretti, 2000). Bradykinin (1-9; nine amino acid; nonapeptide) are more potent at the B2-receptor, furthermore bradykinin (1-9) can be metabolized by kininase I (carboxypeptidase) to bradykinin (1-8) which are potent at B1-receptor. The result of liberation bradykinin can activate various of physiological processes like activating endothelial cells, cause vasodilatation resulting in increased vascular permeability, tissue-type plasminogen activator (t-PA) release and production of nitric oxide (NO) (Marceau & Regoli, 2004).

Regulators that control the contact activation system are: C1-inhibitor, α_2M, $\alpha_1$proteinase and more. However, C1-inhibitor, α_2M stand for 90% of the kallikrein inhibitory activity in plasma. And C1-inhibitor is the major regulator in the intrinsic system referred to activity with fXIIa and of kallikrein (Marceau & Regoli, 2004).

1.4.2 Complement system

The complement system consists of nine major components, the complement factors C1-C9. Substances derived from microorganisms can activate this pathway. The balance of the complement factors is controlled by inhibitors. FXII has been demonstrated to activate the classic pathway through C1, C2 and C4 (Rang, Ritter, Flower, & Henderson, 2014).

The complement system consists of three pathways which are: the classical, alternative and lectin-binding pathways. The classic pathway is activated by antigen-antibody (immune) complexes. The process includes C1q, C1r and C1s that activates C4 and C5. The alternative pathway is directly initiated by surface molecules containing carbohydrates and lipids after contact with foreign surface or spontaneously occurring. The process includes C3 and C5. The lectin-binding pathway is triggered by binding to either mannose-binding lectin (MBL) or MBL-associated serine proteinases (MASPs). The process includes C2 and C4.

In summary, complement system activates inflammation and immunological processes. C1-inhibitor plays an important role in the suppression of inflammation through many mechanisms in the complement system, included controlling activation of the complement system through C1r, C1s and MASP2 (A. E. Davis, Mejia, & Lu, 2008).

1.5 The ischemia-reperfusion injury model in pigs

A novel pig model of multiorgan IRI has been established in a prospective randomized pilot project performed at the Nordland hospital by prof. Erik Waage Nielsen et al. Thoracic aortic clamping was performed on the aorta above diaphragm for 45 minutes followed by reperfusion (opening of the clamp). The animals were observed for the following five hours. The model aims to investigate selected topics concerning modulation of innate immunity during IRI. In this thesis we will focus on the contact system, including kinin formation.

2 Aims of the present study

The aim in this thesis is to investigate whether activation of the contact system happens during ischemia-reperfusion injury (IRI) and whether C1-inhibitor can reverse contact activation during IRI. To test this hypothesis whole blood and plasma samples from an IRI model in pigs will be used and different methods will be utilized.

The specific aims are:

- To study the activity of plasma kallikrein during IRI.
- To study whether C1-inhibtor attenuate the activity of plasma kallikrein during IRI.
- To study whether cleavage products of high molecular weight kininogen (HK) are produced during IRI.
- To identify whether there are C1-inhibitor complexes present in plasma from C1-inhibitor treated pigs.
- To study whether the stabile metabolite of bradykinin, the pentapeptide (BK1-5), is present in blood from pigs, and whether C1-inhibitor reduces the concentration of this metabolite.
- To evaluate different substrates for measuring plasma kallikrein.

3 Materials and methods

3.1 Chemicals and reagents

Chemicals and reagents	Supplier
Albuminstandard (23209)	Thermo Scientific, USA
Bio-Rad Protein Assay Dye Reagent Concentrate (500-0006)	Bio-Rad Laboratories, USA
Dried milk	Normilk, Norway
DTT, ditiotreitol, C4H10O2S2 (438117)	Sigma-Aldrich, USA
Ethanol, rectified (200-578-6)	Kemetyl AS, Norway
Formic acid EMSURE	Merck KGaA EMD Millipore Corp, Germany
H–D–Pro–Phe–Arg–p–nitroanilide (S-2302)	Chromogenix AB, Sweden
H–Pro–Phe–Arg–AMC (I-1295)	Bachem, Switzerland
Methanol LiChrosolv	Merck KGaA EMD Millipore Corp, Germany
NuPAGE (4-12 % Bis-Tris Gel), Novex (NP0322)	Life Technologies, USA
NuPAGE Antioxidant	Life Technologies
NuPAGE LDS Sample Buffer (4X)	Life Technologies
NuPAGE MOPS Running Buffer (20X) (NP0001)	Life Technologies
Ponceau solution (P7170IL)	Sigma-Aldrich
Precision Plus Protein™ Dual Color Standards (161-0374)	Bio-Rad Laboratories
Protein Assay Dye Reagent Concentrate	Bio-Rad Laboratories
Restore™ Western Blot Stripping Buffer (21059)	Thermo Scientific
Tween 20 (170-6531)	Bio-Rad Laboratories

3.2 Equipment list

Equiptment	**Supplier**
Aluminium folie	Duni AS, Norway
Belly Dancer	Stovall Life Science Grensboro, USA
Blotting Paper 703 (0,38 mm)	VWR International, UK
Corning selfstanding centrifuge tube (50 ml)	Corning Inc, Mexico
Costar 96-well microplate (transparent, black)	Corning Incorporated
Costar stripette (5mL, 10ml, 25 ml)	Corning Incorporated Corning, USA
Digital dry bath	Labnet International Inc, USA
Dri-block DB 3D	Techne, UK
Dynabeads Mx Mixer	Invitrogen Corporation by AIR Inc, USA
Eppendorf tubes (0.5, 1.5 and 2 mL)	Eppendorf AG, Hamburg, Germany
Heraeus Fresco 21 Centrifuger	Thermo Fischer Scientific, USA
Incubator Galaxy 170S	Eppendorf, USA
Incubator rocker	VWR
Invotrogen Novex Mini Cell (electrophorese chamber)	XCell Surelock, China
Megafuge 16R Centrifuge	Therumo Scientific, Germany
MS2 Minishaker	IKA, Germany
Nitrocellulose Blotting Membrane	GE Healthcare Life Science Amersham, Germany
NuPAGE 4-12 % Bis-Tris Gel	Invitrogen by Therumo Fischer Scientific, USA
Odyssey® CLx Near-Infrared Imaging System	Li-Cor Biosciences, USA
Sample concentrator	Techne
SPE C18	Corning Inc, USA
Stripettes (5, 10 and 25 mL)	Corning Inc
Trans-Blot Turbo Transfer System	Bio-Rad Laboratories Inc, Singapore
Vacuum pump	Vacuubrand, Germany
Victor™ X4 Multimode Plate Reader	PerkinElmer, USA

3.3 Plasma samples

This thesis is based on materials received from a prospective, double-blinded and randomized animal study performed at Nordland hospital, during autumn 2015 (Appendix 1). The study included 24 pigs that underwent aortic cross clamping for 45 minutes causing ischemia and followed by 5 hours of reperfusion. The 24 pigs were randomized into two groups, a control group which received infusion of saline (control; n=12) and a treatment group which received infusion of C1-inhibitor (250 IU C1-inhibitor/kg; n=12). Also included were three sham pigs that did not undergo aortic cross clamping. All pigs received saline during the procedure to keep them alive. Human plasma samples from a HAE patient (HAE; n=1) and healthy controls (control; n=2) were also included.

The material studied in this thesis consisted of the following samples from pigs:

- Two plasma samples from each pig, added inhibitor cocktail to prevent *in vitro* activation of the contact system. The inhibitor cocktail consisted of EDTA, polybrene and a commercial Complete Mini Protease Inhibitor tablet (Roche). Plasma samples were collected immediately after the clamp opened (T-0 reperfusion) and five hours afterwards (T-5 reperfusion). Blood (4.5 mL) was drawn to vials containing the inhibitor cocktail and centrifuged (1500 g) for 15 minutes at 4°C. The plasma samples were transferred to Matrix vials with 500 µL in each vial and stored at -70°C until analysis.

- Citrated plasma was collected at nine points in time: Before clamp and before injection (T-basis and 0 minute), before clamp and after injection (T-0 ischemia and 10 minutes), right before the clamp opens (T-45 ischemia at 55 minutes), immediately after the clamp opens (T-0 reperfusion at 60 minutes), and then every 60 minutes until five hours reperfusion (T-5 reperfusion; 360 minutes). About 2 mL blood was drawn to vacutainer vials containing citrate and centrifuged (1500 g) for 15 minutes at 4°C. The plasma samples were transferred to Matrix vials and stored at -70°C until analysis.

- Two ethanolic extracts of blood were available from each pig. To prepare for the collection of sample, 50 ml centrifuge vials (Corning) were filled with 15 mL 96% ethanol and placed in the freezer at -20°C. Before blood sample collection, prepared cold ethanol was placed on ice and blood samples were collected from pigs. Blood (6

mL) was drawn into silicon vacutainer vials with EDTA and 5 mL blood was immediately added to the cold ethanol. The vials were shaken vigorously and put on ice for one hour. This procedure causes denaturation of enzymes and thus prevent both generation and metabolism of bradykinin (Murphey, Hachey, Vaughan, Brown, & Morrow, 2001). Afterwards the vials were centrifuged (1500 g) for 25 minutes at 4°C. The supernatant was transferred to three 15 mL vials (Corning) on ice and stored at -70°C until analysis. Samples were collected at time T-0 reperfusion and T-5 reperfusion.

3.4 Activity of plasma kallikrein measured by chromogenic substrate S-2302

The assay uses the simple chromogenic substrate H–D–Pro–Phe–Arg–p–nitroanilide (S-2302) as described by M. J. Gallimore and Friberger (1982). Plasma kallikrein cleaves the substrate and releases paranitroaniline (pNA) and the leaving group turns yellow. The more enzyme activity present the more yellow it gets. Absorbance at 405 nm was measured in a microplate reader (Victor™ X4 Multimode Plate Reader, PerkinElmer) at 37 °C.

Frozen samples of citrate plasma from pigs were heated to 37°C on a heat block for 6 minutes. Duplicates of each sample (10 µL) were added to a transparent 96-well plate (Corning) by manual pipetting. Then, 90 µL of 0.05 M Trisbuffer (50 mM Tris base, 360 mM NaCl, 20 mg/mL Polybrene, pH 7.8) (Appendix 2) was added to dilute the plasma. Finally, 100 µL of 2 mM S-2302 was added to the microplate and the mixture was incubated at 37°C for 6 hours and measured repeatedly. The increase in absorbance (O.D.) was calculated as increase in µO.D./sec.

Spontaneous activity of plasma kallikrein was measured in the human sample plasma (EDTA plasma and heparin plasma). The same procedure as for citrated plasma from pigs was performed. Plasma samples were also incubated with 12.5 mg/L dextran sulphate (DXS) for 7 minutes at 37°C and measured.

3.5 Activity of plasma kallikrein measured by a fluorogenic substrate

This method also measures the enzyme activity of plasma kallikrein, but is using the fluorogenic substrate Bz–Pro–Phe–Arg–AMC. The peptide sequence is the same as in S-2302 but differs in the leaving group which is AMC (7-amino-4-methylcoumarin). Plasma kallikrein will cleave the substrate and release AMC which generates an increase in fluorescence (exciting light at wavelength 360 nm and emitting light at wavelength 460 nm).

Samples of citrated plasma were prepared the same way as with the S-2302 substrate, and heated to 37℃ on the heat block for 6 minutes. Ten µL sample was added in three parallels to a black 96-well plate (Corning). Automatic injectors were used to add 140 µL 0,05 M Trisbuffer (50 mM Tris base, 360 mM NaCl, 20 mg/mL Polybrene, pH 7,8) (Appendix 2) and 50 µL substrate (10 µM) to each well. The microplate was shaken for 10 seconds after each addition and incubated at 37℃ for 1 hour. The microplate was then measured repeatedly over the following 2 hours. The increase in fluorescence was calculated as delta increase in fluorescence per second (Δ F/sec).

3.6 Immunoblotting (Western blotting)

This method is based on interactions between antibodies to detect a specific protein. Techniques performed are: electrophoresis, blotting, blocking, incubation with primary antibody, incubation with secondary antibody, and lastly detection by Odyssey® CLx Near Infrared Imaging System (Li-Cor).

Plasma samples were first diluted in distilled water (dH_2O)(1:50). Each diluted sample (13 µL) was mixed with 5 µL commercial Sample buffer (NuPAGE LDS Sample buffer (4X), NOVEX) and added 2 µL reducing agent dithiotreitol (DDT, Sigma-Aldrich) before heated at 70°C for 10 minutes to denaturate the proteins. The Sample buffer contained the detergent lithium dodecyl sulfate (LDS) and glycerol to make the sample heavier (i.e. make it easier to load on the gel). Heating and LDS unfold the proteins and gave the proteins a uniform negative charge. DDT will further break disulphide bonds and destroy 2D and 3D structure of the proteins. Samples and a commercial color standard (Precision Plus Protein™ Dual Color Standards, Bio-Rad) were loaded on a gradient polyacrylamide gel (4-12 % polyacrylamide,

NOVEX), placed in an electrophoresis chamber with electrophoresis buffer (Appendix 3). Electrophoresis was performed at 200 V for 50 minutes and the proteins migrated towards the positive electrode and were separated according to size.

After separation, proteins were blotted onto a nitrocellulose membrane (GE Healthcare Life Science) in order to make the proteins accessible to antibodies. The polyacrylamide gel was placed in a sandwich with the following order: three blotting papers (0.38 mm, VWR) soaked with Transfer buffer (Appendix 4), membrane, gel and lastly three soaked blotting papers (0.38 mm, VWR) on top of the gel. To prevent any bubbles and overheating of the gel the whole sandwich was soaked in Transfer buffer as mentioned above and clamped tightly together with a roller. The sandwich was then placed in the blotting machine (Trans-Blot Turbo Transfer System, Bio-Rad) at 25 Volt for 30 minutes.

To verify that proteins were transferred to the membrane a diazo dye of red color agent was added to the membrane (Ponceau, Sigma-Alderich) for 1 minute and washed with dH_2O a few times. The diazo dye of red colors the proteins red and a photo was taken.

Subsequently the membranes were blocked with 5 % dried milk powder dissolved in 1X PBS (Appendix 5) for one and a half hours at room temperature with gentle shaking. This was to prevent non-specific binding of antibody to the membrane by milk proteins blocking the free binding sites on the membrane. Afterwards the membrane was transferred to 50 mL vials (Corning) with 5 mL primary antibody against the protein of interest diluted in 1X TTBS (Appendix 6), and incubated overnight at low temperature (4°C). The concentration of primary antibodies that were used in this thesis is listed in Table 3.1.

Table 3.1. Primary antibodies utilized in immunoblotting

Antibody	**Dilution**
Human Kininogen Light Chain (MAB15691, R&D Systems)	1:500
Human Kininogen (AF1396, R&D Systems)	1:2000
Human C1 Inhibitor (AF2488, R&D Systems)	1:1000
Human Legumain (AF2199, R&D Systems)	1:200

After incubation with primary antibody the membrane was washed with 10 mL 1X TTBS 4-5 times. The membrane was then incubated with secondary antibodies against the respective

primary antibody for one hour. The secondary antibody was diluted in 1X TTBS to 10 mL. After this step, the membrane was protected from light by wrapping the container in aluminum foil. The membrane was further washed with 10 mL 1X TTBS 4-5 times before detection. The secondary antibodies used in this thesis are listed in table 3.2.

The secondary antibodies utilized are conjugated with an infrared fluorescent dye ("IRDye"). The Odyssey® CLx Near Infrared Imaging System uses a near-infrared (NIR) laser that gives stabile fluorescent signals of the specific protein of interest. Secondary antibodies marked with "IRDye" can be detected either at 700 nm (red channel) or 800 nm (green channel). If two target proteins were detected at the same time, co-localisation will give yellow signal. The protein standard (Precision Plus Protein™ Dual Color Standards, Bio-Rad) was visualized by the 700 nm channel and contains proteins with known sizes, which were used to estimate the size of the detected protein bands.

Table 3.2. Secondary antibodies utilized in immunoblotting

Antibody	**Dilution**
800 CW – IRDye Donkey Anti-Mouse IgG (926-32212, Li-Cor)	1:15000
800 CW - IRDye Donkey Anti-Goat IgG (926-32214, Li-Cor)	1:15000

3.7 Total protein measurements

For quantification of total proteins, a colorimetric assay described by Bradford (1976) is performed. It is a rapid and accurate method and relies on the bonding of a dye agent to proteins (Protein Assay Dye Reagent Concentrate, Bio-Rad). The dye agent is redish brown, acidic and has maximum absorbance at 465 nm. Binding of the dye to proteins (forms a stabile complex to the dye and gives a blue color) causes a shift in absorption of the dye to a maximum absorbance at 595 nm. The amount of protein-bound dye can be quantified by measuring the absorbance (O.D.) at 595 nm.

Plasma samples with inhibitor cocktail were first diluted in 1X PBS buffer (1:100)(Appendix 5). For a standard curve, duplicates (10 µL) of standard concentrations 0, 50, 100, 200, 300 and 400 µg/mL of albumin were added to a 96-well plate (Corning). Triplicates of 10 µL diluted plasma were added to the microplate and finally 200 µL dye agent was added to all

wells. The plate was incubated for 5 minutes at room temperature before the absorbance was measured at 595 nm.

3.8 Enzyme-linked-immunosorbent assay (ELISA)

Concentration of legumain in plasma samples were analyzed by enzyme-linked-immunosorbent assay (DY4749, R&D Systems, Minneapolis, MN, USA) according to the manufacturer´s protocol.

3.9 Bradykinin analysis

To determine a stable metabolite of bradykinin (bradykinin 1-5; BK1-5) in whole blood, a recently established LC-MS/MS method was used (Seip et al., 2014). Sampling of ethanolic extracts of blood is extremely critical and described above.

Ethanolic extracts of blood (4 mL) was added to 15 mL Corning vials. Then, 20 µl of internal standard (Phe-labelled (13C915N) BK1-5)(0.5 µg/mL) was added and the ethanolic extract evaporated to dryness under a stream of nitrogen at 60˚C for approximately 4 hours. The dried sample was redissolved in 3 mL 20 mM formic acid. Solid phase extraction on a C18 SPE cartridge (1 ml) was performed. Methanol (1 mL) was added to the cartridge followed by addition of 1.0 mL 20 mM formic acid under vacuum. Then 3.0 mL sample was added to the SPE cartridge followed by washing of the SPE cartridge with 3 X 1 ml 20 mM formic acid. Eppendorf tubes were placed under the SPE cartridge to collect the eluate. A mixture of methanol and 20 mM formic acid (1:1; 1.0 ml) was used to elute the sample from each cartridge. Again the eluate was evaporated to dryness under a stream of nitrogen at 60˚C for 3-8 hours and stored at -20˚C. After storage at -20°C the samples were redissolved in 50 µL mobile phase A (20 mM formic acid : methanol 95:5) and transferred to LC-MS vials and analyzed as previously described (Seip et al., 2014).

3.10Statistical analysis

In this thesis the data is presented as the mean ± standard error of the mean (SEM). Significant differences are noted when $p < 0.05$. Statistical analyses performed were non-parametric statistical test two-tailed (unpaired test, Mann-Whitney test) in datasets with assumption of none normal distributions, and ANOVA two-ways in datasets containing different categorical independent variables against one continuous dependent variable.

In the present study the statistical software used to calculate was GraphPad Prism 6 (GraphPad Software, Inc, San Diego, CA, USA).

4 Results

4.1 Determination of plasma kallikrein activity in plasma samples from pigs

The precursor prekallikrein is converted to plasma kallikrein after proteolytic cleavage by factor XIIa. C1-inhibitor and α2-macroglobulin constrain this cleavage, but with different mechanisms. Both inhibitors undergoes major conformational change during the process of inhibition and C1-inhibitor forms irreversible complexes with the target proteinase (Gál et al., 2013). When plasma kallikrein is inhibited by α2-macroglobulin, the enzyme is still active inside a complex with α2-macroglobulin. This plasma kallikrein can still be measured by using small synthetic tripeptides such as the chromogenic substrate S-2302 or a fluorescent substrate with AMC as a leaving group (M. J. Gallimore & Friberger, 1982).

4.1.1 Proteolytic activity measured by the chromogenic substrate S-2302

Plasma samples from sham pigs that did not undergo cross clamp of aorta were used as a reference to pigs with aorta cross clamps. The activity of plasma kallikrein in sham operated pigs was stable over the 9 points in time as seen in Fig. 4.1. There was a maximum activity of plasma kallikrein at 60 minutes when the pigs underwent opening of the abdomen.

During ischemia with cross clamp on aorta for 45 minutes, the activity of plasma kallikrein increased substantially in both control and C1-inhibitor treated groups. When the cross clamp was opened (after 60 minutes), the activity of plasma kallikrein in the control group and the C1-inhibitor group were approximately at the same level and significantly increased when compared to both initial activities and activities in sham operated animals. It was concluded that ischemia caused by cross clamping of aorta caused increased activity of plasma kallikrein.

In the control group, the activity of plasma kallikrein was still increasing after one hour reperfusion and had a maximum activity at one hour after reperfusion (120 min). In contrast, the C1-inhibitor group showed no further increase of the activity of plasma kallikrein at one hour after reperfusion. Prolonged reperfusion caused a gradual reduction of plasma kallikrein

activity in both the C1-inhibitor treated pigs and controls. However, the activity of plasma kallikrein was significantly higher in the control pigs when compared to those infused with C1-inhibitor(p=<0,001).

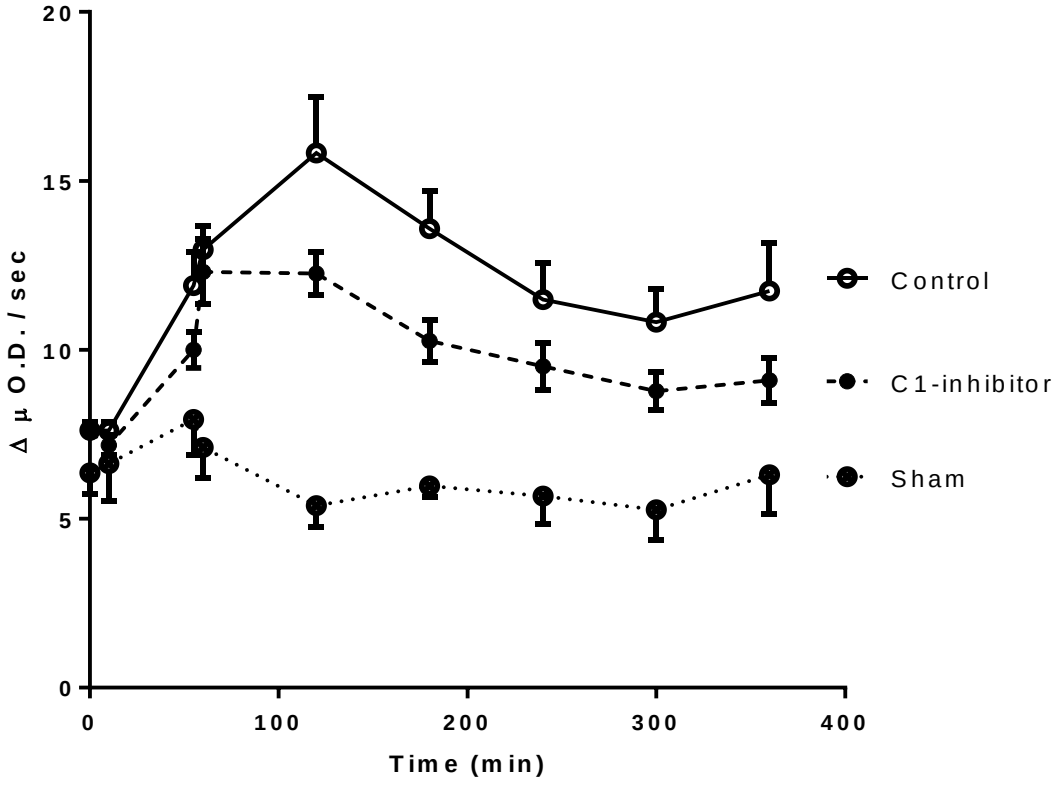

Figure 4.1. Activity of plasma kallikrein in citrate plasma from pigs measured with the chromogenic peptide substrate S-2302. In this model of ischemia-reperfusion injury, pigs were randomized to either receive saline (control, n=12) or C1-inhibitor (250 IU/kg)(C1-inhibitor, n=12). Sham pigs (Sham, n=3) did not undergo aortic cross clamping. Plasma samples were taken at nine points in time: before clamp and before injection (time 0), before clamp after injection (control/C1-inhibitor; 10 minutes), immediately before the clamp was opened (55 minutes), after the clamp was opened (1 hour) and then every 60 minutes up to 360 min. The figure shows the activity measured as cleavage of the substrate S-2302 (HD-Pro-Phe-Arg-pNA), recorded as increase in absorbance at 405 nm (delta micro optical density per second, ΔµO.D./sec). The data represents mean +/- SEM. Two-ways ANOVA test showed significant difference between the control and C1-inhibitor group.

4.1.2 Proteolytic activity measured by a fluorogenic substrate

A selection of plasma samples was used in this measurement of kallikrein activity using a fluorogenic substrate (Bz–Pro–Phe–Arg–AMC). Plasma samples from sham pigs that did not undergo cross clamp were used as reference. During ischemia followed by prolonged reperfusion the activity of plasma kallikrein still remained unaffected in the sham group (Fig 4.2).

The activity of plasma kallikrein in the control group was on the other hand increased substantially with an increase at 45 minutes ischemia and at one hour reperfusion. The activity of plasma kallikrein still increased to the highest level at five hours reperfusion. The same observation was seen in the C1-inhibitor group, but the C1-inhibitor group showed significantly lower activity of plasma kallikrein throughout the course (p=<0,001).

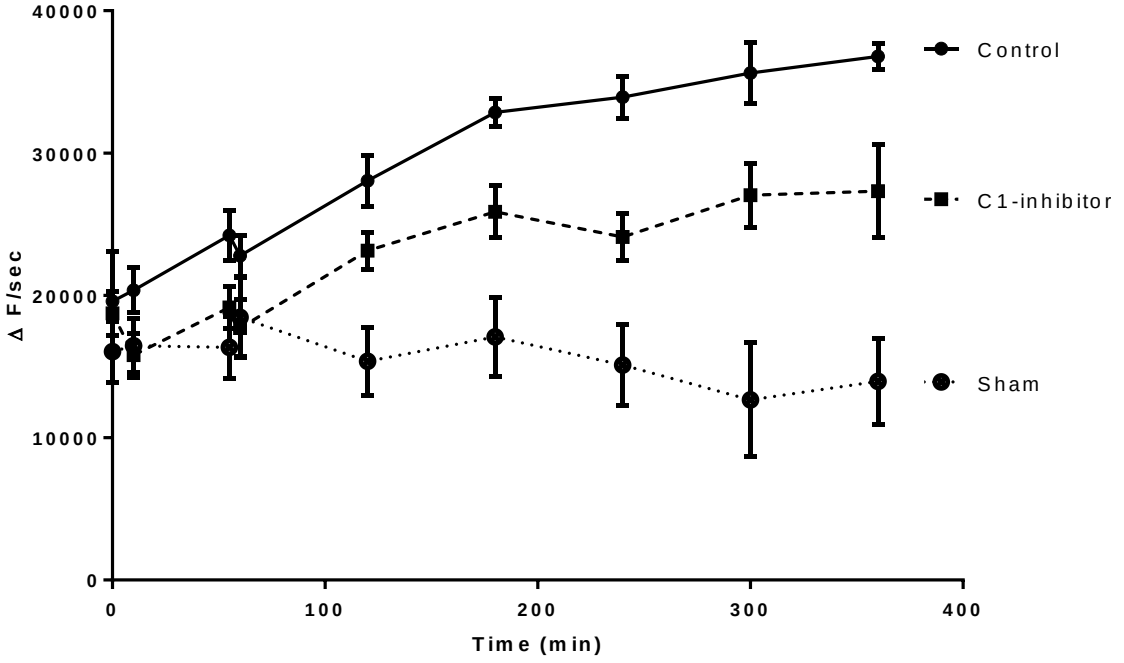

Figure 4.2. Activity of plasma kallikein in citrate plasma from pigs measured with an AMC-containing fluorogenic peptide substrate. Selected samples from both control group (control, n=3), C1-inhibitor group (C1-inhibitor, n=3) and sham group (sham, n=2) were measured. Plasma samples were taken at nine point in time: before clamp and before injection (time 0), before clamp after injection (control/C1-inhibitor; 10 minutes), immediately before the clamp was opened (55 minutes), after the clamp was opened (60 minutes) and then every 60 minutes up to 360 min. The figure shows the activity measured as delta fluorescence unit per second (Δ F/sec). The data represents mean +/- SEM of three parallels. Two-ways ANOVA test showed significant difference between the control and C1-inhibitor group.

A further comparison of the two peptide substrates, HD-Pro-Phe-Arg-pNA and Bz-Pro-Phe-Arg-AMC, was performed with experiments on human plasma samples. Figure 4.3 shows the spontaneous activity of plasma kallikrein in a plasma sample from one HAE-patient compared with two healthy controls. Plasma samples collected with both EDTA and heparin were studied and in all experiments a continuous increase in absorbance of pNA (Fig. 4.3A) or

fluorescence of AMC (Fig. 4.3B) were measured over a period of approximately 6 hours. In table 4.1 increase in ΔO.D./sec or ΔF/sec, respectively, were calculated. Surprisingly, the EDTA-plasma from the HAE-patient had lower kallikrein activities than the two controls. However, it should be noted that this patient informed that she had received a concentrate of C1-inhibitor only 2 hours prior to the sampling of plasma. When comparing activities in EDTA-plasma to heparin-plasma it was clear that higher activities were consistently found in EDTA-plasma when using HD-Pro-Phe-Arg-pNA, while the opposite was the case when using Bz-Pro-Phe-Arg-AMC.

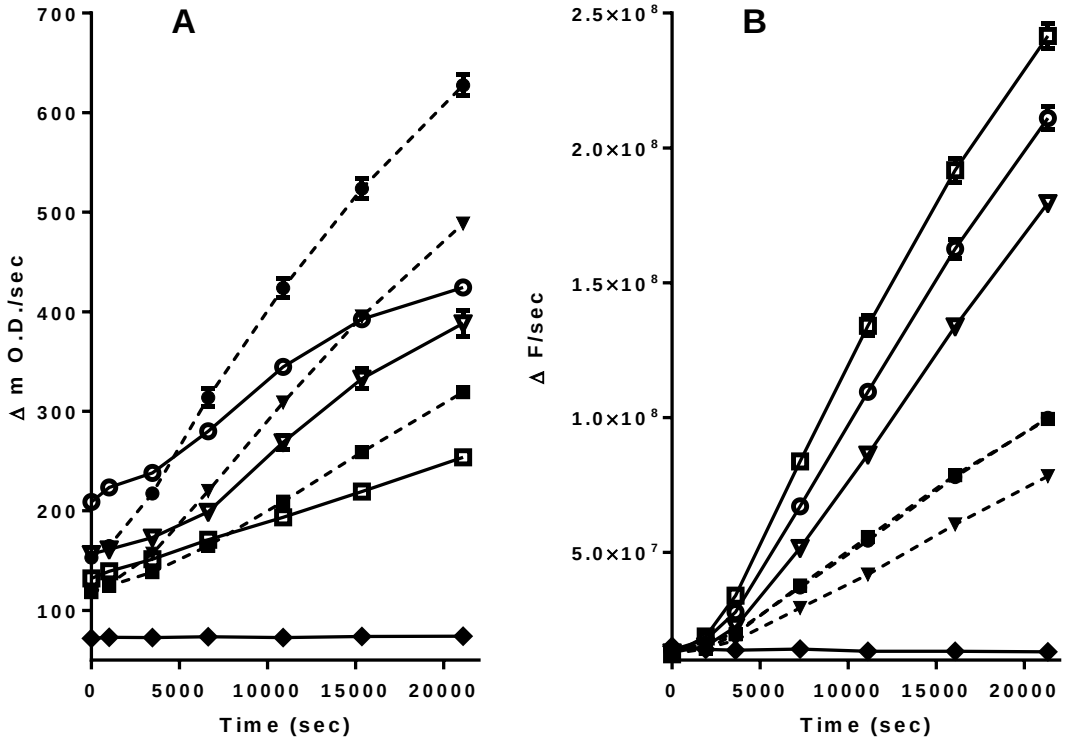

Figure 4.3. Measurements of plasma kallikrein in human plasma comparing two peptide substrates. **A.** Plasma samples from one patient with HAE and two healthy controls were collected with either EDTA or heparin as anticoagulant. **A**: Increase in mO.D. using the substrate HD-Pro-Phe-Arg-pNA (HAE (■); control 1 (▼); control 2 (●)). Solid lines are heparin-treated plasma (open symbols), dotted lines are EDTA-plasma (filled symbols). **B**. Increase in fluorescence (ΔF/sec) using the substrate Bz-Pro-Phe-Arg-AMC. Samples symbols as in A.

To further estimate how much the measured spontaneous plasma kallikrein represented compared to a complete conversion of all prekallikrein to kallikrein, an activation of the contact system was performed with dextran sulphate (DXS) to cause a complete conversion of

prekallikrein to plasma kallikrein. As shown in table 4.1 the spontaneous activity in EDTA-plasma represented only about 1 % of the activity that could be generated by DXS when using the substrate HD-Pro-Phe-Arg-pNA. When using the substrate Bz-Pro-Phe-Arg-AMC, the spontaneous activity accounted for 4 – 6 % of total activity after activation.

Table 4.1.Activation of plasma prekallikrein with or without dextran sulphate (DXS). Plasma samples described in Fig. 4.3 were incubated with 12.5 mg/L DXS for 7 minutes at 4 °C. Plasma kallikrein activity was measured against the substrates HD-Pro-Phe-Arg-pNA (S2302) and Bz-Pro-Phe-Arg-AMC. Right column in each substrate shows the calculated percentage of spontaneous kallikrein activity compared to total activity after activation, respectively as ΔmO.D./sec or ΔF/sec.

	HD-Pro-Phe-Arg-pNA (S-2302)			**Bz-Pro-Phe-Arg-AMC**		
	Spontaneous activity (Δ mO.D./ sec)	Activity after activation with DXS (Δ mO.D./ sec)	Percent spontaneous activity in plasma (%)	Spontaneous activity (ΔF/sec)	Activity after activation with DXS (ΔF/sec)	Percent spontaneous activity in plasma (%)
HAE EDTA	9,82	1350	**0,7**	4202	64188	**6,5**
HAE Heparin	6,13	1983	**0,3**	11322	71542	**15,8**
Control 1 EDTA	16,9	1534	**1,1**	3195	73904	**4,3**
Control 1 Heparin	9,85	1876	**0,5**	8554	74402	**11,5**
Control 2 EDTA	22,26	1857	**1,2**	4168	65767	**6,3**
Control 2 Heparin	9,16	2618	**0,3**	9902	90829	**10,9**

4.2 Determination of C1-inhibitor in plasma samples from pigs

To investigate if C1-inhibitor was present in the plasma samples and if there was complex binding, quantification of immunobands on immunoblots was performed.

Immunoblotting (Fig. 4.4A) illustrated the presence of C1-inhibitor in pigs receiving C1-inhibitor (left panel) or not (right panel) using a human C1-inhibitor primary antibody. Pigs that had received human C1-inhibitor expressed a strong immunoband of 95 kDa representing C1-inhibitor that was not found in plasma from the control pigs. No endogenous C1-inhibitor was detected before clamp and before injection of C1-inhibitor (left panel, lane 1, T-0 reperfusion)) did. In contrast, strong expression was seen after injection of C1-inhibitor (left panel, lanes 2-9). No C1-inhibitor was observed in control pigs (right panel). The weaker immunobands were unspecific binding of previous primary antibody to proteins that were left after stripping. The results indicated that the used antibody against human C1-inhibitor only detected the injected human C1-inhibitor and did not bind to C1-inhibitor from pigs.

Figure 4.4B is showing the quantification of the 95 kDa C1-inhibitor immunoband in three C1-inhibitor-treated pigs. Left panel shows fluorescence A.U. of C1-inhibitor, and the right panel shows the result as merged normalized in percentage. The amount of C1-inhibitor remained stable after C1-inhibitor was injected (5 - 360 minutes).

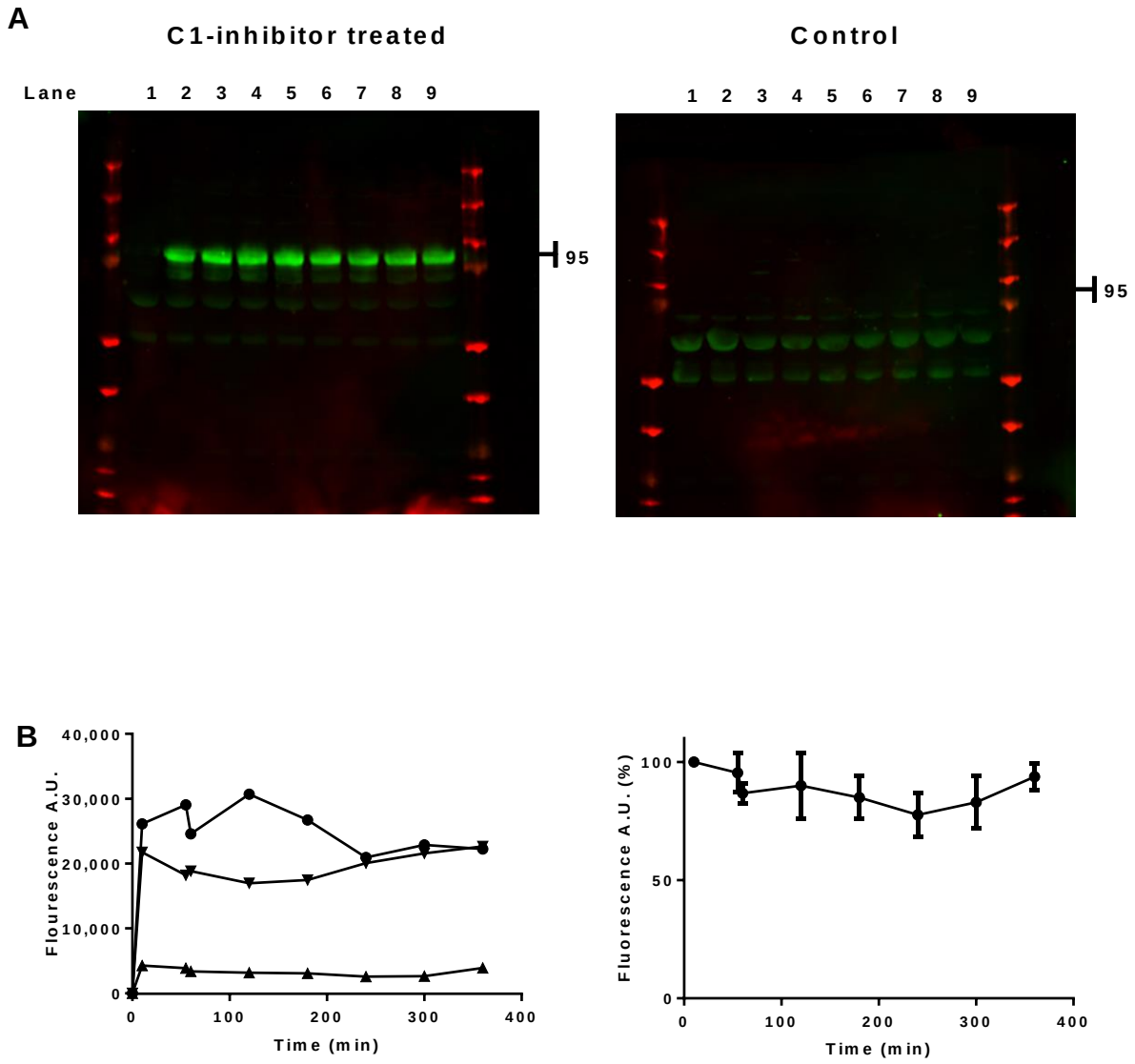

Figure 4.4. C1-inhibitor in citrate plasma from pigs. A. Immunoblots of C1-inhibitor in plasma samples from one pig treated with human C1-inhibitor (C1-inhibitor; left panel) or saline (control; right panel) before, during and after ischemia for 45minutes. The first lane (T-basis) is before C1-inhibitor or saline was administrated intravenously. Samples in lanes 2-9 (T-0 ischemia to T5) is after the pig has received C1-inhibitor or saline. Samples in lane 1-9 represent the 9 points in time which are: first lane (T-basis), second lane (T-0 ischemia), third lane (T-45 ischemia), fourth (T-0 reperfusion) and etc until the last lane, lane 9 (T-5 reperfusion). The two lanes at both sides which are not numbered shows standard proteins according to size (from heaviest protein size: 250, 150, 100, 75, 50, 37, 25, 20 and 15 kDa). **B.** Quantification of the immunoband for C1-inhibitor (95 kDa) measured with Image Studio Ver 5.2. The figures show fluorescence A.U. (arbitrary units) of three pigs receiving C1-inhibitor and samples taken at nine points in time as described above; as individual measurements (left panel) and merged as normalized in percentage (right panel).

4.3 Determination of high molecular weight kininogen (HK)

Immunoblotting was performed to quantify high molecular weight kininogen (HK) in pig plasma samples.

Immunoblotting (Fig. 4.5A) illustrated the presence of HK in pigs receiving C1-inhibitor (left panel) or not (right panel) using a human HK primary antibody. Pigs expressed a 120 kDa protein representing endogenous HK. The panels represent two pigs.

To adjust the different volume of saline given to all pigs during surgery, total protein measurement was performed on the plasma samples. The HK immunoblot was adjusted to the total protein concentration in each sample and is shown in Fig. 4.5B, left panel. Plasma samples containing inhibitor cocktail collected at T0 (immediately after reperfusion) and T5 (five hours reperfusion) were also analyzed. These samples were available from all pigs included in the study. After quantification of HK, the concentration of total proteins was measured. The level of HK was measured as percentage of fluorescence in sample T5 versus T0.

The results of total protein measurement of plasma samples indicated that the plasma had been diluted during the operation procedure because of lower mean of total protein concentration at T5 in all plasma samples compared to T0. After correction with total protein there was no significant change in HK in control group versus C1-inhbitor-treated group (p=NS).

ELISA measurements of plasma samples using an antibody against human legumain was also performed, but no results were obtained. This indicated that the antibody against human legumain did not recognize legumain from pigs or there was no legumain in the samples.

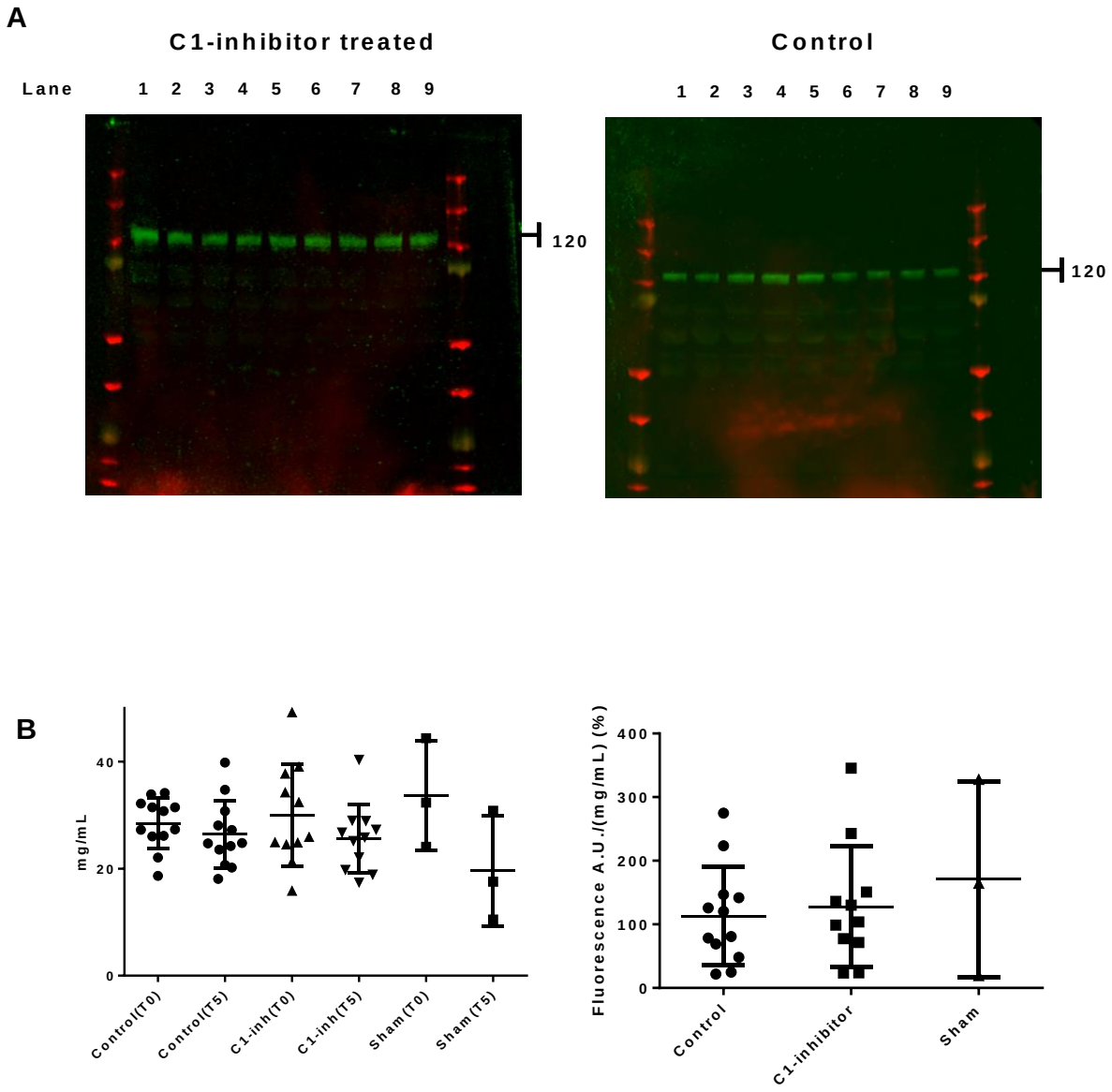

Figure 4.5. High molecular weight kininogen (HK) in plasma from pigs. A.Immunoblots of HK in citrated plasma samples from one pig treated with C1-inhibitor (C1-inhibitor; left panel) or saline (control; right panel) before, during and after ischemia for 45 minutes. The description of the lanes is the same as seen in Fig 4.4. **B.** Quantification of the immunoband for HK (120 kDa). Left panel shows quantification of total protein in plasma samples from 27 pigs containing an inhibitor cocktail. The figure shows total protein concentrations in mg/mL Right panel shows quantification of HK in immunoblots of plasma with inhibitor cocktail correlated with total protein concentration measured with Image Studio Ver 5.2 showing fluorescence A.U./(mg/mL) in percentage. The data represents the mean +/- SEM.

4.4 Determination of concentration of BK 1-5

To quantify the stable metabolite of bradykinin (BK1-5), ethanolic extracts of whole pig blood samples were analyzed by LC-MS/MS. Very low or zero concentration of BK1-5 was found in pig blood. The median concentration in control pigs at T0 was 11.85 pM while median concentrations in the other groups at both T0 and T5 were zero. There appeared to be a higher concentration of BK1-5 in control pigs at T0 when compared with pigs treated with C1-inhibitor, although not significant. However, the reduction in BK1-5 from T0 to T5 in the control group was significant (p=0.007), indicating the disappearance of BK1-5 during the five hour interval.

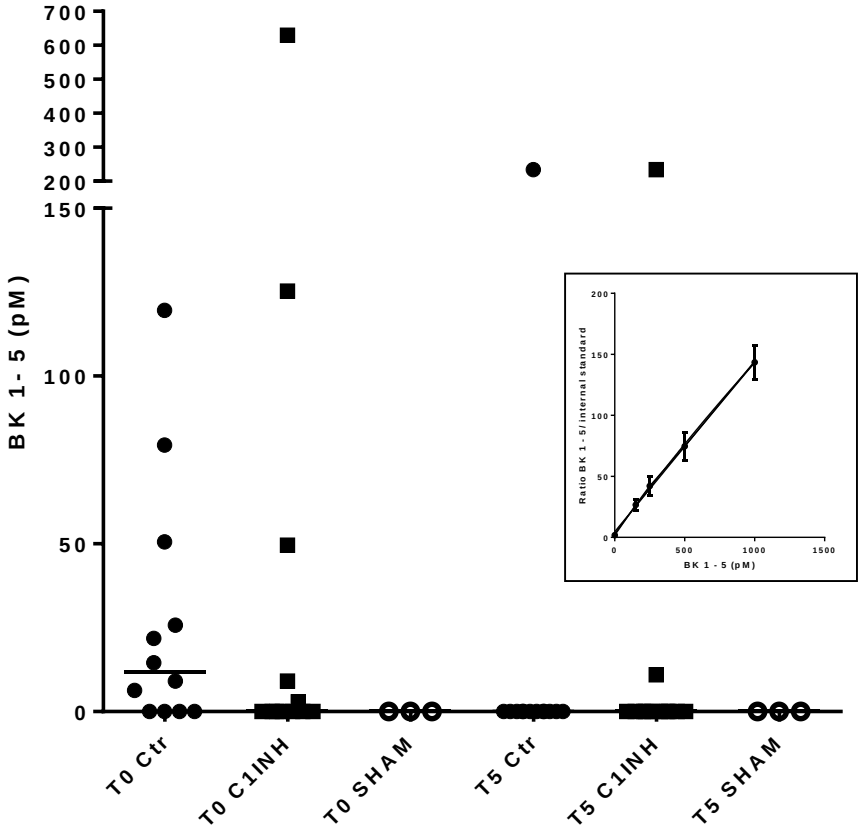

Figure 4.6. Quantification of BK1-5 in plasma. The concentration of BK1-5 was measured by LC-MS/MS of ethanolic extracts of pig blood. Two samples were available from each pig: T0 collected immediately after 45 minutes of ischemia and T5 collected after 5 hours of reperfusion. Twelve pairs of samples were from control pigs (Ctr; saline) and from pigs treated with C1-inhibitor (C1INH), and tree pairs of samples were from pigs that were not exposed to ischemia (SHAM). The insertion represents a standard curve of purified BK1-5.

5 Discussion

Ischemic reperfusion injury (IRI) occurs when an affected tissue is deprived of oxygen and nutrients and followed by reperfusion. This can cause a strong inflammatory response depending on duration and severity of the ischemia. The complement system can be activated and numerous pro-inflammatory mediators can be generated locally in affected tissues, eventually leading to organ failure (Dong, Pratt, Smith, Dodd, & Sacks, 1999).

The involvement of the complement system is well documented in IRI, but a complete understanding of how IRI triggers the immune system is still lacking. Clinical and experimental studies in different animal models and in several organs have shown that IRI activation of the complement system involves both the classical and alternative pathways (Dong et al., 1999; Weiser et al., 1996).

In the complement system, proteolytic enzymes are regulated by proteinase inhibitors in plasma. The most prominent of these is the C1-inhibitor, which is the major inhibitor of C1s and C1r (Davis et al., 2008). The functions of C1-inhibitor are not restricted to the complement system, but it also plays an important role in controlling the contact system (Maas et al., 2011). C1-inhibitor is an inhibitor of plasma kallikrein, fXIIa, fXIIf and fXIa. By controlling the activity of these enzymes, C1-inhibitor is able to inhibit both the activation of the internal coagulation cascade and also the biosynthesis of the biological active peptide bradykinin. This peptide may cause edema and life threatening situations (Joseph & Kaplan, 2005). The *in vivo* function of bradykinin is best illustrated by the autosomal genetic disorder hereditary angioedema (HAE), which is caused by a partial deficiency of the inhibitor (Massimo Cugno et al., 2009). Although complement activation does take place in these patients, their clinical symptoms are primarily caused by the uncontrolled production of bradykinin, resulting in attacks of edema (Nussberger et al., 1998).

The present study intended to explore the hypothesis that IRI not only causes activation of complement and inflammation, but also activate the contact system. The model used applied aortic cross clamping in pigs for 45 minutes, followed by 5 hours of reperfusion. One group of 12 animals was given an infusion of C1-inhibitor, while 12 animals were given infusion of saline and served as the control group. Different plasma samples were collected throughout

the experiments as described in more detail in the Materials and methods section (Chapter 3: Plasma sample).

The main objective in this study was to establish if contact activation did take place during the IRI procedure, and if C1-inhibitor could prevent this.

Contact activation causes several changes in plasma, so we decided to study these changes, expecting that this would reflect and provide evidence of potential contact activation. Contact activation will generate active proteinases such as plasma kallikrein, and some of this activity can be measured in stable complexes with alpha-2-macroblobulin. Also, contact activation can result in stable complexes between C1-inhibitor and plasma kallikrein, and the degradation products from H-kininogen can be detected after electrophoresis and immunoblotting. Finally, the release of bradykinin (BK) can be detected by the presence of the stable pentapeptide metabolite (BK1-5).

5.1 Plasma kallikrein activity during IRI

The well-established assay using the chromogenic peptide substrate HD–Pro–Phe–Arg–pNA (S-2302) was first described by M. Gallimore, Amundsen, Larsbraaten, Lyngaas, and Fareid (1979) and has since been used in both clinical trials and animal models to monitor activity of kallikrein in plasma. The method is simple, sensitive, specific, rapid and convenient. It has therefore often been used in studies to investigate the contact system and disorders or conditions associated with disruption in the contact system, such as in HAE (Defendi et al., 2013; Suffritti et al., 2014).

One may assume that ischemia activates the contact system, resulting in inflammation. To investigate this, we chose to explore whether plasma kallikrein increases during IRI based on the fact that kallikrein is the biological precursor of bradykinin. Plasma kallikrein in plasma was measured with the substrate S-2302. The results indicated that the contact system was activated, as shown by an increase in activity of plasma kallikein during ischemia in the control group. This confirmed our hypothesis that ischemia causes activation of the contact system.

Previous studies of contact system activation during IRI are limited. There are however a few animal studies of ischemia in heart, intestine and brain (Groger et al., 2005; Souza et al.,

2004; Storini et al., 2006; Veeravalli & Akula, 2004). A novel study used a mice stroke model, resulting in strong and rapid activation of the contact system, which lead to inflammation and progressive thrombosis in the brain (Göb et al., 2015). Mombouli and Vanhoutte (1995) have confirmed activation of plasma kallikrein during myocardial infarction in humans, and in a myocardial ischemic dog model. Also, during angioedema attacks in HAE patients there was an indication of reduced levels of prekallikrein, respectively high molecular weight kininogen (HK) and elevated bradykinin (BK) levels (Schapira et al., 1983). The study resulted in proteolytic cleavage of prekallikrein, increasing level of plasma kallikrein and rapid HK clearance.

Further, the activity of plasma kallikrein was also measured with a new fluorogenic peptide substrate, containing AMC as the leaving group (Bz–Pro–Ph–Arg–AMC). It contained the same tri-peptide sequence as S-2302 but differed in the leaving group which causes an increase in fluorescence when released and had a benzo group blocking end, which is lacking in the S-2302. Activity measurements with this substrate gave results similar to those described above with S-2302.

We expected similar kallikrein activity profiles for individual plasma samples when comparing results for the two substrates. This was not always the case. While measurements with S-2302 were done at 1 mM concentration of substrate, the concentration of substrate containing AMC was only 10 µM. This was as expected, based on Km values reported for S-2302 (0.2 mM) and the AMC-substrate. It is possible that the AMC-substrate measured activity of other enzymes present in addition to plasma kallikrein. However, the substrate containing AMC is a sensitive and good substrate for plasma kallikrein, which was seen by the 10-fold increase in activity after activation of plasma with dextran sulphate (Table 4.1).

5.2 Spontaneous plasma kallikrein activity in human plasma

In a study which included 162 HAE -patients, patients showed a higher spontaneous plasma kallikrein activity, compared to the control group (Suffritti et al., 2014). In the present study, we measured spontaneous plasma kallikrein activity in plasma samples from one HAE-patient and two controls. We expected higher spontaneous plasma kallikrein activity in plasma sample from the HAE -patient, although this was not observed. This can be explained

by the fact that the HAE-patient received an infusion of C1-inhibitor just a few hours before the blood sample collection.

5.3 C1-inhibitor attenuates activation of the contact system

The knowledge of C1-inhibitor pathology has increased in the past years. C1-inhibitor has been used in numerous studies *in vivo*, *in vitro* and *ex vivo* to examine both vascular permeability and inflammation (A. E. Davis et al., 2008). The focus in this thesis was on contact system, also known as the kallikrein-kinin system. This thesis demonstrated that infusion of C1-inhibitor attenuated activation of plasma kallikrein in pigs. The effects of C1-inhibitor are best illustrated in the clinical picture of HAE characterized as attacks with local increase in vascular permeability.

The first evidence to observe effects of C1-inhibitor was Landerman et al., who observed that plasma from hereditary angioedema (HAE) patients had increased activity of plasma kallikrein and postulated whether deficiency of components in blood caused the increase (Landerman, Webster, Becker, & Ratcliffe, 1962). One year later Donaldson and Evans discovered that patients with HAE genetically lacked C1-inhibitor. Nowadays we know that the contact system is activated during attacks in HAE patients through increase in "active" kallikrein (plasma kallikrein) and reduced prekallikrein during attacks (Curd, Prograis, & Cochrane, 1980; Schapira et al., 1983). A series of experimental study designs to isolate and characterize bradykinin in C1-inhibitor depleted plasma has provided additional strong evidence for the generation of bradykinin in HAE patients (Shoemaker et al., 1994).

C1-inhibitor knock-out mice, both homo- and heterozygous, do not have obvious angioedema episodes but present increased vascular permeability. The increased vascular permeability was reversed by intravenous C1-inhibitor (Han et al., 2002; Han Lee et al., 2003). Such increase in vascular permeability is depended on contact activation and is mediated by the bradykinin receptor 2 (B2) (Cicardi et al., 2005). In conclusion, lack of C1-inhibitor generates bradykinin release due to contact activation, which stimulates B2-receptor.

The observation study in C1-inhibitor knock-out mice also showed that these mice did not have phenotypical abnormalities. Patients with HAE also appear normal except during attacks, which raise the question whether C1-inhibitor has other pathologic effect. Various

animal studies on ischemia-reperfusion injury (IRI) have shown beneficial effect of C1-inhibitor treatment. A myocardium IRI study has shown that C1-inhibitor reverse necrosis and maintain normal cardiac performance (Buerke, Murohara, & Lefer, 1995). These studies provide strong evidences to support the findings in this thesis, and therefore it may be concluded that C1-inhibitor attenuate contact activation.

5.1 C1-inhibitor complexes

Serine proteinase inhibitors have remarkable features of inhibitory mechanisms, which are (I) conformation change, (II) irreversible trapping and (III) trapping complexes, which are covalent and effectively irreversible in nature. C1-inhibitor belongs to this group of inhibitors and inhibits C1r, C1s and plasma kallikrein. The results of C1-inhibitor covalently binding to target proteinases, leads to loss of functions for both C1-inhibitor and the targeted proteinase. This was earlier referred to as a suicide inhibitor (Gettins, 2002; Patston, 1991).

A complex between C1-inhibitor and plasma kallikrein has been verified through previous studies (de Agostini, Schapira, Wachtfogel, Colman, & Carrel, 1985; Patston, 1991). In the present study, we investigated whether there actually was a complex between C1-inhibitor and plasma kallikrein. We observed C1-inhibitor in the pigs that had received C1-inhibitor, but no C1-inhibitor complex binding was observed. Because of these results, we suggest that the level of contact activation was not sufficient to observe such C1-inhibitor complex. But it does not exclude that contact activation has happened to some degree, but rather slightly. The same observation has been seen in a study of patients with HAE (M. Cugno et al., 1990).

5.2 High molecular weight kininogen (HK)

C1-inhibitor is a major regulator of the contact system, but other regulators have also been identified, such as α2-macroglobulin (α_2M), antitrombin III, α1-proteinase inhibitor and several others (Moreau et al., 2005). In the present study, we confirmed that C1-inhibitor treatment attenuated the activity of plasma kallikrein during IRI, whereas increased activation of plasma kallikrein was observed during IRI in the control group. Thus, the cleavage product of high molecular weight kininogen (HK) will appear and be a valuable product to measure. The presence of C1-inhibitor will limit the cleavage of HK. Measurements of HK were performed in immunoblots, and HK-expression showed no change in control group versus

C1-inhibitor treated group. Also, no difference in HK was observed in samples collected immediately after reperfusion (T0 reperfusion) versus five hours after reperfusion (T5).

Minor differences in concentrations of plasma proteins could result from different volumes of saline administered to the individual pigs. To calibrate for this, the quantification of HK in immunoblots was correlated to the total concentration of plasma proteins. After correction for total protein concentration the results were still the same. There was no significant difference in the control group versus the C1-inhibitor group, and also no significant change between plasma samples collected immediately after reperfusion (T0) compared to after five hours of reperfusion.

Cleavage of HK could occur by *in vivo* activation of the contact system at the time blood was drawn, and can even occur when the samples are added inhibitor cocktail (used in our samples) that can minimize such activation. This technical problem has been discussed by Konings et al. (2013).

5.3 The stable metabolite of bradykinin -BK 1-5

Bradykinin is a biologic active small peptide formed in plasma, and it is rapidly inactivated by several endogenous enzymes, such as kininase I (carboxypeptidase) and kininase II (angiotensin-converting enzyme; ACE). The fraction of bradykinin which is not inactivated by these enzymes reaches peripheral tissue and binds to its receptors B1 and B2. There is strong evidence that bradykinin enhances vascular permeability by binding to receptor (B2-receptor) as mentioned earlier (Han et al., 2002; Nussberger et al., 1998; Shoemaker et al., 1994).

The half-life of bradykinin is very short *in vivo* (a matter of seconds). Measurements of bradykinin are difficult, not just because of the rapid metabolism, but also because of a possible generation of BK during sampling. Previous data has shown that it is possible to measure bradykinin (1-9) in plasma samples, but with limited sensitivity (Massimo Cugno, Nussberger, Cicardi, & Agostoni, 2003; Nussberger et al., 1998). Recently, Seip et al. (2014) have discussed these challenges and suggested measurement of a stable pentapeptide metabolite of bradykinin (BK1-5) as a diagnostic tool for HAE. However, the disadvantages with measuring BK1-5 are: (I) the method requires expensive instruments (i.e. LC-MS), (II) time-demanding sampling preparation and (III) limited sensitivity.

Previous reports has demonstrated that the difference in concentration of bradykinin between HAE-patients in remission and healthy controls are statistically significant, but a substantial rise of bradykinin has been described during acute attacks of HAE, and acquired or captropril-induced angioedema(Nussberger et al., 1998).

When plasma kallikrein cleaves HK, the released bradykinin is rapidly metabolized to the pentapeptide BK1-5. The LC-MS/MS method for measuring BK1-5 used in this thesis has been validated with a limit of detection at 35.4 pM (Seip et al., 2014). Most measurements in this study were below this detection limit. However, there was a clear indication of BK1-5 being present in plasma immediately after 45 minutes of aortic cross clamping. Also, at this point in time there seemed to be less BK1-5 present in plasma samples from pigs treated with C1-inhibitor although this difference was not significant. Still, this observation is in agreement with lower activity of plasma kallikrein in pigs treated with C1-inhibitor. Reduced activity of plasma kallikrein is expected to release less bradykinin from HK and thus lower levels of the metabolite BK1-5. When comparing T0 with T5, there was observed a significant decrease in the concentration of BK1-5. This could reflect clearance of the metabolite during the 5 hours. The published $t_{1/2}$ of BK1-5 is 86-101 minutes, so a significant amount of the metabolite generated during ischemia is expected to be cleared if generation of BK/BK1-5 takes place during ischemia and not during reperfusion (Murphey et al., 2001). No BK1-5 was found in sham pigs, and nearly all measurements at T5 were zero. This indicates that no measurable concentrations of BK1-5 is normally present in pig plasma, but also that the observed concentrations at T0 in control pigs reflects a genuine release of BK during ischemia.

5.4 Evaluation of the ischemia reperfusion injury (IRI) model in pigs

The contact system, also known as the kallikrein-kinin system, consists of three serine proteinases: coagulation factor fXII and fXI, plasma kallikrein and the nonenzymatic cofactor high molecular weight kininogen (HK). This system has been heavily investigated for the last 50 years. The components of this system and their interactions have been elucidated from *in vitro* experiments, which indicate that this system is prothrombotic by activating the intrinsic pathway of coagulation, and pro-inflammatory by producing the bioactive peptide bradykinin. Our understanding of the contact system has greatly increased in the last 10 years through

investigations using gene-modified animal models, such as the C1-inhibitor knock-out mice. The contact system is implicated in various human diseases, such as hereditary angioedema, however the physiological functions remain a mystery.

C1-inhibitor, which is an inhibitor of these contact proteinases mentioned above, seems to reverse the effect of contact activation. Various studies, such as the C1-inhibitor knock-out mice and the autosomal genetic disorder HAE, have provided strong evidence that C1-inhibitor has a major role in controlling this system.

Ischemia-reperfusion injury (IRI) results in activation of the contact system due to increase in plasma kallikrein and generation of bradykinin. Cleavage of the chromogenic substrate S-2302 and fluorogenic substrate containing AMC (Bz–Pro–Ph–Arg–AMC) indicate activation of plasma kallikrein. Increased activity of plasma kallikrein was observed during ischemia for 45 minutes. Also increase in pentapeptide BK(1-5) during IRI indicates contact activation. Previous studies mostly based on animal studies have concluded the same. Although we observed increase in plasma kallikrein, the results in this thesis of measuring plasma kallikrein was insufficient to affect the visual observation of cleaved HK or complex binding to C1-inhibitor.

The peptide substrate S-2302 is the most selective to use when measuring contact activation due to plasma kallikrein. The substrate containing AMC (Bz–Pro–Ph–Arg–AMC) is also sensitive, but the results in this thesis indicate that it also is recognized and cleaved by other enzymes.

Our findings are relevant to give more knowledge of one aspect of the immune system, particularly the contact system. Hopefully these findings will be useful for further investigations.

6 Conclusion

- Increased activity of plasma kallikrein was observed after ischemia for 45 minutes.
- C1-inhibitor attenuated the activation of plasma kallikrein.
- Bradykinin was generated during ischemia as shown by the presence of the metabolite BK1-5.
- Bradykinin was not generated during five hours of reperfusion but BK1-5 disappeared.
- Cleavage products of high molecular weight kininogen during ischemia-reperfusion injury (IRI) were not observed.
- C1-inhibitor complexes were not observed.
- The chromogenic substrate (S-2302; H–D–Pro–Phe–Arg–p–nitroanilide) is the most selective substrate to measure activity of plasma kallikrein.

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Appendix

1. Appendix (two pages from the protocol (Appendix 2 and 3) performed at Nordland hospital during autumn 2015) with a detailed description of the plasma samples collected from pigs written in Norwegian.

Appendix 2	Dato	Kjønn	Vekt kg	Alder	*Clamp tid*	Release tid	Team		Intervensjon
			11,5						Volum C1inh/NaCl(ml)
Definisjon av TBasis: Etter arteriekran er lagt inn						BLODVOLUM hos GRISEN		Volum (ml)	57,5
Definisjon av T0 Tang: Like før start ischemi						Vekt denne grisen (kg)	11,6		
Ischemitid: 45 min						Total blodvolum denne grisen (ml	(Vekt x 7%) x 1000 gir ml	805	
Forslag til ny definisjon T0 reperfusjon (150414 EWN): Tidspunkt når aortatang slippes opp og trykk MAP har falt > 40 mmHg						Plasmavolum pr gris (ml)	(70% av Blodvolumet)	563,5	
						MAX prøvetakingsvolum (ml)	(15% av blodvolumet)	120,75	

Bare systemiske prøver	Prøvetakning Blodvolum		Syrebase	Hematologi/Advia	Koag	Cytokiner, TCC	Med biokjemi analyser	cPCR	Bradykinin (BK 1-6)	Prøver til Immunelektrofor	
Tid (h)	ml	Tid	Heparin-sprøyte(ml)	EDTA (ml) Mikrotainer	Citrat-fullblod (ml)	EDTA-fullblod (ml)	Fullblod (ml) vacutainer med gel	Tempus-rør (ml)	6 ml blod i sprøyte	Inhibitorplasma	Urin
1. TBasis (etter arteriekran)	9,3		0,5	*0,5*	1,8	*2,0*	*2*	*3*			
Injeksjon C1inh/saltvann over ca 15 min											
2. T0 Tang(rett før start ischemi)	6,3		0,5	*0,5*	1,8	*2,0*	*2,0*				
Ekstra T15 min Tang			0,5								
Ekstra T30 min Tang			0,5								
3. T45 min Tang(ischemi, rett før tang åpnes)	6,3		0,5	*0,5*	1,8	*2,0*	*2,0*				
4. T0 reperfusjon (så snart som mulig etter start åpning)	9,3		0,5	*0,5*	1,8	*2,0*	*2,0*	*3*	*5*	*4,5*	
Ekstra T15 min e reperfusjon			0,5								
Ekstra T30 min e reperfusjon			0,5								
5. T1 h reperfusjon	5,8		0,5		1,8	*2,0*	*2,0*				
6. T2 h reperfusjon	9,3		0,5	*0,5*	1,8	*2,0*	*2,0*	*3*			
7. T3 h reperfusjon	5,8		0,5		1,8	*2,0*	*2,0*				
8. T4 h reperfusjon	9,3		0,5	*0,5*	1,8	*2,0*	*2,0*	*3*			
9. T5 h reperfusjon (= TDØD)	9,3		0,5	*0,5*	1,8	*2,0*	*2,0*	*3*	*5*	*4,5*	*5 mlx2*
Totalvolum (ml) (utenom TDØD)	76,9										

Prøvetaking alle tidspunkt:

All prøvetaking skjer med sprøyte:
Ta ut 2-5 ml blod som settes tilbake etter prøvetaking. Syre-base sprøyte taes først.
Ta ut blodvolum i hht skjema og fordel i hhv. mikrotainer EDTA (NB må fylles først), Citrat, EDTA rør og serumrør.
Ved T0 reperf også i ethanol og proteasehemmersprøyte
Bruk gjerne BD Microlance 3 (21 G, 2" 0.8x50mm, grønn) til overføring av blod til vacutainer. *OBS: Etter prøvetaking: Ikke skyll etter med ringer.*

Sentrifugering :

EDTA og Citrat: Program 1 (Tid: 15min., Temp: 4°C, RCF: 1.500): Avpipetter plasma i Matrix-rør på is.
SERUM/ Vacutainer: Program 2 (Tid 10min, Temp: 4 °C, RCF:2.000), sentrifugeres etter ca 30min.
SERUM/Mikrotainerrør Liten sentrifuge: 10.000 g i 1.5 min., sentrifugeres etter ca 30min.
Ca. 250µl serum overføres til 5 ml rør merket med barkodelapp. Resten på 2 eller flere Matrix-rør.
Bradykinin/ethanol: Sentr. NB! 25min, 4°C, RCF: 1.500 Avpipetter plasma, 6-7 ml overføres 2xCorningrør (15ml) på is. Se egen prosedyre.
Proteasehemmer cocktail: Overføres 4,5 ml Nunc. Sentr 15 min, 4°C, RCF: 1500 Avpipetter plasma, matrix-rør 500 µl x 4. Se egen prosedyre.

Oppbevaring:

Hematologirør og Tempus oppbevares ved RT.
Citrat / EDTA plasma og serum oppbevares på tørris.
Vev: På tørris (2 ml matrix-rør)
Tempus Blood RNA Tube: Rist kraftig umiddelbart etter prøvetaking, ev. vortex. La stå på benk v/RT. Ved ankomst sykehus fryses prøven v/-20C.

TDØD: Biopsier (se egen prosedyre)	Nyre	Lever	V.Lunge	Tynntarm	Tykktarm	Hjerte HV	Hjerte VV	Milt	
1. Tørre BIOPSIER (min 3 småbiter overføres til 2 ml matrix rør)	3	3	3	3	3	3	3	3	
2. Formalinfixering (2 biter på ca 0.5x0.5x0.5cm i 10 ml rør)	1	1	1	1	0	0	0	0	
3. Frysesnitt (OCT ev Iso-pentane) (lever+tynntarm kan fryses sammen	2	2*	1	2*	0	0	0	0	*fryses i samme form

Ved ankomst sykehus:

1. Hematologi	Microtainer leveres hematologi-lab. Kjøres på Advia.
2. Plasma/serum og biopsier	Fryses ved - 80 ° C. Legges inn i freezerpro neste dag
3. Tempus fullblod	Fryses ved - 20 ° C. Legges inn i freezerpro neste dag
4. Biopsier formalin	Oppbevares RT. Forsendelse patolog helst innen 1 uke.

APPENDIX 3

Time	ID	Citrat (ml)	EDTA (ml)	Serum vacutainer m/gel (ml)	Serum vacutainer m/gel (ml)
Analyse		TAT/PAI-1	Cytokiner m.m	Med Biokjemi	
TBasis (når anestesi er på plass)	1	500 x 2	500 x 2	250	500 x 2
T0 (ved ischemi)	2	500 x 2	500 x 2	250	500 x 2
T45 min. ischemi (rett før ballong fjernes)	3	500 x 2	500 x 2	250	500 x 2
T0 reperfusjon (rett etter ballong er fjernet)	4	500 x 2	500 x 2	250	500 x 2
T1h reperfusjon	5	500 x 2	500 x 2	250	500 x 2
T2h reperfusjon	6	500 x 2	500 x 2	250	500 x 2
T3h reperfusjon	7	500 x 2	500 x 2	250	500 x 2
T4h reperfusjon	8	500 x 2	500 x 2	250	500 x 2
T5h død reperfusjon	9	500 x 2	500 x 2	250	500 x 2

Citrat/EDTA: 500µl x2 fordeles i merkede matrixrør
Serum: 250 µl i 1x5ml rør (med.biokjemi). Dessuten 500 µl x2 i matrixrør

2.

0.05 M Trisbuffer

50 mM Tris base

360 mM NaCl

20 mg/mL Polybrene

pH = 7,8

3.

Electrophoresis buffer

40 mL NuPAGE running buffer (20X)

dH_2O up to 800 mL

500 µL NuPAGE Antioxidant added to the electrophoresis chamber

4.

Transfer buffer

400 mL Tris/Glycine

800 mL methanol

dH_2O up to 4 L

5.

10X PBS

80 g NaCl

2 g KCl

14,4 g Na_2HPO_4 X H_2O

2

2 g KH_2PO_4

HCl, pH adjusted to 7,3

dH_2O up to 1,0 L. Filtrate through filter 0,45 µm. Dilute 1:10 with dH_2O before use

6.

10X TTBS

48 g Tris

220,2 g NaCl

20 mL Tween

HCl, pH adjusted to 7,5

dH_2O up to 2,0 L. Dilute 1:10 with dH_2O before use