# Kinin System Activation in Vasculitis

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# Contents

# List of papers

This thesis is based on the following papers, referred to in the text by their Roman numerals:

I. **Kahn R**, Herwald H, Müller-Esterl W, Schmitt R, Sjögren A-C, Truedsson L, Karpman D. Contact system activation in children with vasculitis. *Lancet* 2002, 360: 535-541.

II. **Kahn R**, Hellmark T, Leeb-Lundberg LMF, Akbari N, Todiras M, Olofsson T, Wieslander J, Christensson A, Westman K, Bader M, Müller-Esterl W, Karpman D. Neutrophil-derived proteinase 3 induces kallikrein-independent release of a novel vasoactive kinin. *J Immunol* 2009, 182: 7906–7915.

III. **Kahn R**, Hellmark T, Karpman D. Proteinase 3-ANCA inhibits proteinase 3-induced high molecular weight kininogen cleavage. Manuscript.

IV. **Kahn R**, Mossberg M, Ståhl A, Sandén C, Tarkowski A, Qadri F, Bader M, Leeb-Lundberg F, Karpman D. The kinin $B_1$-receptor is upregulated in mice with vasculitis. Manuscript.

The following paper has been published but was not included in this thesis:

Karpman D, **Kahn R**. The contact/kinin and complement systems in vasculitis. *APMIS Suppl. 2009;(127):48-54.*

# Abbreviations

| | |
|---|---|
| $\alpha_1$-AT | alpha-$_1$-antitrypsin |
| ANCAs | Anti-neutrophil-cytoplasmic antibodies |
| Arg | arginine |
| B1KO | $B_1$-receptor-knock-out |
| B2KO | $B_2$-receptor-knock-out |
| CK-1 | cytokeratin-1 |
| EGF | epidermal growth factor |
| eNOS | endothelial nitric oxide synthase |
| fXI, fXIa | factor XI, activated factor XI |
| fXII, fXIIa | factor XII, activated factor XII |
| gC1qR | the receptor for the globular head of C1q |
| HBP | heparin binding protein |
| HK | high-molecular-weight kininogen |
| HSP | Henoch Schönlein Purpura |
| IL | interleukin |
| IFN-γ | interferon-γ |
| LAMP-2 | lysosomal membrane protein-2 |
| LPS | lipopolysaccharide |
| LK | low-molecular-weight kininogen |
| Lys | lysine |
| MAPK | mitogen-activated protein kinase |
| Met | Methionine |
| MP | microparticle |
| MPO | myeloperoxidase |
| NETs | neutrophil extracellular traps |
| NF-κB | nuclear factor-κB |
| NO | nitric oxide |
| NOD | non-obese-diabetic |
| PI | phosphatidylinositide |
| PR3 | proteinase 3 |
| PRCP | prolylcarboxypeptidase |
| SCID | severe combined immunodeficiency |
| TNF-α | tumor necrosis factor-α |
| uPAR | urokinase plasminogen activator receptor |

# Abstract

The kinin system is activated when high-molecular-weight kininogen (HK) is cleaved by plasma kallikrein thus generating bradykinin. Bradykinin is a potent proinflammatory peptide that induces plasma leakage, blood pressure drop, liberation of inflammatory cytokines and pain. Vasculitis is an autoimmune systemic inflammatory disease, characterized by leukocyte inflammation in and around vessel walls leading to perturbed vessel patency and tissue damage. Many different organs may be afflicted but most commonly the kidney, respiratory tract and skin are involved. Some patients with severe vasculitis have circulating antibodies against neutrophil enzymes called anti-neutrophil-cytoplasmic antibodies (ANCAs). Theoretically kinin system activation may explain some of the inflammation seen during vasculitis.

In this thesis we demonstrate, for the first time, activation of the kinin system in patients with vasculitis. Elevated kinin levels were demonstrated in the circulation and kinins were detected at sites of inflammation. We also found that neutrophil-derived proteinase 3 (PR3) cleaves HK liberating a novel vasoactive kinin, termed PR3-kinin. PR3-kinin binds to and activates kinin $B_1$-receptors both *in vitro* and *in vivo*. In addition, PR3-ANCAs from patients with vasculitides inhibit PR3-induced HK proteolysis and subsequent PR3-kinin release. In the $MRL^{lpr/lpr}$ mouse, that develops systemic inflammation and vasculitis, we demonstrate $B_1$-receptor upregulation both systemically, on circulating leukocyte-derived microparticles (MPs), and locally, in the renal vasculitic lesions.

In conclusion, we demonstrate kinin system activation in vasculitis and suggest that it may partake in the pathogenesis of this inflammatory condition. We therefore propose that inhibiting kinin system activation by blockage of $B_1$-receptors may prove to be effective by reducing the inflammatory response during vasculitis.

# Introduction

Patients with autoimmune disorders typically present with the classic signs of inflammation: rubor (redness), tumor (swelling), calor (heat) and dolor (pain). These typical signs of inflammation could theoretically be caused by kinin system activation. The kinin system, found at the interface between the coagulation and the innate immune system, has been shown to be activated in a few autoimmune inflammatory conditions. Systemic autoimmune inflammatory conditions comprise a wide range of diseases but in this thesis we focused on conditions affecting the vessel wall, termed vasculitis, leading to perturbed vessel patency, plasma leakage and leukocyte influx. Vasculitis may affect both children and adults with high morbidity and mortality. We hypothesized that inappropriate kinin system activation may contribute to the inflammatory state during vasculitis. We therefore set out to investigate if the kinin system is activated in patients with vasculitis, and how this activation may occur as well as to study if kinin receptors are upregulated both systemically and locally in an animal model of vasculitis.

# The kinin system

The kinin system is found at the interface between the coagulation system and innate immunity with components present in both systems, although studies performed during the last decades have suggested that the kinin system is more involved in inflammation than in coagulation.

The kinin system has many different names, it is sometimes termed the contact system, as activation occurs via auto-activation of factor XII when in contact with negatively charged surfaces, or the kallikrein-kinin system, as activation in plasma may commence by activation of prekallikrein. In this thesis, the term kinin system will be used.

## The components of the kinin system

The kinin system in plasma consists of high-molecular-weight kininogen (HK), plasma prekallikrein, factor XII (fXII) and factor XI (fXI).

### *High-molecular-weight kininogen*

HK is produced mainly in the liver although it may also be produced by other cells such as neutrophils, endothelial cells and platelets (Colman and Schmaier 1997; Joseph and Kaplan 2005). The plasma concentration of HK is about 80 μg/ml and it has a molecular weight of 120 kD. It consists of a heavy and light chain with the bradykinin sequence in between. The heavy chain is composed of three domains (D1 – D3) and the light chain of two domains (D5 – D6). The D4 domain is 21 amino-acids long and contains the nine amino-acid long bradykinin sequence that is liberated through proteolytic cleavage by plasma kallikrein or activated factor XII (fXIIa) during activation of the kinin system. HK binds to a receptor complex on endothelial cells mainly via the D3 and D5 domains and to plasma prekallikrein and fXII via the D6 domain (Herwald et al. 1995; Colman and Schmaier 1997).

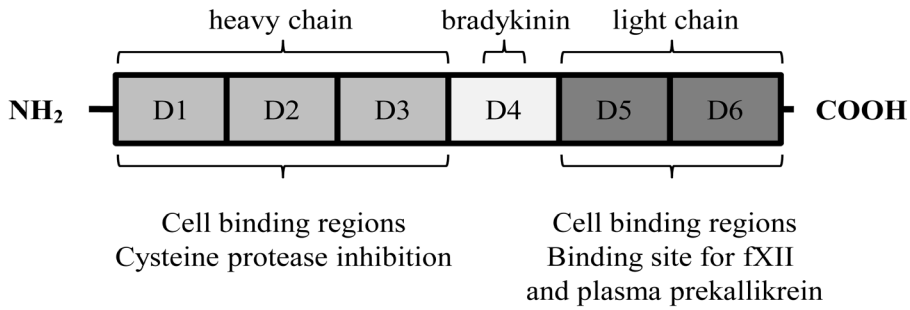


**Figure 1: Domains of high-molecular-weight kininogen (HK).** HK consists of six domains. Domains 1 – 3 (D1 – D3) constitute the heavy chain and comprise cell binding regions. The heavy chain also has cysteine inhibitory effects, although the relevance of this *in vivo* is unclear. D4 is 21 amino-acids long and contains the nine amino-acid long bradykinin sequence. Bradykinin is liberated by proteolytic cleavage by plasma kallikrein or activated factor XII during activation of the kinin system. D5 – D6 constitute the light chain that has binding sites for plasma prekallikrein and fXII as well as cell binding regions.

## *Low-molecular-weight kininogen and tissue kallikrein*

By alternative splicing of the gene encoding for HK, low-molecular-weight kininogen (LK) is produced, consisting of the same D1 – 3 domains and bradykinin sequence as HK with the addition of a truncated form of the light chain (Kitamura et al. 1985). LK has a plasma concentration of 160 µg/ml and a molecular weight of 66 kD. As LK lacks large parts of the light chain it has no binding site for plasma prekallikrein or for fXII. Instead, LK is the substrate for tissue kallikrein (also termed KLK1) and when cleaved by tissue kallikrein it generates lys-bradykinin (kallidin) which may be processed to lys-des-arg$^9$-bradykinin (des-arg$^9$-kallidin) (Lottspeich et al. 1984; Muller-Esterl et al. 1985) (Figure 2B).

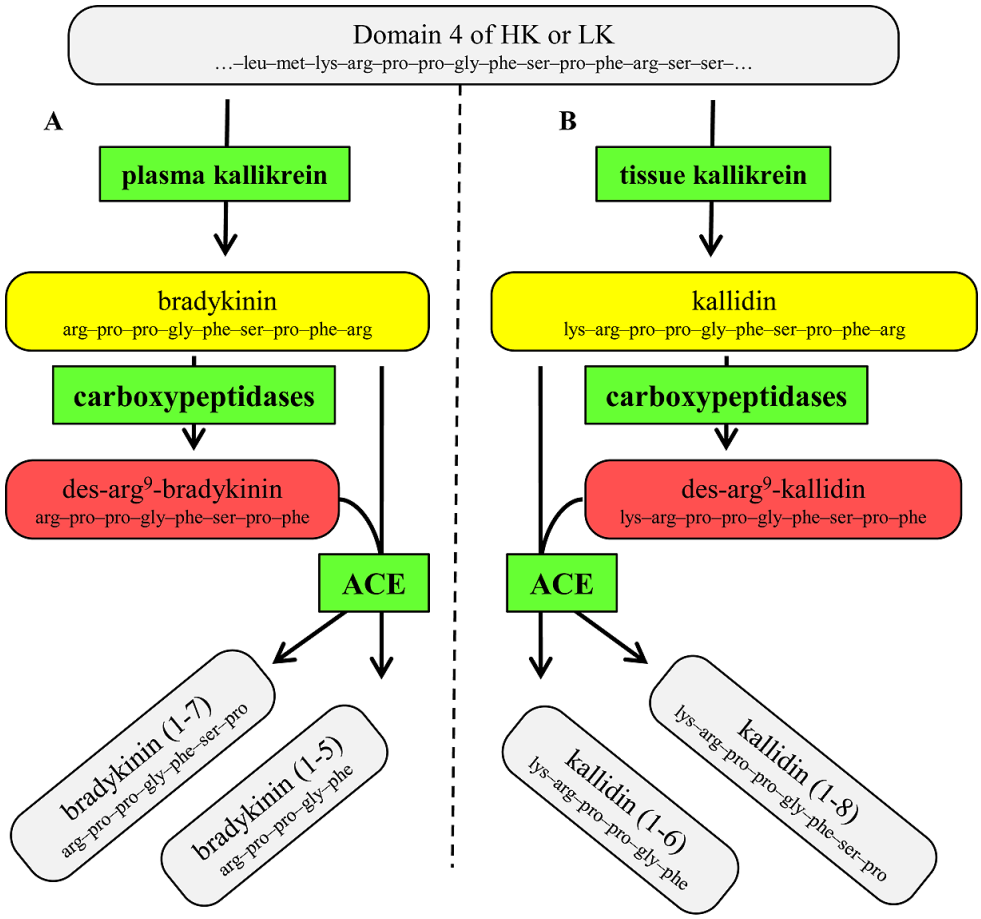


**Figure 2: Kinins. A:** Domain 4 of high-molecular-weight kininogen (HK) is cleaved by plasma kallikrein generating bradykinin. Bradykinin may be further processed by carboxypeptidases to des-arg$^9$-bradykinin. Both bradykinin and des-arg$^9$-bradykinin are degraded by angiotensin-converting enzyme (ACE) to smaller inactive metabolites. **B:** Domain 4 of low-molecular-weight kininogen (LK) is cleaved by tissue kallikrein, generating kallidin (also called lys-bradykinin). Kallidin may be further processed by carboxypeptidases to des-arg$^9$-kallidin (also called lys-des-arg$^9$-bradykinin). Both kallidin and des-arg$^9$-kallidin are degraded by ACE to inactive metabolites. Kinins acting via kinin $B_1$-receptors are marked in red and kinins acting via $B_2$-receptors are marked in yellow, whereas inactive kinins are gray.

## *Plasma kallikrein*

Plasma prekallikrein is a serine protease with a molecular weight of 88 kD with certain homology to fXI (Asakai et al. 1987). It has a plasma concentration of approximately 35 – 50 µg/ml (Colman and Schmaier 1997). The main source of

plasma prekallikrein production is the liver. In the circulation, plasma prekallikrein binds to HK via D6, forming a complex. Plasma prekallikrein is activated either by activated fXII (fXIIa) or by cell bound prolylcarboxypeptidase (PRCP) to plasma kallikrein. Plasma kallikrein cleaves HK, triggering the kinin system cascade (see below) (Colman and Schmaier 1997).

### *Factor XII and factor XI*

FXII is a serine protease that is produced in the liver. It auto-activates in contact with negatively charges surfaces such as glass (Silverberg et al. 1980), hence the term "the contact system". However, *in vivo*, no evidence for auto-activation of FXII has been found but FXII may bind to receptors on endothelial cells and thus participate in the kinin system cascade by activating plasma prekallikrein. Activated fXII (fXIIa) may also cleave fXI to fXIa, thereby initiating the intrinsic pathway of coagulation. *In vitro* FXIa has been shown to play the same part as plasma kallikrein in the kinin system, but the *in vivo* relevance of this is unclear (Colman and Schmaier 1997).

## Activation of the kinin cascade

*In vivo* in plasma, activation of the kinin system commences when HK and plasma prekallikrein, circulating in complex (Mandle et al. 1976), bind to a receptor complex on endothelial cells consisting of the gC1q-receptor (gC1qR, originally identified as the receptor for the globular head of C1q) (Herwald et al. 1996; Joseph et al. 1996), urokinase plasminogen activator receptor (uPAR) (Colman et al. 1997) and cytokeratin-1 (CK-1) (Hasan et al. 1998; Joseph et al. 1999). After binding, plasma prekallikrein can be activated to plasma kallikrein by two means, either by activated fXII (fXIIa), which may bind to the same receptor complex as HK (Mahdi et al. 2002), or by the cell bound protease prolylcarboxypeptidase (PRCP) (Shariat-Madar et al. 2002; Shariat-Madar et al. 2002). Plasma kallikrein then cleaves HK into a heavy and a light chain and the vasoactive nonapeptide bradykinin (Colman and Schmaier 1997; Joseph and Kaplan 2005). Plasma kallikrein may also generate fXIIa from fXII thereby initiating a positive feedback loop, generating more plasma kallikrein and thereby more bradykinin (Figure 3). Bradykinin is further processed by carboxypeptidases (Erdos and Sloane 1962; Sheikh and Kaplan 1986) to des-arg$^9$-bradykinin and both bradykinin and des-arg$^9$-bradykinin are degraded to inactive metabolites by angiotensin-converting enzyme (ACE) (Sheikh and Kaplan 1986). The major inhibitor of the kinin system is C1 inhibitor that inhibits plasma kallikrein and fXIIa (Colman and Schmaier 1997; Joseph and Kaplan 2005) (Figure 2A and 3).

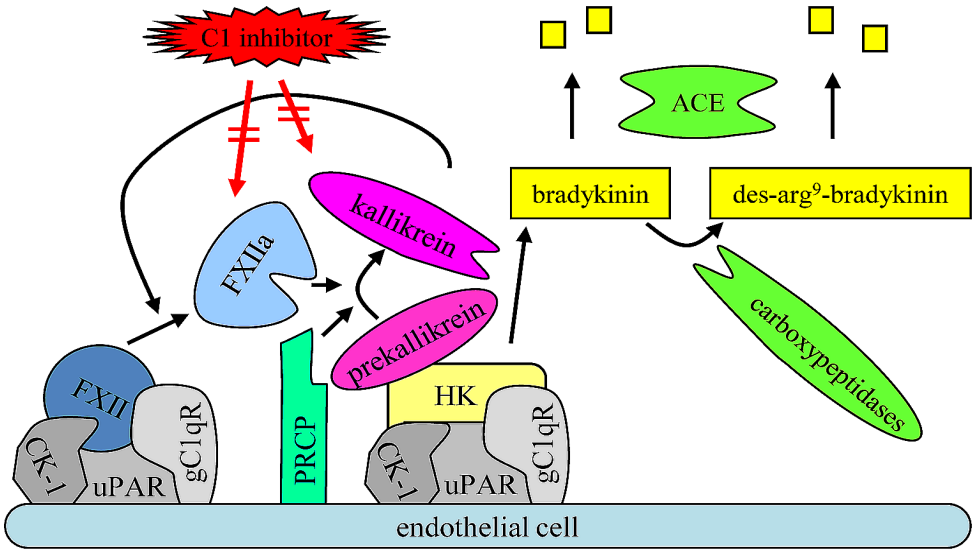


**Figure 3: Activation of the kinin system.** Activation of the kinin system commences when circulating complexes of plasma prekallikrein and high-molecular-weight kininogen (HK) bind to a receptor complex present on endothelial cells consisting of urokinase plasminogen activator receptor (uPAR), cytokeratin-1 (CK-1) and gC1q-receptor (gC1qR). Once bound plasma prekallikrein can be activated to plasma kallikrein either by prolylcarboxypeptidase (PRCP) or by activated factor XII (fXIIa). Plasma kallikrein may activate factor XII, thus initiating a positive feedback loop, resulting in the conversion of plasma prekallikrein to plasma kallikrein. Plasma kallikrein rapidly cleaves HK into a heavy and a light chain, and liberates the nonapeptide bradykinin. Bradykinin is degraded by carboxypeptidases to des-arg$^9$-bradykinin and both bradykinin and des-arg$^9$-bradykinin are degraded to inactive metabolites by angiotensin-converting enzyme (ACE). C1 inhibitor inactivates both plasma kallikrein and fXIIa, thereby inhibiting activation of the kinin system.

# Properties of the components of the kinin system

## *Kinins*

The kinins exert their effects via activation of two distinct kinin receptors (described separately below). Kinins have multiple effects *in vivo*, such as local regulation of blood pressure, promotion of inflammation, inhibition of platelet aggregation, induction of fibrinolysis and capillary leakage as well as stimulation of pain.

### *Bradykinin*

Bradykinin stimulates endothelial cells to produce nitric oxide (NO) leading to an immediate and transient blood pressure fall due to vasodilatation induced by relaxation of smooth muscle cells (Cockcroft et al. 1994). Bradykinin induces release of inflammatory mediators, such as IL-1β (Pan et al. 1996), IL-6 and IL-8 (Hayashi et al. 2000) from fibroblasts and stimulates endothelial cells to form superoxide (Holland et al. 1990) and induces prostacyclin expression (Hong 1980; Crutchley et al. 1983), which will, in turn, lead to both vascular relaxation and inhibition of platelet aggregation. Bradykinin exerts its profibrinolytic properties by inducing release of tissue plasminogen activator from endothelial cells (Smith et al. 1985). Bradykinin induces pain by stimulating kinin receptors on neural cells (Couture et al. 2001).

### *Des-arg$^9$-bradykinin*

Des-arg$^9$-bradykinin shares many of the same features as bradykinin. It induces NO production (Tsutsui et al. 2000) and smooth muscle relaxation. Prostacyclin is released by endothelial cells after stimulation with des-arg$^9$-bradykinin (Levesque et al. 1993; Duchene et al. 2009), whereas prostacyclin production in inhibited by des-arg$^9$-bradykinin in neural glia cells (Levant et al. 2006).

## *High-molecular-weight kininogen*

The main function of HK is the release of bradykinin after enzymatic degradation by plasma kallikrein, although HK has some additional properties. It inhibits thrombin-induced platelet activation and the heavy chain of HK has cysteine inhibitory effects, as D2 inhibits calpain whereas D2 and D3 both inhibit papain and cathepsin L, but the *in vivo* relevance of this is unclear (Colman and Schmaier 1997).

## *Plasma kallikrein*

The major function of plasma kallikrein is enzymatic cleavage of HK and subsequent bradykinin release. In addition, plasma kallikrein is chemotactic for neutrophils (Kaplan et al. 1972) and induces the secretion of neutrophil elastase (Wachtfogel et al. 1983). It is profibrinolytic, as it cleaves plasminogen to plasmin (Colman and Schmaier 1997) and activates pro-urokinase, a potent plasminogen activator (Ichinose et al. 1986).

## *Factor XII and factor XI*

In addition to their role in the kinin system, both fXII and fXI partake in the intrinsic pathway of coagulation. This pathway of coagulation commences by

auto-activation of fXII with subsequent activation of fXI. FXI in turn activates factor IX that activates factor X triggering the common pathway of coagulation resulting in the formation of fibrin from fibrinogen. The relevance of the intrinsic pathway of coagulation *in vivo*, is uncertain, however recently it has been suggested to have a role in thrombus formation (Gailani and Renne 2007).

# Bradykinin receptors

Kinin receptors can be expressed on many different cells, such as vascular endothelial cells, smooth muscle cells, fibroblasts, epithelial cells, neural cells and leukocytes (Leeb-Lundberg et al. 2005). There are two distinct kinin receptors: the $B_2$-receptor binds bradykinin and lys-bradykinin (kallidin) whereas the $B_1$-receptor binds des-arg$^9$-bradykinin and lys-des-arg$^9$-bradykinin (des-arg$^9$-kallidin) (Figure 2) (Leeb-Lundberg et al. 2005). The kinin receptors are encoded on the same locus, 14q32, by three exons each. Both kinin receptors belong to the group of G-coupled receptors that span the membrane seven times with the N-terminal region extending extracellularly (Leeb-Lundberg et al. 2005).

## *The $B_1$-receptor*

### *$B_1$-receptor expression and regulation*

In resting cells, the $B_1$-receptor is localized intracellularly in the endoplasmic reticulum and only minute amounts are present on the cell surface (Enquist et al. 2007). The receptor is expressed during inflammation and several inflammatory cytokines, such as interleukin 1 (IL-1), IL-2, epidermal growth factor (EGF) and interferon-γ (IFN-γ), may induce $B_1$-receptor expression, via the nuclear factor-κB (NF-κB) pathway (Leeb-Lundberg et al. 2005). *In vivo*, both lipopolysaccharide (LPS) treatment and ischemia induce $B_1$-receptor expression (Marceau et al. 1998; McLean et al. 1999; Schanstra et al. 2000; Mazenot et al. 2001).

When activated, the $B_1$-receptor is not phosphorylated and accordingly not desensitized (Blaukat et al. 1999; Leeb-Lundberg et al. 2005), resulting in sustained signaling. The cell surface expression of the receptor is upregulated when the receptor is stimulated (Schanstra et al. 1998) as ligand binding slows down the spontaneous internalization of the receptor seen under resting conditions (Enquist et al. 2007). Thus, stimulation of the $B_1$-receptor leads to both a prolonged and sustained signal.

## *$B_1$-receptor signaling*

When activated, the $B_1$-receptor initiates several proinflammatory and vasoactive events. $B_1$-receptor stimulation leads to G-protein induced phosphatidylinositide (PI) hydrolysis. Stimulation will result in prolonged elevation of intracellular $Ca^{2+}$ leading to a sustained signal. $B_1$-receptor stimulation activates endothelial nitric oxide synthase (eNOS) resulting in NO formation. In addition $B_1$-receptor stimulation induces proinflammatory eicosanoid production (Levesque et al. 1993; Leeb-Lundberg et al. 2005). In vitro, $B_1$-receptor-stimulation has been shown to be both proliferative and antiproliferative, effects mediated via the mitogen-activated protein kinase (MAPK) pathway. However, which effect that predominates *in vivo*, and under what circumstances, is unclear.

## *The (patho)physiological properties of the $B_1$-receptor*

The $B_1$-receptor is essential for inflammation and, in addition, it plays a role in tissue damage after ischemia as well as in nociception.

The $B_1$-receptor has been shown to be essential for neutrophilic inflammation in several animal models. $B_1$-receptor agonists induce neutrophil migration and chemotaxis (Ahluwalia and Perretti 1996; Ehrenfeld et al. 2006), an effect in part due to $B_1$-receptor induced ELR-CXCL chemokine production by endothelial cells (Duchene et al. 2007). The necessity of $B_1$-receptors for neutrophil inflammation has also been demonstrated in $B_1$-receptor-knock-out (B1KO) mice, in which neutrophil inflammation was almost totally abolished using a carrageenan-induced pleurisy model (Pesquero et al. 2000).

Another model for inflammation, in which streptozotocin-treated mice developed inflammation of the pancreatic islets, leading to insulin-dependent diabetes, a $B_1$-receptor antagonist was effective in attenuating the inflammation and thus preventing the development of diabetes (Zuccollo et al. 1999). These studies demonstrate the crucial role of $B_1$-receptors in mediating inflammation.

$B_1$-receptors have been suggested to play a part in attenuating the tissue damage after ischemia/reperfusion. This was shown in a mouse model of ischemia/reperfusion induced by bilateral renal artery occlusion followed by reperfusion. In this model, mice lacking both the $B_1$- and the $B_2$-receptor showed an increased degree of apoptosis, tissue damage and mortality as compared to $B_2$-receptor-knock-out (B2KO) and wild-type mice (Kakoki et al. 2007). However, the role of the $B_1$-receptor in ischemia/reperfusion is debated, as others have shown that B1KO mice have reduced pathology after ischemia/reperfusion both in the intestine and the heart (Lagneux et al. 2002; Souza et al. 2004).

The $B_1$-receptor also partakes in nociception. $B_1$-receptor mRNA can be found in neural sensory cells (Seabrook et al. 1997) and $B_1$-receptor agonists stimulate C-fibers in mouse spinal cords. *In vivo*, selective $B_1$-receptor antagonists have shown to attenuate diabetic hyperalgesia in mice (Gabra et al. 2006) and B1KO mice have reduced nociception in response to chemical and thermal stimulation (Pesquero et al. 2000).

## *The $B_2$-receptor*

### *$B_2$-receptor expression and regulation*

The $B_2$-receptor is ubiquitously and constitutively expressed. When the $B_2$-receptor is stimulated it is phosphorylated and desensitized followed by internalization and recycling of the receptor to the plasma membrane (Leeb-Lundberg et al. 2005). The rapid desensitization results in a transient signal.

During chronic inflammation, such as renal transplant rejection (Naidoo et al. 1996), $B_2$-receptors may be down-regulated. Similarly, in vitro experiments using human fibroblasts showed that prolonged stimulation with bradykinin resulted in down-regulation of both cell surface expression and de novo synthesis of $B_2$-receptors (Blaukat et al. 2003). In contrast, in fibroblast cell lines, inflammatory chemokines such as tumor necrosis factor-α (TNF-α) and IL-1β upregulated the $B_2$-receptor, an effect inhibited by dexamethasone (Haddad et al. 2000; Phagoo et al. 2000). The clinical significance and under which circumstances $B_2$-receptor up- or down-regulation occur are, as yet, unclear.

### *$B_2$-receptor signaling*

$B_2$-receptor signaling acts mainly via G-proteins leading to PI hydrolysis and an increase in intracellular free $Ca^{2+}$, although in contrast to the $B_1$-receptor the increase is transient (Leeb-Lundberg et al. 2005). Stimulation of the $B_2$-receptor also leads to translocation of protein kinase C to the cell membrane (Tippmer et al. 1994; Ross and Joyner 1997; Leeb-Lundberg et al. 2005). In similarity to $B_1$-receptor stimulation, $B_2$-receptor activation leads to eNOS stimulation and subsequent NO production (Busse and Fleming 1995; Leeb-Lundberg et al. 2005). Stimulation of the $B_2$-receptor results in several proinflammatory events mediated via the NF-κB pathway, such as expression of IL-1β, IL-6, IL-8 (Pan et al. 1996; Phagoo et al. 1999; Hayashi et al. 2000) as well as $B_1$-receptor upregulation (Phagoo et al. 1999), demonstrated in vitro in human fibroblasts.

$B_2$-receptor-stimulation may both induce proliferative or antiproliferative responses in vitro. The proliferative response may involve growth-factor dependent pathways and stimulation of MAPKs (Blaukat et al. 1999; Leeb-

Lundberg et al. 2005), whereas the antiproliferative response may be prostaglandin-mediated (Leeb-Lundberg et al. 2005). Whether $B_2$-receptors mediate proliferative or antiproliferative actions *in vivo* and under which circumstances is, as yet, unclear.

### *The (patho)physiological properties of the $B_2$-receptor*

When bradykinin stimulates $B_2$-receptors many of the cardinal symptoms of inflammation may arise, namely vasodilatation, edema and pain, as described above. However $B_2$-receptor stimulation may also have protective roles *in vivo* and it has been demonstrated that B2KO mice exhibit increased senescence-related changes (Kakoki and Smithies 2009).

In similarity to $B_1$-receptors, $B_2$-receptors have been shown to have a protective role in a mouse model of ischemia/reperfusion damage induced by bilateral renal artery occlusion followed by reperfusion. A contrary result was achieved in mouse intestine after ischemia/reperfusion in which $B_2$-receptor antagonists were shown to be protective (Souza et al. 2004; Kakoki et al. 2007).

$B_2$-receptors partake in nociception as they are present in neural cells, including sensory ganglions. When $B_2$-receptors are stimulated in blisters, in humans, they induce pain (Whalley et al. 1987) and $B_2$-receceptor signaling via protein kinase C induces activation of sensory neurons. In vitro, $B_2$-receptor stimulation has been shown to induce releases of sensory neuropeptides such as substance P. Stimulation of $B_2$-receptors in the brain has been shown to produce an immediate nociceptive response accompanied by a longer period of antinociception, due to acute stimuli of the sensory fibers followed by activation of inhibitory neurons (Couture et al. 2001).

## *Kinins and kinin receptors in disease*

### *Rheumatoid arthritis*

The kinin system has been proposed to partake in the inflammation during rheumatoid arthritis (RA) (Cassim et al. 2002). In patients with RA, kinin levels have been shown to be increased (Hargreaves et al. 1988) Neutrophils from the circulation and from synovial fluid express high levels of $B_1$- and $B_2$-receptors and surface immunolabelling of kinins was reduced indicating consumption of HK and subsequent kinin release (Cassim et al. 2009). $B_2$-receptors have been shown to be upregulated in synovial tissue from patients with RA (Bathon et al. 1992).

In animal models of arthritis, plasma kallikrein inhibitors and $B_2$-receptors antagonists have been shown to attenuate the symptoms in the joints and a $B_2$-

receptor antagonist was effective in reducing plasma extravasation early in the inflammation whereas a $B_1$-receptor antagonist was more effective later in the inflammation, indicating that $B_2$-receptors are important in early inflammation whereas $B_1$-receptors partake in chronic inflammation (Hargreaves et al. 1988; Fujimori et al. 1993; Sharma and Wirth 1996).

### *Inflammatory bowel disease*

Patients with ulcerative colitis have been shown to have kinin system activation as measured by reduced levels of plasma kallikrein and HK (Stadnicki et al. 1997) whereas patients with Crohn's diseases had no sign of systemic kinin system activation (Devani et al. 2002). In experimental colitis in mice, the $B_1$-receptor has been shown to be crucial for the development of inflammation as inflammation was markedly reduced using $B_1$-receptor antagonists and in B1KO mice (Hara et al. 2008).

### *Sepsis*

In severe sepsis the kinin system is activated (Colman and Schmaier 1997; Oehmcke and Herwald 2009) as exemplified by consumption of the kinin cascade proteins prekallikrein and fXII (Mason and Colman 1971; Wuillemin et al. 1995; Sriskandan and Cohen 2000). However, a double-blind randomized multicenter study using a $B_2$-receptor antagonist in patients with systemic inflammatory response syndrome and sepsis showed no improvement in outcome (Fein et al. 1997). This disappointing result may be explained by the complexity of inflammatory cascades where blockage of only $B_2$-receptors may be insufficient to achieve the expected results, inhibiting both $B_2$- and $B_1$-receptors would presumably be a better approach.

### *Atherosclerosis*

The kinin system is believed to play a role in the development of atherosclerosis (Ahluwalia et al. 2009). Kinin receptors have been shown to be upregulated in the atherosclerotic plaque in humans (Raidoo et al. 1997). In ApoE-KO mice, prone to develop atherosclerosis, $B_1$-rececptors were shown to be upregulated when these mice were feed a high-fat diet (Duchene et al. 2009). Low laminar-shear stress, believed to be present at sites of atheroma formation, induced $B_1$-receptor expression and increased both prostaglandin release and chemokine expression in endothelial cells in response to a $B_1$-receptor agonist (Duchene et al. 2009). Thus the authors concluded that drugs targeting the $B_1$-receptor could potentially be useful in treating atherosclerosis. On the other hand, others have shown that, when ApoE-KO mice were crossed with B1KO mice, these mice suffered more aortic lesions as compared to ApoE-KO mice with the $B_1$-receptor, suggesting that

upregulation of the $B_1$-receptor in atherosclerotic plaques could be protective (Merino et al. 2009).

Recently neutrophil inflammation was implicated in the development of atherosclerosis, as neutrophil depletion reduced plaque formation in ApoE-KO mice (Zernecke et al. 2008). Neutrophil inflammation has been shown to be dependent on $B_1$-receptors stimulation as shown using a $B_1$-receptor antagonist and B1KO mice (described above). Thus, as neutrophil inflammation has been suggested to partake in the pathogenesis of atherosclerosis a pathogenic role of $B_1$-receptors in atherosclerosis may be implied. Nevertheless, the role of $B_1$-receptors in atherosclerosis in humans is still unclear.

### *Hereditary angioedema*

Patients with hereditary angioedema have mutations in the gene encoding for C1 inhibitor leading to reduced levels of the inhibitor in the circulation. As C1 inhibitor is the major inhibitor of plasma prekallikrein and fXII, reduced levels of C1 inhibitor will lead to inappropriate kinin system activation and bradykinin release, accounting for the acute swelling seen during angioedema attacks. The pathogenic role of bradykinin is apparent as the $B_2$-receptor antagonist HOE-140 is effective in abrogating the attacks and is used in management of the disease (Zuraw 2008; Cugno et al. 2009).

### *$B_1$-receptor polymorphisms in clinical conditions*

Some clinical conditions such as hypertension (Dhamrait et al. 2003), inflammatory bowel disease (Bachvarov et al. 1998) and end-stage renal disease (Bachvarov et al. 1998), have been associated with reduced frequency of the $B_1$-receptor $G^{-699}\rightarrow C$ promoter polymorphism. The $G^{-699}\rightarrow C$ promoter polymorphism, more common in the control population, leads to increased promoter activity and therefore increased receptor transcription. Hence the patients with these conditions who have the $GG^{-699}$ sequence, instead of $CG^{-699}$ which was more frequent in the controls, would have reduced transcription of the receptor. This result could support the theory that $B_1$-receptors are protective, although the clinical relevance of the polymorphism is unclear.

### *$B_2$-receptor polymorphisms in clinical conditions*

$B_2$-receptor polymorphisms, resulting in lower concentrations of the receptor, have been associated with increased cardiac ventricular growth in response to exercise (Brull et al. 2001), poorer response to treatment in patients with left ventricular hypertrophy (Hallberg et al. 2003), hypertension (Dhamrait et al. 2003) and lower urinary albumin:creatinine levels in patients with diabetes (Maltais et al. 2002), but the clinical relevance of this is unclear.

### *Vasculitis*

Vasculitis is a systemic inflammatory disease affecting the blood vessels (described below). When the project leading to this thesis commenced no studies had previously addressed the role of the kinin system in this systemic inflammation. This thesis therefore addressed kinin system activation in patients with vasculitis and upregulation of $B_1$-receptors in an animal model mimicking the systemic inflammation seen in vasculitis.

# Vasculitis

## *Characteristics of vasculitis*

Vasculitis is the hypernym for diseases characterized by inflammation in and around vessel walls, accompanied by neutrophil influx leading to perturbed vessel patency and secondary tissue damage. The clinical presentation depends on which vessels are afflicted and the severity of the inflammation.

## *Classification of vasculitis*

Vasculitis it commonly classified according to the size of the predominantly affected vessels, dividing the vasculitides into large-vessel, medium-sized-vessel and small-vessel vasculitis (Hunder et al. 1990) (Table 1). Consideration is also taken based on histopathology and clinical symptoms. The presence and type of anti-neutrophil cytoplasmic antibodies (ANCAs; described below) in serum may be of guidance in classification of vasculitides (Jennette et al. 1994) and is clinically a valuable diagnostic tool. In this thesis the focus will be on small-vessel vasculitides, with special reference to ANCA-associated vasculitides.

Table 1: Classification of systemic vasculitides

| **Large-vessel vasculitis** | **Medium-sized-vessel vasculitis** | **Small-vessel vasculitis** |
|---|---|---|
| Temporal Arteritis | Polyarteritis Nodosa | Wegener's Granulomatosis |
| Takayasu Arteritis | Kawasaki Disease | Microscopic Polyangiitis |
| | | Henoch Schönlein Purpura |

## *Clinical presentation of vasculitis*

In vasculitis, various organs may be afflicted, but most commonly the kidneys, respiratory tract and skin are involved. Patients may exhibit various symptoms such as renal dysfunction, respiratory symptoms, sinus inflammation, purpura, joint pain and swelling, abdominal pain, gastrointestinal bleeding, as well as other symptoms depending on which organs are afflicted. Specific vasculitides may have a predilection for particular organs, for example, Wegener's Granulomatosis commonly affects the upper airways, lungs and kidneys, whereas microscopic

polyangiitis commonly affects kidneys and lungs, but the upper airways are spared. A special feature of Wegener's Granulomatosis is the formation of granulomas consisting of leukocytes. The organization of these granulomas resembles lymphoid structures (Savage et al. 1997; Voswinkel et al. 2005).

In children, a milder and often transient, vasculitis is more common. Henoch-Schönlein Purpura (HSP) presents as petechial skin rash, arthralgia or arthritis, hematuria and bowel pain and/or gastrointestinal bleeding. The disease is usually self-limited and often resolves within a few months, although some patients may develop a severe form of glomerulonephritis (Tizard and Hamilton-Ayres 2008).

# The pathogenesis of vasculitis

## Neutrophils and neutrophil-related proteins in vasculitis

Neutrophil extravasation is a key finding in vasculitis and much of the damage seen in the vessels in vasculitis is believed to be due to neutrophil activation and degranulation. The pathogenic role of the neutrophil has been shown in an animal model of vasculitis in which neutrophil depletion resulted in total abrogation of disease activity (Xiao et al. 2005). Neutrophils contain enzymes believed to be important in the pathophysiology of vasculitis. Proteinase 3 (PR3) and myeloperoxidase (MPO) have been extensively studied, as certain subsets of patients with vasculitides have ANCAs directed to either or both of these enzymes. Both the enzymes and the antibodies are believed to be important in the pathogenesis of vasculitis, as they possess several proinflammatory properties.

### *Proteinase 3*

PR3 is a serine protease with high sequence homology to human neutrophil elastase. The gene coding for PR3 is located on chromosome 19p13.3 and consists of five exons and four introns (Zimmer et al. 1992). The molecular weight of PR3 is between 29-32 kD depending on the degree of glycosylation (van der Geld et al. 2001). In plasma, the activity of PR3 is inhibited by α-$_1$-antitrypsin ($\alpha_1$-AT) (Rao et al. 1991).

#### *Expression of proteinase 3*

PR3 is expressed by granulocytes and monocytes and stored in the azurophilic granules, the specific granules and the secretory vesicles. In the azurophilic granules, PR3 is kept in a conformationally inactive form, due to the acidic environment. Upon degranulation, translocation to an environment with neutral pH allows PR3 to become enzymatically active (van der Geld et al. 2001). PR3 is also expressed in cell-bound form on the membrane of neutrophils and the number of neutrophils expressing membrane-bound PR3 is genetically determined and varies

between individuals (Halbwachs-Mecarelli et al. 1995). Nevertheless, isolated neutrophils from different individuals, regardless of genetic background can upregulate membrane-bound PR3 when primed with TNF-α or IL-8 (Csernok et al. 1994; van der Geld et al. 2001).

Whether endothelial cells express PR3 or not is debated as some studies have shown PR3 expression after stimulation with TNF-α, IL-1α or IFN-γ treatment (Mayet et al. 1993) although others have not been able to detect any PR3 expression (King et al. 1995; Pendergraft et al. 2000). PR3 has, however, been shown to bind to endothelial cells in vitro (Ballieux et al. 1994; Taekema-Roelvink et al. 2000).

## *Functions of proteinase 3*

The main functions of PR3 are related to inflammatory processes. PR3 has antimicrobial properties, facilitates neutrophil migration, activates several proinflammatory proteins, induces apoptosis, and regulates granulocyte and monocyte growth and differentiation during hematopoiesis.

### *Antimicrobial properties of proteinase 3*

PR3, as well as other neutrophil-derived serine proteases, has antimicrobial properties and killing of bacterial is believed to be one of the major functions of PR3. PR3 is effective against both Gram-positive and Gram-negative bacteria (van der Geld et al. 2001).

### *Neutrophil migration*

Neutrophil migration through the basement membrane is facilitated by PR3, as it degrades several extracellular matrix proteins, such as elastin, proteoglycans, collagen IV and fibrinogen (van der Geld et al. 2001).

### *Modulation of inflammation*

PR3 may modulate inflammation as it induces secretion and activation of several inflammatory proteins.

*TNF-α*: In vitro, PR3 has been shown to cleave off TNF-α from the cell surface releasing it into the circulation and thereby activating TNF-α (Robache-Gallea et al. 1995).

*IL-8*: PR3 both enhances the production of IL-8 from endothelial cells (Berger et al. 1996) and processes it to become more active (Padrines et al. 1994). This would theoretically lead to increased neutrophil chemotaxis.

*IL-1β and IL-18*: The proinflammatory cytokines IL-1β and IL-18, synthesized as proforms, may be cleaved and activated by PR3 (Sugawara et al. 2001; Wiedow and Meyer-Hoffert 2005).

*IL-2 and IL-6 receptors*: PR3 may cleave off the IL-2 receptor and the IL-6 receptor from cell membranes and thereby modulate inflammation but whether this accounts for a pro- or anti-inflammatory effect *in vivo*, is uncertain (Bank et al. 1999).
*Progranulin*: PR3 has been shown to inactivate the anti-inflammatory protein progranulin both *in vitro* and *in vivo*. *In vitro*, purified PR3 rapidly degraded and inactivated progranulin. Neutrophil lysates from wild-type mice degraded progranulin whereas neutrophil lysates from PR3 and elastase double knock-out mice did not. In the PR3 and elastase double knock-out mice, progranulin was not degraded in neutrophil lysates harvested from peritoneal fluid after induction of peritonitis by casein injection. In addition, neutrophil infiltration was markedly reduced in an immune complex-mediated disease model in the PR3 and elastase double knock-out mice (Kessenbrock et al. 2008), indicating that cleavage of progranulin by PR3 may be relevant in enhancing inflammation *in vivo*.

### *Induction of apoptosis*

PR3 has been shown to induce apoptosis in endothelial cells by cleavage of p21, resulting in loss of function and subsequent apoptosis (Pendergraft et al. 2004). *In vitro*, PR3 has been shown to bind to and cause endothelial cell detachment and cytolysis (Ballieux et al. 1994; Ballieux et al. 1994).

### *Proteinase 3 and the kinin system*

PR3 may facilitate kinin system activation as it cleaves and inactivates C1 inhibitor, the major inhibitor of the kinin system (Leid et al. 1993). Other interactions between PR3 and the kinin system will be addressed in this thesis.

### *Regulator of hematopoiesis*

During hematopoiesis PR3 may serve as regulator of granulocyte and monocyte growth and differentiation. In early myeloid hematopoiesis, PR3 is upregulated and induces proliferation, but at a later stage, when PR3 is down-regulated, differentiation will occur (Bories et al. 1989; van der Geld et al. 2001).

## *Proteinase 3 in vasculitis*

Patients with active vasculitis have more cell-bound PR3 on their neutrophils than the same patients during remission as well as healthy controls (Muller Kobold et al. 1998). The plasma level of PR3 is also elevated in patients with vasculitis (Baslund et al. 1994; Henshaw et al. 1994). PR3 has multiple immunomodulating effects demonstrated both *in vitro* and *in vivo*. These effects may be of relevance in the pathogenesis of vasculitis. For example IL-8, of importance for neutrophil chemotaxis, is activated by PR3. IL-8 has been detected in affected tissues during vasculitis and endothelial cells express IL-8 in response to PR3. PR3-induced endothelial cell apoptosis could further aggravate the endothelial cell injury seen

in vasculitis. The proteolytic effect of PR3 on C1 inhibitor, leading to inactivation of C1 inhibitor, could result in uncontrolled kinin system activation, which would in turn intensify the inflammatory response. Other specific interactions between PR3 and components of the kinin system are addressed in this thesis.

## *Myeloperoxidase*

MPO is a peroxidase with a molecular weight of approximately 140 kD. It is stored, along with PR3 and elastase, in the azurophilic granules of neutrophils. *In vivo*, MPO is inhibited by ceruloplasmin (Segelmark et al. 1997). MPO catalyzes the production of the highly toxic hypochlorous acid from hydrogen peroxide and halides and it oxidizes tyrosine to tyrosyl radical (Klebanoff 1968; Heinecke et al. 1993). Both hypochlorous acid and tyrosyl radical are cytotoxic and consequently one major function of MPO is the killing of pathogens. In addition, MPO may inactive $\alpha_1$-AT, the main inhibitor of PR3, and hence facilitate uninhibited PR3 activity (Weiss 1989).

MPO is not expressed by endothelial cells (Pendergraft et al. 2000) but it may bind to the cells (Savage et al. 1993).

## *Proteinase 3 - Anti-Neutrophil Cytoplasmic Antibodies*

PR3-ANCAs are IgG autoantibodies directed against PR3. PR3-ANCAs are associated with Wegener's Granulomatosis and a rise in PR3-ANCA titers may precede a relapse of the disease.

### *Epitope specificity*

PR3-ANCAs are oligo-clonal and the antibodies recognize different epitopes on PR3 *in vivo*. During active disease and remission PR3-ANCAs have been shown to exhibit epitope shifting and spreading. PR3-ANCAs may bind to or near the enzymatic site of PR3, thereby inhibiting the enzymatic activity. However, the antibodies may bind to PR3, without interfering with the enzymatic site, but inhibiting the binding of $\alpha_1$-AT, the main inhibitor of PR3, resulting in loss of inhibition of PR3 in the circulation. There have even been reports of PR3-ANCAs that enhance the enzymatic activity of PR3 (van de Wiel et al. 1992; Daouk et al. 1995). The pathophysiological role of this epitope shifting and spreading is unclear, however, it may be of importance as it has been shown, on a molar basis, that during active disease PR3-ANCAs are relatively less efficient in inhibiting the enzymatic activity of PR3 than during remission (van der Geld et al. 2002). This may indicate that, during active disease, PR3-ANCAs may not be able to neutralize the increased levels of PR3 expressed on and secreted from activated neutrophils, resulting in uninhibited PR3 activity.

### *Induction of PR3-ANCA production*

There is no clear evidence yet as to what initiates ANCA production and if they are secondary to neutrophil influx and secretion of PR3 during vasculitis or the primary cause of vasculitis. A theory for PR3-ANCA production in patients with Wegener's Granulomatosis has been proposed. In the granulomas, detected in patient tissues, neutrophils, dendritic cells, B and T cells are organized resembling lymphoid tissue. The presence of neutrophils expressing PR3 in close proximity to dendritic cells, B and T cells may sever tolerance and lead to subsequent ANCA production by plasma cells (Voswinkel et al. 2005; Mueller et al. 2008). However, the events initiating and maintaining ANCA production *in vivo* are still uncertain.

Others have proposed a different theory for the production of PR3-ANCAs. It has been demonstrated that patients with PR3-ANCA also have antibodies against the peptide transcribed from the antisense DNA. The sequence from the antisense DNA was found to mimic sequences in bacteria, such as *Staphylococcus aureus* (Pendergraft et al. 2004). In patients with Wegener's Granulomatosis, chronic nasal carriage of these bacteria is related to higher frequency of relapses (Stegeman et al. 1994). When mice were immunized with the peptide transcribed from the antisense DNA they developed antibodies against the peptide as well as against PR3 (Pendergraft et al. 2004).

However intriguing these theory are, it is still unclear what initiates and maintains ANCA production *in vivo*.

### *Leukocyte activation by PR3-ANCA in vasculitis*

When primed with inflammatory mediators such as TNF-α, neutrophils and monocytes upregulate the expression of PR3 on their surface, this may serve as a binding site for PR3-ANCA. PR3-ANCA may then further activate the leukocytes, requiring both PR3 and Fc receptor binding for full effect, as $F(ab')_2$-fragments are only able to induce activation to a lesser extent (Porges et al. 1994; Kettritz et al. 1997). The activation of neutrophils by PR3-ANCA manifests in different ways.

*Degranulation and oxidative burst*: PR3-ANCA stimulates neutrophils to degranulate and undergo oxidative burst *in vitro*, an effect that is increased when the neutrophils are primed with TNF-α (Falk et al. 1990; Charles et al. 1991).

*Interleukin expression*: When neutrophils are stimulated by PR3-ANCAs (and MPO-ANCAs), *in vitro*, they release IL-1 (Brooks et al. 1996) and IL-8 (Cockwell et al. 1999; Hsieh et al. 2007). Monocytes release TNF-α, IL-1β, IL-6 and IL-8 when stimulated with PR3-ANCA (Ralston et al. 1997; Hattar et al. 2002). IL-8 is believed to be important in the inflammation seen in vasculitis and is present at the

site of damage (Cockwell et al. 1999), but whether this is a secondary phenomenon due to increased inflammation or a primary event, is, as yet, unclear.

*Complement activation*: PR3-ANCA binding to neutrophils *in vitro* has been shown to cause release of unidentified factors from neutrophils that induce complement activation via the alternative pathway (Xiao et al. 2007).

*Neutrophil extracellular trap formation*: When neutrophils are stimulated they release neutrophil extracellular traps (NETs). NETs may trap and kill microbes but have also been shown to cause tissue damage during sepsis (Brinkmann et al. 2004; Clark et al. 2007). Primed neutrophils expressing PR3 and MPO on their surface have been shown to release NETs when stimulated with either PR3- or MPO-ANCA. The NETs have been shown to be deposited in the glomeruli of patients with vasculitis and these NETs contain PR3 and MPO which may serve as binding sites for ANCAs. This could represent a novel pathway for neutrophil-induced tissue damage in vasculitis (Kessenbrock et al. 2009).

*Opsonization*: PR3-ANCA may bind to primed or apoptotic neutrophils expressing PR3 and thereby facilitate phagocytosis by macrophages. When the macrophages phagocytize opsonizied neutrophils they secrete TNF-α, which may further promote inflammation (Moosig et al. 2000; Hsieh et al. 2007)

In conclusion, PR3-ANCA has multiple proinflammatory effects on leukocytes, including degranulation, interleukin expression and complement activation and is thus believed to partake in the pathophysiology of vasculitis.

### *Endothelial cell activation by PR3-ANCA in vasculitis*

In addition to activating leukocytes, which may induce endothelial cell damage (Savage et al. 1992), PR3-ANCA may bind to and activate endothelial cells. Whether endothelial cells themselves express PR3 is controversial (Mayet et al. 1993; King et al. 1995; Pendergraft et al. 2000), but PR3 may bind to endothelial cells and thereby serve as a binding site for PR3-ANCA (Ballieux et al. 1994; Taekema-Roelvink et al. 2000). PR3-ANCA has been shown to directly activate the endothelium resulting in IL-1 and tissue factor expression (de Bandt et al. 1997). PR3-ANCA also induces IL-8 release from endothelial cells (Mayet et al. 1999), this may further aggravate the inflammation occurring during vasculitis.

### *Neutrophil – endothelial cell interactions induced by PR3-ANCA*

*In vitro* experiments were carried out in which neutrophils were flowed over endothelial cells. When the neutrophils were stimulated with PR3-ANCA they started to roll, an effect mediated via $\beta_2$ integrins, and migrate, mediated via

chemokine receptors (Calderwood et al. 2005). This would indicate that PR3-ANCA may induce neutrophil extravasation in vasculitis. In addition, PR3-ANCA also induced actin polymerization and alteration of neutrophil cell shape. This change in shape could, theoretically, lead to deposition in small arteries and might explain why ANCA-associated vasculitides have a preference for small vessels (Tse et al. 2005).

In conclusion, PR3-ANCA stimulation leads to upregulation of several proinflammatory peptides from both endothelial cells and neutrophils, and PR3-ANCA induces rolling and migration of neutrophils. Neutrophils degranulate in response to PR3-ANCA. This causes tissue damage and induces additional inflammatory signals, thus further recruiting inflammatory cells. These properties are believed to be important in the pathogenesis of vasculitis.

## *Myeloperoxidase - Anti-Neutrophil Cytoplasmic Antibodies*

MPO-ANCAs are IgG antibodies directed against MPO. MPO-ANCAs are associated mainly with microscopic polyangiitis.

### *Epitope specificity*

MPO-ANCAs are mono- or oligoclonal and may bind to different epitopes on MPO, although the antibodies have been shown to recognize a restricted number of epitopes on MPO (Audrain et al. 1997; van der Geld et al. 2004). Depending on the recognized epitope, different MPO-ANCAs have been shown to inhibit the oxidation activity of MPO (Zhang et al. 2007), interfere with the binding of its inhibitor ceruloplasmin (Griffin et al. 1999; van der Geld et al. 2004), or enhance the oxidative activity of MPO (Guilpain et al. 2007).

### *Leukocyte activation*

When neutrophils are activated they express MPO on their cell membrane, and thus MPO-ANCA may bind to the cells. In similarity to PR3-ANCA, activation of neutrophils by MPO-ANCA involves Fc receptor binding, whereas $F(ab')_2$-fragments are only able to induce limited activation (Porges et al. 1994; Kettritz et al. 1997). Binding of MPO-ANCA will lead to cell activation manifested in several ways.

*Degranulation and oxidative burst*: In similarity with PR3-ANCA, MPO-ANCA induces neutrophil degranulation and oxidative burst (Falk et al. 1990; Charles et al. 1991).

*Interleukin expression*: When neutrophils are stimulated with MPO-ANCAs, *in vitro*, they release IL-1 (Brooks et al. 1996) and IL-8 (Cockwell et al. 1999; Hsieh et al. 2007).

*Complement activation*: MPO-ANCA, like PR3-ANCA, has been shown to induce neutrophils to secrete factors that induce complement activation via the alternative pathway (Xiao et al. 2007).

*Apoptosis and opsonization:* MPO-ANCA has been shown to induce neutrophil apoptosis, but the mechanisms by which this occurs are unclear. Phagocytosis of neutrophils by macrophages is facilitated by binding of MPO-ANCA to the neutrophils (Harper et al. 2001; Hsieh et al. 2007).

### *Neutrophil – endothelial cell interactions induced by MPO-ANCA*

MPO-ANCA induces neutrophil activation and subsequent endothelial cell damage (Savage et al. 1992). In similarity to PR3-ANCA, MPO-ANCA induces neutrophil rolling and migration through endothelial cell monolayers *in vitro* (Calderwood et al. 2005) and MPO-ANCA induces actin polymerization accompanyed by a conformational change of neutrophils, that could lead to neutrophil deposition in the small arteries (Tse et al. 2005). Complexes of MPO and MPO-ANCA may bind to endothelial cells and could lead to complement activation (Savage et al. 1993).

In conclusion, MPO-ANCA binding to neutrophils will lead to several proinflammatory events. MPO-ANCA may induce oxidative burst, interleukin expression and release of complement activating factors. MPO-ANCA may also induce neutrophil apoptosis and facilitate neutrophil phagocytosis by macrophages. In similarity to PR3-ANCA, MPO-ANCA will induce neutrophil rolling and migration through endothelial cells into the tissue. Several *in vivo* models for vasculitis, described in detail below, have demonstrated the pathogenic potential of MPO-ANCA and thus MPO-ANCAs are believed to be of major importance in the pathogenesis of vasculitis.

## *Lysosomal membrane protein-2 antibodies*

Antibodies against lysosomal membrane protein-2 (LAMP-2) have been demonstrated in patients with vasculitis (Kain et al. 1995). These antibodies are believed to arise due to cross-reactivity against the bacterial adhesin, FimH, which shares 100% homology with the epitope that the LAMP-2 auto-antibodies are directed towards. Almost all patients tested in one cohort had antibodies against LAMP-2 (Kain et al. 2008) although this finding has, as yet, not been confirmed by others. LAMP-2 antibodies activate neutrophils *in vitro* and induce

degranulation as demonstrated by shape changes and myeloperoxidase release. In addition, LAMP-2 antibodies induced endothelial cell apoptosis, *in vitro*, as detected by cells expressing cleaved caspase-3. Finally, *in vivo* injection of rats with antibodies against human-LAMP-2 resulted in renal pathology resembling human vasculitis (Kain et al. 2008). Thus, antibodies against LAMP-2 may be involved in the pathogenesis of small-vessel vasculitis.

# T cells in vasculitis

The interest in T cell involvement in vasculitis has increased during recent years and evidence for a pathogenic role of T cells in vasculitis is mounting.

## *In the tissue*

The number of T cells is increased in the interstitium of kidneys from patients with ANCA-associated vasculitides. T cells are predominantly found in the interstitium around sclerotic and crescent-forming glomeruli but not within the glomeruli (Weidner et al. 2004).

T cells are also present in the granulomas of patients with Wegener's Granulomatosis, where PR3-expressing neutrophils are surrounded by T cells, B cells and dendritic cells. As described above, these granulomas resemble lymphoid structures and are hypothesized to be the site for break of tolerance and induction of antibodies against PR3, providing a potential role for T cells in the induction of ANCA production (Voswinkel et al. 2005; Mueller et al. 2008), although the mechanism for this is still unclear.

## *In the circulation*

T cell activation markers, such as soluble IL-2 receptor and soluble CD30, are increased in patients with PR3-ANCA-associated vasculitis and high levels of theses markers correlate with PR3-ANCA titers (Sanders et al. 2006). It has been shown that T cells from patients with PR3- or MPO-ANCA-associated vasculitides proliferate in response to PR3 or MPO, respectively (Brouwer et al. 1994; Griffith et al. 1996), and expansion of cytotoxic effector memory T cells, probably due to repeated antigen stimulation (Abdulahad et al. 2006), has been documented. The number of T regulatory cells was reduced in patients with vasculitis. In addition, the function was impaired as the cells were not able to suppress T cell proliferation in response to PR3. The reduced concentration and impaired function of T regulatory cells in patients with vasculitis has been proposed to be of importance in the pathogenesis of vasculitis (Morgan et al. 2010). In the circulation patients have a relative increase in T cells producing IL-17 in response to PR3 stimulation (Abdulahad et al. 2008). IL-17 is believed to

participate in the pathogenesis of other autoimmune diseases, such as rheumatoid arthritis and systemic lupus erythematosis (Abdulahad et al. 2009), and may be of importance in vasculitis.

A model was proposed for the pathophysiological role of T cells in vasculitis (Abdulahad et al. 2009). It was suggested that a chain of events commencing with T cells producing IL-17, thus activating macrophages to produce IL-1β and TNF-α, that in turn prime neutrophils and upregulate membrane expression of PR3. Repeated antigen stimulation by PR3 and reduced function of regulatory T cells would result in B cell stimulation with subsequent PR3-ANCA production and differentiation of T cells to cytotoxic effector memory T cells, which would result in tissue damage (Abdulahad et al. 2009). However exciting this proposed model for T cell participation in the pathogenesis of vasculitis is, the exact role of T cells in vasculitis remains to be clarified.

## B cells in vasculitis

B cells are believed to partake in the pathogenesis of vasculitis in two different ways. B cells may differentiate to plasma cells which produce ANCAs as well as partake as antigen-presenting cells. B cells are present in granulomas (described above). Drugs aimed at depleting B cells (such as Rituximab) are sometimes used in autoimmune diseases, such as rheumatoid arthritis, but only a limited amount of studies have been performed in patients with vasculitis, although with encouraging results, thus suggesting a role for B cells in the pathogenesis of vasculitis (Hinze and Colbert 2008; Walsh and Jayne 2009).

## Microparticles in vasculitis

Microparticles (MPs) may be released from a great variety of cells including leukocytes, endothelial cells and platelets. Although MPs are released under resting conditions, cell activation as well as apoptosis increase the release of MPs (Distler et al. 2006). MPs are defined as small membrane vesicles with a size of 0.1 – 1 μm carrying cellular membrane markers (Distler et al. 2006). When MPs bud off, the cell membrane of the MP may "flip-flop" leading to expression of negatively-charged phospholipids which are procoagulant (Nomura et al. 2008), although not all MPs turn their membrane inside-out (Burnier et al. 2009). As MPs are cell-derived, their properties reflect the status of the parent cells and consequently MPs share many of the same properties as their origin cells.

### *Neutrophil-derived MPs*

Neutrophil-derived MPs may influence vascular biology by inducing endothelial cell activation, demonstrated by IL-6 expression from the endothelial cells (Mesri and Altieri 1998; Mesri and Altieri 1999). Neutrophil-derived MPs may carry both PR3 and MPO on their surface (Gasser et al. 2003), which may serve as binding sites for ANCA. Leukocyte-derived MPs have been shown to transfer membrane-bound chemokine receptors between cells (Mack et al. 2000), providing evidence for MPs as mediators of inter-cellular crosstalk.

### *T cell-derived MPs*

T cell-derived MPs have been shown to impair endothelial functions, such as prostacyclin and NO formation (Martin et al. 2004). These deleterious effects were speculated to be of importance during cardiovascular and immune disorders.

### *Endothelial-derived MPs*

Endothelial-derived MPs have been demonstrated to bind C1q on their surface. This may result in complement activation via the classical pathway (Nauta et al. 2002), leading to enhanced inflammation via complement-induced chemotaxis.

### *The role of MPs in vasculitis*

MPs from endothelial cells, neutrophils and platelets are found in increasing numbers during active vasculitis but not during remission (Brogan and Dillon 2004; Brogan et al. 2004; Daniel et al. 2006). Endothelial-derived MPs express activation markers, suggesting that the endothelium releases MPs when activated rather than when undergoing apoptosis. Whether MPs participate in the pathogenesis of vasculitis or whether they are merely epiphenomena of cellular activation is unclear. However, endothelial-derived MP levels may be useful for evaluation of the degree of inflammation in vasculitis as levels have been shown to correlate with clinical assessment scores (Brogan et al. 2004).

## Animal models of vasculitis

In recent years several new animal models of vasculitis based on the production of auto-antibodies have emerged. Animal models of vasculitis based on auto-antibodies as well as other models will be presented below.

### *PR3-ANCA in animal models of vasculitis*

Until recently, there was no animal model for PR3-ANCA-associated vasculitis. However, immunizing non-obese-diabetic (NOD) mice, predisposed for autoimmunity, with recombinant mouse PR3 resulted in the development of anti-

mouse PR3-antibodies. Although these mice did not develop disease, when splenocytes from the immunized mice were transferred to NOD – severe combined immunodeficiency (NOD-SCID) mice, the mice developed renal failure and crescentic glomerulonephritis (Primo et al. 2010). This constitutes the first animal model, in which PR3-ANCA has been shown to induce disease, but this animal model for vasculitis has some limitations. Disease was only demonstrated in NOD-SCID mice that have a severely deficient immune system, thus the development of vasculitis in this animal model may not resemble the pathogenesis of human PR3-ANCA-associated vasculitides.

### *MPO-ANCA in animal models of vasculitis*

In the first MPO-ANCA model of vasculitis, MPO knock-out mice were immunized with mouse MPO and developed anti-MPO antibodies. When IgG from these mice was transferred to recombinase-activating gene-2–deficient mice, which lack functional B and T cells, or to wild-type C57BL/6J mice, they developed pauci-immune glomerular necrosis and crescent formation, features that are typical for ANCA-associated vasculitides (Xiao et al. 2002). Manifestations of vasculitis were less prominent in the wild-type C57BL/6J mice. This constituted the first *in vivo* model of vasculitis that focused on the pathogenic role of MPO-ANCA. It has further been established, in this animal model, that bacterial LPS aggravates the disease, in part via TNF-α. TNF-α levels increased after administration of LPS and anti-TNF-α treatment attenuated disease development (Huugen et al. 2005). The necessity of neutrophils for mediating disease was demonstrated by neutrophil depletion, which abrogated disease activity (Xiao et al. 2005). It has also been shown that complement activation, via the alternative pathway, plays a crucial role in this model, as factor B-KO or C5-KO mice did not develop disease (Xiao et al. 2007). Likewise, inhibition of C5 with a monoclonal antibody prevented the development of disease if administered early (Huugen et al. 2007) and mice deficient in C5a-receptors on neutrophils did not develop disease (Schreiber et al. 2009). These results demonstrate the importance of pathophysiological interactions between neutrophils and complement in ANCA-associated vasculitides, as both neutrophils and complement activation via the alternative pathway are a prerequisite for disease development. This opens for therapies, targeting neutrophils, complement or the interactions between them.

Another animal model for MPO-ANCA-associated vasculitis has been presented. In this animal model rats were immunized with human MPO and subsequently developed anti-human-MPO antibodies that cross-reacted with rat MPO as demonstrated by binding of the antibodies to rat neutrophils. The rats developed pauci-immune crescentic glomerulonephritis and pulmonary hemorrhage, which are key findings in a subset of human MPO-ANCA-associated vasculitides (Little et al. 2005). Treating the rats with TNF-α inhibitors, starting 28 days after

immunization, markedly reduced glomerulonephritis and pulmonary hemorrhage, indicating that TNF-α plays a pivotal role in the pathogenesis of MPO-ANCA-associated vasculitis in this model (Little et al. 2006). These results further demonstrated the pathogenic potential of MPO-ANCA and stress the importance of inflammatory mediators in diseases development.

In conclusion, animal models of MPO-ANCA-associated vasculitides are well-established, as compared with PR3-ANCA-associated vasculitides. Important data have been assembled from these animal models regarding the pathogenic potential of MPO-ANCA, the role of neutrophils, T cells and complement, as well as the importance of inflammatory mediators, such as TNF-α, on induction and maintenance of inflammation in vasculitis.

### *LAMP-2 in an animal model of vasculitis*

When human LAMP-2 antibodies were injected into rats, they rapidly developed signs of renal affection, such as hematuria and increased urine albumin:creatinine ratio. Renal pathology showed leukocyte infiltration, focal capillary necrosis and crescent formation starting after 24 hours. The authors did not find any IgG deposited in the glomeruli. Thus, this animal model mimics the pauci-immune glomerulonephritis seen during human vasculitis (Kain et al. 2008).

### *MRL$^{lpr/lpr}$ mouse model of vasculitis*

The MRL$^{lpr/lpr}$ mouse is a well established animal model of systemic inflammation and is commonly used as a model for systemic lupus erythematosis. The MRL$^{lpr/lpr}$ mouse spontaneously develops an immune complex mediated renal vasculitis. The vasculitic lesions are believed to consist mainly of T cells and, to a lesser extent, B cells (Tarkowski et al. 1988). However, others have demonstrated that the mice have two different types of inflammatory lesions, one type with mononuclear cell infiltrates and the other with polymorphonuclear cells (Alexander et al. 1985). In addition, a subpopulation of MRL$^{lpr/lpr}$ mice may present with MPO-ANCAs (Harper et al. 1998). Thus the MRL$^{lpr/lpr}$ mouse model has some, but not all, of the features of human small-vessel vasculitis, and may be useful for studying the pathogenesis of vasculitis.

# Present investigation

## Aims

- To investigate if the kinin system is activated in patients with vasculitis
- To investigate if neutrophil-derived PR3 cleaves HK and liberates a vasoactive kinin
- To investigate if PR3-ANCA inhibits PR3-induced HK proteolysis
- To investigate if $B_1$-receptors are upregulated in the circulation and in the kidney in mice with systemic inflammation and vasculitis

# Experimental conditions and results

## *Paper I: The kinin system is activated in children with vasculitis*

At the start of this investigation there were no reports on kinin system activation in patients with vasculitis and activation had only been demonstrated in a limited number of clinical inflammatory conditions, such as ulcerative colitis and sepsis. We set out to demonstrate kinin system activation in vasculitis, as several of the features of vasculitis, such as inflammation, plasma leakage and pain, could theoretically be explained by kinin system activation.

Seventeen children, aged 4 – 19 years, with various small-vessel vasculitides, including HSP and ANCA-associated vasculitis were included in the study and compared with 21 pediatric controls. Plasma samples as well as kidney and skin biopsies were taken at the acute onset of disease. The patients had no immunosuppressive treatment at time of sampling.

Proteolysis of HK in plasma, indicating kinin system activation, was demonstrated in 13/17 patients but only in one control ($p < 0.0001$), using immunoblotting. In the patients' plasma, bradykinin levels were elevated (median 320 ng/L) as compared to the controls (median 11 ng/L, $p = 0.0004$), measured by ELISA. *In situ* staining of kinins revealed local release of kinins at sites of inflammation both in the kidney and skin biopsies from patients. Levels of heparin binding protein (HBP) were measured in the circulation of the patients. HBP is stored in the azurophilic granules of the neutrophils and induce vascular permeability (Gautam et al. 2001). HBP was used as a marker for neutrophil activation and degranulation. Patients had increased levels of HBP (median 17.4 µg/L) compared to controls (median 6 µg/L, $p = 0.008$), quantified by ELISA.

## *Paper II: Proteinase 3 liberates a novel vasoactive kinin from high-molecular-weight kininogen*

Paper I had shown kinin system activation in children with vasculitis. As neutrophil-derived proteases are believed to partake in the pathogenesis of vasculitis and patients with certain small-vessel vasculitides have circulating antibodies against PR3, we set out to investigate if PR3 may cleave HK and liberated kinins, thus activate the kinin pathway. Furthermore, we wanted to investigate if kinin system activation occurred in adults with vasculitis.

Incubating HK with purified PR3 resulted in a dose-dependent degradation of HK, as detected by immunoblotting. The degradation was inhibited by $\alpha_1$-AT as well as rabbit anti-human PR3 antibodies. Incubating HK with recombinant wild-type

PR3 resulted in HK proteolysis whereas recombinant mutated PR3, lacking enzymatic activity, had no effect on HK proteolysis.

Using mass spectrometry, PR3 was shown to releases a novel tridecapeptide, termed PR3-kinin, from HK. This novel kinin consists of the bradykinin sequence with two additional amino acids on each terminus ($NH_2$-MK**RPPGFSPFR**SS-COOH, the bradykinin sequence marked in bold). In plasma, PR3-kinin was processed to bradykinin, a potent $B_2$-receptor agonist, demonstrated using mass spectrometry.

PR3-kinin bound to human $B_1$-receptors, but not $B_2$-receptors, as demonstrated by competition binding assays using transfected HEK293 cells. PR3-kinin also activated $B_1$-receptors on the HEK293 cells as quantified by PI hydrolysis.

Administrating PR3-kinin *in vivo* resulted in transient hypotension mediated via both $B_1$- and $B_2$-receptors. This was demonstrated using both $B_1$-receptor over-expressing rats as well as wild-type and B2KO mice. $B_2$-receptor binding was also inhibited using HOE-140, a potent $B_2$-receptor antagonist.

Plasma samples from adults and children with vasculitis exhibited HK degradation and elevated kinin levels in plasma. Purified neutrophils from both patients with vasculitis and healthy controls were lyzed and the amount of PR3 in the extracts was measured by ELISA. Patients with ANCA-associated vasculitis and controls had comparable amounts of PR3 in their extracts. The neutrophil extracts from both patients and controls induced HK proteolysis, this effect was abrogated when PR3 was immunoadsorbed.

## *Paper III: Proteinase 3 - Anti-Neutrophil Cytoplasmic Antibodies inhibits proteinase 3-induced high-molecular-weight kininogen proteolysis*

Based on our previous results showing that PR3 liberates the vasoactive kinin, PR3-kinin, from HK, we set out to investigate the effect of PR3-ANCA from patients with vasculitis on PR3-induced HK proteolysis.

Affinity purified PR3-ANCAs were available from six patients with Wegener's Granulomatosis.

When PR3 was preincubated with affinity-purified PR3-ANCA from the different patients before adding HK, proteolysis was inhibited to various degrees in all patients, as detected by immunoblotting.

To examine the inhibitory potential of PR3-ANCA on PR3 using the known substrate casein, fluorescent-labeled casein was added to PR3 preincubated with PR3-ANCA. The various PR3-ANCAs demonstrated 34 – 69 % inhibitory effect on casein degradation by PR3.

## *Paper IV: The $B_1$-receptor is upregulated in vasculitis*

We had demonstrated that patients with vasculitides have high levels of kinins in the circulation as well as kinins present at the site of inflammation and that PR3 liberated PR3-kinin, a $B_1$-receptor agonist, from HK. The aim of this study was to investigated if $B_1$-receptors were upregulated, both systemically on MPs and locally in the kidneys, during vasculitis.

$MRL^{lpr/lpr}$ mice, presenting with chronic inflammation and vasculitis, were used as a model for vasculitis. The $MRL^{lpr/lpr}$ mice were compared with $MRL^{+/+}$ mice, that have milder symptoms, and healthy C3H/HeN mice.

Levels of leukocyte-derived MPs in the plasma of $MRL^{lpr/lpr}$ mice were increased compared with $MRL^{+/+}$ mice and C3H/HeN mice, as analyzed by flow-cytometry. The leukocyte-derived MPs were predominantly of neutrophil and T cell origin. The $MRL^{lpr/lpr}$ mice demonstrated high numbers of neutrophil- and T cell-derived MPs expressing $B_1$-receptors as compared with $MRL^{+/+}$ mice and C3H/HeN mice.

In the kidneys of $MRL^{lpr/lpr}$ mice, leukocyte infiltrates were present, both in the interstitium and in the glomeruli, and expressed $B_1$-receptors, demonstrated by immunohistochemistry. The leukocyte infiltrates were shown to consist mainly of T cells, as they expressed CD3. In the $MRL^{+/+}$ mice $B_1$-receptor expressing leukocytes in the kidney was demonstrated, albeit to a lesser extent.

# Discussion

In this thesis we demonstrate, for the first time, kinin system activation in vasculitis, both systemically in plasma and locally in kidney and skin biopsies. We also demonstrate that PR3 cleaves HK, liberating the novel kinin, termed PR3-kinin, thereby initiating kinin system activation. PR3-kinin binds to and activates $B_1$-receptors both *in vitro* and *in vivo*. In addition, we demonstrate $B_1$-receptor upregulation in an animal model of systemic vasculitis both in the circulation and in the kidneys. Thus we propose that kinin system activation may be of relevance in the pathogenesis of vasculitis and that $B_1$-receptor antagonists may be useful in abrogating the inflammation seen during vasculitis.

In paper one, we demonstrated, for the first time, that the kinin system is activated during the acute onset of vasculitis. In the circulation children with vasculitis had high levels of bradykinin and extensive proteolysis of HK, and biopsies from vasculitic lesions, in the kidneys and skin, showed deposition of kinins. Thus the kinin system was activated both systemically and locally. Several of the characteristics of vasculitis, such as inflammation, plasma leakage, edema, bleeding and pain, could theoretically be explains by kinin system activation. We therefore propose that kinin system activation may be of relevance in the pathogenesis of vasculitis.

In paper two, we demonstrated a novel pathway for kinin system activation, in which PR3-induced proteolysis of HK leads to generation of a vasoactive kinin, termed PR3-kinin. PR3-kinin binds to and activates $B_1$-receptors both *in vitro* and *in vivo*. In addition, PR3-kinin is further processed in plasma to bradykinin which may activate $B_2$-receptors. This novel pathway of kinin generation circumvents the known inhibitors of the kinin system and would be controlled *in vivo* by $\alpha_1$-AT, the major inhibitor of PR3. However, at inflammatory sites, where levels of $\alpha_1$-AT would presumably be low, membrane-bound or PR3 secreted from neutrophils could generate active kinins, and thereby enhance inflammation.

Others have shown that activated neutrophils generate active kinins from HK. These kinins act via $B_2$-rececptors and were believed to be bradykinin and met-lys-bradykinin. Tissue kallikrein were responsible for the generation of these kinins as antibodies blocking the activity of tissue kallikrein inhibited the reaction (Wintroub et al. 1974; Movat et al. 1976; Figueroa et al. 1999; Stuardo et al. 2004). It has also been shown that elastase cleaves HK and liberates a bradykinin-like peptide (Kleniewski and Donaldson 1988; Imamura et al. 2002; Imamura et al. 2004). This peptide, which lacks kinin properties when tested *in vitro*, consists of the bradykinin sequence with four additional amino-acids on each terminus (SLMK**RPPGFSPFR**SSRI, the bradykinin sequence is marked in bold). However

it is capable of lowering blood pressure in an animal model (Imamura et al. 2002), probably due to C-terminal processing *in vivo* by carboxypeptidases into smaller fragments with intact kinin activity. HK proteolysis induced by neutrophil-derived elastase and kallikrein may be of some relevance *in vivo*, however in our hands PR3 was almost exclusively responsible for kinin generation by neutrophil extracts.

Thus, we demonstrate a novel function of neutrophil-derived PR3, namely generation of the vasoactive kinin, PR3-kinin. This could be relevant during inflammatory conditions, such as vasculitis, in which neutrophils expressing PR3 infiltrate the affected tissue, leading to PR3-kinin formation which may further enhance the inflammation and capillary leakage acting via $B_1$-receptors. $B_1$- and $B_2$-receptors ligands known to-date are summarized in Table 2.

Table 2: Kinin receptor ligands

| **$B_1$-receptor ligands** | **$B_2$-receptor ligands** |
|---|---|
| PR3-kinin | bradykinin |
| des-arg$^9$-kallidin | kallidin |
| des-arg$^9$-bradykinin | met-lys-bradykinin |

In paper three, we demonstrated that PR3-ANCA inhibits PR3-induced HK proteolysis. We therefore propose that PR3-ANCA may have a regulatory function on PR3-kinin generation.

It is known that PR3-ANCAs in vasculitis change epitope specificity during active disease and remission. PR3-ANCAs have been shown to inhibit the enzymatic activity, interfere with inhibition of PR3 by $\alpha_1$-AT and even enhance the enzymatic activity of PR3 on known substrates, such as casein (van de Wiel et al. 1992; Daouk et al. 1995). We demonstrate that PR3-ANCA, from patients with Wegener's Granulomatosis, inhibits PR3-induced degradation of HK. This demonstrates a novel role of PR3-ANCA in regulation of kinin system activation by inhibiting PR3-kinin release, which may be relevant during PR3-ANCA-positive vasculitis.

In paper four, we investigated the presence of $B_1$-receptors both systemically and locally in MRL$^{lpr/lpr}$ mice known to develop systemic inflammation and vasculitis. Leukocyte-derived MPs have been shown to be elevated during vasculitis (Brogan and Dillon 2004) and are believed to be a sign of leukocyte activation and apoptosis. However $B_1$-receptors have never before been described on MPs. In this study we demonstrated that, in plasma from MRL$^{lpr/lpr}$ mice, levels of leukocyte-

derived MPs were increased and that MPs, mainly of neutrophil and T cell origin, expressed $B_1$-receptos on their surface. This represents the first evidence that $B_1$-receptors are expressed on MPs.

We also demonstrate $B_1$-receptor upregulation on leukocytes present in the renal vasculitic lesions in the $MRL^{lpr/lpr}$ mice. The infiltrates consisted mainly of T cells, although other cells may also be seen. This represents the first evidence of $B_1$-receptor upregulation in vasculitis.

In vasculitis both T cells and neutrophils are believed to be of great importance. In animal models, T cells have been shown to be of importance in glomerulonephritis, a common feature of renal vasculitis, as T cell depletion attenuated the development of glomerulonephritis (Ruth et al. 2006) and in another animal model neutrophil depletion abrogated the development of vasculitis (Xiao et al. 2005). In addition, $B_1$-receptors have been shown to be of great importance in neutrophil migration and chemotaxis during inflammation (Ahluwalia and Perretti 1996; Pesquero et al. 2000; Ehrenfeld et al. 2006; Duchene et al. 2007), as demonstrated in B1KO mice. Thus we suggest that $B_1$-receptors, present both systemically on MPs and locally in the inflammatory lesion, could be of importance in the pathogenesis of vasculitis.

In conclusion, in this thesis we demonstrated kinin system activation both systemically and locally in patients with vasculitis. $B_1$-receptors were shown to be upregulated both on circulating MPs and in the vasculitic lesions of $MRL^{lpr/lpr}$ mice. We also demonstrated a novel vasoactive kinin PR3-kinin, produced by proteolytic cleavage of HK by PR3, which binds to and activates $B_1$-receptors. In addition, PR3-ANCAs, present in some patients with vasculitis, could inhibit PR3-kinin production.

The results of this thesis indicate a role of the kinin system in vasculitis, whereby activated neutrophils express and secrete PR3, which may cleave HK liberating PR3-kinin. PR3-kinin may then bind to and activate $B_1$-receptors, which were shown to be upregulated during vasculitis, and induce inflammation, chemotaxis and vascular leakage. This will attract and facilitate leukocyte extravasation into inflamed tissues. The neutrophils will induce PR3-kinin generation, by membrane bound or secreted PR3, thus triggering a vicious circle of inflammation. We therefore propose that inhibiting the kinin system, by blocking $B_1$-receptors, could be an effective treatment for inflammation in vasculitis.

# Conclusions

- The kinin system is activated both systemically and locally in children with vasculitis
- PR3 liberates a vasoactive kinin, termed PR3-kinin, by proteolytic cleavage of HK, thereby initiating kinin system activation.
- PR3-kinin binds to and activates $B_1$-receptors both *in vitro* and *in vivo*.
- PR3-ANCA inhibits PR3-induced HK proteolysis and subsequent PR3-kinin release
- $B_1$-receptors are upregulated both on circulating MPs, mainly of neutrophil and T cell origin, and in the leukocyte infiltrates in the kidneys of mice with systemic inflammation and vasculitis.
- Kinin system activation may be of importance in the pathogenesis of vasculitis.
- Targeted therapies aimed at inhibiting kinin system activation in patients with vasculitis could be effective in abrogating the inflammation seen in these patients.

# Populärvetenskaplig sammanfattning

Vaskulit är en inflammatorisk sjukdom som drabbar vävnader i och kring blodkärl vilket leder till att dessa skadas och blodkroppar och vätska läcker ut i vävnaden. Det är en allvarlig sjukdom som ofta leder till långt lidande och för tidig död. Orsaken till sjukdomen är okänd men vita blodkroppar spelar en avgörande roll i den skada som uppstår. Många patienter har antikroppar riktade mot proteiner som finns på och i de vita blodkropparna. Flera olika organ kan drabbas vid vaskulit men vanligast är att njurar, hud och luftvägarna skadas.

Kininsystemet är ett proteinsystem som aktiveras vid inflammation. När systemet aktiveras frisätts det kininer som leder till inflammation. Kininer binder till receptorer på cellytan, vilket leder till att signaler skickas in i cellen. Med kännedom om kininsystemets verkningar skulle kininer teoretiskt kunna orsaka många av de symtom som ses vid vaskulit, såsom smärta, inflammation och blödningar.

I vår första artikel beskrev vi, för första gången, att barn med vaskulit har aktivering av kininsystemet och frisättning av kininer både i blodet och lokalt i de vävnader som drabbats av vaskulit. Vi menar att detta kan förklara flera av de symtom som ses hos dessa barn.

I nästa artikel gick vi vidare och visade att ett protein, proteinas 3, som finns på och i vita blodkroppar, direkt kan frisätta en av oss nyupptäckt kinin och därmed leda till aktivering av kininsystemet. Eftersom proteinas 3 inte kan hämmas av de proteiner som kontrollerar kininsystemet så skulle detta kunna leda till okontrollerad kininfrisättning, vilket skulle leda till ökad inflammation och försämring hos patienten.

I artikel tre visade vi att de antikroppar som en del patienter med vaskulit har i blodet, och som hämmar aktiviteten av proteinas 3, även hämmar frisättningen av denna nya kinin. Detta talar för att dessa antikroppar kan vara skyddande hos vissa patienter med vaskulit.

I artikel fyra visade vi att möss, som spontant utvecklar systemisk inflammation och vaskulit, har ökad mängd kininreceptorer både i blodet och i inflammatoriska härdar i njurarna.

I denna avhandling demonstrerar vi, för första gången, att kininsystemet är aktiverat både i blodet och i vävnader vid vaskulit. Vi visar att proteinas 3 frisätter en aktiv kinin som aldrig tidigare beskrivits. Vi visar också att möss med systemisk inflammation och vaskulit har en ökad mängd kininreceptorer både i blodet och i den drabbade vävnaden. Vaskuliter är sjukdomar med hög sjuklighet och dödlighet och det saknas en specifik behandling. Vi tror att läkemedel som hämmar kininsystemet skulle vara effektiva vid behandlig av vaskulit.

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