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Inflammation, kinins, and kinin receptors

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Abstract

Evidence is accumulating that the pathogenesis of many diseases is triggered by inappropriate inflammatory responses. These serious complications may occur when the tightly regulated inflammatory network is out of balance and it is now clear that this can eventually cause substantial harm to own cells and tissues. Successful infectious agents have evolved an enormous repertoire of modulatory mechanisms that allow the evasion, re-direction, dampening, over-stimulation, and even the use of inflammatory responses for their own purposes. The contact system, which is studied in the present thesis, is part of this inflammatory network and it has been shown that its systemic activation contributes to an exacerbation in many disease areas.

Bradykinin (BK) belongs to the group of vasoactive peptides, so-called kinins, which are potent inflammatory mediators. BK, once released from the human contact system, elicits a transient inflammatory response via activation of the constitutively expressed B2 receptor, which desensitizes and is internalized upon ligand binding. The kininase I metabolite of BK, desArg9BK, on the other hand mediates chronic deleterious inflammatory responses by interacting with the B1 receptor. Although normally absent, B1 receptor expression can be induced under inflammatory conditions.

The aim of the present thesis was to explore and analyze modifications in the regulation of kinins and their receptors in different settings of inflammation. It is known that BK is generated in the airways of asthmatic subjects and that disease symptoms are exacerbated during respiratory viral infections. In paper I, we report that BK induces an up-regulation of B2 receptors in human airway epithelial cells, which is in sharp contrast to other investigated cells. Further more, rhinovirus induces up-regulation of functional B1 receptors in the same cell type (paper II). Both mechanisms may render the respiratory tract more responsive to generated kinins and can significantly influence the inflammatory response and thereby symptom severity. In paper III and IV we demonstrate that the important human pathogens S. aureus and S. pyogenes can induce profound inflammatory reactions in the human host by secreting toxins that via stimulation of monocytic cells induce up-regulation of B1 receptors. Concurrently, both bacterial species are able to induce release of BK and its subsequent conversion to desArg9BK, which can evoke an over-amplification and prolongation of the inflammatory response.

Taken together, the present thesis demonstrates that kinin receptor regulation and kinin generation is affected during different settings of inflammation and we conclude that this may have a great impact on disease progression.

Key words: B1 receptor, B2 receptor, bradykinin, desArg9bradykinin, airway epithelial cells, Staphylococcus aureus, Streptococcus pyogenes, TAFI

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Inflammation, kinins, and kinin receptors

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Cover image: Bacterial-bound gold-labeled thrombin activatable fibrinolysis inhibitor (TAFI) (large spot) interacting with gold-labeled bradykinin (BK) (small spot) at the surface of *Streptococcus pyogenes,* visualized by electron microscopy. Picture provided by courtesy of Dr. Matthias Mörgelin.

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Till mamma och pappa

"Med båda fötterna på jorden kommer man inte långt"
Okänd

"The microorganisms… turn out … to be rather more like bystanders. It is our response to their presence that makes the disease. Our arsenals for fighting off bacteria are so powerful… that we are more in danger from them than the invaders."

Lewis Thomas

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List of papers

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

I **Bengtson S.H, Eddleston J., Mörgelin M., Zuraw B.L.*, and Herwald H**.* (2007) Regulation of Kinin B_2 receptors by bradykinin in human lung cells. *Under consideration*

II **S.H. Bengtson*, J. Eddleston*, S.C. Christiansen, B.L. Zuraw.** (2007) Double-stranded RNA Increases Kinin B1 Receptor Expression and Function in Human Airway Epithelial Cells. *International Immunopharmacology* 2007 Dec 20;7(14):1880-7

III **Bengtson SH, Phagoo SB, Norrby-Teglund A, Påhlman L, Mörgelin M, Zuraw BL, Leeb-Lundberg LMF, and Herwald H.** (2006) Kinin receptor expression during *Staphylococcus aureus* infection. *Blood* 2006 Sep 15;108(6):2055-63

IV **Bengtson SH, Sandén C, Mörgelin M, Leeb-Lundberg LMF, Meijers JC, and Herwald H**. (2008) Activation of TAFI on the surface of *Streptococcus pyogenes* evokes inflammatory reactions by modulating the kallikrein/kinin system. *Innate Immunity*, *In press*

Abbreviations

APC	antigen presenting cell
BK	bradykinin
B_1R	B_1 receptor
B_2R	B_2 receptor
desArg^{9}BK	desArg^{9}bradykinin
HMWK	high molecular weight kininogen
IL	interleukin
KD	kallidin
LPS	lipopolysaccaride
MHC	major histocompatibility complex
NO	nitric oxide
ROS	reactive oxygen species
TAFI	thrombin activatable fibrinolysis inhibitor
TF	tissue factor
TLR	toll-like receptor
S. aureus	*Staphylococcus aureus*
Scl (A and B)	streptococcal collagen-like surface protein
S. pyogenes	*Streptococcus pyogenes*
tPA	tissue plasminogen activator
uPA	urokinase

Introduction

The immune system is essential for our survival as it protects us from foreign invaders and participates in repair of damaged tissues. It is activated upon injury or infection and it initiates a series of inflammatory reactions that involves a complex network of mediators such as cytokines and complement. So-called plasma cascade systems such as the contact, coagulation, and fibrinolytic systems are also part of this network and their activation triggers additional inflammatory reactions. Contact system activation results in release of bradykinin (BK), which is a potent pro-inflammatory peptide. Its metabolite, desArg^{9}bradykinin (desArg^{9}BK), is also biologically active and the two kinin peptide variants exert their effects via binding to kinin receptor B_2 and B_1, respectively. Activation of B_2 receptors mediates a quick inflammatory reaction, whereas B_1 receptor activation is involved in a more sustained response (1).

An inflammatory reaction aims at rapid destruction and removal of the initial insult. Once repair of the damaged tissue has been initiated, the inflammatory response has fulfilled its functions and is turned off under normal circumstances. Although inflammation is fundamentally protective, it may, if inappropriately regulated, be harmful and itself induce cell destruction and eventually disease. In fact, inflammation contributes to the pathogenesis of almost all maladies (2).

The present thesis aims to explore how different causes of inflammation such as bacterial and viral infections can influence the release of BK and desArg^{9}BK, as well as the expression of their respective receptors, and thereby control the course of inflammation.

1 Immune responses

We are constantly being exposed to a universe of threatening pathogens. Without an immune system we would rapidly succumb to severe infections. The ability of the immune system to distinguish between self and non-self structures allows it to control and eliminate an invading microbe without causing excessive damage to the host's own tissues. All immune responses must therefore rely on pathogen recognition. Mechanisms for sensing and reacting to microbial structures are divided into two general classes called innate and adaptive immunity. Recognition mechanisms in innate immunity are encoded by genes in the germ line and this part of the immune system recognizes conserved structural features shared by many microbes. The adaptive immune system on the other hand, has specificity for unique foreign structures. This is achieved by recognition mechanisms, encoded by gene elements that somatically rearrange to build millions of different antigen-binding molecules (3). However, even though the immune system generally can be divided into innate and adaptive immunity which involve somewhat different effectors, the two arms of immunity should be viewed as complementary and cooperating. They both serve to activate protective reactions of which inflammatory responses (described in more detail in chapter 2) are an important part.

1.1 Innate immunity

The first line of defense consists of physical barriers such as the skin and mucosal membranes. Any penetrant microbe that breaches these barriers is greeted by the innate immune system, which is mobilized within minutes. Apart from battling the intruder it also has an important role in activating the slower but more specific adaptive immune system. The importance of innate immunity is reflected in the very conserved phylogenecity of the system (4).

In humans innate immunity consists for example of antimicrobial peptides, complement proteins, and most importantly myeloid cells. Myeloid cells include mononuclear- and polymorphonuclear phagocytes (5). Phagocytes, such as neutrophils and macrophages (described in more detail below), engulf pathogens into a membrane bound compartment known as the phagosome. The phagosome fuses with intracellular

granules where the microbes are killed via a number of mechanisms including oxidative damage via reactive oxygen species (ROS), enzymes, and antimicrobial peptides (6). As ROS are non-specifically cytotoxic it may, ones it is released from the phagocyte, cause substantial injury to healthy host tissues.

Another type of immune cell, called the mast cell, is found at mucosal surfaces and in connective tissue. Its primary function is to protect the host against parasites by releasing for example histamine which enhances inflammation. However, in addition to inducing protective responses, histamine may also cause the adverse symptoms of allergy (7).

1.1.1 Polymorphonuclear neutrophils

Neutrophils are specialized killers, and the most abundant immune cell in humans. They circulate in the bloodstream in an inactive state and have a half-life of only a few hours (6). Upon infection neutrophils are rapidly guided to the inflamed site by chemotactic agents such as factors derived from the fibrinolytic and kinin systems, products of other immune cells such as chemokines and cytokines, and bacterial components. Temporary expressed structures on the neutrophil and on the vessel lining at the infected site, mediates migration out of the vessel into the affected tissue. Apart from pre-stationed immune cells in the tissue, neutrophils are the first arriving at the site of infection. They have a large arsenal of antibiotic proteins stored in two main types of granules called azurophilic and specific granules. Apart from ingesting and killing microbes inside these compartments, extracellular release of granules and cytotoxic substances may also occur (7).

1.1.2 Monocytes and macrophages

Monocytes are very versatile phagocytes derived from the bone marrow. They circulate in the blood for several days, before eventually migrating into tissues throughout the body. There, they differentiate into a very heterogeneous spectra of tissue-resident macrophages as well as specialized cells such as the dendritic cell (8). Under normal conditions these cells maintain tissue homeostasis by collecting garbage such as apoptotic

cells. However, macrophages also have an important supervisory function. They sense microbial material through a limited number of receptors including mannose and fucose receptors and a specialized class of molecules called toll-like receptors (TLRs). These receptors recognize molecules that are indispensable components of a microbe and therefore not readily altered, such as components of the cell wall and bacterial and viral nucleic acids. In response to sensing foreign material, macrophages become important phagocytozing effector cells of innate immunity. They also recruit other myeloid cells, in particular neutrophils, to the site of infection, through the release of chemokines such as IL-8 and chemotactic cytokines (5), including interleukin (IL)-1β, IL-6, and tumor necrosis factor α (TNF-α), which are inflammatory mediators.

Macrophages are so-called antigen presenting cells (APC) and can initiate an adaptive immune response by presenting antigens to T-lymphocytes. Further, depending on the initial stimulus macrophages induce polarization of the adaptive immune system towards cell- or antibody-mediated responses. In addition, macrophages can up-regulate a molecule called tissue factor (TF) on their surfaces, which induces blood coagulation and further amplifies inflammatory responses.

1.2 Adaptive immunity

An adaptive immune response is initiated when an antigen (usually a non-self structure recognized by immune cells) is recognized by cells of the lymphoid system, i.e. T- and B-lymphocytes. Recognition relies on a highly diversified repertoire of antigen specific receptors. Upon antigen stimulation lymphocytes, expressing receptors with relevant specificity, proliferate and differentiate into effector cells, a process which typically takes 4-10 days. Selected clones are preserved even after clearance of the initial stimulus and constitute an immunological memory.

Adaptive immunity is directed by a type of T-lymphocytes called T-helper (T_H) cells (9) which in turn are educated by APCs such as the macrophage, as mentioned in the section above. When the T-cell receptor (TCR) of a naive T-cell (T_H0) recognizes an antigen presented by an APC on a structure, called major histocompatibility complex (MHC) II, it starts to differentiate into a T_H1 or a T_H2 cell depending on the co-

stimulatory signal supplied by the APC. By secreting different sets of cytokines T_H1 cells promote cellular immunity whereas T_H2 cells favor humoral responses. Cellular immunity involves special T-cells, termed cytotoxic T-lymphocytes that directly can lyse cells that are infected, or of foreign origin, or have signs of cancer. In addition, cytotoxic T-cells can secrete cytokines that enhance immune responses such as phagocytosis and inflammation. The humoral part of adaptive immunity is based on antibodies produced by activated B-lymphocytes. Antibodies serve to neutralize certain toxins, opsonize threatening agents (mark target to increase the activity of phagocytozing cells), and form aggregates of antigens, which also enhance the clearance by phagocytozing cells (85). Antibodies are also involved in allergic reactions (see section 6.1) where they participate in the induction of inflammation.

2 Inflammation

Inflammation is Latin for “set on fire” and it refers to the basic process whereby tissues of the body respond to harmful stimuli such as injury, irritants, or pathogens. It is a protective non-specific response. Key features are alterations of local blood flow and accumulation and activation of inflammatory cells. This is followed by removal of the initial stimulus, cell debris, and the inflammatory cells themselves, once the healing process has been initiated. Local inflammation normally leads to repair of tissue structure and function and is therefore a key mechanism of tissue homeostatic maintenance.

Inflammation is tightly associated with both innate and adaptive immunity, and can be considered as the main effector process upon antigen recognition (2). Inflammation is also considered to be an important link between innate and adaptive immune processes. It has powerful effects and is therefore normally tightly regulated. In its absence infections and wounds would never heal. On the contrary, if run unchecked or if resolution does not occur at the right time point, inflammation has an autotoxic character that may cause substantial harm to the involved tissues. If activated systemically (such as by a severe infection) the inflammatory response may cause more damage than the microbe itself and threaten the survival of the host (10).

Inflammation is driven by a complex network of soluble mediators of both exogenous and endogenous extraction. Examples of exogenous mediators of inflammation are bacterial products and toxins, whereas endogenous mediators are derived from inactive precursors present in plasma, such as components of the complement, coagulation, and contact system. Of special interest to the present thesis are small potent pro-inflammatory peptide fragments called kinins derived from the human contact system (described in section 4.1.1). Depending mainly on the duration and character of the mediated response, inflammation can be classified as either acute or chronic (11).

2.1 Acute inflammation

There are five classical signs of acute inflammation including redness (rubor), heat (calor), pain (dolor), swelling (tumor), and decreased function (functio laesa). An inflammatory response is initiated when chemical messengers are released from injured tissue, resident immune cells, blood plasma, or even from infecting microbes. These inflammatory signals activate endothelial cells of nearby capillaries, and generate chemotactic gradients (11). The resulting change in the endothelial lining makes it adhesive to neutrophils, which subsequently attach and squeeze through the endothelial layer and migrate towards the affected site. If needed, the recruitment of neutrophils is followed by other immune cells such as monocytes/macrophages and lymphocytes. When inflammation makes the vascular barrier permeable to immune cells, plasma proteins may also leak out. The resulting extravasation into the tissue causes the swelling and an increased blood flow in the area, due to a temporary extension of the diameter of affected vessels, gives rise to the redness and heat. Inflammatory mediators binding to nerve endings cause the pain, and damage of the tissue may lead to a decreased function. Once the injurious stimulus has been removed, degraded, or walled off by scarring the acute inflammatory response ceases and the signs weaken and disappear (12).

2.2 Chronic inflammation

As the name implies chronic inflammation is a prolonged process, which lasts anywhere from a week to an indefinite time. It may develop as a progression from an acute inflammatory response if the offending agent persists or after repeated episodes of acute responses, but it can also occur as a distinct process without preceding acute inflammation (11). Chronic inflammation is characterized pathologically by a dense infiltration of immune cells, tissue destruction and evidence of prior attempts at repair as shown by fibrosis and angiogenesis. In contrast to acute inflammation, immune cells are not cleared from the site but are trapped in place and new cells are continuously recruited. This condition usually causes permanent tissue damage (10).

3 Coagulation

The coagulation system serves to prevent blood loss and maintain blood pressure in case of injury to the vasculature, and has traditionally been considered to be entirely separate from the immune system. It has become increasingly clear, however, that the coagulation system and innate immunity communicate at many levels and have developed through evolution to function as a highly integrated unit for survival defense following tissue injury and inflammation (13). During infection, coagulation may facilitate the defense by containing the intruder and the consequent inflammatory response to a limited area within a fibrin net. In the case of an insufficiently controlled or systemic inflammatory response, however, the activated coagulation system may substantially contribute to disease (14). A well-known pathological phenomenon is systemic activation of coagulation during an acute inflammatory response to sepsis (bacteria in the bloodstream). This gives rise to disseminated intravascular coagulation (DIC) leading to consumption of clotting factors and subsequent bleeding, which may progress into multiple organ dysfunction and eventually death (15).

Inflammation initiates coagulation primarily through the exposure of TF (further described below), but pro-inflammatory cytokines and endothelial cells may also play central roles (14). Moreover, during inflammation there is an accompanying suppression

of the natural anticoagulant systems as well as the fibrinolytic system which further facilitate clot formation (16). The most important mechanism by which coagulation proteases (clotting factors) influence inflammation is through activation of so-called protease activated receptors (PARs) that are expressed by many cells, including endothelial cells and mononuclear cells (17). Activation of PAR receptors by coagulation proteases leads to production of pro-inflammatory cytokines and growth factors as well as up-regulation of inflammatory responses in macrophages (14). Hence, there is a reciprocal activation between coagulation and inflammation leading to an amplification loop.

3.1 The coagulation cascade

The coagulation system consists of a number of serine proteases, or coagulation factors, that circulate in the vasculature in inactive forms. Coagulation occurs when activated coagulation factor X (FXa) cleaves pro-thrombin to thrombin, which converts fibrinogen to insoluble fibrin and activates platelets. This constitute the common pathway of coagulation which is initiated by either the intrinsic or extrinsic pathway (Fig. 1). The extrinsic route is the most important for coagulation *in vivo* and is activated by TF, a cellular receptor (18) expressed for example by fibroblasts surrounding blood vessels (19). Upon damage to the vessel, TF is exposed to blood and interacts with its ligand, clotting factor VII, which becomes activated. The resulting FVIIa/TF complex initiates the common pathway of coagulation by catalyzing the activation of FX. Although normally not expressed by cells in contact with blood circulation, TF can be induced in monocytes under inflammatory conditions (15).

The intrinsic pathway of coagulation, starting with activation of the so-called contact system, is considered to be of subordinate importance for clot formation (20). Instead, it may have important functions in innate immunity and inflammation (see chapter 4). The intrinsic pathway consists of the pro-enzymes prekallikrein, coagulation factor XII and XI, and high molecular weight kininogen (21). Contact activation occurs upon exposure to a negatively charged surface leading to a bidirectional activation of FXII and

prekallikrein. FXIIa triggers a sequential activation of FXI, FIX, and FX, which initiates the common pathway of coagulation (Fig 1).

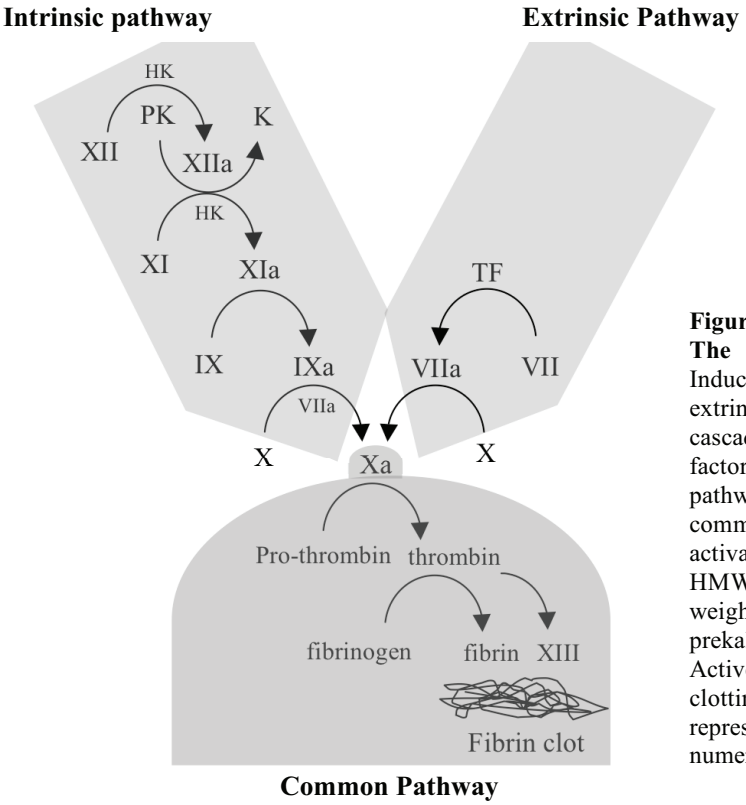

Figure 1.
The coagulation cascade. Induction of the intrinsic or extrinsic pathway triggers a cascade of coagulation factor activation. Both pathways initiate the common pathway by activating factor X.
HMWK; high molecular weight kininogen, PK; prekallikrein, K; kallikrein. Active (a) and inactive clotting factors are represented by their roman numerals.

3.2 Fibrinolysis

Coagulation is followed by a process known as fibrinolysis, serving to lyse the clot once the bleeding has stopped and repair of the vessel has begun. Plasmin is the major fibrinolytic protease, which circulates in blood in its inactive pro-form - plasminogen. Fibrinolysis is initiated when plasminogen is converted to plasmin by either tissue

plasminogen activator (tPA), secreted by endothelial cells, or urokinase (uPA) which is produced in the liver. Conversion of plasminogen is enhanced in the presence of fibrin ensuring that plasmin is mostly generated during blood clotting (22). Once formed, plasmin cleaves fibrin, which leads to the exposure of carboxy-terminal lysine residues and generation of soluble fibrin degradation products. Both tPA and plasminogen have binding sites for lysine, which mediate further binding to fibrin and enhanced formation of plasmin (23). Fibrinolysis is counteracted by inhibitors of plasmin and plasminogen activation, such as α_2-plasmin inhibitor and plasminogen activator inhibitor-1, as well as by an enzyme termed thrombin activatable fibrinolysis inhibitor (TAFI) described in more detail below.

3.2.1 Thrombin activatable fibrinolysis inhibitor (TAFI)

Activated TAFI (TAFIa) is an enzyme able to prevent clot degradation. It was discovered in the 1990s by several different groups (24-27). TAFI is synthesized in the liver and is secreted into plasma where it circulates as a zymogen. Its most potent activator is the thrombin/thrombomodulin complex, although plasmin and neutrophil elastase also can cleave TAFI into its active form (23)(28). Once activated, TAFIa is inactivated within a few minutes by a conformational change (29).

TAFIa is a peptidase with specificity for carboxy-terminal arginine and lysine residues. It is therefore able to attenuate fibrinolysis by removing carboxy-terminal lysine residues from fibrin that are required for efficient plasmin formation (22). Surprisingly, TAFI-knock-out mice do not exhibit any hemostatic abnormalities (30) such as excessive bleeding that might be expected in the absence of a fibrinolysis inhibitor. Instead, this model show impaired wound healing (31), increased recruitment of neutrophils to an infectious site (32), and a lethal inflammatory response to venom in mice primed with bacterial lipopolysaccaride (LPS) (33), suggesting an important role for TAFI in inflammatory conditions. This role is further supported by the fact that TAFIa can degrade inflammatory mediators such as the leukocyte chemoattractants C3a and C5a (34) and the potent pro-inflammatory peptide BK (33). Taken together, these activities

suggest that TAFI may have an important function in the cross-talk between coagulation, fibrinolysis, and inflammation.

4 Contact system

Like TAFI, the contact system, also known as the kallikrein/kinin system or the intrinsic pathway of coagulation, might have important functions at the interface between coagulation, fibrinolysis and inflammation. The system was first described in the 1950's by Ratnoff (35). In addition to initiating the intrinsic pathway of coagulation, proteins of the contact system have anticoagulant, profibrinolytic, antiadhesive, and proinflammatory properties (36). As mentioned in section 3.1, the contact system consists of the proteinases prekallikrein, FXII, and FXI and the non-enzymatic co-factor high molecular weight kininogen (HMWK). This group of plasma proteins are called contact factors because they require contact with a negatively charged surface for zymogen activation *in vitro* (21). Activation of the contact system leads not only to the initiation of the intrinsic pathway of coagulation but also to the release of the potent pro-inflammatory and vasoactive mediator bradykinin (BK) (further described in section 4.1.1) from HMWK.

Kininogens are synthesized in the liver and due to alternative splicing of a single kininogen gene, two variants exist in humans called low- and high-molecular weight kininogen (36). However, only the heavy form (HMWK) is part of the contact system. Plasma kallikrein zymogen, also known as Fletcher factor (37), circulates in blood mostly in complex with HMWK (36). The major substrates for plasma kallikrein are FXII, HMWK, and pro-urokinase. The third member of the contact system, FXII, also known as Hageman factor, is a zymogen susceptible to activation by plasma kallikrein, plasmin, and by its own active form, FXIIa (36).

It is well known that the contact system can be activated on an artificial negatively charged surface such as kaolin, dextran, or glass, resulting in auto-catalytic activation of FXII. Subsequently FXI is cleaved, initiating the intrinsic pathway of coagulation, and in addition prekallikrein is activated, which both activates further FXII in a positive feedback loop and cleaves HMWK resulting in release of BK (38). How the

system is activated *in vivo* is not completely understood. It can assemble on the surface of platelets, monocytes, neutrophils (39), and endothelial cells (40)(36). It has further been reported that if the endothelial lining changes from an anti-coagulant to a pro-coagulant stage, for instance upon vessel injury, the contact system can be initiated via activation of PK in a FXII independent manner (41,42).

Although traditionally regarded as part of the coagulation cascade, deficiencies in proteins of the contact system are not associated with bleeding disorders (20). However, FXII-deficient mice are protected from ischemic stroke by vessel-occluding fibrin formation, indicating that FXII is involved in pathologic thrombus formation (43).

It is currently believed that the contact system has important functions in the induction of inflammatory reactions and local regulation of blood pressure via the release of BK (36,41) and it could even be regarded as a part of innate immunity. For instance, it has been found that activated plasma kallikrein is chemotactic for neutrophils (44) and that cleavage of HMWK can generate an antimicrobial peptide (45). However, the system is also thought to be involved in several pathologic conditions of inflammatory character such as allergic asthma, rheumatiod arthritis and interstitial cystitis (21,46)(47)(48).

Interestingly, the contact system is assembled and activated on the cell wall of several pathogenic bacterial species including *Escherichia coli, Salmonella, Staphylococcus aureus,* and *Streptococcus pyogenes* (49-52), leading to the release of BK at the infectious site. In severe infections such as sepsis, a massive activation of the contact system may occur resulting in pathological levels of kinins and a consumption of contact factors, contributing to the deleterious hypotension and coagulopathy associated with these conditions (53).

4.1 Kinins

The sole sources of kinin peptides are the kininogens, and since kininogen deficiency in humans is reported to be relatively asymptomatic (54), kinin peptides seem to have minor functions in normal physiology. However, kinins are typically liberated at injured or inflamed tissue and have a prominent role in the inflammatory process.

Kinins are a family of peptides that contain the full-length sequence of BK or part thereof. They include BK and kallidin (KD) derived from high- and low-molecular weight kininogen by the action of plasma- and tissue-kallikrein respectively, as well as desArg^{9}BK and desArg^{10}KD which are truncated versions generated by carboxypeptidases (21) (Fig 2). Under normal conditions, the kinin level in plasma is very low (femtomolar to picomolar range) and the ratio between kinins and kininogen is 1:1000 implicating that kinin release is very tightly controlled. Once released, kininases present in plasma, on endothelial cells, or in the tissues degrade kinins and thereby regulate their functions in the body. Kininases are classified as type I or II, depending on their cleavage sites (see fig 2) Angiotensin-converting enzyme (ACE) is a membrane-bound type II kininase whereas carboxypeptidase of the N and M type, found in plasma and on cell membranes respectively, belong to the kininase I group. They are all zinc-dependent metalloproteases and may therefore be inhibited by metal chelators (38). Interestingly, TAFIa, described earlier in section 3.2.1 also display kininase activity (55). Of special interest to the present thesis are the two kinins BK and its kininase I metabolite desArg^{9}BK that have been found to be generated during inflammation (56).

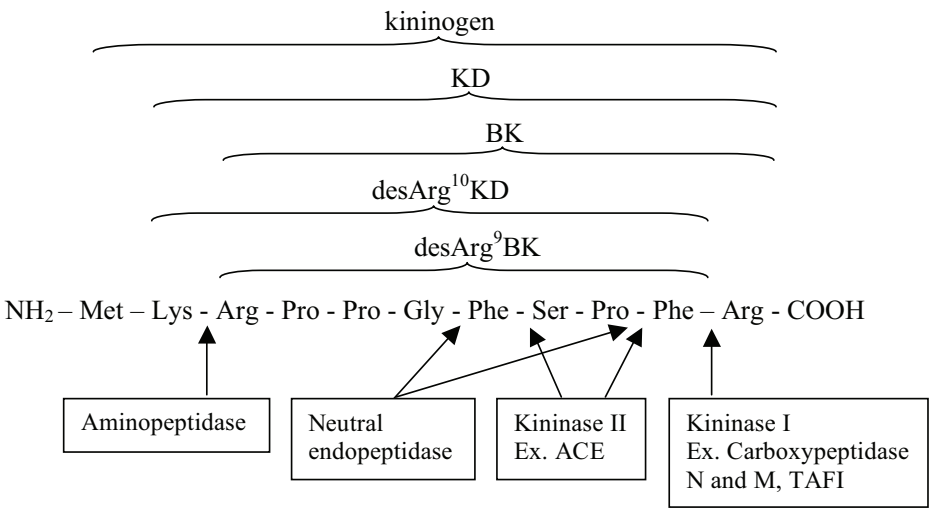

Figure 2. Kinins and degradation enzymes
KD-kallidin, BK-bradykinin, ACE-angiotensin-converting enzyme, TAFI-thrombin activatable fibrinolysis inhibitor

4.1.1 BK and desArg^{9}BK

In 1949 Rocha e Silva et al noticed that incubation of plasma with venom extracted from the Brazilian snake *Bothrops jararaca* resulted in the release of a potent vasodilating and smooth muscle stimulating substance (57). In isolated guinea pig ileum, the substance produced slow, delayed contractions when compared to those obtained with the neurotransmittors histamine and acetylcholine. The substance was then given the name bradykinin from the Greek words *brady* meaning slow and *kinin* indicating movement. Since then, numerous investigations have been performed to characterize BK and its effects. It is now well established that this nine amino acid long peptide, derived from the fourth domain of kininogen by the action of kallikrein, can induce all classical signs of inflammation (58-60). If the carboxyterminal arginine residue is removed from BK it is transformed into another biologically active kinin called desArg^{9}BK. The two kinins possess similar pharmacological actions although their biological effects are mediated via separate specific receptors (38).

5 Kinin receptors

In the 1970's Regoli and coworkers found that two distinctive kinin receptors exist, differing in their pharmacological profile as well as in their expression patterns (61,62). One type was stimulated by the full-length peptide of BK and KD whereas the other type was selectively sensitive to kinins lacking the carboxy-terminal arginin residue like desArg^{9}BK and desArg^{10}KD. The two receptors were called B_2 and B_1, respectively.

5.1 B_1- and B_2-receptors

The biological effects of BK are mediated by the B_2 receptor (B_2R) whereas responses to desArg^{9}BK are induced via signaling through the B_1 receptor (B_1R). This discrimination has been suggested to partly depend on the B_1R binding site being positively charged

which therefore repels ligands like BK that possess a positively charged carboxy-terminal Arg (63). The genes coding for the two kinin receptors have been found to lie in close proximity, indicating that they have evolved from a common ancestor (64). The receptors have been isolated and sequenced in several mammalians including e.g. mouse, rat, rabbit and human and found to have only 80% identity, when the amino acid sequence for the B_2R was compared, suggesting a fairly rapid evolution of the gene (1). Both kinin receptor subtypes can be expressed by the same cells including endothelial, smooth muscle, fibroblasts, epithelial, nervous, and various cancer cells (65-67). In addition, the B_1R has been reported to be expressed by various leukocytes including e.g. neutrophils and T-lymphocytes (68,69).

The amino acid sequence for B_1R and B_2R share only 36% homology, although the receptors have many similar features. They both belong to the family of G-protein coupled receptors consisting of a single polypeptide that transverse the cell membrane seven times with the amino terminal end on the extracellular side and the carboxy-terminus on the inside of the cell (70). The receptors mediate similar responses by inducing some identical second messenger systems including phosphoinositol-hydrolysis, elevation of intracellular Ca^{2+}, arachidonic acid release, eicosanoid production, endothelial nitric oxide synthase (eNOS) activation, and nitric oxide (NO) production (1). Through a chain of events, these signaling cascades give rise to increased vascular permeability, venoconstriction, arterial dilatation, and pain, leading to the classical signs of acute inflammation (71).

Although the transduction pathways of the B_1R and B_2R are very similar, their signaling pattern differs as a consequence of their distinct mode of regulation. Whereas the B_2R is constitutively expressed, the B_1R is generally absent in normal tissue. However, the B_1R is induced under inflammatory conditions such as during tissue injury, infections, and treatment with cytokines including IL-1β and TNFα (63,72). The induction of B_1R is controlled by mitogen-activated protein (MAP) kinase and the transcriptional nuclear factor κB (NF-κB) which in turn is activated by many inflammatory cytokines, inflammatory mediators, and toll-like receptors (TLRs), including the receptor for LPS (TLR-4) (73-75).

In addition to the rapid ligand degradation, B_2R signaling is limited to a quick transient response since ligand binding is followed by a rapid desensitization and internalization of the receptor (76-78). Although the B_2R normally recycles to the plasma membrane it may be long-term down-regulated by prolonged agonist exposure (79). In contrast, the B_1R elicit persistent responses as this receptor type is subjected to only very limited desensitization and receptor internalization. In addition, prolonged agonist exposure leads to up-regulation of the B_1R (80,81).

Kinins are very potent vasodilators via signaling through the kinin receptors, which explains their hypotensive physiological effect. Hence, the normal hypotensive response to bacterial LPS was found to be significantly decreased in a B_1R gene knockout mouse. Inflammatory responses were further affected in this model, by a reduced neutrophil accumulation to the inflammatory site (82,83). Mice with genetic deletion of the B_2R still show hypotensive responses to kinins due to a compensatory up-regulation of B_1Rs in the vasculature (84).

Taken together, in normal conditions cardiovascular actions of kinins are mediated by the preformed B_2R. However, during tissue insult the situation changes as the B_1R receptor is induced (63). B_2R then mediate a quick response as part of the acute phase of inflammation whereas the B_1R, with its sustained signaling, participate in the deleterious chronic phase of inflammation (71).

6 Causes of inflammation

An inflammatory response, involving kinins and signaling through their receptors, can have many different causes. The following sections give a brief introduction to some of them which are of special interest to this thesis.

6.1 Allergy

An allergic reaction is the result of an inappropriate immune response triggering inflammation. It occurs when an individual encounters an antigen (allergen) a second

time, to which it has produced IgE antibodies against at the previous exposure. The generated antigen specific IgE binds to receptors on mast cells (as well as certain other cells like basophils and eosinophils), thereby sensitizing the cells. When the next allergen exposure occurs, the allergen binds to and cross-links the IgE on the mast cells, triggering degranulation with subsequent release of both preformed and newly generated inflammatory mediators (85).

A generalized allergic response is called systemic anaphylaxis. It usually results in respiratory impairment due to smooth muscle constriction in the bronchioles and a rapid loss of fluids into the tissues depending on dilated arterioles and increased capillary permeability (11). The drastic physiological changes can result in a circulatory shock, which may be fatal.

A localized anaphylaxis is called an atopic reaction. It generates symptoms that primarily depend on the route by which the allergen enters the body. Hay fever (allergic rhinitis) and asthma are examples of atopic diseases involving the respiratory tract (86). Generation of kinins as well as regulation of their receptors have been shown to be involved in allergic conditions in the airways (87-91) and they have a potential role in allergic rhinitis, asthma, and anaphylaxis in contributing to tissue hyperresponsiveness, local inflammation, and hypotension. Release of kinins in these conditions are caused by secondary effects of endothelial-cell activation and other pathways of inflammation (92). Substantial evidence also points at an involvement of viral infections in the onset and pathogenesis of allergic hyperresponsiveness in the respiratory tract (93,94).

6.2 Viral infection

Virus is the Latin word for toxin or poison. It is an acellular infectious agent consisting of genetic material, DNA or RNA, surrounded by a protective protein coat called the capsid. Viruses are obligate intracellular parasites and reproduces therefore only within host cells. The general life cycle of an eukaryotic virus has five phases including, adsorption to host cell, penetration, replication of virus nucleic acids and synthesis of viral proteins, assembly of the capsid, and virus release (11).

Resistance to viral infections involves sensitization of infected cells with interferons, which are cytokines that are produced by a wide variety of cells in response to the

presence of double-stranded RNA, a key indicator of viral infection. Interferons assist the immune response, involving both cell mediated and humoral immunity (85).

Viral infections are common in the respiratory tract and have been shown to increase airway inflammation (94). Interestingly, rhinovirus infection (common cold) results in increased local kinin production and the symptom severity is directly correlated with the kinin content measured in nasal secretions (95), indicating a role of kinins in the pathogenesis of viral disease.

6.3 Bacterial infection

Bacteria are unicellular so-called prokaryotes, which unlike eukaryotic cells do not contain a nucleus. Outside the cell membrane bacteria have a cell wall and depending on its structure most bacteria can be divided into two broad groups called Gram-positive and Gram-negative bacteria, referring to a staining procedure. The Gram-positive cell wall contains many layers of the glycoprotein peptidoglycan and teichoic acids whereas the Gram-negative wall has less peptidoglycans but is, unlike the Gram positive bacteria, surrounded by LPS and lipoproteins (11).

Although the vast majority of bacteria are harmless or even beneficial, some bacteria are pathogenic and constitute a major cause of human disease and death. Gram-positive cocci, in particular *Staphylococcus aureus* (*S. aureus*) and *Streptococcus pyogenes* (*S. pyogenes*) are important human pathogens since they may cause a variety of serious invasive infections (96). These two bacterial species are of special interest to the present thesis and are further described below.

In order to establish an infection, pathogenic bacteria have evolved a multifold repertoire of virulence factors. They help the bacteria to adhere to host tissues as well as to degrade host structures and penetrate epithelial and endothelial barriers to allow spreading. To restrain bacterial invasions, an inflammatory response is of great importance as it facilitates resistance against the pathogen and recruits neutrophils and macrophages to the site of infection. In addition to combat the germs, macrophages release pro-inflammatory cytokines such as IL-1β, and activate T-lymphocytes, which in turn help to induce antibody production in B-lymphocytes (11). To survive such powerful

defense mechanisms the bacteria have evolved strategies to evade or modify the host immune responses (97-100). To this end, bacteria interfere with different host systems including coagulation, fibrinolysis, and even the immune system itself to subvert their functions (99,101-104). The contact system has been found to be a target of such corruption for several different species including *S. aureus* and *S. pyogenes* and this may significantly contribute to serious complications such as hypovolemic hypotension and coagulopathy seen in severe infections (41,50,51,105).

6.3.1 Staphylococcus aureus

As implicated by the name *S. aureus* is a yellowish (aureus) spherical bacterium (coccus) that usually form grape-like clusters (staphylo) (106). *S. aureus* can harmlessly colonize skin and mucus membranes but may occasionally cause a range of illnesses ranging from minor skin infections, such as impetigo, cellulitis, and abscesses, to scalded skin syndrome, pneumonia and life-threatening diseases, such as meningitis, and toxic shock syndrome (107). It expresses a wide array of cell-surface associated and secreted virulence factors including superantigenic toxins (97). Superantigens are among the most potent inflammatory mediators known and they significantly contribute to the induction of life-threatening conditions, such as cardiovascular shock in humans. They corrupt normal immune responses by their ability to crosslink MHC class II proteins with the T-cell receptor in the absence of a presented antigen. As a consequence up to 30% of the T-lymphocytes are activated in this non-specific manner (versus less than 0.01% by a normal antigen), leading to the release of pathologic amounts of inflammatory cytokines. Eventually this over-stimulation of the immune system may lead to immunosupression (97,108,109).

S. aureus has become an increasing threat due to its developing resistance to antibiotics. Methicillin- and vancomycin resistant strains are among the most dangerous antibiotic-resistant pathogens known (11).

6.3.2 Streptococcus pyogenes

S. pyogenes is a spherical bacterium that grows in pairs or chains. It is a strictly human pathogen that mainly causes skin- and throat infections such as pharyngitis, scarlet fever, and erysipelas. Although most diseases are mild, they may evolve to life-threatening invasive infections of deeper tissues, the blood stream, and multiple organs (110). Like *S. aureus*, *S. pyogenes* has a multifold repertoire of virulence factors, both secreted such as superantigens, and surface bound such as the multifunctional M-proteins of which more than 100 serotypes have been identified to date (96,111). M-proteins are able to interact with a large number of host proteins, including e.g. fibrinogen, fibronectin, albumin, and IgG, an important feature to be able to infect different sites in the host such as mucosal surfaces, skin and connective tissue, and blood and lymphatic systems (112). Interestingly, streptococcal M-proteins have also been found to bind human kininogen (49). Two other surface proteins, termed streptococcal collagen-like surface protein A and B (SclA and SclB), have recently been identified (113,114). Their physiological role is not well known. However, they have been suggested to mediate adherence to human cells (113,115) and more recent studies have showed interactions between Scl proteins and components of human plasma, intriguingly including TAFI (116,117).

Present investigation

Paper I: BK differently affects expression of B_2R in fibroblasts and epithelial cells of the human lung

BK is generated in the airways of asthmatic subjects and has been suggested to contribute to the pathogenesis of chronic allergic inflammation (48,89) and B_2R is thought to be the major kinin receptor involved in airway responses to kinins. According to the common understanding that BK causes a rapid internalization of the B_2R, one would expect chronic asthmatic subjects to be insensitive to BK. However, inhalation of BK causes bronchospasm in asthmatics but not normal subjects (87,88). We therefore hypothesized that not all airway cell types react in the same way as the commonly used model cell line IMR-90 (a lung fibroblast) does, by rapid internalization of B_2R upon exposure to BK. Thus, the aim of the study was to compare the regulation of B_2R by BK in fibroblasts and epithelial cells from the human lung. To this end, we examined the induction of B_2R mRNA expression and stabilization, as well as prevalence of the B_2R at the cell surface in response to BK treatment in IMR-90 cells and BEAS2B cells, which is a human lung epithelial cell line.

By radio-ligand binding assays and electron-microscopy analysis we found that B_2Rs were down-regulated from the cell surface of IMR-90 cells but strongly up-regulated in the BEAS2B cells, in response to BK. The same tendency, although less pronounced, was observed at the mRNA level by quantitative RT-PCR analysis and this was at least partly due to an increased stabilization of B_2R mRNA by BK in the BEAS2B cells. The results provide a possible explanation for the sensitivity to BK in airway inflammation.

Paper II: Double-stranded RNA induces up-regulation of B_1Rs in human airway epithelial cells

Asthma symptoms exacerbate during respiratory viral infections such as common colds caused by rhinoviruses (118). Interestingly, increased levels of kinins have been found in the airways during such conditions and it has been reported that kinin generation is directly correlated to symptom severity (95). Since the effects of kinins are mediated by

their respective receptors, we asked whether kinin receptors are up-regulated during viral infections and whether this leads to increased inflammatory responses.

To this end, we stimulated BEAS2B, a human lung epithelial cell line, with Poly I:C, a double stranded (ds)RNA analog, in order to mimic a viral infection. Quantitative RT-PCR analysis revealed that poly I:C induced a modest increase of B_2R mRNA expression whereas the generation of B_1R mRNA was significantly boosted. By using a radio-ligand binding assay and by investigating activation of ERK (an intracellular second messenger) we further found up-regulated functional B_1Rs but not B_2Rs on the BEAS2B cell surface in response to poly I:C treatment. Poly I:C also induced B_1R mRNA expression in primary human normal bronchial epithelial cells. In addition, increased B_1R mRNA expression was found in nasal tissues of human subjects suffering from an upper respiratory viral infection.

The findings suggest that viral infections in the respiratory tract can lead to increased expression of B_1Rs in the airway epithelium, which might amplify detrimental inflammatory responses to generated kinins.

Paper III: *S. aureus* bacteria cause an up-regulation of B_1Rs during infection

In order to restrain a bacterial infection an efficient inflammatory response is of great importance. However, if the inflammatory process is not tightly balanced, an exaggerated or prolonged response may be detrimental to the host's own tissues and significantly contribute to severe symptoms of the disease. Pathogenic bacteria may take advantage by disturbing the delicate equilibrium of the inflammatory response. For instance, some bacterial pathogens trigger inflammatory reactions to open the vascular barriers, which eventually will promote influx of nutritious plasma to the site of infection and facilitate bacterial spreading.

Interestingly, *S. aureus* has been found to assemble the human contact system resulting in its activation and a continuous release of the potent vasoactive inflammatory mediator BK, from the bacterial surface (51). However, since the effects of kinins are mediated by their receptors, we hypothesized that an impact on the inflammatory response, by kinins, relies on kinin receptor regulation. Notable, up-regulation of B_1Rs, which are absent under normal conditions, has a potential impact on the inflammatory

reaction since they induce a more sustained response than the B_2R due to their lack of ligand induced desensitaization.

Here we investigated whether *S. aureus* can influence the regulation of kinin receptors in order to employ kinins, generated upon bacterial-induced contact activation, for induction of inflammatory reactions in the human host.

First we found that *S. aureus* secrete several exotoxins, which are known superantigens. We then showed that these substances strongly induce the release of pro-inflammatory cytokines, especially IL-1β, from human monocytic cells. IL-1β is known to induce up-regulation of B_1Rs (119). Thus, when the supernatants from bacterial-treated monocytic cells were added to human fibroblasts (IMR-90 cells), B_1R induction was observed both at the mRNA and the protein level. When kinins were added together with the monocytic exudates, B_1R surface expression was further increased and a clear shift in receptor expression (B_1R versus B_2R) was observed since the B_2Rs were down-regulated from the surface. Especially desArg^{9}BK acted in synergy with IL-1β to induce B_1R. Interestingly, BK, which is released from the surface of *S. aureus* during infection, was found to be processed into desArg^{9}BK by the action of carboxypeptidases located on eukaryotic cells. Further, an up-regulation of B_1Rs was also seen at the infectious site in a patient suffering from a soft-tissue *S. aureus* infection, implicating that the *in vitro* findings have clinical relevance.

Our findings suggest that *S. aureus* not only has the possibility to cause a continuous generation of desArg^{9}BK via contact activation and further processing by host carboxypeptidases, but also cause a prominent shift in kinin receptor surface expression towards B_1R. The *S. aureus*-induced up-regulation of B_1Rs may significantly extend the inflammatory response and thereby cause detrimental effects in the human host.

Paper IV: BK is processed to desArg^{9}BK by TAFI, bound to the surface of *S. pyogenes*

S. pyogenes is an important human pathogen that occasionally cause serious invasive infections, partially because the bacterium has evolved sophisticated mechanisms to evade or modulate the host's threatening immune responses (100). Depending on the progression stage of the infection, these modulations can diminish, over-activate, or

prolong the inflammatory response, which in all cases might be devastating to the human host. Like other pathogenic bacteria, *S. pyogenes* interacts with several host proteins involved in the host defense with the purpose to corrupt their functions. One example is human fibrinogen, which can be converted into a fibrin network around the bacteria and possibly providing protection from host defense mechanisms by shielding the bacteria from recruited phagocytes (96).

A recent study showed that also TAFI binds the surface of *S. pyogenes* bacteria (117). In addition, *S. pyogenes* efficiently bind human HMWK, which is cleaved at the bacterial surface resulting in release of BK (49). BK may be transformed into a B_1R agonist by the action of carboxypeptidases and interestingly, activated TAFI is such an enzyme.

The aim of the present study was to examine whether *S. pyogenes* can generate a B_1R ligand by hijacking TAFI, as well as evoke an up-regulation of B_1Rs in the human host and thereby amplify the inflammatory response.

First we detected degradation of HMWK and release of BK from the surface of *S. pyogenes* following pre-incubation of bacteria in human plasma. Then, by employing HPLC and electron microscopy analysis, we found that TAFI binds to the bacterial surface, where it is prone to activation by its natural activators. As a consequence, desArg^{9}BK is formed as the activated TAFI interacts with BK. Notable, the generated desArg^{9}BK was proven to be a functional B_1R ligand in functional assays. Finally, via stimulation of human monocytic cells by streptococcal supernatants, we found that *S. pyogenes* bacteria are able to induce up-regulation of B_1Rs, which was further amplified in the presence of desArg^{9}BK.

The results indicate that *S. pyogenes* can modulate an inflammatory response towards a chronic state, by employing TAFI to generate a B_1R ligand from BK and by inducing up-regulation of functional B_1Rs in the human host.

Conclusions

- Contradictory to other investigated cell types, human airway epithelial cells up-regulate surface expression of B_2R in response to BK. This phenomenon could potentiate the effects of generated BK in inflamed respiratory tracts and substantially contribute to symptom severity.

- Double stranded RNA, which is produced during viral replication, boosts biosynthesis of B_1R and its expression on the cell surface of human airway epithelial cells. As rhinovirus infection of the human airway is known to induce production of kinins, increased B_1R expression will render the airway epithelium more responsive to the increased levels of kinins, which could contribute to symptom exacerbation in subjects with pre-existing asthma.

- *S. aureus* can induce a shift in kinin receptor expression, from B_2Rs to B_1Rs, on human cells. *S. aureus* further induce release of BK, which via host enzymes is converted to desArg^{9}BK. Therefore, substantial signaling through B_1Rs are likely during *S. aureus* infections, which could, at least partly, account for development of a deleterious state of inflammation associated with *S. aureus* infections.

- *S. pyogenes* induce, via stimulation of host immune cells, a pro-found inflammatory state which provoke an up-regulation of B_1Rs in the human host. Further, a B_1R ligand is generated via a sophisticated mechanism involving binding of human TAFI, activation of the contact system, and truncation of released BK to generate desArg^{9}BK by activated TAFI. Subsequent B_1R signaling might re-direct inflammation from a transient to a chronic state.

Discussion

It seems contradictory that inflammation, which is vital for defense against invading microbes and injury, itself is a major cause of disease. However, the effects of inflammation are so powerful that they, if not precisely regulated, substantially can damage own cells and tissues and even threaten the survival of the host - making inflammation a double-edged sword.

Inflammation can be elicited by the contact system, which upon activation releases the potent vasoactive inflammatory mediator BK. One may speculate that the contact system primarily is part of the innate immune system as it adheres to several bacteria where it is activated and releases antimicrobial peptides as well as induces inflammation (45). However, even if such a function generally should be beneficial for the host and facilitate clearance of the microbe, pathogens are masters of manipulating host defenses and might corrupt the system to direct the inflammatory response and even small modifications in the inflammatory process may have great consequences.

Although or maybe because BK is a very potent inflammatory mediator, its activity is tightly regulated. Specific proteolytic activity is required for its liberation from HMWK, and once released it is rapidly degraded by kininases, giving BK a half-life of less than 15 s in plasma. Further, its receptor, B_2R, is subjected to rapid desensitization and internalization upon ligand binding (71). Therefore, effects mediated via B_2R signaling are self-limiting and participate in a short-term inflammatory response. However, as shown in paper I, a rapid B_2R internalization in response to ligand binding might not occur in all cell types.

The kininase I product of BK, desArg^{9}BK, is significantly more stable than BK and has been found to be increased at sites of inflammation, possible due to an induction of carboxypeptidase M under such conditions (120). The same conditions induce B_1R expression, and this receptor subtype elicits persistent responses and its up-regulation is further boosted by ligand binding. Hence, a switch from surface expressed B_2Rs to B_1Rs might have a great impact on the duration of the inflammatory response and thereby its deleterious side effects.

Kinins are generated during allergic- and virus-induced rhinitis and asthma as well as in several bacterial infections. The present thesis demonstrates that a receptor

subtype switch, form B_2R to B_1R, is induced under these pathologic conditions and it further provide insights into how B_1R ligand generation can occur. Up-regulated B_1R and an accumulation of its ligand provide perfect prerequisites for long-term noxious inflammatory responses. As such responses, often to a greater extent than the initial insult, contribute to morbidity and mortality there is an obvious need to regulate the pathological effects of inflammation and for that purpose B_1Rs should be an interesting drug target.

Taken together, the present thesis demonstrates that kinin receptor regulation and kinin generation is affected during different settings of inflammation, which possible can have a great impact on disease progression. The findings suggest that kinin receptors, especially B_1R, provide a potential target to treat as diverse pathologies as bacterial infections, asthma and viral infections of the human airways.

Swedish summary –

Populärvetenskaplig sammanfattning

Utan ett skyddande immunförsvar hade vi inte överlevt länge eftersom den mänskliga kroppen ständigt utsätts för potentiella hot såsom sjukdomsframkallande bakterier och virus. Immunförsvaret består av många olika komponenter som grovt sett kan delas in i två grupper som kallas det medfödda respektive det förvärvade (adaptiva) immunförsvaret. Det medfödda försvaret består bl.a. av antimikrobiella substanser och immuncellerna monocyter och neutrofiler. De är snabbt på plats vid en infektionshärd och bekämpar inkräktarna genom att äta upp dem och utsätta dem för toxiska substanser. Monocyterna, som kallas makrofager när de har vandrat ut från blodbanan till det infekterade stället, är duktiga på att tillkalla förstärkning från det adaptiva immunförsvaret. Den delen av immunförsvaret består till största delen av T-celler som kan ses som dess chefer och B-celler som med T-cellernas tillåtelse bildar antikroppar mot främmande substanser och patogener. Antikropparna märker ut och klumpar ihop det som är främmande och underlättar således arbetet för stridande immunceller.

Immunförsvaret inducerar inflammation vilket kan ses som vävnadens svar på en skada eller infektion. Inflammationsprocessen innebär en rad förändringar, bland annat i kärlen, som skall underlätta bekämpandet av inkräktare genom att leja immunceller till rätt plats och göra kärlen genomträngliga så att de stridande cellerna kan ta sig ut till infektionshärden. Inflammationsprocessen är så tätt sammanflätad med det medfödda immunförsvaret att de ibland används synonymt.

Då infektionen har bekämpats är det viktigt att inflammationen lägger sig och istället för att rekrytera immunceller inducerar läkning av skadad vävnad. De effekter som inflammationsprocessen har, bl.a. dess kärlpåverkan och utsöndringen av toxiska substanser som den inducerar, är nämligen så kraftiga att de förutom att förgöra inkräktaren även allvarligt kan skada den egna kroppens celler. Om en överaktivering sker lokalt i kroppen skadas den berörda vävnaden. Riktigt illa blir det om ett inflammatoriskt respons induceras systemiskt i kroppen eftersom det kan leda till så lågt blodtryck, pga kärlpåverkan, att följden blir multiorgansvikt och i värsta fall döden. Det är därför av yttersta vikt att inflammationsprocessen är noga kontrollerad.

Bradykinin (BK) är ett ämne som kraftigt inducerar inflammation genom att binda till sin receptor som kallas för B_2. Frisättning av BK är därför normalt sett noga kontrollerat. Vidare tillåter B_2-receptorn bara en kort signalering, dvs ett kort övergående inflammatoriskt svar, eftersom den snabbt blir okänslig för BK och dessutom nedregleras från cellytan och blir således otillgänglig. Dessutom klyvs BK snabbt sönder av olika s.k. proteaser i kroppen och blir inaktiv. Dock kan vissa proteaser, s.k. karboxypeptidaser, klyva BK på ett sådant sätt att ett annat aktivt ämne bildas som kallas för desArg^{9}bradykinin (desArg^{9}BK). Detta ämne förmedlar också inflammation och är betydligt stabilare än BK. Det har en annan typ av receptor som kallas B_1 och som under normala förhållanden inte finns tillgänglig, men som under vissa förutsättningar kan uppregleras till cellytan så att desArg^{9}BK kan komma åt att binda och på så sätt signalera inflammation. När B_1 väl finns på cellytan och aktiveras förmedlar den ett inflammatoriskt svar som blir beständigt. Detta pga att B_1 inte nedregleras efter en första aktivering utan fortsätter att vara mottaglig för aktivering via bindning av desArg^{9}BK. B_1 och desArg^{9}BK anses därför delta i utvecklingen av kronisk inflammation som kan åsamka mycket skada på kroppsegen vävnad.

Inflammation är ett stort problem i många, faktiskt de flesta, sjukdomstillstånd och orsakar ofta större skada än det ursprungliga hotet som kanske var en bakterie eller ett virus. Särskilt i vissa svåra bakteriella infektioner förekommer kraftig överaktivering av det inflammatoriska svaret och detta tillstånd har en hög dödlighet. Det samma gäller systemiska allergiska reaktioner.

Sjukdomsframkallande bakterier är mästare på att interagera med människans olika försvarsmekanismer och på så sätt undkomma dem genom att förändra, försvaga, förstärka eller omrikta dem, samt till och med använda dem för sina egna syften. Det kan tex vara en fördel för en bakterie att i vissa lägen inducera inflammation eftersom det gör kärlväggen mera genomsläpplig vilket medför att näringsrik plasma läcker ut till de infekterande bakterierna samt underlättar bakteriens spridning eftersom de lättare kan ta sig in och ut ur kärl.

Det är känt sedan tidigare att vissa mycket framgångsrikt infekterande bakterier tex *Streptococcus pyogenes* och *Staphylococcus aureus* kan inducera en frisättning av BK hos människa. *S. pyogenes* kan orsaka allt från ringa hudinfektioner och halsfluss till

mycket svåra infektioner och är känd i pressen som den fruktade ”köttätande mördar bakterien”. *S. aureus* kan också orsaka ett brett spektrum av infektioner och utgör ett allt större hot pga en snabbt ökande resistensutveckling mot antibiotika. Frisättning av kininer har också uppmärksammats i andra sjukdomstillstånd där inflammation är ett problem, tex i andningsvägarna hos astmatiker (astma är en form av allergi) och i luftvägsinfektioner orsakade av förkylningsvirus.

Syftet med den här avhandligen var att undersöka om frisättning av BK och bildandet av desArg^{9}BK samt tillgängligheten av deras respektive receptor påverkas vid infektion med *S. pyogenes* eller *S. aureus*. Vi undersökte också huruvida virus och förekomsten av BK påverkar luftvägscellers uttryck av B_1 och B_2.

I artikel I beskriver vi att BK faktiskt själv inducerar ett ökat uttryck av sin receptor, B_2, i luftvägarnas ytceller. Detta är i bjärt kontrast till andra undersökta celltyper där uttrycket av B_2 snabbt försvinner i närvaro av BK. Eftersom BK genereras hos astmatiker kan uppregleringen av B_2 ha stor betydelse för inflammationsnivån och därmed svårighetsgraden på sjukdomssymptomen pga att BK tillåts fortsätta signalera inflammation.

Artikel II beskriver att virus ger upphov till att B_1 receptorn kommer upp och blir tillgänglig på cellytan i luftvägarnas ytceller. Detta kan förklara varför astmatiker upplever förvärrade symptom vid förkylningar eftersom de kininer som frisätts vid en virusinfektion således kan signalera inflammation via B_1 receptorerna som annars inte finns tillgängliga.

Artikel III beskriver hur *S. aureus* kan orsaka ett totalt förändrat uttryck av kinin-receptorerna på ytan av mänskliga celler från att endast ha varit B_2 till uteslutande de mer långtidssignalerande B_1-receptorerna. Dessutom kan den här bakterien frisätta BK som via karboxypeptidaser på de mänskliga cellerna omvandlas till B_1-receptoraktiveraren desArg^{9}BK. Således modifierar *S. aureus* det inflammatoriska svaret mot ett mer långvarit respons som kan har stor betydelse för sjukdomsutvecklingen och skadeomfattningen på den mänskliga värden.

Artikel IV visar hur *S. pyogenes* på ett mycket sofistikerat sätt kan modifiera det inflammatoriska svaret genom att generera desArg^{9}BK i den mänskliga värden. Den ”kidnappar” nämligen ett mänskligt karboxypeptidas kallat TAFI som den binder till sin

yta. Eftersom den här bakterien även kan binda de komponenter som behövs för att lösgöra BK så frisättas BK från bakterieytan och klyvs direkt av bakterie-bundet TAFI till desArg^{9}BK. Dessutom, genom att utsöndra flera toxiner som inducerar inflammation i människa så förändras uttrycket av kinin-receptorer och B_1 kommer upp till ytan och görs synlig för desArg^{9}BK. Följdaktligen styrs det inflammatoriska svaret, via B_1 receptor-signalering, om mot ett mer kroniskt respons som utgör en ökad risk för skada på den mänskliga värden.

Sammantaget visar den här avhandligen att regleringen av kininer och deras receptorer är påverkat i olika inflammationssammanhang, vilket kan ha en stor betydelse för den aktuella sjukdomens utveckling. Forskningsresultaten tyder på att kinin-receptorer, särskilt B_1, är ett intressant mål för framtida läkemedel mot så vitt skilda åkommor som bakteriella infektioner, astma och virus-infektioner i luftvägarna.

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I

Regulation of Kinin B_2 receptors by bradykinin in human lung cells

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Running title: B2R regulation in human lung cells

Abstract

Bradykinin is a potent mediator of inflammation that has been shown to participate in allergic airway inflammation. The biologic effects of bradykinin are mediated by binding and activating its cognate receptor, the B_2 receptor (B2R). In the lung fibroblast cell line IMR-90, binding of bradykinin to the B2R triggers the down-regulation of the receptor surface expression suggesting that bradykinin-induced inflammation is transient and self-limited. Notably, subjects with chronic airway inflammation continue to respond to BK following a first challenge. B2Rs are expressed on many different lung cell types, including airway epithelial cells. We therefore compared IMR-90 cells with the human lung epithelial cell line BEAS2B and found that B2R expression in the two cell types is differently regulated by BK. While BK induces a down-regulation of B2R in IMR-90 cells, the same treatment leads to an up-regulation of the receptor in BEAS2B cells. These results provide a possible explanation for the potency of bradykinin in inducing ongoing airway inflammation.

Key words;
Asthma; BEAS2B cells; IMR-90 cells; Inflammation

Introduction

The nonapeptide bradykinin (BK) was described for the first time in 1949 (Rocha e Silva, et al., 1949) and is now considered a classical and one of the most potent promoters of inflammation in humans (for a recent review see (Leeb-Lundberg, et al., 2005)). Studies performed over the last six decades have shown that BK evokes a number of different inflammatory responses, including vasodilatation, increased vascular permeability and edema, hypotension, and pain (Leeb-Lundberg, et al., 2005). BK also activates pro-inflammatory signaling pathways and stimulates the expression of cytokines and chemokines (El-Dahr, et al., 1998; Pan, et al., 1996). The significance of the effects mediated by BK is reflected in the tightly regulated generation and catabolism of the peptide as well as in the expression of its receptor, the kinin B_2 receptor (B2R). The B2R belongs to the family of G-protein-coupled seven transmembrane receptors and is expressed in many cell types including epithelial, endothelial, smooth muscle, nerve cells, and fibroblasts (Marceau and Bachvarov, 1998).

BK is a short-lived peptide as it is processed and inactivated by kininases within seconds. Despite the short half-life, it has been found that BK levels can increase dramatically under systemic inflammatory conditions such as hereditary angioedema or severe infectious diseases (Bork, et al., 2007; Mattsson, et al., 2001). There is also substantial evidence that BK contributes to pathological inflammatory conditions in chronic allergic diseases (Farmer, et al., 1992). For instance, acting through the B2R, BK has been suggested to play a key role in the pathogenesis of asthmatic inflammation (Abraham, et al., 2006; Proud, 1998). Interestingly, not only is BK generated in the airway of asthmatic subjects (Christiansen, et al., 1987; Christiansen, et al., 1992), but also an up-regulation of the B2R in asthmatic inflammation has been observed which can be caused by inflammatory cytokines such as transforming growth factor-β (TGF-β), tumor

necrosis factor-α (TNF- α), and interleukin 1β (IL-1 β) (Schmidlin, et al., 1998; Zhang, et al., 2007). Taken together, these observations suggest that the generation of BK under chronic inflammatory conditions combined with an up-regulation of B2R could constitute an important patho-mechanism that contributes to some of the complications observed in asthmatic patients.

Most *in vitro* studies on the regulation of B2R have been performed with IMR-90 cells, a human lung myofibroblast cell line (Ehler, et al., 1996), or with transfected cells. In these cells, the B2R desensitizes rapidly upon ligand binding and is internalized (Blaukat, et al., 1996; Simaan, et al., 2005). If BK is involved in the pathogenesis of chronic asthmatic inflammation, however, one would expect airway cells to respond with a sustained activity that involves up-regulation rather than down-regulation of the receptor. Thus, it is tempting to speculate that not all airway cells down-regulate B2R to the same extent as has been shown for IMR-90 cells. In order to address this question, we compared the effects of long-term BK exposure on cell surface expression of B2R in human lung fibroblasts and human lung epithelial cells.

Results

Influence of BK on specific B2R binding

We compared BEAS2B cells, a human epithelial cell line, to IMR-90 cells with respect to their response to BK treatment. In the first set of experiments, IMR-90 and BEAS2B cells were pre-treated in the absence or presence of BK for 6 h. The number of B_2 receptors on the cell surface was then determined by radiolabeled ligand assay using [^{3}H]BK. Figure 1a shows that exposure of IMR-90 cells to BK caused an almost complete down regulation (>90%) of available B2R, while the same treatment resulted in a significant up-regulation of B2R in BEAS2B cells (Figure 1b). The data show that the surface expression of B2R is differently regulated in IMR-90 and BEAS2B cells.

BK induces reorganization of B2R between the cell surface and the cytosol

As the radioligand binding experiments showed a clear difference in surface expression of B2R in response to BK in the IMR-90 and BEAS2B cells, we assessed the relationship between internalized and cell surface B2R after treatment with BK in these cell types. To investigate this, we used transmission electron microscopy to visualize the B2Rs in the cell. Briefly, cells were incubated with or without BK for 6 hours, embedded, thin sectioned, incubated with primary antibodies against B2R followed by gold-labeled secondary antibodies and subjected to transmission electron-microscopical analysis. Figure 2a depicts that the majority of the B2Rs were found in the plasma membrane of untreated IMR-90 cells, while receptors were preferentially localized intracellularly in cells treated with BK. This is in contrast to BEAS2B cells, where an increase of B2Rs at the cell surface is seen upon exposure to BK (Figure 2b). Taken together, the electron microscopical results suggest that IMR-90 and BEAS2B cells have

different mechanisms by which they regulate the density of the B2R on their surfaces in response to BK.

BK increases the expression of B2R mRNA in BEAS2B cells but not in IMR-90 cells

Next, we explored whether the increase in BK-induced B2R cells surface expression in BEAS2B cells was associated with an induction in B2R gene expression in BEAS2B cells and compared this with the effect of BK on B2R gene expression in IMR-90 cells. To do so, BEAS2B and IMR-90 cells were incubated for 6 hours with or without BK. RNA was isolated and B2R mRNA and GAPDH mRNA levels were assessed by quantitative real-time PCR. In IMR-90, cells BK caused a 50% decrease in B2R mRNA levels, while in BEAS2B cells BK induced a 50% increase in B2R mRNA levels (Figure 3). The same trend was seen at 2 hours post BK stimulation (data not shown).

BK-induced post transcriptional regulation of B2R mRNA in BEAS-2B cells

In the next series of experiments we analyzed whether BK can influence the stability of B2R mRNA in IMR-90 and BEAS2B cells. For this purpose, both cell types were incubated with Actinomycin D, a transcription inhibitor, in the presence or absence of BK. Total RNA was harvested from the cells at different time points (0 to 6 h) and B2R mRNA was measured by quantitative real-time PCR. As shown by the representative result in Figure 4b, BK-treatment clearly extended the half-life of B2R mRNA in BEAS2B. On the other hand, BK had no significant effect on the B2R mRNA stability in IMR-90 cells (Figure 4a). Therefore, increased B2R mRNA levels in BEAS2B cells following BK treatment are, at least in part, due to an extended stability of B2R mRNA.

Discussion

Bradykinin is a potent inflammatory mediator that has been suggested to play an important role in allergic airway diseases (for reviews see (Abraham, et al., 2006) and (Proud, 1998)). The molecular mechanisms that lead to the generation of kinins are not completely understood, however, it has been found that kinin levels are significantly increased under pathological conditions, for instance in bronchoalveolar lavage fluids from asthmatic patients (Christiansen, et al., 1992). It is now generally believed that most of the effects evoked by kinins in human airways involve B2R (for a review see (Abraham, et al., 2006)). Furthermore, it has been reported that B2R antagonists effectively block the development of the late phase airway response as well as the development of bronchial hyperresponsiveness following allergen challenge in a number of different experimental animal models (Abraham, et al., 2006). In order to become hyper-reactive to BK, many cell types, including airway smooth muscle cells and bronchial epithelial cells, up-regulate B2R on their cell surface once they have been stimulated with pro-inflammatory mediators such as transforming growth factor-β (TGF-β) tumor necrosis factor-α (TNF- α) and interleukin 1β (IL-1 β) (Kim, et al., 2005; Schmidlin, et al., 1998; Zhang, et al., 2004; Newton, et al., 2002). Interestingly, this is in contrast to IMR-90 cells (a lung myofibroblast cell line), which do not respond with a dramatic up-regulation of B2R when treated with these cytokines (Bengtson, et al., 2006; Phagoo, et al., 1999). The regulation and expression of the B2R has been most extensively investigated in IMR-90 cells, a lung myofibroblast cell line. Many studies have shown that after ligand binding the B2R undergoes rapid phosphorylation, desensitization and internalization (Blaukat, et al., 1996; Munoz and Leeb-Lundberg, 1992). Most of the phosphorylated B2R is then dephosphorylated and re-expressed at the cell surface; however prolonged exposure to BK has been reported to result in sustained down-regulation of B2R

expression in human fibroblasts (Blaukat, et al., 2003). Based on these findings it is now believed that IMR-90 cells utilize B2R activation for causing a transient inflammatory response. However, as allergic airway diseases are often combined with a persistent inflammatory phase, we speculated that not all airway cell types control B2R expression in this manner. In particular, cells that induce an up-regulation of the receptor would contribute to a sustained inflammatory response, if the receptor remains at the cell surface upon agonist treatment.

To specifically address the potential impact of prolonged BK exposure on B2R expression in the inflamed airway, we compared the effect of BK on B2R expression in human airway fibroblasts (IMR-90 cells) and human airway epithelial cells (BEAS2B). We chose to investigate this in airway epithelial cells as these cells respond with a massive up-regulation of B2R expression when exposed to inflammatory mediators, thus rendering them more responsive to BK during inflammation. Moreover, airway epithelial cells play a significant role in airway diseases since they are among the first cells to come into contact with inhaled substances, they produce a large array of inflammatory cytokines upon stimulation and their damage is a typical feature in many airway diseases.

In this study, we find that the B2R is differently regulated in BEAS2B and IMR-90 cells. Indeed, while BK triggers a down regulation of B2R cell surface expression in IMR-90 cells, as determined by radioligand binding and transmission immuno electron microscopy, BEAS2B cells respond to the same treatment with an up-regulation of the receptor cell surface expression. Subsequently we observed that the rise of the B2R density at the cell surface correlates well with an increased expression of B2R mRNA in BEAS2B cells following stimulation with BK.

Moreover, the BK-induced increase in B2R mRNA in the BEAS2B cells is at least in part due to an extended half-life of B2R mRNA. In contrast, we observed in IMR-90 cells that BK treatment resulted in a decrease in B2R mRNA levels and had no effect on the half-life of B2R mRNA. Upon B2R activation the myofibroblast cell line IMR-90 presumably mediates an inflammatory response that is transient and rapidly down-regulated. It should be mentioned that the origin of myofibroblasts, produced following injury, is not completely understood and it has been reported that they may derive from a variety of sources including transdifferentiation of fibroblasts and smooth muscle cells or dedifferentiation of epithelial cells (McAnulty, 2007). However, it should be noted that during the metamorphosis to myofibroblasts, the cells change their phenotype (Willis, et al., 2006), which may explain, why IMR-90 cells regulate B2R expression differently than BEAS2B cells. Keeping in mind that myofibroblasts have an important function in wound healing, it is tempting to speculate that a transient signal is sufficient to prepare the cells for their task. For epithelial cells, on the other hand, it might be beneficial to sense and respond to BK as long as an inflammatory reaction is ongoing in order to guarantee an efficient elimination of the cause of the inflammation. The present study clearly shows that B2R expression is increased upon ligand activation in airway epithelial cells. Under chronic disease conditions sustained signaling via B2R in airway epithelial cells could potentially contribute to prolonged, uncontrolled inflammation leading to additional complications such as bronchoconstriction and tissue remodeling.

Materials and Methods

Culture of BEAS2B and IMR-90 cells.

The human airway epithelial cell line BEAS2B (CRL-9609) and the human fetal lung fibroblasts cell line IMR-90 (CCL-186) (ATCC Manassas, VA, USA) were cultured as previously described (Eddleston, et al., 2002; Phagoo, et al., 1999). Cells were plated at a density of 1.5 x 10^5 cells/well in 6-well plates (35 mm well) and experiments were conducted after 3-4 days when cells were confluent. Cells were washed and treated in media free from supplements, antibiotics and serum. Experiments with BEAS2B were conducted in supplement free Keratinocyte-SFM media except for treatment prior to binding experiments, which was done in Minimum Essential Medium (MEM) containing L-glutamine alone.

Radioligand binding.

IMR-90 and BEAS2B cells were treated with or without 10μM BK for 6h, where after they were washed and subjected to binding analyzes. The binding of 1 nM [^{3}H]BK (90.0 Ci/mmol) (PerkinElmer Life Science Products, Boston, Massachusetts, USA) to IMR-90 or BEAS2B cells was performed as described earlier (Phagoo, et al., 1999). Binding assays were conducted on ice in triplicate, and nonspecific binding was defined as the amount of radiolabeled ligand bound in the presence of 1 μM non-radiolabeled BK. Specific binding (non-specific binding subtracted from total binding) was expressed in relation to specific binding of non-stimulated cells which was considered as 100%.

Thin-sectioning and transmission electron microscopy.

IMR-90 and BEAS2B cells were incubated in the presence or absence of 10μM BK for 6h before they were imbedded. Thin sections were subjected to immuno-labeling as described (Roth, 1986) with the modification that Aurion-BSA (Aurion, Wageningen, the Netherlands) was used as a blocking agent. Briefly, the sections were placed on grids and were incubated with primary antibodies against B2R, followed by immuno-detection with a 5 nm gold bead on a secondary antibody against rabbit IgG (Agar Scientific ltd. Stansted, UK). Samples were finally stained with uranyl acetate and lead citrate and observed in a Jeol JEM 1230 electron microscope, operated at 80 kV accelerating voltage. Images were recorded with a Gatan Multiscan 791 CCD camera. Evaluation of the data, numbers of gold particles, was determined for 50 cellular profiles for each condition.

RNA isolation and reverse transcription (RT).

IMR-90 and BEAS2B cells were treated with or without 10μM BK for 6h before total RNA was extracted from the cells using the RNA STAT-60™ reagent method (Tel-Test Inc., Friendswood, Texas, USA) as described by the manufacturer. Isolated RNA was treated with DNase (DNA-free™) (Ambion, Austin, Texas, USA) according to the instructions of the manufacturer. 1 μg RNA/sample was denatured at 65°C for 5 min, to avoid hairpin structures, and then reverse transcribed in a total volume of 20 μl using the Omniscript™ RT Kit (Qiagen, Valencia, California, USA). The reaction was carried out for 1h at 37°C and stopped by a 3 min-incubation at 95°C. A 1/20 aliquot of the RT product was used for subsequent quantitative real-time PCR.

Quantitative real-time PCR.

The 7300 real time PCR system (Applied Biosystems, Foster City, CA, USA) was used for the real-time quantitative PCR analyzes. The primers used were as follows; B2R forward primer, 5'-gggcacactgcggacct-3'; B2R reverse, 5'-gcgtttgctcactgtctgctc-3'; GAPDH forward primer, 5'-gggaaggtgaaggtcggagt-3'; and GAPDH reverse primer, 5'-tccactttaccagagttaaaagcag-3'. The following dual-labeled probes were obtained from BioSearch Technologies (Novato, California, USA): B2R 5' FAM-tccgtggaacgccagattcacaaac-TAMARA; GAPDH 5' FAM-accaggcgcccaatacgaccaa-BHQ. Standards, from 10 to 0.0001 attomoles of the PCR product cloned into pGEMTeasy (Promega, Madison, Wisconsin, USA), were run alongside the samples for the generation of a standard curve. All samples and standards were analyzed in triplicates. The PCR reaction mixture consisted of 1.5 mM Tris-HCl, 5 mM KCl, 0.2 mM dNTPs, 4 ng/μl of forward and reverse primers and 0.1 pg/μl of B2R probe, 3 mM Mg^{2+} and 0.025 U/μl of Amplitaq gold (Applied Biosystems, Foster City, California, USA) and 0.25 μl Reference Dye for Quantitative PCR (Sigma, St. Louis, Missouri, USA) in a total volume of 25 μl. For comparison, 0.06 pg/μl of the GAPDH probe was employed instead of the B2R probe. Samples were incubated for 10 min at 95°C, followed by 50 cycles of 15 s at 95°C and 60 s at 60°C. The starting amount of specific cDNA in each sample was calculated using the 7300 real time PCR system software package (Applied Biosystems, Foster City, California, USA).

Stability of B2R mRNA.

To measure the influence of BK on the half-life of B2R mRNA present in the cells, IMR-90 cells or BEAS2B cells were incubated with 10 μg/ml of the transcription inhibitor Actinomycin D (*Streptomyces sp.*) (Calbiochem, San Diego, CA, USA) in the presence or absence of 10 μM BK

(Bachem, Torrance, California, USA) at 37°C. Total RNA was harvested from the cells at 0, 0.5, 1, 2, 4 and 6 hours. B2R mRNA as well as GAPDH mRNA levels were measured in all samples by quantitative real-time PCR, and B2R mRNA levels were normalized to GAPDH mRNA levels. The level of B2R mRNA at 0 hours was considered 100%.

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Figure legends

Figure 1.

B2R cell surface expression in IMR-90 and BEAS2B cells upon BK treatment.

IMR-90 (a) and BEAS2B (b) cells were treated with either BK (10μM) or media alone for 6 hours and then analyzed for B2R cell surface expression by radiolabeled ligand binding assay. Each binding experiment was performed in triplicate and the figure shows the mean ± SEM of three independent experiments. Specific B2R binding equals non-specific binding subtracted from total binding and the specific binding to non-treated cells is considered as 100%. $*P < 0{,}001$ by *t-test*.

Figure 2.

Distribution of B2R in IMR-90 and BEAS2B cells before and after BK treatment.

IMR-90 and BEAS2B cells were treated with either BK (10μM) or media alone for 6 hours, where after they were sliced and immunolabelled with specific B2R antibodies that were detected with secondary antibodies marked with gold beads. Figure 2a shows detected B2R, one representative picture for each condition, where panel (A) is non-treated IMR-90 cells and (B) IMR-90 cells treated with BK, (C) is non-treated BEAS2B cells, and (D) BK-treated BEAS2B. Arrows point at examples of B2R marked with gold-labeled antibodies. Scale bar indicate 250 nm. Figure 2b shows the distribution of B2R between the cell surface and the cytosol in % in BK and non-treated IMR-90 and BEAS2B cells. Statistics were performed on 50 cellular profiles.

Figure 3.

Induction of B2R mRNA expression in IMR-90 and BEAS2B cells following BK treatment.

IMR-90 and BEAS2B cells were treated with either BK (10μM) or media alone for 6 hours. Total RNA was extracted and reverse transcribed. B2R mRNA levels and GAPDH mRNA levels were measured using specific dual labeled probes and primers. B2R mRNA levels were normalized to GAPDH mRNA levels in each sample. A standard was run alongside. All samples and standards were analyzed in triplicate and the bars show 3 independent experiments ± SEM. As shown, BK induced a 50% decrease in B2R mRNA levels in IMR-90 cells (a), while a 50% increase was seen in the BK-treated BEAS2B cells (b). *$P<$ 0,001 by *t-test*.

Figure 4.

BK induces increased B2R mRNA stability in BEAS2B cells

To measure the influence of BK on the half-life of B2R mRNA, IMR-90 cells or BEAS2B cells were incubated with the transcription inhibitor Actinomycin D in the presence or absence of BK (10μM). Cells were incubated for 0h, 0.5h, 1h, 2h, 4h, or 6h before total RNA was extracted. B2R mRNA was measured by quantitative real-time PCR. BK (dashed line) did not have a significant effect on the B2R mRNA stability in IMR-90 (a) cells whereas it clearly extended the B2R half-life in BEAS2B cells (b).

Figures

Figure 1

Figure 1a

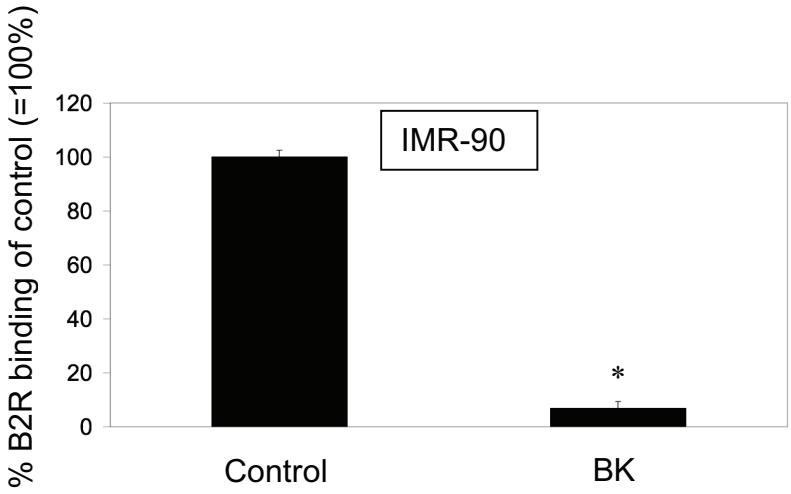

Figure 1b

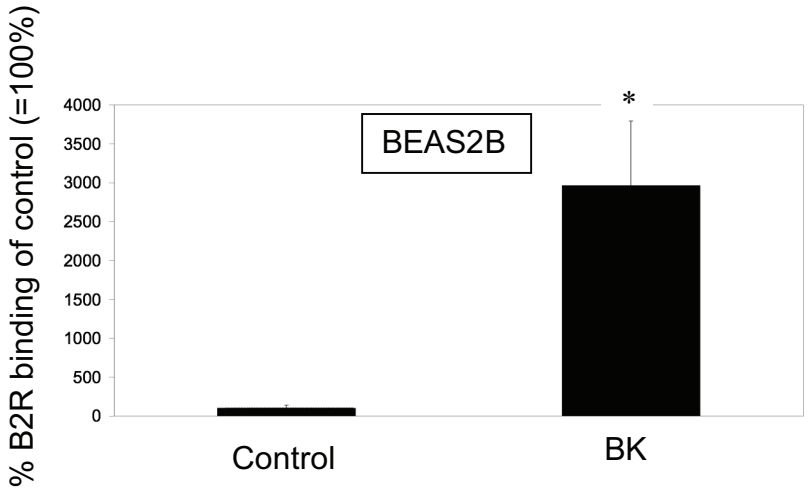

Figure 2

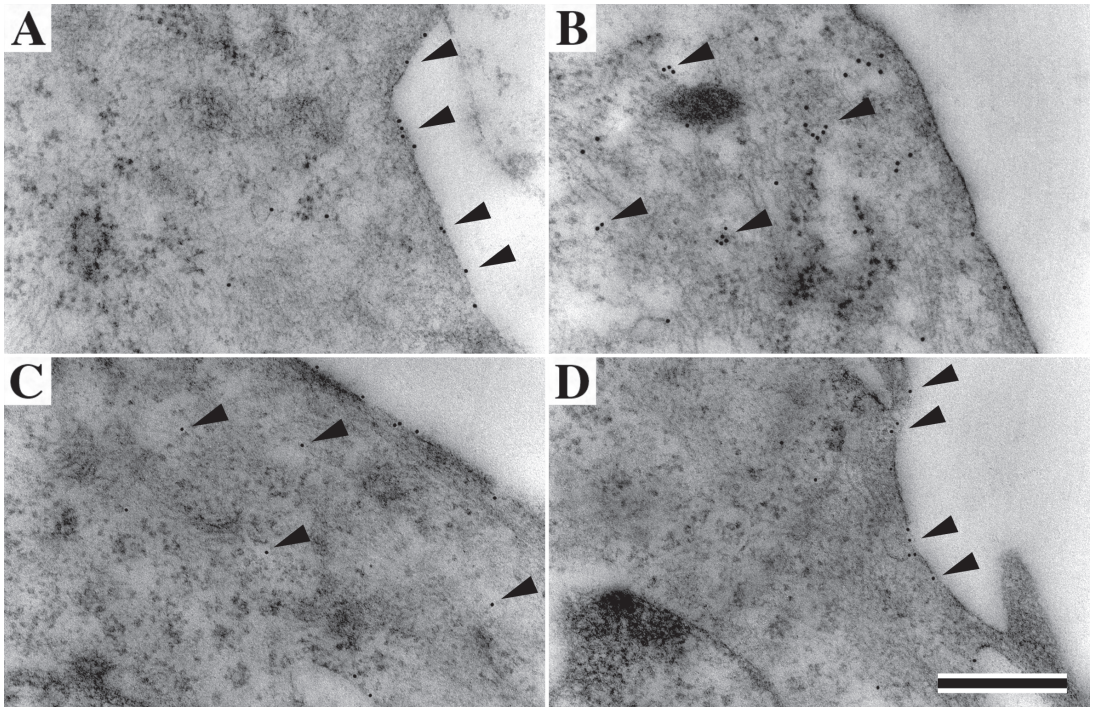

Figure 2b

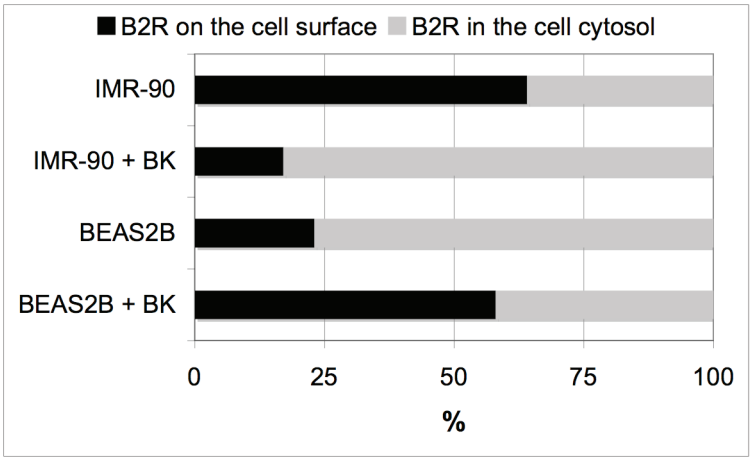

Figure 3

Figure 3a

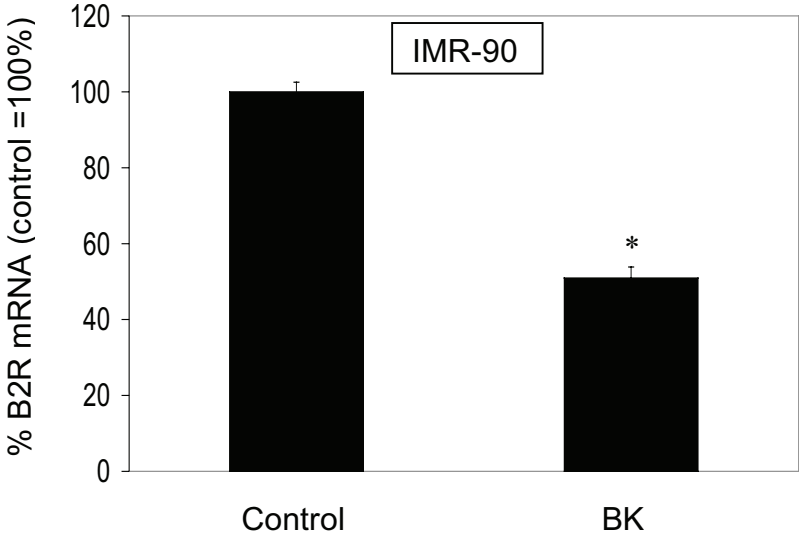

Figure 3b

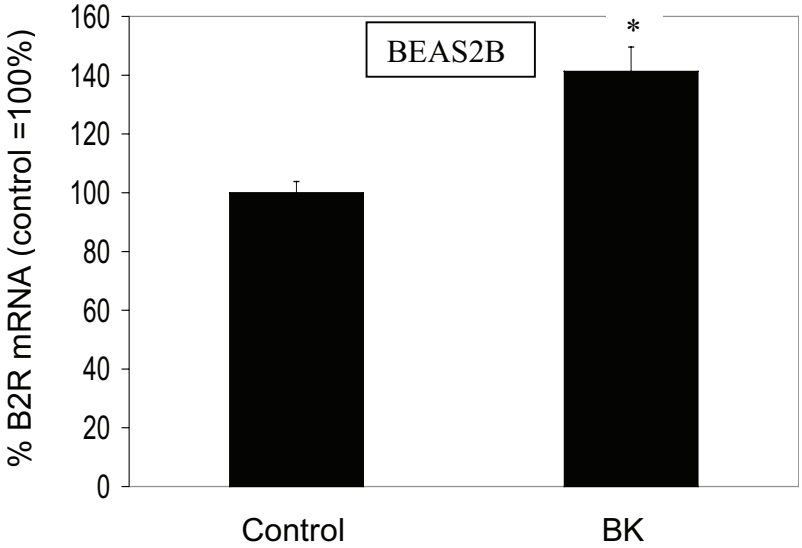

Figure 4

Figure 4a

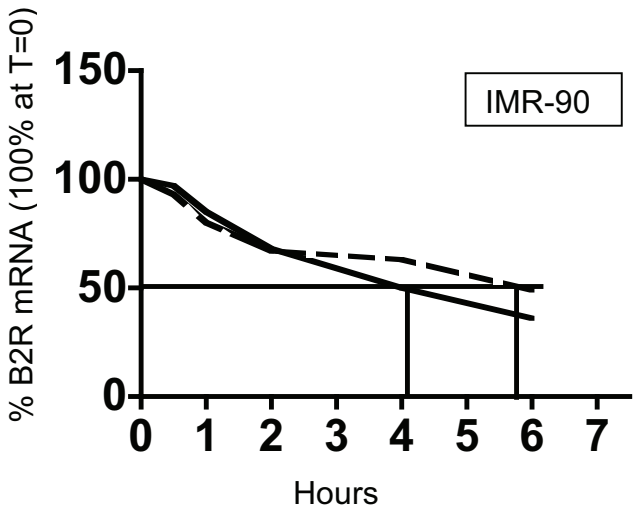

Figure 4b

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Double-stranded RNA increases kinin B1 receptor expression and function in human airway epithelial cells ☆

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Abstract

Increased levels of kinins have been detected within the airways during upper respiratory viral infections (URIs). Rhinovirus, the major URI associated with acute exacerbations of asthma, is an ssRNA virus that primarily infects the airway epithelium and produces dsRNA during replication. We asked whether dsRNA could increase the expression of kinin receptors in airway epithelial cells, thereby potentiating the inflammatory consequences of kinin generation. Human airway epithelial cell line BEAS-2B was stimulated with the dsRNA analog Poly I:C and kinin receptor expression detected by quantitative RT-PCR as well as radioligand binding. Poly I:C induced an increase in B1 and B2 receptor mRNA levels in BEAS-2B and primary human normal bronchial epithelial cells. At the cell surface, only B1 receptor expression was increased by Poly I:C. Furthermore, pretreatment of BEAS-2B cells with Poly I:C enhanced the induction of phospho-ERK following B1 receptor ligand stimulation. To investigate whether these finding had potential *in vivo* relevance, we assessed B1 receptor expression in nasal tissue obtained from 8 normal human subjects with URIs and 3 control subjects. Five of the URI subjects demonstrated increased B1 receptor mRNA compared to the 3 control subjects. We suggest that increased expression of B1 receptor in the human airway following a URI could increase the risk of an exacerbation of asthma by contributing to increased inflammation in the airway.
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Keywords: Airway; dsRNA; Epithelial; Kinin; Viral

1. Introduction

Respiratory viral infections are the major cause of asthma exacerbations [1,2]. Rhinovirus (RV) infections account for more than 50% of colds during the peak fall season and are a major cause of asthma exacerbations and morbidity in children and adults [3]. The mechanisms leading to viral-induced asthma exacerbations, however, have not been fully elucidated. Consequently there is a need to understand more thoroughly the pathways that are activated during respiratory viral infections.

Kinins are sentinel mediators that are rapidly generated in response to diverse stimuli then participate in the activation of the inflammatory response [4–7]. Several observations suggest that respiratory viral infection activates the kinin system. Experimental rhinovirus infection of human volunteers resulted in increased local kinin (both bradykinin and Lys-bradykinin) levels,

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with symptom severity directly correlating with the level of kinin generation measured in nasal secretions [8,9]. Animal models also suggest a participatory role for kinin generation during respiratory viral infections. Objective signs of respiratory disease in ferrets inoculated with influenza virus corresponded with the appearance of kinins in the nasal lavages, and airway hyper-responsiveness following parainfluenza-3 infection in guinea pig correlated with the generation of bradykinin [10,11].

Additional evidence suggests that there may be a link between the activation of the kinin system and activation of the innate immune response during viral infection of the airway. Toll-like receptors (TLRs) sense bacterial and viral components and upon contact activate the innate immune system [12]. TLR3, which recognizes dsRNA, has been associated with the immune response of lung epithelial cells to RNA viruses [13–15]. During replication of ssRNA viruses, such as rhinovirus, dsRNA is produced within the infected cell as a viral replicative intermediate and can potentially activate TLR3 as well as the double-stranded RNA-activated protein kinase (PKR) [16]. Interestingly a recent study reported that activation of TLR2, TLR4 and TLR3 in cultured murine tracheal segments increased the sensitivity of the murine trachea to agonists for both the kinin B1 receptor and kinin B2 receptor, potentially by increasing their expression [17].

We hypothesized that during human respiratory viral infection, viral dsRNA upregulates kinin receptor expression. This mechanism could thereby enhance the detrimental effects of kinins that are generated through virus-induced activity of tissue kallikrein. To address our hypothesis we investigated the effect of the dsRNA analog Poly I:C on the expression of kinin receptors by airway epithelial cells in vitro, as well as assessing the level of B1 receptor mRNA in the nasal tissue of subjects with cold symptoms.

2. Methods

2.1. Subject characteristics and nasal sample collection

Eight non-smoking, non-atopic individuals with symptoms of active upper respiratory infections (URI) and 3 subjects without allergy or URI symptoms were recruited to participate in the study. Informed consent was obtained in accordance with the Human Subjects Committee at The Scripps Research Institute. Nasal samples were collected by gentle scraping of the inferior turbinate using a Rhinoprobe curette (Arlington Scientific Inc., Springville, Utah). The sample was placed directly into RLT buffer (Qiagen Inc., Valencia, California) and placed immediately at − 70 °C.

2.2. Cell culture conditions and treatments

The human airway epithelial cell line BEAS-2B (CRL-9609, ATCC Manassas, VA, USA), and normal human bronchial epithelial cells (NHBEs, Clonetics-BioWhittaker Walkersville, MD, USA) were cultured as previously described [18,19]. Cells were treated in supplement free keratinocyte media (Invitrogen, Carlsbad, CA, USA) with Poly I:C (polyinosinic:polycytidylic acid, Invivogen, San Diego, CA, USA).

2.3. RNA isolation and reverse transcription

Total RNA was extracted from nasal tissue samples and NHBEs using the RNeasy mini kit (Qiagen Inc.). DNA was digested during the RNA isolation procedure using the RNase-Free DNase 1 treatment kit (Qiagen Inc.). Total RNA from BEAS-2B cells was isolated using RNA-STAT 60 (Tel-TEST, Friendswood, TX) following the manufacturers' directions. A third of the nasal RNA or 1 μg of either NHBE or BEAS-2B RNA was reverse transcribed using the Omniscript RT kit (Qiagen Inc.). A 1/20th aliquot of the RT product was used for subsequent PCR.

2.4. Quantitative real-time PCR

Quantitative real-time PCR was performed as previously described [5] to assess the levels of B1 receptor (B1R), B2 receptor (B2R) and the housekeeping gene *β-actin*. The primers used were as follows: B2R forward primer, 5′-gggcacactgcggacct-3′; B2R reverse primer 5′-gcgtttgctcactgtctgctc-3′; B1R forward primer, 5′-caactgaacgtggcagaaatctac-3′; B1R reverse primer, 5′-caagcccaagacaaacaccag; β-actin forward primer 5′-tgcgtgacattaaggagaag-3′; β-actin reverse primer 5′-gtcaggcagctcgtagctct-3′. The following dual-labeled probes were obtained from Biosearch Technologies (Novato, CA): B2R, 5″-FAM-tccgtggaacgccagattcacaaac-3′BHQ (black hole quencher); B1R, 5′-FAM-tggccaacctggcagcctctga-3′BHQ; β-actin 5′-FAM-cacggctgcttccagctcctc-3′BHQ.

2.5. Ribonuclease protection assay (RPA)

Specific mRNA levels for the *B1R* and *IL-8* gene in the nasal samples were measured by multiprobe RPA as previously described [5]. Briefly, antisense RNA probes were prepared by in vitro transcription of PCR products of each gene cloned into pGEM3Z plasmid using either T7 or SP6 RNA polymerases (Promega) with the incorporation of UTP (800 Ci/mmol; Amersham, Arlington Heights, IL). 1 μg of RNA per sample was heated at 95 °C for 5 min then hybridized with 1 μl of labeled riboprobe mix for 16 h at 55 °C in a total volume of 10 μl. Unhybridized single-stranded RNA was digested with 100 μl of RNase digestion mixture for 1 h at 30 °C. Undigested RNA was purified by ethanol precipitation and separated on a 6% acrylamide/urea sequencing gel. The protected bands were visualized and quantified by scanning the gels using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

2.6. Radioligand binding

BEAS-2B cells were grown to confluency in 6-well culture plates. Cells were washed twice with ice-cold Hank's balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+} (BioWhittaker). Binding assays were performed on ice in triplicate in a final volume of 1 ml/well. For B1R binding BEAS-2B cells were incubated for 90 min with 1 nM [^{3}H]desArg^{10}kallidin (77.5 Ci/mmol, PerkinElmer Life Science Products, Boston, Massachusetts, USA) in binding buffer [20 mM HEPES, pH 7.4, 5 mM KCl, 125 mM *N*-methyl-D-glucamine (Sigma), 0.14 g/l bacitracin (Sigma), 1 mM 1,10-phenanthroline(Sigma)] with or without 1000-fold excess of desArg^{10}kallidin. For B2R binding BEAS-2B cells were incubated for 90 min with 1 nM [^{3}H]bradykinin (90.0 Ci/mmol, PerkinElmer Life Science Products, Boston, Massachusetts, USA) in binding buffer with or without 1000-fold excess of bradykinin. After incubation, the cells were washed twice with ice-cold PBS containing 0.3% BSA (Sigma) and then lysed with 100 μl of 2% SDS. Cell lysates were added to 6 ml scintillation cocktail (Lefko-Fluor, Research Products International Corp., Mt. Prospect, Illinois, USA) and the [^{3}H]desArg^{10}kallidin or [^{3}H]bradykinin binding quantitated using a Packard Tri-Carb 2100TR liquid scintillation analyzer (Global Medical Instrumentation, Inc., Albertville, Minnesota, USA). Non-specific binding was defined as the amount of radiolabeled ligand bound in the presence of excess non-radiolabeled ligand. Specific binding (non-specific binding subtracted from total binding) was expressed in relation to specific binding of non-stimulated cells that was considered as 100%.

2.7. Lys-des-Arg-bradykinin treatment and protein isolation

BEAS-2B cells were grown to confluence in 6-well plates, either untreated or stimulated with Poly I:C (1 μg/ml) for 24 h, then were treated with 10^{-7} M Lys-des-Arg-bradykinin (Bachem California, Inc., Torrance, CA) for 5 and 20 min. The cells were washed twice in ice-cold PBS containing 1 mM Na_3VO_4. Cells were incubated for 10 min in lysis solution (20 mM Tris pH 7.5, 120 mM Na Cl, 10% glycerol, 1% Triton X100, 1 mM Na_3VO_4, 1 mM PMSF) and then the lysates were centrifuged for 20 min at 12,000 rpm at 4 °C. The supernatants were collected and stored at − 70 °C. Protein concentrations were determined using the Bradford Assay kit (Bio-Rad, Hercules, CA).

2.8. ERK immunoblotting

15 μg of protein was separated by SDS-PAGE on a 12% (for ERK and p38 detection) or a 15% (for SAP/JNK detection) acrylamide gel and electrophoretically transferred to a nitrocellulose membrane. The membrane was blocked with 5% non-fat milk in 1× TTBS (1× Tris-buffered saline, 0.1% Tween-20) for 1-h shaking at room temperature. ERK, SAPK/JNK and p38 phospho-specific antibodies were used as directed by the manufacturer (Cell Signaling Technology™, Beverly, MA). After detection of the phospho-protein the blot was stripped and hybridized with antibodies specific for total ERK, SAPK/JNK or p38 as appropriate.

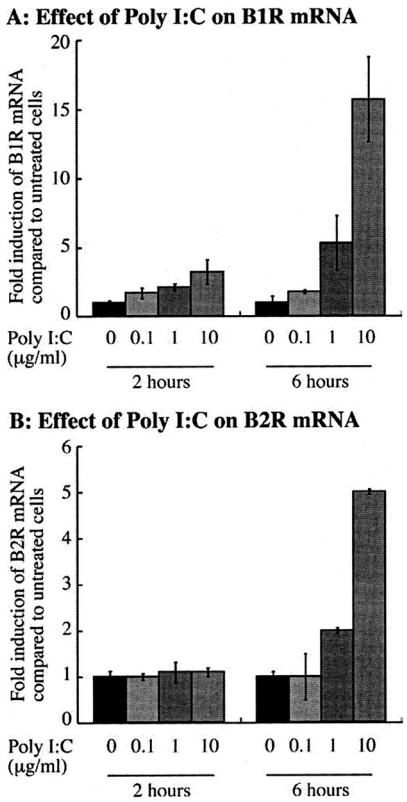

Fig. 1. Poly I:C increases bradykinin B1 and B2 receptor mRNA levels in BEAS-2B cells. BEAS-2B cells were stimulated with 0.1, 1 or 10 μg/ml of Poly I:C or media alone for 2 and 6 h. Total RNA was extracted and assessed in triplicate by quantitative real-time RT-PCR for B1 receptor, B2 receptor and b-actin mRNA levels. B1 receptor and B2 receptor mRNA levels were normalized to b-actin mRNA levels. The data were calculated as the fold induction of B1 receptor (A) or B2 receptor (B) in Poly I:C-treated cells compared to the level in untreated cells.

3. Results

3.1. Poly I:C induces an increase in B1 receptor and B2 receptor mRNA levels in a human airway epithelial cell line

BEAS-2B cells were stimulated with 0.1, 1 and 10 μg/ml of Poly I:C for 2 and 6 h. B1 receptor, B2 receptor and β-actin mRNA levels were measured by quantitative real-time PCR,

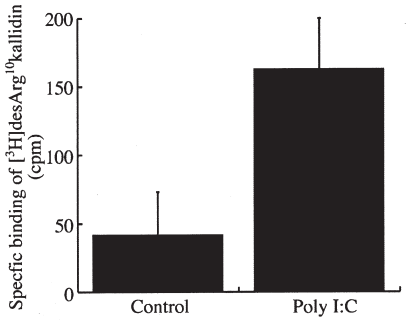

Fig. 2. Induction of bradykinin B1 receptor cell surface expression by Poly I:C. BEAS-2B cells were stimulated with 10 μg/ml of Poly I:C or media alone for 16 h prior to the radioligand binding assays. BEAS2B cells were incubated for 90 min with 1 nM [^{3}H]desArg^{10}kallidin with or without 1000-fold excess of desArg^{10}kallidin. Specific binding (non-specific binding subtracted from total binding) was expressed in relation to specific binding of non-stimulated cells was considered as 100%.

with B1 and B2 receptor mRNA levels normalized to β-actin mRNA levels in each sample. Poly I:C increased B1 receptor mRNA by 15-fold at 6 h post stimulation (Fig. 1A). In contrast, B2 receptor mRNA levels were only increased by 5-fold (Fig. 1B). In addition, we confirmed the induction of chemokine gene transcription following Poly I:C treatment of airway epithelial cells as previously demonstrated [20]. By quantitative real-time PCR, we observed a rapid and dramatic increase in IL-8 and RANTES mRNA levels induced by Poly I:C (data not shown).

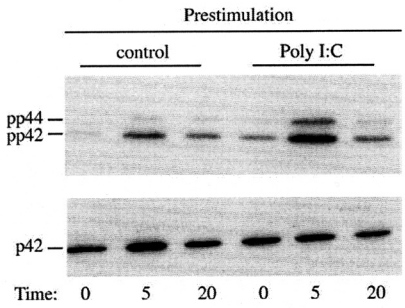

Fig. 3. Pretreatment of BEAS-2B cells with Poly I:C enhances Lys-Des-Arg-BK induced phosphorylation of ERK. BEAS-2B cells were grown to confluence in 6-well plates, cultured for 4 h on basal media prior to stimulation with or without Poly I:C (10 mg/ml) for 16 h. The cells were then stimulated with 10^{-7} M Lys-des-Arg-BK for 5 or 20 min. Cell lysates were harvested and total and phospho-ERK was detected by Western analysis.

3.2. Poly I:C increases cell surface expression of B1 receptor on BEAS-2B cells

Next we assessed whether Poly I:C treatment of BEAS-2B cells also resulted in the upregulation of cell surface expression of the kinin receptors. To explore this we performed radioligand binding assays for both B1 receptor and B2 receptor expression on Poly I:C-treated cells and control/untreated cells. As expected, BEAS-2B cells expressed abundant B2 receptor but only barely detectable B1 receptor on the cell surface at baseline. Treatment with Poly I:C induced a 5-fold increase in B1 receptor cell surface expression (SEM of ± 1.03) (Fig. 2) whereas B2 receptor cell surface expression was unchanged (data not shown).

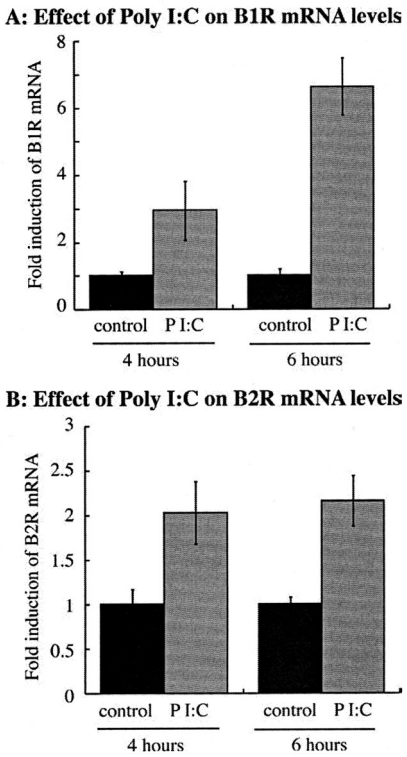

Fig. 4. Poly I:C induces increased levels of bradykinin B1 and B2 receptor mRNA expression in NHBEs. NHBEs were grown to confluence in 6-well plates, culture in basal media overnight and treated with either Poly I:C (10 μg/ml) or media alone for 4 and 6 h. Total RNA was extracted and assessed in triplicate by quantitative real-time RT-PCR for B1 receptor, B2 receptor and β-actin mRNA levels. B1 receptor and B2 receptor mRNA levels were normalized to β-actin mRNA levels. The data were calculated as the fold induction of B1 receptor (A) or B2 receptor (B) in Poly I:C treated cells compared to the level in untreated cells.

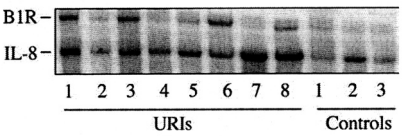

Fig. 5. Increased B1 receptor mRNA levels in nasal tissue of subjects with URIs. Total RNA was isolated from nasal tissue from 8 non-atopic subjects with URI symptoms (URIs) and 3 non-atopic control subjects (controls) and assessed by RPA for B1 receptor and IL-8 mRNA.

3.3. Pretreatment of BEAS-2B cells with Poly I:C enhances B1 agonist induced ERK activation

We next investigated whether Poly I:C induced an increase in functional B1 receptors on the airway epithelial cell surface. In a previous study our group has reported the activation of the MAP kinase ERK in airway epithelial cells following stimulation of the B1 receptor [5]. Therefore, we assessed the effect of LDA-bradykinin, a B1 receptor ligand, with respect to the phosphorylation of ERK in airway epithelial cells pretreated with Poly I:C for 16 h compared to control untreated cells. LDA-bradykinin caused a pronounced increase in phospho-ERK in cells pretreated with Poly I:C compared to control cells pretreated with media alone (Fig. 3).

3.4. Poly I:C increases B1 receptor mRNA levels in normal human primary bronchial epithelial cells

To assess whether Poly I:C affects the expression of kinin receptors in primary human airway epithelial cells we treated NHBEs with Poly I:C for 4 and 8 h. By quantitative PCR we observed a 6-fold increase in B1 receptor mRNA levels following Poly I:C treatment (1 μg/ml) (Fig. 4A). As with the BEAS-2B cells, Poly I:C induced a smaller increase (2-fold) in B2 receptor mRNA levels in NHBES (Fig. 4B).

3.5. Bradykinin B1 receptor expression is increased in the nasal tissue of subjects with upper respiratory infections

In order to determine if naturally occurring upper respiratory infection (URI) induces bradykinin B1R expression in the airway, we assessed the levels of B1 receptor mRNA in nasal samples from subjects with active cold symptoms. Nasal samples were obtained using the Rhinoprobe curette from 8 non-atopic subjects with active symptoms of URIs and 3 non-atopic control subjects. mRNA levels of the bradykinin B1 receptor as well as the mRNA levels of IL-8 (a marker known to be induced during URI infections) were measured by multiprobe RPA. We have previously found that the bradykinin B1 receptor is either undetectable or very lowly expressed at the mRNA level in the nasal tissue of non-atopic individuals [5]. In the RNA from the 3 control subjects B1 receptor was barely detectable, while IL-8 mRNA levels were very low. Of the 8 URI subjects, 5 (subjects 1, 3, 6, 7 and 8) had marked increase in B1 receptor compared to the normal controls (Fig. 5). In addition IL-8 mRNA levels were increased in 7 out of the 8 subjects with URIs compared to control subjects.

4. Discussion

A substantial body of research has implicated the kinin system in playing an important role in the pathogenesis of allergic airway inflammation and respiratory viral infections. Nasal insufflation of bradykinin causes acute symptoms of nasal congestion, drainage, and throat irritation; while inhalation of bradykinin in asthmatic but not normal subjects results in cough, chest tightness, and measurable drops in flow rates indicative of bronchoconstriction [21,22]. Other cardinal features of asthma induced by bradykinin include mucus hypersection, airway edema, activation of inflammatory cells and the secretion of histamine, lipid-derived mediators and neuropeptides. Moreover, levels of kinins are increased in nasal secretions following allergen provocation as well as in the BAL fluid of patients with active asthma and chronic bronchitis [23,24]. In addition, increased levels of kinins are detected in the airways during experimental rhinovirus infection, with their generation directly correlating with symptom severity [8,9].

Kinins are generated in the airway through the cleavage of kininogen by tissue kallikrein (hTK2). We recently observed that experimental rhinovirus infection in atopic subjects leads to increased hTK mRNA as well as hTK activity in vivo (unpublished data). The increase in hTK activity was significantly more robust for subjects with asthma than for subjects with allergic rhinitis without asthma.

Human tissue kallikrein is primarily synthesized in the submucosal glands, then retained at the apical surface of the airway epithelium by binding to surface-associated hyaluronan that immobilizes hTK and inhibits its catalytic activity [25]. This pool of tissue kallikrein becomes activated following degradation of the high molecular weight hyaluronan by stimuli such as reactive oxygen species [26], as likely occurs during viral infection. Activated tissue kallikrein generates Lys-bradykinin, an agonist of the kinin B2 receptor. Lys-bradykinin may then be further metabolized by carboxypeptidase N (kininase 1) thereby generating des-Arg-Lys-bradykinin, an agonist for the kinin B1 receptor. While bradykinin and Lys-bradykinin have essentially equivalent effects at the human B2 receptor, des-Arg-Lys-bradykinin is a much more potent B1 receptor agonist than is des-Arg-bradykinin [27,28].

The B2 receptor is constitutively expressed in airway epithelial cells, however, the B1 receptor is not expressed to an appreciable extent in normal airway

epithelial cells, but its expression is inducible [5,29]. Importantly, the B1 receptor is also constitutively active and is only poorly desensitized by ligand. Recent papers have linked expression of the B1 receptor to airway inflammation in both animal models and human disease. Previously our group reported the upregulation of the bradykinin B1 receptor in human airway epithelial cells of allergic rhinitis subjects following allergen provocation. In addition we reported that nasal instillation of the bradykinin B1 receptor in patients with allergic rhinitis, but not normal control subjects, leads to activation of the ERK pathway. Moreover, in OVA-sensitized mice allergic lung inflammation was significantly reduced by a bradykinin B1 receptor antagonist [30], while disruption of the bradykinin B1 receptor gene in mice reduced inflammatory responses caused by carrageenan induced pleurisy [31].

Evidence suggests that the B1 receptor may also be upregulated during respiratory viral infection. A recent report showed that exposure of mouse trachea to the Toll-like receptor (TLR)-3 agonist Poly I:C increased bradykinin B1 receptor expression and enhanced sensitivity of the tissue to both bradykinin B1 and B2 receptor ligands, suggesting that viral infections of the airway could lead to increased sensitization of the airways to kinins [17]. In addition, it has been noted that the bradykinin B1 receptor appears to behave like an inducible enzyme, in that it is upregulated under the same conditions as the inducible proinflammatory enzymes COX-2 and iNOS [32]. Interestingly, during rhinovirus infection of airway epithelial cells iNOS is induced as part of the anti-viral response. Considering all the data, we anticipated that rhinovirus infection of human airway epithelial cells would result in an upregulation of the expression of the inducible bradykinin B1 receptor.

In this report we present the observation that the dsRNA analog, Poly I:C, is capable of regulating kinin receptor levels in human airway epithelial cells. Initially we observed increased mRNA levels of both the bradykinin B1 receptor, as well as the constitutive bradykinin B2 receptor in a human airway epithelial cell line following treatment with Poly I:C. Bradykinin B1 receptor mRNA levels were increased 15-fold while bradykinin B2 receptor mRNA levels were induced 5-fold by Poly I:C. While Poly I:C correspondingly increased the cell surface expression of B1 receptors, it had no effect on the level of B2 receptor cell surface expression. Corroborating the expression data, we found that stimulation with Poly I:C also upregulated functional B1 receptor signaling in response to ligand. Following incubation with Poly I:C for 16 h, treatment of BEAS-2B cells with the bradykinin B1 receptor agonist caused an increase in ERK activation compared to cells incubated with media alone for 16 h. Furthermore, we observed that Poly I:C also induced an increase in bradykinin B1 receptor mRNA levels in primary human normal bronchial epithelial cells. Additionally, we show an upregulation of the inducible B1 receptor and the proinflammatory chemokine IL8 during naturally occurring 'common colds' in human subjects, suggesting that our in vitro observations have clinical relevance.

dsRNA produced by ssRNA viruses, such as rhinovirus, as a replicative intermediate is detected by the innate immune receptor Toll-like receptor (TLR)-3. Human airway epithelial cells have been shown to constitutively express TLR-3 [33]. Activation of TLR3 by both viral and synthetic dsRNA in airway epithelial cells induces an anti-viral response as well as an inflammatory response [34]. Regarding the anti-viral response, a recent study showed that blocking TLR3 during RV infection of airway epithelial cells in vitro resulted in increased replication of the virus. However, activation of TLR3, as well as viral infection, induces airway epithelial cells to produce many cytokines, including RANTES and IL-8, that would be expected to exacerbate airway inflammation by recruiting and activating neutrophils, lymphocytes, monocytes and eosinophils [20,35–37]. As rhinovirus infection of the human airway is known to induce the production of kinins, increased bradykinin B1 receptor expression following viral infection via activation of TLR3 will render the airway epithelium more responsive to the increased levels of kinins.

Considering that B1 receptor expression and activation is associated with lung inflammation in animal models, our data suggest a mechanism via which respiratory virus infection can contribute to asthma severity. Lys-bradykinin is elevated in lavage fluid following experimental rhinoviral colds and during allergen provoked rhinitis and asthma [23,24,38,39]. The principle influence of Lys-bradykinin generation in the airway is increasingly accepted as proinflammatory. We have reported that the B1 receptor is upregulated in vivo in allergic inflammation and actively signals with exposure to its ligand Lys-des-Arg^{9}BK [5]. Murine tracheal explants display enhanced contractile responses to Lys-des-Arg^9-bradykinin and bradykinin following long term culture with IL-4, mediated by upregulation of B1 receptor [40]. In animal models, inhibition of the B1 receptor with desArg^{10} [HOE 140] dose dependently inhibited allergen-induced bronchial hyper-responsiveness to acetylcholine, a cardinal feature of asthma [41].

In conclusion, we have provided in vivo and in vitro evidence that viral infection as well as Poly I:C, the

synthetic analog for viral double-stranded RNA, can lead to increased expression and signaling of the B1 receptor in airway epithelium. We speculate that the increased expression of B1 receptors in the setting of enhanced kinin generation during viral infection will amplify the inflammatory response in the human airway thus increasing the risk of asthma exacerbation.

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III

Kinin receptor expression during *Staphylococcus aureus* infection

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An inappropriate host response to invading bacteria is a critical parameter that often aggravates the outcome of an infection. *Staphylococcus aureus* is a major human Gram-positive pathogen that causes a wide array of community- and hospital-acquired diseases ranging from superficial skin infections to severe conditions such as staphylococcal toxic shock. Here we find that *S aureus* induces inflammatory reactions by modulating the expression and response of the B1 and B2 receptors, respectively. This process is initiated by a chain of events, involving staphylococcal-induced cytokine release from monocytes, bacteria-triggered contact activation, and conversion of bradykinin to its metabolite desArg^{9}bradykinin. The data of the present study implicate an important and previously unknown role for kinin receptor regulation in *S aureus* infections. (Blood. 2006;108:2055-2063)

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Introduction

Staphylococcus aureus, an important opportunistic Gram-positive human pathogen, is the most common organism isolated from soft-tissue and wound infections. The bacterium can cause a variety of community- and hospital-acquired diseases ranging from relatively benign skin infections, such as furuncles and subcutaneous abscesses, to more severe conditions, including scaled skin syndrome, necrotizing pneumonia, endocarditis, sepsis, and staphylococcal toxic shock syndrome (for reviews, see Lowy[1] and Yarwood and Schlievert[2]). In severe conditions, staphylococci may evoke an inappropriate inflammatory host response by modulating so-called host effector systems. For instance, *S aureus* produces a diverse range of virulence factors contributing to the inflammatory response, among others the enterotoxins and toxic shock syndrome toxin-1 (TSST-1) that form a class of substances also known as pyrogenic toxin superantigens or PTSAgs (for a review, see Balaban and Rasooly[3]). PTSAgs can induce a profound inflammatory reaction by interacting with MHC class II molecules and T-cell antigen receptors disengaged from the normal antigen-specific signal transduction of T cells.[4,5] The resulting inflammatory response is by far greater than antigen-specific activation and leads to pathologic levels of proinflammatory cytokines.[6]

The human contact system, also known as the kallikrein-kinin cascade or intrinsic pathway of coagulation, is another example of a system that can be targeted and affected during infection.[7] The contact system consists of 4 factors, 3 serine proteinases (coagulation factors XI and XII, and plasma kallikrein), and 1 nonenzymatic cofactor (high-molecular-weight kininogen). Normally, these factors circulate as zymogens in the bloodstream. Contact activation can occur for instance on newly exposed cellular surfaces and is regulated by limited proteolysis. The initial step is activation of coagulation factor XII, which converts plasma kallikrein into the active form. Active kallikrein in turn amplifies the activation of factor XII, eventually resulting in clot formation, and the release of bradykinin (BK) from the precursor molecule, high-molecular-weight kininogen. Previous studies have shown an interaction between *S aureus* and the contact system leading to its activation at the bacterial surface.[8] As a result, BK is generated and continuously released from the bacterial cell wall over an extended period of time.[8] Of interest, this does not apply to all bacterial species. For instance, *Streptococcus pneumoniae* was not able to activate the contact system in this study.[8] BK and its metabolite desArg^{9}BK are potent inflammatory mediators, causing hypotension, increased vascular permeability, edema formation, fever, and pain (for a review, see Mahabeer and Bhoola[9]). Conversion of BK to desArg^{9}BK involves the cleavage of a carboxy-terminal arginine by carboxypeptidases of the N and M type, also known as kininases type I.[10] There are 2 kinin receptors described in humans, B_1 receptor (B1R) and B_2 receptor (B2R) (for a review, see Leeb-Lundberg et al[11]). While BK interacts mainly with B2R, desArg^{9}BK is selective for B1R. The 2 receptors differ also in their expression

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pattern and pharmacologic profile. B2R is constitutively expressed on most cell types and is rapidly internalized upon agonist binding, followed by its recycling to the cell membrane. B1R, on the other hand, is expressed in very low numbers under physiologic conditions, but is induced upon pathologic insults and autologously in response to agonist binding.[12] Upon expression on the cell surface, for instance following stimulation with interleukin 1β (IL-1β) or endotoxin, B1R exhibits high ligand-independent, constitutive activity that is further enhanced by agonist binding.[13]

The present investigation was undertaken to examine whether *S aureus* can use the contact system for the induction of inflammatory reactions in the human host. In particular, we wished to analyze the regulation of B1R and B2R at the cellular level in response to treatment with staphylococcal toxins. Our results show that the induction of kinin receptors and their respective ligands is modulated by *S aureus* and its secreted products. The proposed mechanism may play an important role in severe infections caused by this pathogen.

Materials and methods

Materials

IL-1β was from R&D Systems (Minneapolis, MN); [2,3-Prolyl-^{3}H]BK (2.9×10^{12} – 3.5×10^{12} Bq [79-96 Ci]/mmol), des-Arg^{10}-[3,4-prolyl-3,4-^{3}H]kallidin (3.9×10^{12} Bq [107 Ci]/mmol), and [^{3}H]thymidine (3.0×10^{12} Bq [80.4 Ci]/mmol) were purchased from PerkinElmer Life Sciences (Wellesley, MA). BK, desArg^{9}BK, desArg^{10}kallidin, BK(1-5), BK(1-6), BK(1-7), and BK(2-7) were from Bachem (Torrance, CA), and staphylococcal enterotoxins A and B (SEA and SEB, respectively) and toxic shock syndrome toxin I (TSST-I) were obtained from Sigma (St Louis, MO). Note that commercially available superantigens are truncated versions of the respective staphylococcal toxins produced in *Escherichia coli* containing the active domain of the protein.

Cell culture

Human fetal lung fibroblasts (IMR-90 cells) CCL-186 (American Type Culture Collection, Manassas, VA) were cultured in minimum essential medium as described earlier.[12] Cells were plated at a density of 1.5×10^5 cells/well in 6-well plates (35-mm well) and used at confluency after 3 to 4 days. All stimulations (and controls) of IMR-90 cells were conducted in culture media containing L-glutamine in the absence of antibiotics and FCS. Human peripheral blood mononuclear cells (PBMCs) were isolated as described.[14] Smooth muscle cells were isolated from rabbit superior mesenteric artery and cultured as described.[15]

Stimulation of PBMCs

PBMCs were incubated with SEA, SEB, or TSST-I at a final concentration of 100 ng/mL or 1% (vol/vol) *S aureus* Wood supernatants (obtained from overnight cultures of single colonies in 50 mL Todd Hewitt Broth [TH] media [Becton Dickinson, Sparks, MD]) in RPMI 1640 in the presence of 2 mM L-glutamine for 24 hours at 37°C. The cytokine content in PBMC exudates was measured by enzyme-linked immunosorbent assay (ELISA, Quantikine immunoassay kit; R&D Systems). To exclude a possible endotoxin contamination of the purchased purified staphylococcal toxins produced in *E coli,* toxins were incubated with polymyxin B (PMB; Sigma), a specific LPS antagonist, at a final concentration of 20 μg/mL for 30 minutes before experiments were started. The LPS contents in the stock solutions of the exotoxins (which were diluted 1:10 000 for the assays) were less than 1 ng/mL as determined by the Limulus test.

Intracellular cytokine staining

Purified PBMCs were adjusted to 6×10^6 cells/mL in RPMI medium. Cells were stimulated with 1% of supernatant from an overnight culture of *S aureus* (Wood 46) in the presence of brefeldin A (3 μg/mL, final concentration). Unstimulated cells and bacterial medium alone were used as controls. Cells were fixed and permeabilized as described[16] and then stained with anti–IL-6–FITC, anti–IL-1β–FITC, or anti–TNF-α–FITC (R&D Systems). Samples were analyzed in a FACSCalibur flow cytometer (Becton-Dickinson, Franklin Lakes, NJ). The monocyte population was identified by FSC/SSC characteristics and positive CD14 staining.

SDS–polyacrylamide gel electrophoresis (PAGE), Western blotting, and immunoprinting

Proteins from an overnight culture (*S aureus* Wood) were precipitated with 5% (wt/vol) trichloro-acetic acid (TCA). The precipitates were dissolved in SDS sample buffer and separated by 12.5% (wt/vol) polyacrylamide gel electrophoresis.[17] Commercially available SEA, SEB, and TSST-1 were used as controls. Proteins were transferred onto nitrocellulose membranes as described,[18] and after a blocking step, membranes were probed with antibodies against SEA, SEB, and TSST-I (ViroStat, Portland, ME) diluted 1:200 in the blocking buffer,[19] and bound antibodies were detected as described.[19]

RNA isolation, reverse transcription, and quantitative real-time PCR

Total RNA was extracted from IMR-90 cells using the RNA STAT-60 reagent method (Tel-Test, Friendswood, TX) as described by the manufacturer. Isolated RNA was DNAse treated (Ambion, Austin, TX) and reverse transcribed as described before.[20] Real-time quantitative polymerase chain reaction (PCR) analyses were performed as described before.[21] The primers used were as follows: B1R forward primer, 5′-caactgaacgtggcagaaatctac-3′; B1R reverse primer, 5′-caagcccaagacaaacaccag-3′; B2R forward primer, 5′-gggcacactgcggacct-3′; B2R reverse primer, 5′-gcgtttgctcactgtctgctc-3′; GAPDH forward primer, 5′-gggaaggtgaaggtcggagt-3′; and GAPDH reverse primer, 5′-tccactttaccagagttaaaagcag-3′. The following dual-labeled probes were obtained from BioSearch Technologies (Novato, CA): B1R, 5′-FAM-tggccaacctggcagcctctga-BHQ; B2R, 5′-FAM-tccgtggaacgccagattcacaaac-TAMARA; GAPDH, 5′-FAM-accaggcgcccaatacgaccaa-BHQ.

Radioligand binding

The binding of 1 nM [^{3}H]desArg^{10}kallidin (77.5 Ci/mmol) or 1 nM [^{3}H]BK (90.0 Ci/mmol) (PerkinElmer Life Science Products, Boston, MA) to IMR-90 cells or rabbit smooth muscle cells was performed as described earlier.[12] Binding assays were conducted on ice in triplicate, and nonspecific binding was defined as the amount of radiolabeled ligand bound in the presence of 1 μM nonradiolabeled desArg^{10}kallidin or BK.

Thin sectioning and transmission electron microscopy

Thin sections were subjected to immunolabeling as described[22] with the modification that Aurion-BSA (Aurion, Wageningen, the Netherlands) was used as a blocking agent. Briefly, sections were incubated with primary antibodies against B1R or B2R, followed by immunodetection with a secondary antibody against rabbit IgG labeled with 10 nm colloidal gold (Agar Scientific, Stansted, United Kingdom). Samples were finally stained with uranyl acetate and lead citrate and observed in a Jeol JEM 1230 electron microscope (JEOL, Tokyo, Japan), operated at 80 kV accelerating voltage. Images were recorded with a Gatan Multiscan 791 CCD camera (Gatan, Pleasanton, CA). For evaluation of the data, numbers of gold particles were determined for 30 cellular profiles in each case.

Determination of bradykinin

Bacteria (2×10^{10} cells/mL in 15 mM Hepes, 135 mM NaCl, 50 μM $ZnCl_2$, pH 7.4) were incubated with plasma as described earlier.[8] After 15 minutes of incubation at room temperature, the bacteria were washed, supplemented with new media, and incubated for 15 minutes before being assayed. The bradykinin concentration in the reaction mixture was quantified by the Markit-A kit (Dainippon Pharmaceutical, Osaka, Japan) as described.[23]

[^{3}H]Thymidine incorporation into rabbit smooth muscle cells

Incorporation of [^{3}H]thymidine into DNA expressed by rabbit vascular smooth muscle cells was performed as described earlier.[24] The carboxypeptidase inhibitors DL-2-mercaptomethyl-3-guanidinoethylthiopropionic acid (MGTPA), potato carboxypeptidase inhibitor (PCI), 2-guani-dinoethylmercaptosuccinic acid (GEMSA), and ϵ-aminocaproic acid (EACA) were purchased from Calbiochem (San Diego, CA). The inhibitors were used with a final concentration of 10 μM.

Analysis of BK cleavage products

Rabbit smooth muscle cells grown in 6-well plates were first washed with DMEM to remove any serum. The cells were then incubated with isotopically and chemically pure [^{3}H]BK (PerkinElmer Life Science Products) for various times at 37°C as indicated. The media were then acidified with 50 mL of 2 N HCl/mL, supplemented with 5 nmol BK, and applied on a C18 SepPak cartridge (Waters Associates, Milford, MA). BK cleavage products were then fractionated by high-performance liquid chromatography (HPLC) on a C18 SepPak column as previously described using defined BK fragments as standards.[25]

Immunohistochemical staining of tissue sections

The study was performed in accordance with the Declaration of Helsinki and ethical approval to obtain the biopsies was granted by the Human Subjects Review Committee of the University of Toronto. Biopsies from a local infection site of a patient with soft-tissue infection caused by *S aureus* had been collected at surgery and were immediately snap-frozen and stored at −80°C (kindly provided by Prof Donald E. Low, Mount Sinai Hospital, Toronto, ON). The biopsies were designated epicenter or distal tissue based on the clinical assessment and the level of inflammation. Samples were embedded in OCT-compound (Tissue-Tek; Mites, Elkhart, IN), cryostat sectioned to 8 mm, mounted to HTC glass slides (Novakemi, Stockholm, Sweden), and fixed with 2% freshly prepared formaldehyde in PBS. The immunohistochemical staining was performed as previously described[26] using 2 μg/mL anti–IL-1β (cocktail of 2-D-8 and 1437-96-15, both murine IgG1, from Dr H. Towbin, Ciba-Geigy, Basel, Switzerland), and 1:1000 dilution of B1R- and B2R-specific antibodies,[27] which had been immunopurified from respective polyclonal rabbit antiserum. The color reaction was developed by the addition of 3,3-diaminobenzidine (Vector Laboratories, Burlingame, CT) followed by counterstain of sections with hematoxylin. To control for nonspecific staining, sections that had not been incubated with primary antibodies were included and were always completely negative. Moreover, no staining was observed when the first antibody was replaced by normal rabbit serum or an unrelated antibody (against curli, an *E coli* surface protein). The immunostainings were evaluated in a RXM Leica microscope (Leica, Wetzlar, Germany) with a 40×/0.55 NA oil objective lens and 10% glycerol in PBS as imaging medium. Samples were located and analyzed by acquired computerized imaging analysis (ACIA) with a Quantimet 550 IW image analysis (Leica) as described earlier.[19] The microscope was equipped with a 3-charge couple device color camera (DXC-750p; Sony Sverige, Spanga, Sweden). The whole tissue section was analyzed, which yielded an analyzed cell area (defined by the blue hematoxylin counterstaining) ranging from 1.4×10^5 to 3.4×10^5 mm^2. The results are presented as ACIA value, which equals percent positively stained area times mean intensity of positive signal.

Results

Staphylococcal superantigens induce secretion of proinflammatory cytokines from primary human monocytes

To date, more than 10 different superantigens have been reported to be secreted by *S aureus*,[28] of which enterotoxin A (SEA), enterotoxin B (SEB), and toxic shock syndrome toxin I (TSST-I) are the best characterized.[29] To investigate a possible role of these toxins in the induction of cytokine secretion from immune cells, purified human peripheral blood mononuclear cells (PBMCs) were incubated with SEA, SEB, TSST-I, or culture media alone. After 24 hours, PBMC exudates were collected and analyzed for their IL-1β, IL-6, and TNF-α content, which are the primary cytokines that are produced by these cells.[30,31] Figure 1A shows that the 3

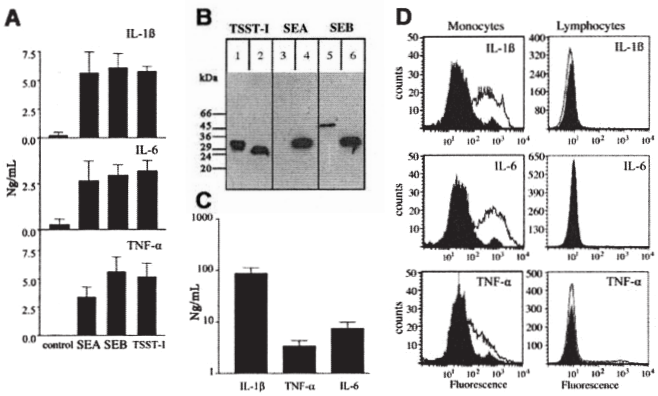

Figure 1. Secretion of proinflammatory cytokines from human peripheral monocytes following stimulation with superantigens from *S aureus*. (A) Human PBMCs were treated for 24 hours with 100 ng/mL staphylococcal enterotoxin A (SEA), staphylococcal enterotoxin B (SEB), toxic shock syndrome toxin I (TSST-I), or culture media alone. PBMC exudates were collected and analyzed for their IL-1β, IL-6, and TNF-α content by ELISA. Results show mean values ± SD of 3 independent experiments for each cytokine. (B) Supernatants from an overnight culture of *S aureus* Wood strain 46 were run on SDS-PAGE. Separated proteins were transferred onto nitrocellulose membranes and probed with antibodies against TSST-I (lanes 1), SEA (lane 3), or SEB (lane 5). Purified toxins were used as controls (lanes 2, 4, and 6). Bound antibodies were detected by peroxidase-conjugated secondary antibodies against rabbit immunoglobulin. It should be noted that size heterogeneity for staphylococcal toxins purified from different isolates has been reported[32] and may explain the different apparent molecular weights observed. (C) Human PBMCs were incubated for 24 hours with 1% (vol/vol) supernatants of an overnight culture from *S aureus* Wood strain 46. Exudates were collected and analyzed for their IL-1β, TNF-α, and IL-6 content by ELISA. Results show the mean ± SD of 3 separate experiments for each cytokine. Background secretion was either below detection level or less than 1% of the stimulated secretion. (D) Human PBMCs stimulated with supernatants from an overnight culture of *S aureus* (open area) and unstimulated cells (filled area) were fixed, permeabilized, and subsequently stained with fluorescent antibodies against IL-1β, IL-6, and TNF-α. The figure shows the monocyte and lymphocyte population gated on SSC and FSC characteristics.

toxins triggered a massive secretion of the analyzed cytokines, resulting in cytokine levels that were 10- to 600-fold higher than the background. Staphylococcal toxins are often encoded by accessory genetic elements, including plasmids, prophages, and mobile pathogenicity islands,[5] leading to a pattern of toxin expression that may differ from strain to strain.[33] We therefore wished to determine whether the tested staphylococcal superantigens are expressed and secreted by the *S aureus* Wood strain 46, which was used throughout this study. Thus, supernatants from overnight cultures of this strain were subjected to immunodetection following separation by SDS-PAGE and Western blotting. Figure 1B shows that SEB and TSST-I, but not SEA, were secreted into the culture medium under the growth conditions used. As these 2 toxins are secreted by the Wood strain, we hypothesized that the bacterial supernatants of overnight cultures should also be able to stimulate the secretion of cytokines from PBMCs. To this end, PBMCs were incubated for 24 hours with 1% (vol/vol) supernatants of overnight cultures of *S aureus* before cytokine measurements were conducted. As shown in Figure 1C, the treatment led to IL-6 and TNF-α levels in the PBMC exudates that were in the same range as those induced by the toxins alone, while the levels of IL-1β were more than 10-fold increased. To confirm whether the 3 cytokines were produced in monocytes and not in copurified B or T cells ("Materials and methods"), cells were stimulated with overnight cultures of *S aureus* in the presence of brefeldin A. Brefeldin A is a fungal toxin that inhibits the transport of secretory proteins between the ER and the *cis*-Golgi, which subsequently leads to an intracellular accumulation of de novo synthesized proteins in the endoplasmic reticulum.[34] As expected, Figure 1D shows that brefeldin A evoked an increase of the intracellular stored cytokines (IL-1β, IL-6, and TNF-α) in monocytes, but not in the B- and T-cell population. Taken together, these data demonstrate that purified staphylococcal toxins and overnight culture supernatants are able to induce a massive inflammatory response by stimulating monocytes to produce high amounts of proinflammatory cytokines.

Induction of B1R and B2R mRNA expression by exudates from PBMCs stimulated with staphylococcal supernatants

The IMR-90 (human fetal lung fibroblasts) cell line is widely used and well studied for the analysis of kinin receptor regulation in response to inflammatory stimuli.[35] IMR-90 cells express B1R and B2R at levels and in a ratio reflecting those in vivo. We therefore used this cell line to investigate B1R and B2R mRNA induction in response to treatment with exudates from PBMCs stimulated with staphylococcal supernatants (subsequently referred to as PBMC exudates). This treatment should mimic an inflammatory environment similar to that found at an infectious site. Prior to extraction of mRNA from IMR-90 cells, cells were incubated for 2 or 6 hours with BK, desArg^{9}BK, IL-1β, or PBMC exudates in the absence or presence of BK or desArg^{9}BK. Expression of mRNA for the B1R and B2R was investigated by quantitative real-time PCR and normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), which was analyzed in parallel. As shown in Figure 2, incubation of cells with BK or desArg^{9}BK for 2 hours did not alter B1R mRNA levels when compared with nonstimulated cells. On the other hand, stimulation with IL-1β or PBMC exudates induced an increase after 2 hours, which was about 7-fold over control (Figure 2A) and even higher (approximately 16-fold) when cells were incubated for 6 hours with PBMC exudates (Figure 2B). Coincubation of PBMC exudates with BK or desArg^{9}BK induced in a time-dependent manner a minor down-regulation of B1R mRNA expression. Figure 2C-D show that

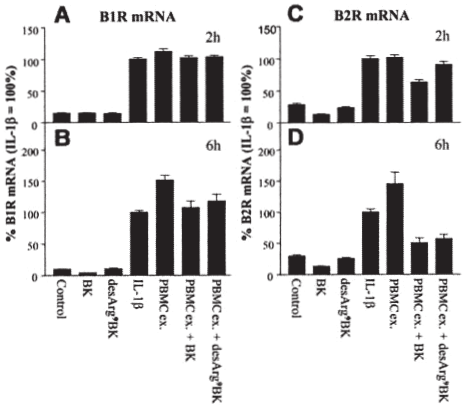

Figure 2. B1R and B2R mRNA expression in IMR-90 cells. IMR-90 cells were treated with 10 μM BK, 10 μM desArg^{9}BK, 500 pg/mL IL-1β, 1% (vol/vol) PBMC exudates (supernatants from monocytes that had been stimulated for 24 hours with 1% *S aureus* overnight culture supernatants), 1% PBMC exudates in the presence of 10 μM BK, 1% PBMC exudates in the presence of 10 μM desArg^{9}BK, or media alone in the absence of serum. Incubation times were 2 hours (A,C) and 6 hours (B,D). B1R (A-B) and B2R (C-D) mRNA expression was measured using quantitative real-time PCR and normalized to GAPDH mRNA levels. B1R and B2R mRNA expression in response to treatment with IL-1β was set to 100%. The figure presents the mean ± SEM of 3 independent experiments each performed in triplicate.

exposure to BK, but not to desArg^{9}BK, led to a decrease in B2R mRNA levels, while treatment with IL-1β or PBMC exudates caused an up-regulation that was approximately 3.5 times higher than the control. Incubation with PBMC exudates in the presence of BK or desArg^{9}BK decreased in a time-dependent manner the exudate effect on B2R mRNA expression. Treatment of IMR-90 cells with purified staphylococcal toxins and culture supernatants did not affect the mRNA levels of B1R and B2R (data not shown). The results show that PBMC exudates have the capacity to induce an up-regulation of B1R and B2R mRNA in IMR-90 cells, which is comparable with that seen from an IL-1β–caused induction. It was also noted that BK and desArg^{9}BK down-regulated this effect significantly in the case of B2R.

Influence of PBMC exudates on specific B1R and B2R binding

A pathophysiologic effect, in respect to kinin receptor regulation, of *S aureus*–stimulated PBMC exudates would require a change in surface expression of kinin receptors. Thus, we measured the ability of PBMC exudates to modulate the number of B1Rs and B2Rs at the surface of IMR-90 cells. To this end, radioligand binding assays were performed using receptor-saturating concentrations of [^{3}H]desArg^{10}kallidin, a B1R agonist, and [^{3}H]BK, a B2R agonist, as previously described.[12] For these analyses, cells were treated for 6 hours with desArg^{9}BK, BK, IL-1β, or PBMC exudates in the presence or absence of BK or desArg^{9}BK, before the radioligand was added. Figure 3A shows that stimulation with desArg^{9}BK caused a 2-fold increase of B1R agonist binding over control, while incubation with IL-1β or PBMC exudates was more efficacious in triggering an up-regulation of approximately 4.5-fold. The number of binding sites was even more increased when IMR-90 cells were incubated with PBMC exudates in the presence of BK or desArg^{9}BK (6- and 11-fold, respectively). In contrast to B1R, none of the stimuli induced an up-regulation of B2R binding sites (Figure 3B). Indeed, incubation of cells with BK or PBMC

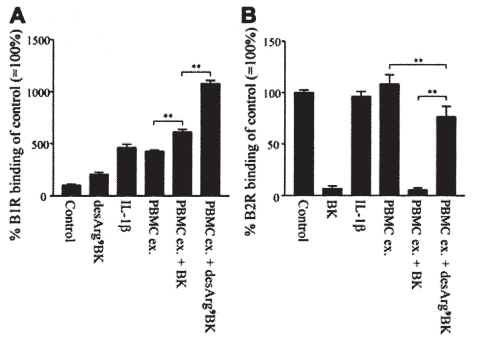

Figure 3. Surface expression of B1Rs and B2Rs on IMR-90 cells. IMR-90 cells were incubated for 6 hours with either 10 μM BK, 10 μM desArg^{9}BK, 500 pg/mL IL-1β, 1% (vol/vol) PBMC exudates (supernatants of monocytes that had been stimulated for 24 hours with 1% *S aureus* overnight culture supernatants), 1% PBMC exudates in the presence of 10 μM BK, 1% PBMC exudates in the presence of 10 μM desArg^{9}BK, or media alone in the absence of serum. After a washing step, cells were assayed for specific [^{3}H]Des-Arg^{10}kallidin (B1R ligand) binding (A) and [^{3}H]BK (B2R ligand) binding (B). Binding of [^{3}H]Des-Arg^{10}kallidin and [^{3}H]BK to nonstimulated cells (control) was normalized to 100% within each experiment. Results represent the mean ± SEM of 3 independent experiments performed in triplicate. $^{**}P < .01$ by analysis of variance followed by Tukey method for pairwise comparisons.

exudates in the presence of BK led to a drastic decrease in specific binding of [^{3}H]BK. No other treatment had a significant effect on agonist binding. The results show that the induction of B1R mRNA is reflected in an increased B1R agonist binding capacity of IMR-90 cells that were stimulated with IL-1β or PBMC exudates. Of interest, the combination of PBMC exudate and kinins, especially desArg^{9}BK, induced a more pronounced up-regulation of B1R compared with the PBMC exudate alone (Figure 3A). However, even though stimulation of IMR-90 cells with various substances led to an up-regulation of B2R mRNA, there was no increase in the binding of [^{3}H]BK. Stimulation with toxins SEA, SEB, and TSST-1 as well as *S aureus* culture supernatants did not cause an up- or down-regulation of B1R or B2R protein expression on IMR-90 cells (data not shown). In order to explain the differences between B1R and B2R up-regulation at the protein level, we used immunoelectron microscopy. Figure 4 shows that treatment of IMR-90 cells with monocyte exudates leads to an up-regulation of the B1R at the cell membrane (approximately 3-fold) and no further enrichment intracellulary, while the B2R accumulates intracellulary (approximately 5-fold), but is not up-regulated at the cell membrane. Thus, the data suggest that even though stimulation of IMR-90 cells leads to increased protein levels of both receptors, only the B1R is up-regulated at the cell surface. The finding can be explained by different trafficking mechanisms that target the receptor to the cell membrane or other yet-unknown mechanism.

Conversion of BK from a B2R agonist to a B1R agonist

Previous work has shown that *S aureus* isolates from patients with septic shock activate the human contact system. As a result, BK is generated and continuously released from the bacterial surface at a rate that is sufficient to induce an activation of B2R in transfected CHO cells.[8] Similar findings were also observed in the present study when the Wood strain was tested. It is important to mention that so far there have been no reports in the literature showing that staphylococcal proteinases are able to release desArg^{9}BK from the precursor molecules or to remove the carboxy-terminal arginine residue from BK, thereby converting the peptide from a B2R to a B1R agonist. Of interest, staphylococcal secretion products were able to stimulate monocytes to release proinflammatory cytokines followed by a massive up-regulation of B1Rs on IMR-90 cells. Moreover, when the stimulation occurred in the presence of desArg^{9}BK, a synergistic effect was recorded. We therefore investigated whether BK released from the staphylococcal surface may be converted to desArg^{9}BK by a eukaryotic-driven mechanism, for instance due to cleavage by a cell surface–bound endopeptidase. In order to address this question, we used rabbit superior mesenteric artery smooth muscle cells, which have been intensively studied in respect to their pharmacology.[15,36] In contrast to IMR-90 cells, the smooth muscle cells are a primary vascular cell line that has retained much of its original phenotype and basally expresses B1Rs and B2Rs at their surface.[15,36]

For further characterization of the vascular smooth muscle cells, we conducted binding assays with saturating concentrations of [^{3}H]desArg^{10}kallidin and [^{3}H]BK. Figure 5A shows that treatment of cells with desArg^{9}BK, IL-1β, or PBMC exudates led to an increase in the number of B1R binding sites but had no effect on the B2R. Furthermore, stimulation with BK caused a down-regulation of the B2R without any effect on B1R. Taken together, the binding assays show that rabbit vascular smooth muscle cells express basal levels of the B1R and B2R and treatment with various stimuli provokes a pattern of receptor expression that resembles that of IMR-90 cells.

To study the conversion of BK to desArg^{9}BK on rabbit smooth muscle cells, cells were incubated with [^{3}H]BK for 3, 6, and 24 hours. Supernatants were then recovered and analyzed by HPLC. Figure 5B-D demonstrates that when isotopically pure [^{3}H]BK (data not shown) was added to the cells, it was converted to [^{3}H]desArg^{9}BK or [^{3}H]BK(1-8) in a time-dependent manner followed by further degradation to other [^{3}H]metabolites. Maximal [^{3}H]desArg^{9}BK formation occurred within 3 hours, and after 24 hours the primary metabolite was [^{3}H]BK(1-5) (Figure 5D).

We next sought to determine whether the response of rabbit smooth muscles cells to treatment with BK is solely BK mediated

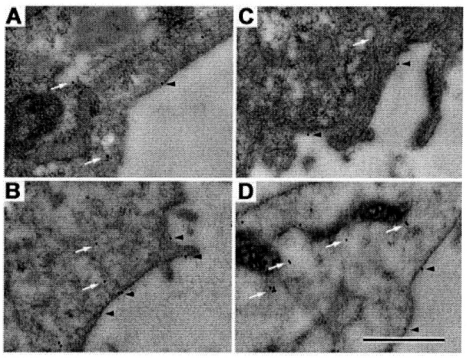

Figure 4. Immunolocalization of B1R and B2R in IMR-90 cells. Ultra-thin sections of unstimulated (A,C) or stimulated with 1% (vol/vol) exudates from monocytes treated with 1% (vol/vol) *S aureus* supernatant from an overnight culture (B,D). IMR-90 cells were incubated with antibodies against B1R (A-B) or B2R (C-D). Bound antibodies were visualized by secondary antibodies labeled with 10-nm gold particles and processed as described in "Materials and methods." Examples of intracellular (arrows) and membrane-associated receptors (arrowheads) are indicated. The scale bar indicates 0.5 μm (magnification ×25 000).

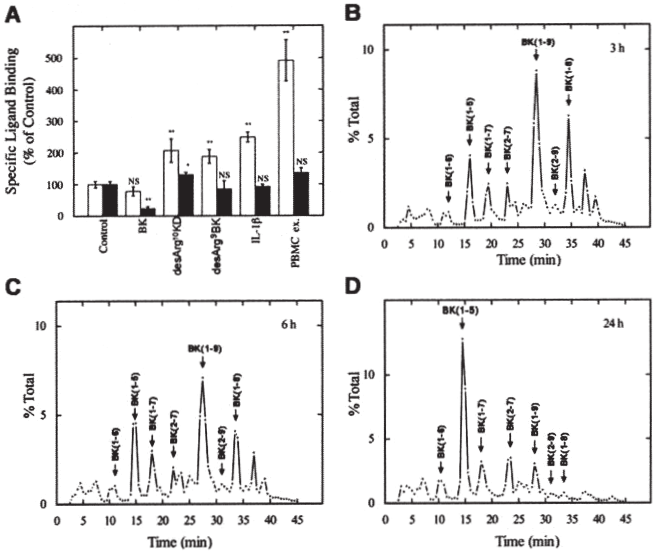

Figure 5. Expression of kinin receptors in response to PBMC exudates and kinins and chromatographic analysis of the hydrolysis of BK on rabbit smooth muscle cells. Rabbit smooth muscle cells were treated for 6 hours with BK, desArg^{9}BK, IL-1β, 1% (vol/vol) exudates from monocytes stimulated with 1% (vol/vol) supernatant from an overnight culture of *S aureus,* or culture medium alone (control) in the absence of serum (A). After a washing step, cells were assayed for specific [^{3}H]desArg^{10}kallidin (B1R ligand; open bars) and [^{3}H]BK (B2R ligand; filled bars) binding as described in "Materials and methods." Specific binding is expressed as percent of control, where 100% is the binding to nontreated cells. The results represent mean ± SEM of at least 3 independent experiments done in triplicate. $^*P < .05$ and $^{**}P < .01$ compared with control values as determined by Student *t* test. NS indicates not significantly different from control values. Isotopically pure [^{3}H]BK was incubated with rabbit smooth muscle cells. Supernatants were recovered after 3 hours (B), 6 hours (C), and 24 hours (D) and analyzed by HPLC as described in "Materials and methods." Results are expressed as percent of total, where total is the total amount of eluted radioactivity. The result is representative of 3 experiments.

or partially caused by the newly formed desArg^{9}BK. To this end, we used a cell proliferation assay, since it has been shown that both kinins (BK and desArg^{9}BK) exert mitogenic effects.[37] The assay was also chosen since it requires a relatively long agonist incubation time and, therefore, provides an opportunity for the formation and action of desArg^{9}BK following BK addition. This is in contrast to other methods such as assays of intracellular Ca^{2+} mobilization, which occurs within seconds. Figure 6A shows that treatment of rabbit smooth muscle cells with desArg^{9}BK or BK caused a significant increase in [^{3}H]thymidine incorporation, which was in the same range as that observed in response to PDGF, one of the most potent vascular smooth muscles mitogens.[38] In order to determine which receptor is activated following desArg^{9}BK or BK addition, rabbit smooth muscles cells were incubated with the agonists in the presence or absence of desArg^9[Leu^8]BK, a selective B1R antagonist, or HOE-140, a selective B2R antagonist. As expected, desArg^{9}BK-induced [^{3}H]thymidine incorporation was significantly reduced in the presence of desArg^9[Leu^8]BK, while HOE-140 was virtually inactive (Figure 6B). In contrast, [^{3}H]thymidine incorporation caused by stimulation with BK was blocked by more than 50% in the presence of desArg^9[Leu^8]BK, whereas the effect of HOE-140 was less pronounced. Of note, the antagonists had no effect on [^{3}H]thymidine incorporation in the absence of kinin peptides (data not shown). To investigate whether the conversion of BK to desArg^{9}BK on rabbit smooth muscle cells was triggered by carboxypeptidases of the kininase I type, rabbit

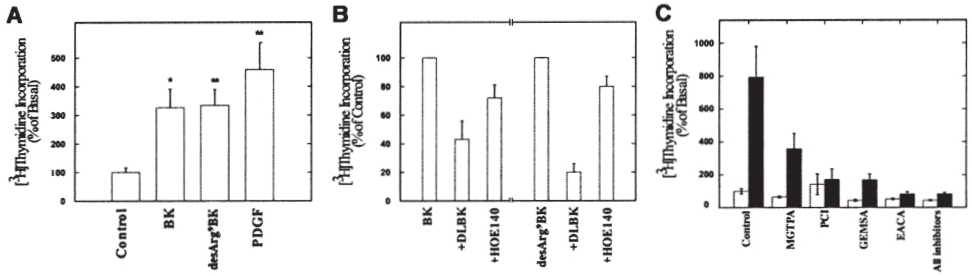

Figure 6. Effect of BK and desArg^{9}BK on [^{3}H]thymidine uptake in rabbit smooth muscle cells. (A) Rabbit smooth muscle cells were treated with BK, desArg^{9}BK, PDGF, or buffer alone (control) for 24 hours and then assayed for [^{3}H]thymidine incorporation as described in "Materials and methods." The results are presented as percent of basal, where 100% basal is the amount of radioactivity in control. (B) Cells were incubated with BK or desArg^{9}BK in the absence and presence of desArg^9[Leu^8]BK (DLBK) (B1 receptor antagonist) or HOE140 (B2 receptor antagonist). [^{3}H]Thymidine incorporation was measured after 24 hours. Results are expressed as percent of control, where 100% control is the incorporation in the presence of BK or desArg^{9}BK. The results represent mean ± SEM of at least 3 independent experiments done in triplicate. $^*P < .05$ and $^{**}P < .01$ compared with the control value as determined by Student *t* test. (C) Rabbit smooth muscle cells were treated for 24 hours with BK (1 μM) in the presence or absence of carboxypeptidase inhibitors of the kininase I type (DL-2-mercaptomethyl-3-guanidinoethylthiopropionic acid [MGTPA], potato carboxypeptidase inhibitor [PCI], 2-guani-dinoethylmercaptosuccinic acid [GEMSA], ε-aminocaproic acid [EACA], or a mix of all inhibitors; black bars). All inhibitors were applied at a final concentration of 10 μM. Control samples in the absence of BK were run in parallel (white bars). The results are presented in percent of thymidine incorporation into the DNA of the rabbit smooth muscle cells, where the incorporation into nontreated cells was set to 100%. The graph represents the mean ± SEM of 2 independent experiments performed in triplicate.

smooth muscle cells were incubated with BK in the presence of the carboxypeptidase inhibitors, MGTPA, PCI, GEMSA, EACA, or with a mix of all inhibitors. Figure 6C shows that all inhibitors clearly impaired BK-prompted thymidine incorporation, while having minimal or no effect on basal incorporation. These data provide direct evidence that the mitogenic effect of BK is partially caused by the conversion of BK to desArg^{9}BK, generated upon interaction of BK with carboxypeptidases from smooth muscle cells.

Western blot analysis of toxins secreted from clinical *S aureus* strains

Next we tested supernatants from overnight cultures of 5 clinical staphylococcal strains for their toxin expression. Figure 7A-B (lanes 2-6) shows that all strains tested produced TSST-1 and SEB, though the amount of secreted toxin seems to vary from strain to strain. Similar results were obtained when the expression of SEA in the clinical isolates was investigated (data not shown). Strain Mu50 (ATCC 700699) whose genome has been completely sequenced, was used as a positive control, and was found to express TSST-1 and SEB (Figure 7A-B lane 1) as well as SEA (data not shown).

Analysis of B1R and B2R expression on tissue biopsies from a patient suffering from a staphylococcal soft-tissue infection

To assess the expression of B1R and B2R in vivo during an infection, we analyzed tissue biopsies collected from a patient with a soft-tissue infection caused by the 9730 strain (Figure 7A-B lane 2). For comparison, samples were collected from 2 sites including the epicenter of the infection and a more distal site. Histologic examination of the biopsies showed that the tissue sections had different morphologies with more signs of inflammation and loss of tissue structure in biopsies obtained from the epicenter of the infectious site (data not shown).

Further immunohistochemical analysis revealed that both B1R and B2R were expressed at the local site of infection. Of interest, the level of B1R expression was more than 3-fold higher at the epicenter compared with the section from the more distal site (Figure 7C). In contrast, equal levels were noted for the B2R in the 2 groups of biopsies. To assess the degree of inflammation in the biopsies, the sections were stained for IL-1β, and in situ imaging analysis revealed a 4-fold higher amount of IL-1β in the epicenter sections. Similar findings were obtained when a biopsy from a patient with staphylococcal-induced erysipelas (an acute superficial form of cellulitis) was analyzed (data not shown). These data are in line with the results obtained from cultured IMR-90 cells and rabbit vascular smooth muscle cells. Taken together, the present investigation shows for the first time that *S aureus* can induce up-regulation of B1R, which combined with its ability to generate BK that is subsequently converted to a B1R agonist may provide a powerful mechanism to evoke pathologic inflammatory response in the human host.

Discussion

In 1972, Lewis Thomas wrote "The microorganisms. . .turn out. . .to be rather more like bystanders. It is our response to their presence that makes the disease. Our arsenal for fighting off bacteria are so powerful. . .that we are more in danger from them than the invaders."[40] One of the most potent inflammatory mediators we have in the human body is BK. Previous work demonstrated that *S aureus* is able to activate the contact system and trigger the release of BK.[8] Based on this observation, the present study was undertaken to examine the influence of kinin receptor regulation and their subsequent activation by their ligands at an infected site. In order to mimic an inflammatory situation in vitro, PBMCs were stimulated with different staphylococcal products and this was found to trigger a massive cytokine response. Moreover, treatment of IMR-90 cells with exudates from monocytes, after incubation with *S aureus* overnight supernatant, induces an up-regulation of the B1 receptor, which was further increased in the presence of BK or desArg^{9}BK. The same treatment had no effect on the expression of the B2R when cells were stimulated in the absence of BK, while in the presence of BK the treatment induced a down-regulation of

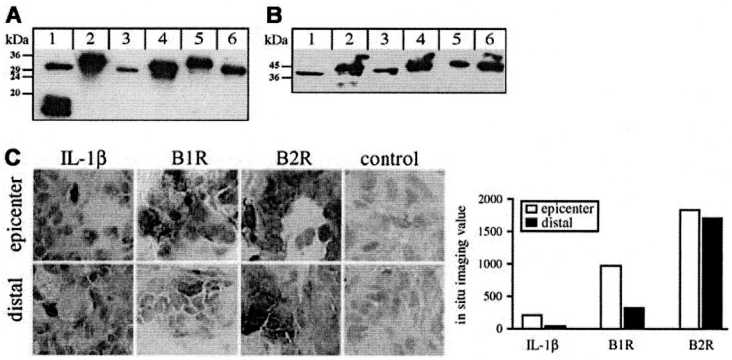

Figure 7. Detection of TSST-1 and SEB in supernatants from clinical *S aureus* isolates and expression of B1Rs and B2Rs in a patient suffering from an *S aureus* soft-tissue infection. (A-B) Supernatants from the clinical isolates 9730, 1878, 2374, 1024, and 15159 (lane 2 to 6) were separated on SDS-PAGE, transferred onto nitrocellulose membranes, and immunostained with antibodies against TSST-1 (A) or SEB (B). The strain ATCC 700699, whose genome has been completely sequenced, was used as a control (lane 1). Note that the apparent molecular weights of toxins vary between the tested strains. Size variation is a common feature of bacterial proteins from different strains that is often caused by homologous recombination between repeated regions within the gene[39] and has normally no influence on the activity of the protein. (C) Tissue biopsies from the epicenter of the infection site and from a distal site were obtained from a patient with a soft-tissue infection caused by *S aureus*. The biopsies were cryosectioned and immunohistochemically stained for IL-1β, B1R, and B2R. Omission of the primary antibody was included as a negative control and was always completely negative. Stainings were quantified by in situ imaging and the results are presented as the imaging value: area and intensity of the positive stain (brown) in relation to the total cell area (blue), as previously described.[26]

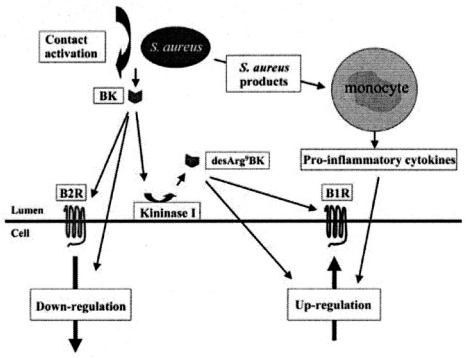

Figure 8. Proposed mechanism used by *S aureus* to interact with B1R and B2R. Based on the results of the present study, the following model is suggested. At the infectious site, invading monocytes become activated by staphylococcal toxins and secrete proinflammatory cytokines that induce an up-regulation of the B1R at the infectious focus. Plasma exudation into the infectious site will trigger contact activation and the formation of BK. BK can bind to B2R and trigger its down-regulation or be converted to the B1R agonist, desArg^{9}BK, which subsequently leads to an activation and an additional up-regulation of B1R.

the receptor. Contact activation by *S aureus* leads to the generation of BK[8] specifically. To test whether BK can be converted to a B1R agonist, HPLC analysis and cell proliferation assays were performed. These experiments demonstrated that a significant portion of BK is converted to desArg^{9}BK on vascular smooth muscle cells. In essence, our data present a chain of events (summarized in Figure 8) initiated by *S aureus* secretion products that leads to the induction of inflammatory reactions through up-regulation of B1Rs on the surface of cells. *S aureus* uses a number of different host systems (ie, contact system, monocytes, and B1Rs/B2Rs) to cause inflammatory reactions that can take place at different time points during an infectious process and lead to different clinical symptoms.

Characteristic signs of inflammation are redness and swelling with heat and pain (Cornelius Celcus, first century AD; for a review, see Nathan[41]), symptoms that all can be induced by BK or its metabolite desArg^{9}BK.[42] Immunohistologic examination of tissue samples from a patient suffering from a soft-tissue infection caused by *S aureus* revealed an up-regulation of B1R at the infectious focus, which coincided with increased IL-1β cytokine levels, while the levels of B2R were not increased. These results confirm our in vitro findings and implicate an important pathophysiologic function for B1R regulation at an inflamed site in bacterial infectious diseases.

Bacteria-provoked kinin generation and the subsequent increase in vascular permeability represent an important pathophysiologic mechanism. The increased permeability and the resulting plasma leakage into the infected site can either serve as a source of nutrients or facilitate dissemination of the infection, which eventually can result in more severe conditions such as sepsis and septic shock.[43,44] Kinin receptors are therefore an interesting target for the development of novel therapies for the treatment of infectious diseases. In this respect, B1R seems more relevant then B2R, since B1R is thought to have an important role in chronic inflammatory responses, while B2R is down-regulated at an early stage of an inflammatory process. So far, deltibant (CP-0127), a B2R antagonist, is the only kinin antagonist that has been tested for the treatment of bacterial infections. In a multicenter, randomized, placebo-controlled trial, the drug was applied to patients with systemic inflammatory response syndrome and presumed sepsis. Even though the drug had no significant effect on risk-adjusted 28-day survival, posthoc analysis revealed a nonsignificant trend toward improvement.[45] Our studies show that *S aureus* not only evokes an up-regulation of B1R, but also has the ability, via contact activation at the bacterial surface and the help of host carboxypeptidases, to allow a sustained generation of desArg^{9}BK, a B1R agonist. Based on our findings, a strategy using a B1R antagonist alone or in combination with B2R antagonist could represent a promising approach for the development of novel therapies for the treatment of severe *S aureus* infections.

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IV

Activation of TAFI on the surface of *Streptococcus pyogenes* evokes inflammatory reactions by modulating the kallikrein/kinin system

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Abstract

Bacteria-controlled regulation of host responses to infection is an important virulence mechanism that has been demonstrated to contribute to disease progression. Here we report that the human pathogen *Streptococcus pyogenes* employs the procarboxypeptidase TAFI (thrombin activatable fibrinolysis inhibitor) to modulate the kallikrein/kinin system. To this end, bacteria initiate a chain of events starting with the recruitment and activation of TAFI. This is followed by the assembly and induction of the contact system at the streptococcal surface, eventually triggering the release of bradykinin (BK). BK is then carboxyterminally truncated by activated TAFI, which converts the peptide from a kinin B_2 receptor (B2R) ligand to a kinin B_1 receptor (B1R) agonist. Finally, we show that streptococcal supernatants indirectly amplify the B1R response as they act on peripheral blood mononuclear cells to secrete inflammatory cytokines that in turn stimulate up-regulation of the B1R on human fibroblasts. Taken together our findings implicate a critical and novel role for streptococci-bound TAFI, as it processes BK to a B1R agonist at the bacterial surface and thereby may re-direct inflammation from a transient to a chronic state.

Introduction

Streptococcus pyogenes is an important human pathogen that normally causes harmless skin and throat infections. Although these conditions often are self-limiting, infections can become invasive and may give rise to severe complications such as streptococcal toxic shock syndrome and necrotizing fasciitis, conditions associated with high mortality [1]. This said, it was recently estimated that more than 500,000 humans die each year from *S. pyogenes* infections, which places this bacterium among the 10 most dangerous pathogens [2]. In order to cause infection, *S. pyogenes* has developed a multifold repertoire of virulence factors [3]. For example, the bacterium has a broad arsenal of secretory proteins, including exotoxins which are powerful inducers of pro-inflammatory cytokines such as interleukin-1β (IL-1β), IL-6, and tumor necrosis factor α (TNF-α) [4]. In addition, streptococci also express a huge panel of surface-bound virulence factors that are involved in many host/parasite interactions such as the recruitment of host proteins to the bacterial surface or bacterial attachment to eukaryotic cells. Among these are M-proteins, which were first described time by Rebecca Lancefield in the 1930's [5]. Today M and M-like proteins are considered as classical virulence factors and are probably the best-characterized surface-bound virulence determinants of *S. pyogenes* [6].

The human contact system (also known as the kallikrein/kinin system or the intrinsic pathway of coagulation) has recently attracted considerable attention, since the recognition pattern of contact factors functions in a manner that is remarkably similar to that seen in other innate immune systems, for instance the complement system (for a review see [7]). Subsequent work has demonstrated that activation of the contact system leads to the generation of antimicrobial peptides derived from high molecular weight kininogen (HK), a co-factor of the contact system [8,9]. Already a decade ago, it was reported that HK has a high affinity for many streptococcal serotypes. Studies with an M1 serotype revealed an interaction of HK with the respective M protein [10]. Interestingly, HK bound to the streptococcal surface via M1 protein is prone to processing by plasma kallikrein (another factor of the contact system) resulting in the release of bradykinin (BK) [11]. BK and the BK metabolite desArg^{9}BK are short peptides of nine and eight amino acids in length, respectively. Both peptides are pro-inflammatory mediators capable of promoting hypotension, increased vascular

permeability, edema, fever, and pain. They achieve these reactions by binding and activating receptors belonging to the family of G-protein coupled seven transmembrane-spanning receptors. While BK has a high affinity for the B_2 receptor (B2R) subtype, desArg^{9}BK preferentially interacts with the B_1 receptor (B1R), which shares only minor sequence homology with the B2R [12]. In contrast to the B2R, which is constitutively expressed by many cells types, the B1R is induced only following inflammatory insult. While activation of B2R gives rise to a transient inflammatory response, stimulation of B1R promotes a sustained response [12].

The conversion of BK to desArg^{9}BK is mediated by carboxypeptidases of the N and M type [13]. Interestingly, another member of the carboxypeptidase family called procarboxypeptidase B, R or U, better known as TAFI (Thrombin Activatable Fibrinolysis Inhibitor), has recently been shown to assemble at the surface of streptococci of the M41 serotype [14], a strain with high affinity for HK [10]. TAFI adheres to the bacterial surface by interacting with the streptococcal collagen-like surface proteins A and B (SclA and SclB) [14]. Subsequent studies have shown that bacteria-bound TAFI is activated by its natural activators, plasmin and thrombin, which are also recruited to the streptococcal surface. As implicated by the name, TAFI impairs fibrinolysis by removing carboxy-terminal lysine residues from partially degraded fibrin that are required for tissue-type plasminogen activator-dependent plasmin formation. However, apart from its role in fibrinolysis, TAFI has been shown to cleave other substrates such as anaphylatoxins C3a and C5a [15], thrombin-cleaved osteopontin [16], and fibrinopeptide B [17]. In addition, TAFI is able to convert BK to desArg^{9}BK which should be associated with a switch from a B2R ligand to a B1R agonist.

The present study was undertaken to determine whether *S. pyogenes* can create prerequisites of inappropriate persistent inflammatory responses by initiating a chain of events including the assembly and activation of the contact system at the bacterial surface, recruitment and activation of TAFI, conversion of BK to desArg^{9}BK by bacteria-bound activated TAFI, and an up-regulation of B1R on human fibroblasts.

Materials and methods

Materials

Bradykinin and desArg^{9}BK were from Bachem (Torrence, CA). Thrombin was from ICN Biomedicals Inc. (Aurora, OH), thrombomodulin from American Diagnostica Inc. (Stamford, CT), and plasmin was purchased from Sigma (St. Louis, MO). Todd Hewitt Broth (TH) media was from Beckton Dickinson (Sparks, MD).

Cell culture.

Human fetal lung fibroblasts (IMR-90 cells) CCL-186 (American Type Culture Collection, Manassas, VA) were cultured in Minimum Essential Medium as described earlier [18]. Cells were plated at a density of 1.5 x 10^5 cells/well in 6-well plates (35 mm well) and used at confluency after 3-4 days. Experiments were conducted in culture media supplemented with L-glutamine alone. Human peripheral blood mononuclear cells (PBMCs) were isolated as previously described [19].

Stimulation of PBMCs.

PBMCs were incubated with 1% (v/v) *S. pyogenes* (M41) supernatants (obtained from over night cultures of single colonies in 40 ml TH media) in RPMI 1640 media (Invitrogen, Paisley, UK) in the presence of 2 mM L-glutamine for 24 h at 37°C. Cells were pelleted by centrifugation and the supernatant was assayed for IL-1β content by ELISA (Quantikine® immunoassay kit; R&D Systems, Minneapolis, MN).

Binding of radiolabeled TAFI to bacterial cells

Binding experiments of radiolabeled TAFI to the surface of the bacterial strains *S. pyogenes* (AP41), *S. aureus* strain Wood 46, Newman, and SH1000 were performed as described before [14].

Degradation of HK at the bacterial surface

S. pyogenes bacteria of the M41 strain were cultured over night in TH-media, washed and diluted to 2 x 10^{10} cells/ml in 10 mM Tris buffer pH 7.4 with 50 μM $ZnCl_2$. The

bacteria were incubated with fresh citrated human plasma (1:1 v/v) by rotation for 1 h at room temperature. Bacteria were then washed and proteins bound to the bacterial surface were dissolved with 0.1 M glycine pH 2.0. The supernatants were separated from the bacteria by centrifugation. Recovered proteins were mixed with SDS sample buffer (reducing conditions) and were separated by 10% (w/v) polyacrylamide gel electrophoresis followed by a transfer onto nitrocellulose and immunodetection with antibodies against HK as described before [20].

Determination of bradykinin.

Bacteria (2 x 10^{10} cells/ml in HEPES buffer; 15 mM HEPES, 135 mM NaCl, 50 μM $ZnCl_2$, pH 7.4) were incubated with fresh citrated plasma (1:1 v/v) or medium. After a 15 min incubation at room temperature, bacteria were washed and resuspended in HEPES buffer followed by another 15 min incubation step. The supernatants were separated from the bacteria and immediately processed for BK concentration measurements with an ELISA kit (Markit-M Bradykinin, Dainippon Sumitomo Pharmaceutical Co., Ltd., Osaka, Japan) according to the instructions by the manufacturer.

Activation of TAFI by plasmin or thrombin/thrombomodulin

Recombinant TAFI was produced as described before [21]. A quadruple mutant of TAFI (T325I, T329I, H333Y, H335Q) was generated and expressed. After activation, this mutant displayed a ~50-fold enhanced stability. Details will be described elsewhere. TAFI (20 nM) was activated by a premixture of thrombin (16 nM) and thrombomodulin (32 nM) or plasmin (10 μg/ml) as described earlier [22].

High pressure liquid chromatography (HPLC)

Bacteria were preincubated with either plasmin (10 μg/ml), thrombin (16 nM) and thrombomodulin (32 nM), or TAFI (20 nM) (wild type or IIYQ) for 1 h on ice in PBSAT (phosphate buffered saline with 0.02% (w/v) sodium azide and 0.05% (v/v) Tween 20). Thereafter, bacteria were washed and re-dissolved in 100 mM HEPES buffer with 0.01% (v/v) Tween 20, pH 8, with 5 mM $CaCl_2$ and incubated with either plasmin (10 μg/ml), thrombin (16 nM) and thrombomodulin (32 nM), or TAFI (20 nM) (wild type or IIYQ). After 20 min in room temperature samples were either

washed or directly further incubated with 1 μM BK for 15 minutes at 37°C. Sample supernatants were then chromatographed by reverse-phase HPLC on a C18 column (5 μm; 250 mm x 4.6 mm) with a linear gradient system, 100% buffer A (0.1% (v/v) trifluoroacetic acid (TFA) in water) to 100 % buffer B (0.1% (v/v) TFA in acetonitril). Samples were run for 45 min with a flow rate of 1 ml/min. The UV absorbance was monitored at 214 nm. Data were analyzed with the software package TotalChrom v6.2.0.0.1 with LC instrument Control (PerkinElmer, Waltham, MA).

Radioligand binding

The binding of 1 nM [^{3}H]desArg^{10}kallidin (77.5 Ci/mmol) (PerkinElmer. Waltham, MA) to IMR-90 cells was performed as described earlier [18]. Binding assays were conducted on ice in triplicate and nonspecific binding was defined as the amount of radio labeled ligand bound in the presence of 1 μM non-radio labeled desArg^{10}kallidin.

Transmission electron microscopy

Proteins and peptides were labeled with colloidal gold as previously described [23]. To study complex formation, gold-labeled TAFI (40 nm) was mixed with either gold-labeled BK (15 nm) or gold-labeled desArg^{9}BK (15 nm). Alternatively, gold-labeled TAFI was incubated with plasmin in the presence of AP41 bacteria (1% bacterial solution) for 20 min at room temperature followed by the addition of gold-labeled BK. Specimens were then adsorbed for 1 min onto carbon-coated grids. The grids had been rendered hydrophilic by glow discharge at low pressure in air beforehand, briefly washed with water, and stained with 0.75% (w/w) uranyl formate in water. The samples were analyzed in a Jeol 1200 EX electron microscope operated at 60 kV accelerating voltage. Evaluation of the data was based on 300 particles from different electron micrographs.

PI hydrolysis

For PI hydrolysis, HEK293 cells (human embryonic kidney cells) stably transfected with B1R were employed [24] and IP hydrolysis was measured as described earlier [25]. Briefly, cells were labeled with 1 μCi/ml myo-[3H]-inositol in DMEM/10% FBS for 20-24 h at 37ºC, washed 4 times in DMEM, and then further incubated in

DMEM for 1 h at 37ºC. This was followed by incubation with various stimuli (10 μM desArg^{9}BK with or without 1 μM [3,4-prolyl-3,4-(3)H(N)]-[des-Arg10, Leu9]kallidin (DLKD) a B1R antagonist, 1 μM DLKD alone, sample supernatants with or without 1 μM DLKD, or media alone) in DMEM supplemented with 50 mM LiCl for 30 min at 37ºC. Cells were then lysed with 0.1 M formic acid for 20 min at 4ºC, transferred to Eppendorf tubes, and centrifuged at 16,100 x g for 5 min at 4ºC. The supernatants were added to anion exchange columns, which were washed two times with a low salt solution (60 mM ammonium formate, 5 mM sodium borate). Inositol phosphates were then eluted with a high salt solution (1 M ammonium formate, 0.1 M formic acid) and counted for radioactivity in a Beckman LS6000 scintillation counter. Each condition was run in triplicate.

Results

Binding of TAFI to the surface of *S. pyogenes*

A common feature of pathogenic bacteria is their ability to induce strong inflammatory reactions in the human host by interfering with so-called host effector systems. In particular, cell-mediated immune systems, complement, coagulation, and fibrinolysis are important targets that, when systemically activated, often significantly contribute to the pathology of the disease. TAFI is considered to be an important inflammatory mediator and previous studies have shown that the protein is absorbed by many different streptococcal serotypes [14]. In the present study we wished to extend these observations and analyze whether TAFI is able to convert BK to desArg^{9}BK at the streptococcal surface. In respect to bacteria-induced contact activation, it is noteworthy to mention that *Staphylococcus aureus*, another important Gram-positive pathogen, has previously been reported to utilize the contact system for the generation of BK [20,26]. Thus, it was tempting to speculate that also *S. aureus* may employ TAFI to generate a B1R agonist by cleaving BK on its surface. To this end, we compared the binding of TAFI to a *S. pyogenes* strain of M serotype 41 (AP41) and three *S. aureus* strains (Newman, Wood 46, and SH 1000). However, while a significant binding of TAFI to *S. pyogenes* (AP41) bacteria was recorded (> 20%, data not shown), no interaction between the three staphylococcal strains and TAFI was found (< 5%, data not shown). These data suggest that the ability of AP41 bacteria to interact with TAFI is not shared by its Gram-positive relative *S. aureus*.

Bradykinin is released from the streptococcal surface after incubation with human plasma

Based on the results obtained from the binding assays, we decided to focus on the streptococcal AP41 strain throughout this study. To investigate whether streptococcal-recruited TAFI can act as a kininase, we tested the ability of AP41 bacteria to assemble and activate the contact system at their surface. By conducting Western-blot analysis we found that upon incubation with plasma, HK is absorbed by AP41 bacteria. Figure 1A depicts that the HK, recovered from the surface of AP41 bacteria by an acid washing step, was processed into heavy and light chains suggesting the release of BK. We therefore analyzed whether the processing of HK into the two

chains is followed by the generation of BK. AP41 bacteria were incubated with plasma for 15 min followed by a washing step to remove unbound plasma proteins. Bacteria were then resuspended in HEPES buffer and incubated for another 15 min to allow contact activation and the release of BK from the bacterial surface into the liquid phase. When the BK content in the resulting supernatants was analyzed by ELISA measurements, we detected increased BK levels in supernatants from plasma, but not buffer-treated bacteria (Fig. 1B). Taken together, the results indicate that the human contact system can be assembled and activated at the surface of *S. pyogenes* bacteria of the M41 serotype, which is followed by the cleavage of HK and generation of BK.

Activation of TAFI at the streptococcal surface and conversion of BK to desArg^{9}BK

We next investigated whether BK is a substrate for activated TAFI in the absence of AP41 bacteria. BK and plasmin-activated TAFI were incubated for 15 min at 37°C followed by HPLC analysis of the resulting BK cleavage products. We found that this treatment led to a complete conversion of BK to desArg^{9}BK (Fig. 2C). Similar findings were recorded, when the experiments were performed with thrombin/thrombomodulin activated TAFI (data not shown). Commercially available BK and desArg^{9}BK were used as controls (Fig. 2A and B). It should also be noted that our results are in line with earlier reports by Myles *et al.* [16]. In the next series of experiments, we analyzed the TAFI-induced generation of desArg^{9}BK at the surface of AP41 bacteria. Thus, bacteria were incubated with plasmin for 1h on ice, followed by a washing step to remove unbound plasmin. TAFI was then added and after 20 minute incubation at room temperature to allow for activation of TAFI by AP41-bound plasmin, BK was introduced to the reaction mixture for 15 minutes at 37°C. Thereafter, supernatants were subjected to HPLC analysis and BK degradation products were measured. As seen before in the absence of bacteria, we found that also under these experimental conditions, BK is readily cleaved by activated TAFI yielding to the generation of desArg^{9}BK (Fig. 2D) and a complete consumption of BK. The same results were also obtained when plasmin was replaced with thrombin/thrombomodulin in these experiments (data not shown). Even though our results show that the TAFI activators (plasmin or thrombin/thrombomodulin) are

bound to the bacterial surface, the experimental settings used did not allow us to distinguish between desArg^{9}BK generation at the bacterial surface or in solution, since activated TAFI could theoretically have been dissociated from the streptococci. This issue could have been solved by introducing a second washing step before adding BK. However, since the half-life time of activated TAFI is rather short, we found that an additional washing step was combined with a complete loss of TAFI's enzymatic activity. To confirm that the interaction between TAFI and BK indeed takes place at the bacterial surface, we employed negative staining electron microscopy. First, we wished to validate the experimental approach and studied the complex formation between colloidal gold-labeled TAFI (40 nm) and colloidal gold-labeled BK (15 nm) in the absence of bacteria. Figures 3A and C depict that most TAFI molecules are in contact with BK whereas gold-labeled TAFI failed to physically interact with gold-labeled desArg^{9}BK (15 nm) (Fig. 3B), implicating that the gold label does not disturb the binding of BK to TAFI nor does it affect the specificity of TAFI. The experiments therefore show that negative staining electron microscopy is a suitable technique for studying the interaction between TAFI and BK. We then tested the complex formation between gold-labeled TAFI and gold-labeled BK at the bacterial surface. To this end, AP41 bacteria were first incubated with plasmin as described before. Afterwards, gold-labeled TAFI was added and after a 20 minute incubation at room temperature gold-labeled BK was given to the mixture. Samples were then adsorbed onto grids and subjected to negative staining electron microscopy analysis. Figure 4 demonstrates that activated TAFI, attached at the streptococcal surface, is able to form complexes with BK.

Having demonstrated that TAFI interacts with BK at the surface of AP41 bacteria, we next wanted to show that this interaction is followed by the generation of desArg^{9}BK. We therefore introduced a TAFI mutant (TAFI-IIYQ), which has been shown to have an extended proteolytic activity (~ 50-fold more stable) when compared with wt TAFI. Thus, experiments with TAFI-IIYQ and BK in the presence of bacteria were performed as described above, with the exception that a washing step was included after incubation with TAFI-IIYQ and before adding BK (data not shown). When analyzing the resulting BK cleavage products by HPLC, it was found that more than half of the BK was converted to desArg^{9}BK under these experimental conditions (Fig. 5A). When the experimental settings were changed in that TAFI-IIYQ was given before the addition of plasmin, we also recorded that most of the BK was cleaved to

desArg^{9}BK (Fig. 5B). Taken together the results demonstrate that the conversion of BK to desArg^{9}BK can be induced by activated TAFI that is attached to the bacterial surface.

TAFI-generated desArg^{9}BK function as a B1R ligand

We next wished to investigate whether desArg^{9}BK, generated by AP41 bacteria-bound TAFI, is a biologically active B1R agonist and measured phosphoinositol (PI) hydrolysis in HEK293 cells, stably transfected with B1R. As a positive control, commercially available desArg^{9}BK was used, which when added at a concentration of 10 μM to the transfected cells, triggered a significant increase in phosphoinositol (PI) hydrolysis (Fig 6A). PI hydrolysis was completely blocked when desArg^{9}BK was co-incubated with a selective B1R antagonist (DLKD; [3,4-prolyl-3,4-(3)H(N)]-[des-Arg10, Leu9]kallidin) (Fig. 6A), implicating that signaling did not involve other receptors, for instance those endogenously expressed by these cells. To test whether bacteria-bound TAFI is able to convert BK to an active B1R agonist, TAFI was added to bacteria and activated with plasmin as described above. This was followed by incubation with BK for 15 min and a centrifugation step to remove bacteria. When the resulting supernatants were added to the B1R transfected cells, we monitored an increase in PI hydrolysis which was not seen when the supernatants were given to the cells in the presence of DLKD (Fig. 6A). Thus, the data show that BK is converted to a biologically active B1R agonist at the bacterial surface.

Up-regulation of the B1R on human fibroblasts upon treatment with exudates from monocytes stimulated with supernatants from AP41 bacteria

In order to cause an inflammatory reaction, desArg^{9}BK, released from the streptococcal surface, requires available B_1 receptors. Most previous studies addressing B1R regulation were performed with IMR-90 cells, a human fetal lung fibroblasts cell line (for a review see [12]) and it has been shown that IL-1β stimulates up-regulation of B1Rs in these cells. Notably, streptococci secrete a number of exotoxins that are able to trigger the release of pro-inflammatory cytokines in human monocytes including IL-1β [4]. We therefore tested whether exudates derived from human peripheral blood mononuclear cells (PBMCs), treated with supernatants from

AP41 bacteria, can induce an upregulation of the B1R on the surface of IMR-90 cells. To this end, supernatants from over night AP41 cultures were collected and incubated with PBMCs for 24h followed by ELISA measurements of the IL-1β content in the PBMC exudates. This bacterial treatment led to a massive release of IL-1β (> 80 ng/ml), which was not seen in untreated cells (< 0.09 pg/ml). With this information in mind it seemed likely that exudates from the streptococcal-activated PBMCs would affect surface expression of B1R on human cells. Thus, we further investigated the ability of PBMC exudates, alone or together with desArg^{9}BK, to modulate the number of available B1Rs on IMR-90 cells. For this purpose, radioligand binding assays were performed using receptor saturating concentrations of [^{3}H]desArg^{10}kallidin, a B1R agonist, as previously described [18]. IMR-90 cells were treated for six hours with PBMC exudates in the presence or absence of desArg^{9}BK. After several washing steps to remove PBMC exudates and desArg^{9}BK, [^{3}H]desArg^{10}kallidin was added. Figure 6B shows that stimulation with exudates from PBMC alone caused a 3-fold increase of B1R agonist binding over control. When desArg^{9}BK was added to the incubation together with PBMC exudates the number of available B1R ligand binding sites on the fibroblasts increased further to 8-fold over control. Taken together the data show that AP41 secreted products induce release of IL-1β from human PBMCs and that these exudates together with desArg^{9}BK have a strong up-regulatory effect on B1R on human cells.

Discussion

Excessive inflammation is a hallmark of the pathology of invasive bacterial diseases. Notably, these severe complications often arise from an inappropriate host response to the infection. *S. pyogenes* has a multifold repertoire of virulence factors that may cause pathologic inflammatory reactions in the human host [3]. Superantigens for instance, of which Streptococci express at least eleven different varients, are highly potent immuno-stimulatory toxins that can induce a massive activation of T-lymphocytes in the absence of a presented antigen. Whereas normal antigen-presenting MHC class II complexes activate 0.01% of the T-cell population, superantigens activate up to 30% by directly crosslinking the T-cell receptor with the MHC II, and this results in the release of pathologic levels of inflammatory cytokines including IL-1β, IL-6, and TNF-α [27]. Since a massive cytokine release is a common feature of many severe bacterial infections, cytokines have been targeted for drug development. However, regardless of the target (IL-6 or TNF-α), all clinical studies involving anti-cytokine treatment have so far failed [28]. The human contact system is another system that once activated, evokes a series of inflammatory reactions [7]. Previous studies have shown that the system can be initiated on the surface of many bacterial species, including streptococci [11,20,26,29], which eventually leads to the release of BK, a B2R agonist. Subsequent animal models of severe bacterial infections demonstrated that the application of contact system inhibitors has a beneficial effect [30,31]. However, so far, only one clinical trial targeting the contact system has been conducted. In this study, the B2R antagonist, deltibant, was tested in a multicenter, randomized, placebo-controlled trial on patients with systemic inflammatory response syndrome and presumed sepsis. It was found that the drug had no significant effect on risk-adjusted 28-day survival, even though posthoc analysis revealed a trend toward improvement [32]. However, a B1R antagonist, which would block sustained inflammatory responses, induced by the BK metabolite desArg^{9}BK, or a combination of a B1R and B2R antagonist have not yet been tested.

Here, we present a novel mechanism by which streptococci convert BK from a B2R to a B1R agonist by recruiting TAFI to the bacterial surface. To achieve this, *S. pyogenes* first have to assemble and activate the contact system at their surface. This is followed by the release of BK which subsequently serves as a substrate for bacteria-

bound TAFI to generate desArg^{9}BK. Moreover, our studies also show that bacteria-released toxins stimulate monocytes to secrete inflammatory cytokines that in turn trigger an increase of surface-expressed B1R, the receptor for desArg^{9}BK. As depicted in figure 7, our findings suggest a chain of events, for which all critical steps are under streptococcal control. The recruitment and activation of TAFI is the crucial step in these processes since the carboxypeptidase drives the host response from a transient inflammatory stage, mediated by activation of B2Rs, to sustained inflammatory conditions involving activation of B1Rs. It should be mentioned that apart from *S. pyogenes*, *S. aureus*, *Escherichia coli*, and *Salmonella* spp have also been shown to generate BK at their surfaces [26,29]. However, we found that *S. aureus* bacteria do not interact with TAFI, and *E. coli* and *Salmonella* spp bacteria have not been reported to interact with this procarboxypeptidase. Thus, it seems likely that these latter bacteria are probably dependent on other mechanisms to allow conversion of BK to desArg^{9}BK, such as eukaryotic membrane-bound carboxypeptidases as shown for *S. aureus* [26].

Apart from targeting BK, streptococci may gain additional advantages by binding and activating TAFI. For instance, activated TAFI has been shown to cleave the anaphylotoxins C3a and C5a, which should lead to an impaired chemotactic activity of these peptides. One can also speculate that bacteria camouflaged with a fibrin network use TAFI to prevent fibrinolysis, which may protect against attacking phagocytic cells. Thus, future work will show whether or not bacteria are using TAFI for these purposes.

A better understanding of the molecular mechanisms behind host/parasite interactions has the potential of discovering important targets in the human host and ultimately new therapeutic approaches for treatment of severe infectious diseases. Here, we present a novel mechanism by which *S. pyogenes* of the M41 serotype may trigger sustained inflammatory reactions in the human host by TAFI mediated conversion of BK to a B1R agonist and by up-regulating B1Rs. Our findings therefore suggest the combined application of B1R and B2R antagonists as a promising approach for treatment of severe infectious diseases.

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Figure legends

Figure 1. Processing of HK at and release of BK from the bacterial surface

(*Panel A*) *S. pyogenes* bacteria of serotype M41 were incubated with human plasma for 60 min followed by a washing step to remove unbound proteins. Bacteria-absorbed proteins were eluted with an acid wash and the recovered proteins were subjected to Western blot analysis and immunodetection with antibodies against HK. *Lane 1*: normal human plasma; *Lane 2*: kaolin-treated human plasma, which leads to a complete cleavage of HK into its heavy and light chain; Lane 3: proteins absorbed from plasma by *S. pyogenes*. (*Panel B*) Bacteria were incubated with medium or plasma for 15 min followed by a washing step to remove unbound proteins. Bacteria were then resuspended in buffer for another 15 minutes to allow an activation of the contact system and the release of BK into the liquid phase. Supernatants were collected and BK concentrations were determined by ELISA. The BK content in the plasma samples that were used for the experiments, were measured and considered as background. Values are means ± standard deviations (n=3).

Figure 2. Detection of BK and desArg^{9}BK by HPLC

Commercially available BK (1 μM; *Panel A*) and desArg^{9}BK (1 μM; *Panel B*) were analyzed by HPLC. (*Panel C*) BK (1 μM) was incubated with plasmin-activated TAFI (20 nM) for 15 min followed by HPLC analysis of BK cleavage products. (*Panel D*) AP41 bacteria (2 x 10^9 cells/ml) were incubated with (10 μg/ml) plasmin for 60 min on ice, followed by a washing step to remove unbound protein. This was followed by the addition of (20 nM) TAFI and after another 20 minute incubation at room temperature, BK was given to the mixture and incubated for 15 min at 37°C. Bacteria were removed by a centrifugation step and the supernatants were analyzed by HPLC. The HPLC chromatograms are representative of at least four separate experiments.

Fig. 3. Transmission electron micrograph of gold-labeled TAFI with gold-labeled BK and desArg^{9}BK

TAFI was labeled with 40 nm colloidal gold and incubated with 15 nm gold-labeled BK (*Panel A*) or desArg^{9}BK (*Panel B*). Samples were prepared for electron microscopy by negative staining with uranyl formate. Low magnification fields of

complexes are shown. Arrowheads point to gold-labeled kinins that are in complex with gold-labeled TAFI. Statistical evaluation revealed that 49% of gold-labeled TAFI were in complex with gold-labeled BK, while only 8% were associated with gold-labeled desArg^{9}BK (*Panel C*) Representative complexes between gold-labeled TAFI and gold-labeled BK are depicted in the inserts. The bar represents 200 nm (A and B) and 25 nm (C).

Fig. 4. Transmission electron micrograph of gold-labeled TAFI with gold-labeled BK and desArg^{9}BK at the surface of AP41 bacteria

(*Panel A*) AP41 bacteria (2 x 10^9 cells/ml) were incubated with (10 μg/ml) plasmin for 60 min on ice, followed by a washing step to remove unbound protein. This was followed by adding gold-labeled TAFI and after another 20 min gold-labeled BK was given to the mixture. Bacteria were pelleted and then subjected to negative staining electron microscopy. Arrows indicate gold-labeled TAFI in complexes with gold-labeled BK attached to the bacterial surface. (*Panel B*) Representative complexes between gold-labeled TAFI and gold-labeled BK are depicted in the inserts. The bar represents 100 nm (A) and 50 nm (B).

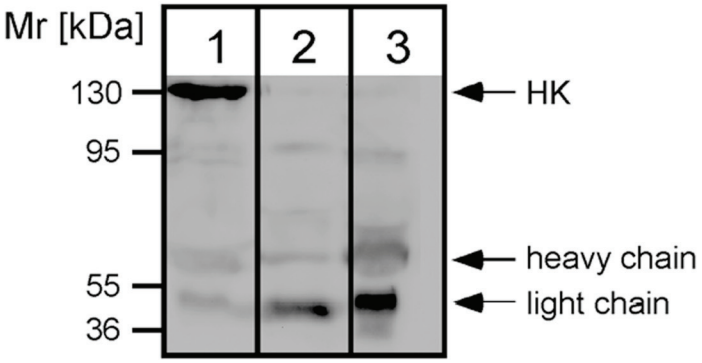
Fig. 5. Detection of BK and desArg^{9}BK released from AP41 bacteria by HPLC

(*Panel A*) AP41 bacteria (2 x 10^9 cells/ml) were incubated with (10 μg/ml) plasmin for 60 min on ice, followed by a washing step to remove unbound protein. Afterwards (20 nM) TAFI-IIYQ was added for 20 min and after a second washing step BK was given to the mixture and incubated for 15 min at 37°C. Bacteria were removed by a centrifugation step and the supernatants were analyzed by HPLC. (*Panel B*) AP41 bacteria were first treated with TAFI-IIYQ and then with plasmin. Washings steps and incubation with BK were performed as described in *Panel A*. The HPLC chromatograms are representative of at least four separate experiments.

Fig. 6. Measurements of the biological activity of desArg^{9}BK released from AP41 bacteria and cell surface expression of B1R

(*panel A*) HEK293 cells stably transfected with B1R were treated with i. buffer, ii. DLKD, iii. desArg^{9}BK, iv a mixture of desArg^{9}BK and DLKD, v. BK cleavage products released from AP41 bacteria, and vi. a mixture of BK cleavage products

A



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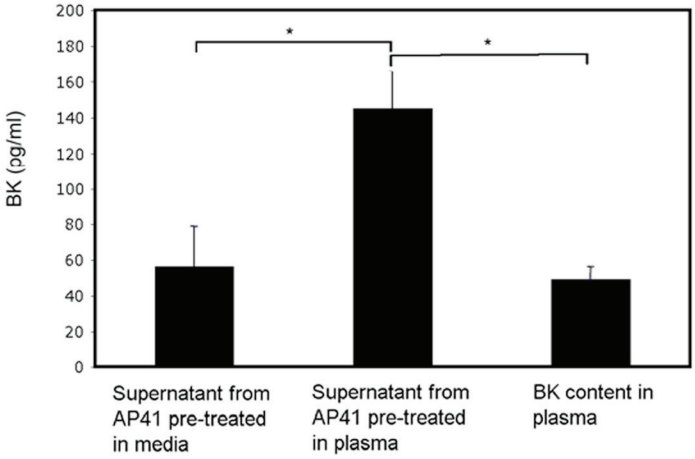

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Fig. 1

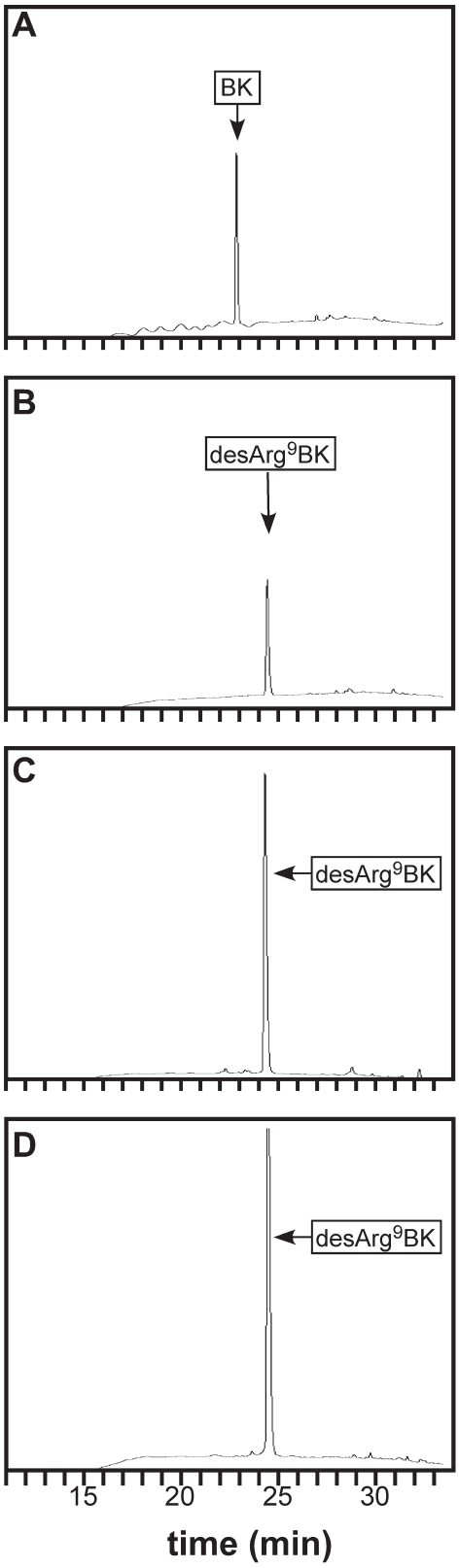

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Fig. 2

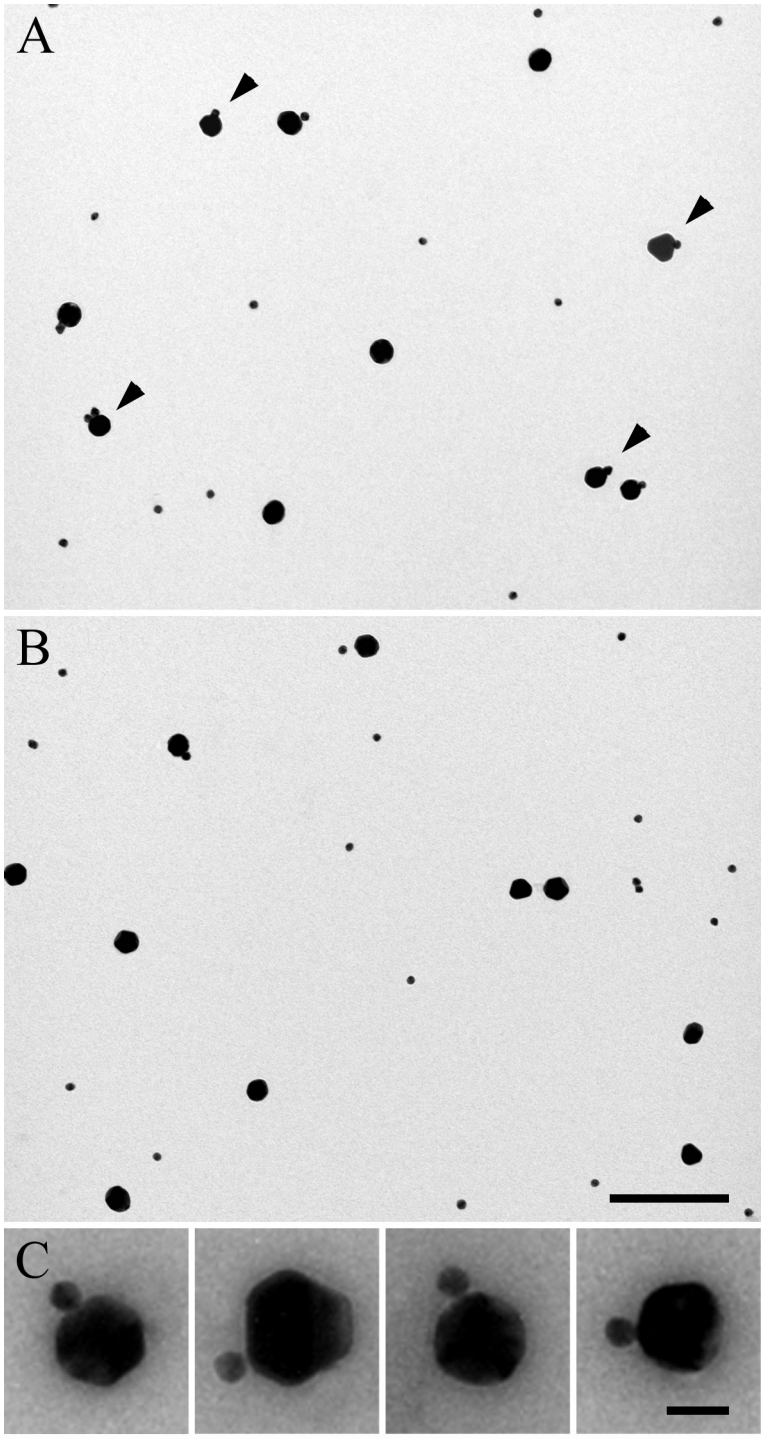

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Fig. 3

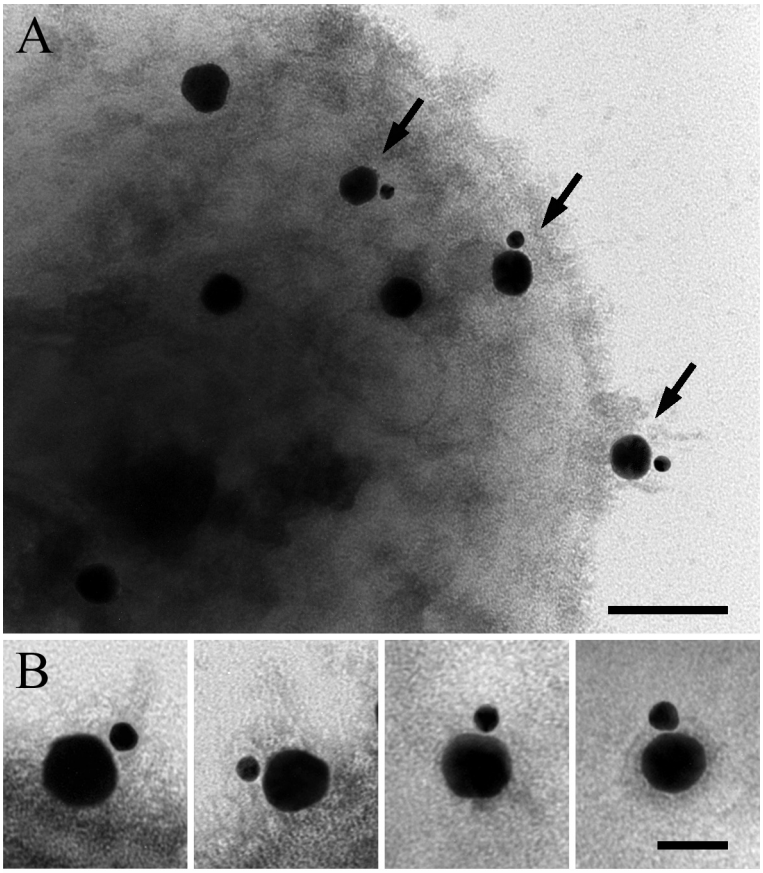

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Fig. 4

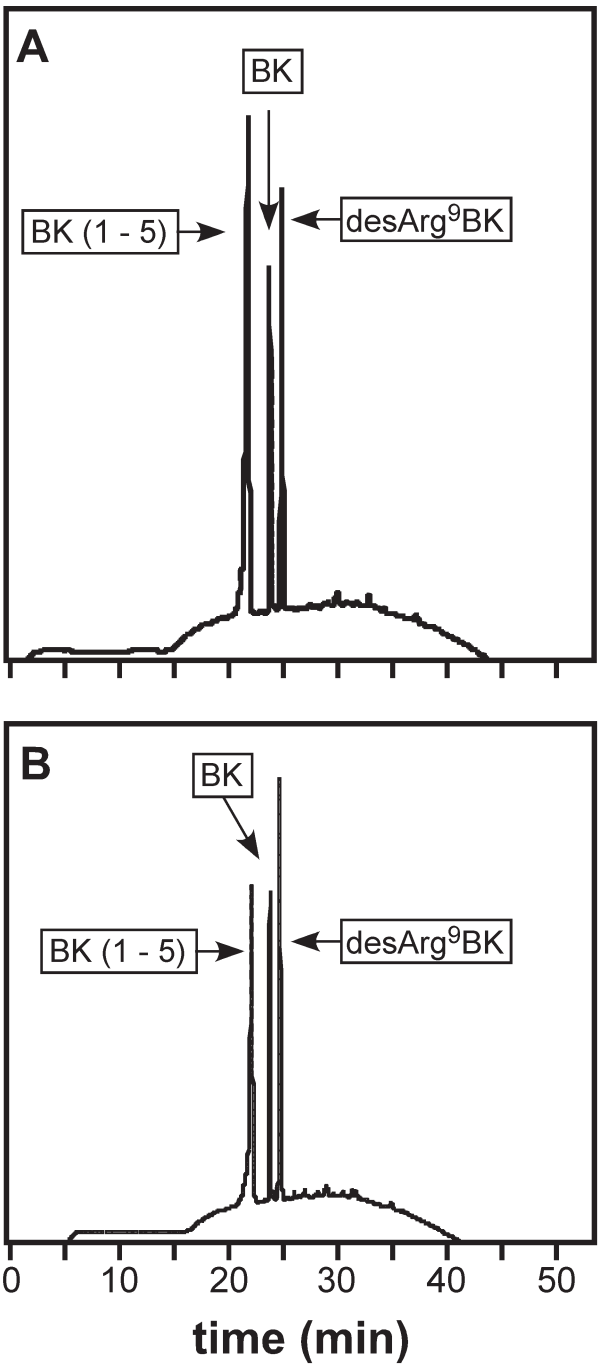

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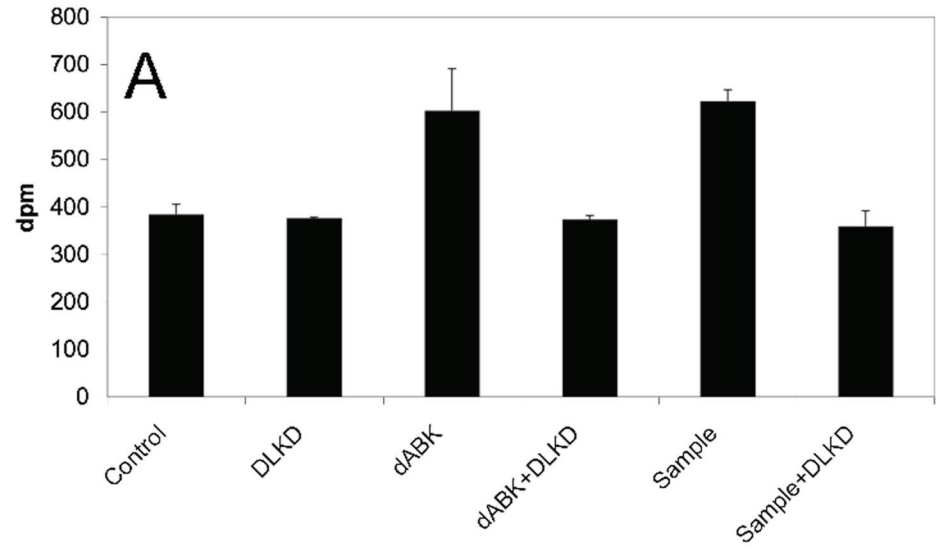
Fig. 5



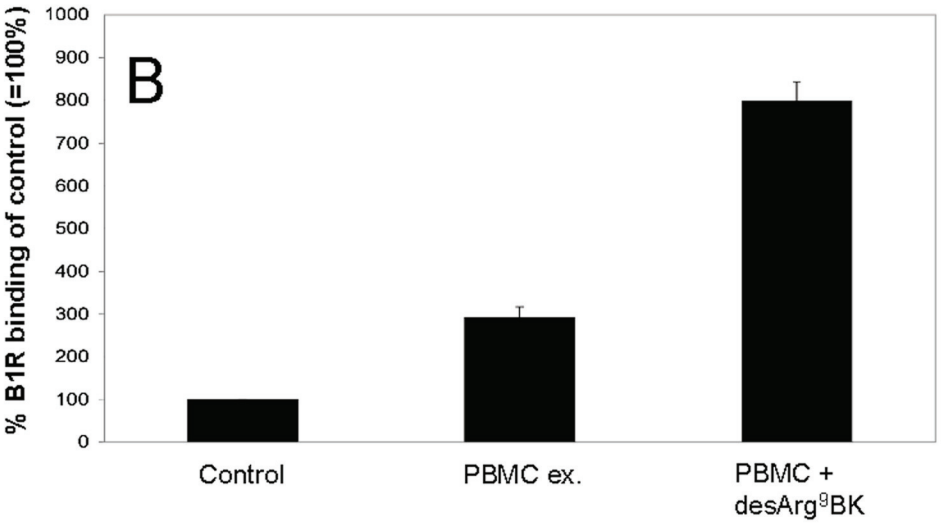


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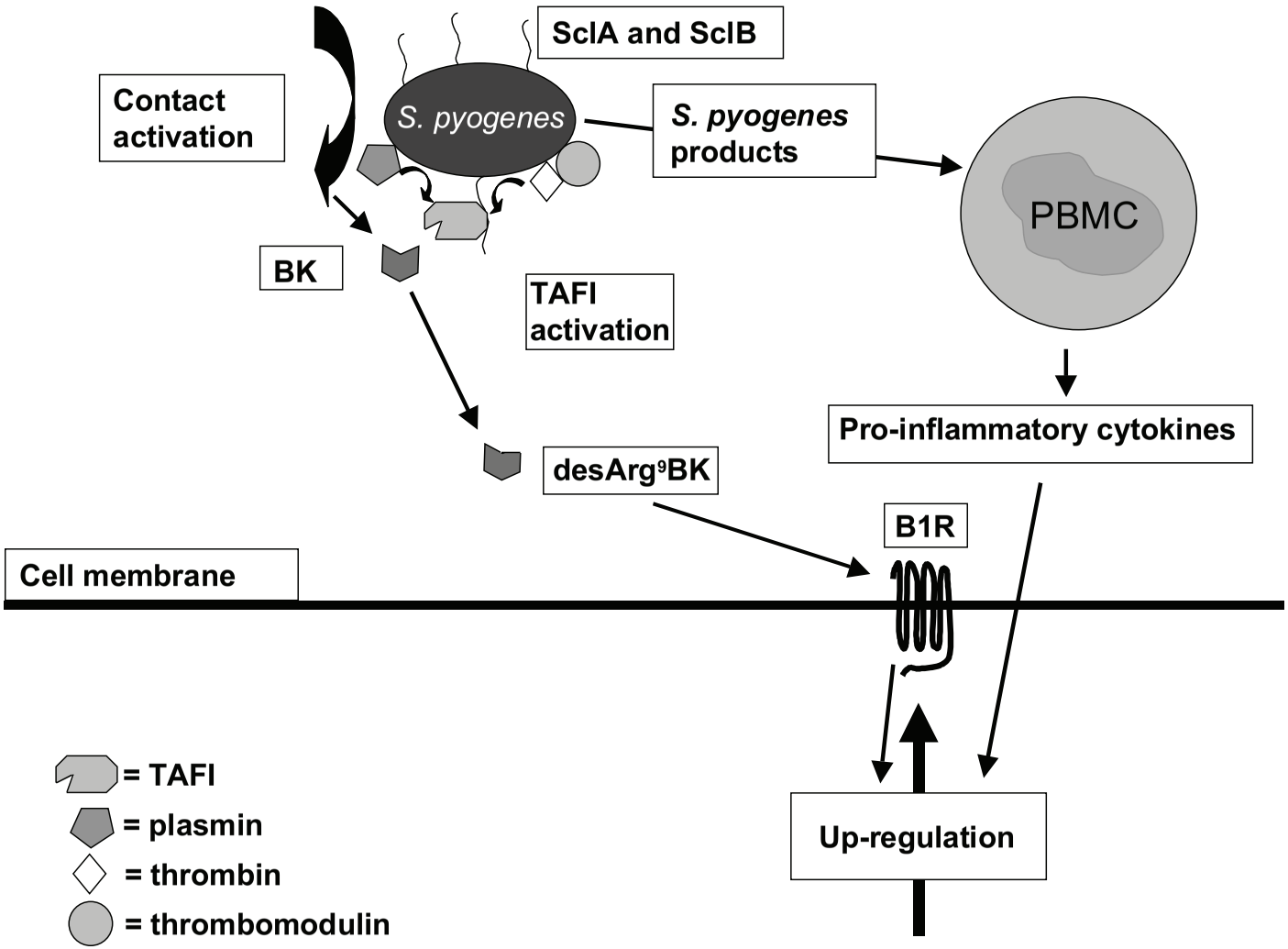
Fig. 6


ScIA and ScIB
Contact activation
S. pyogenes
S. pyogenes products
PBMC
BK
TAFI activation
Pro-inflammatory cytokines
desArg⁹BK
B1R
Cell membrane
= TAFI
= plasmin
= thrombin
= thrombomodulin
Up-regulation