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Bradykinin enhances Sindbis virus infection in human brain microvascular endothelial cells

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ABSTRACT

Sindbis virus (SINV) induces inflammatory and vasoactive responses that are associated with rash and arthritis in human infections. The mechanisms underlying infection-associated microvasculopathy are still unknown. We investigated whether endothelial cells infected by SINV are differentially responsive to bradykinin (BK), a potent inducer of inflammatory edema in a broad range of infectious diseases. Human endothelial cells (HBMECs) infected with SINV presented an upregulation of bradykinin B2 receptors (BK2R) expression. Also, BK reduced SINV-induced apoptosis and enhanced virus replication in HBMECs in a way dependent on BK2R, PI3 kinase and ERK signaling. Strikingly, intracerebral infection of mice in the presence of a BK2R antagonist reduced the local viral load. Our data suggest that SINV infection renders human endothelial cells hypersensitive to BK, which increases host cell survival and viral replication. Ongoing studies may clarify if the deregulation of the kinin pathway contributes to infection-associated vasculopathies in life-threatening arbovirus infections.

Introduction

Sindbis virus (SINV), a prototype member of the Alphavirus genus, is an enveloped, single-strand positive sense RNA virus (Strauss and Strauss, 1994). In mice, SINV infection produces encephalitis and it is considered a model to study the pathogenesis of human arthropod-borne encephalitis caused by other alphaviruses (Johnson et al., 1972; Lustig et al., 1988). Resistance to SINV infection in murine models is age-dependent and involves the participation of multiple mechanisms, such as neuronal maturation (Griffin et al., 1994), variations in cytokine secretion and interferon-induced responses (Labrada et al., 2002; Trgovcich et al., 1999), attenuation of the inflammatory response (Ryman et al., 2007), and differential expression of pro and anti-apoptotic genes (Griffin, 2005; Griffin et al., 1994; Labrada et al., 2002). Noteworthy, apoptosis is often associated to central nervous system (CNS) pathology (Kerr et al., 2002; Lewis et al., 1999; 4 Sarid et al., 2001), albeit it may also represent a cellular defense mechanism to limit viral replication (Teodoro and Branton, 1997).

Although the mechanisms of virus-induced encephalitis are not completely understood, there is strong evidence that it requires leukocyte extravasation through the blood brain barrier (BBB) (Kim et al., 2009). The BBB typically excludes circulating cells in normal conditions, but there is evidence that the presence of SINV in the brains of inoculated mice induces the activation of the endothelium, leading consequently to the upregulation of adhesion molecules (Irani and Griffin, 1996). It was also showed that the expression of these adhesion molecules in the BBB is modulated according to the stage of SINV infection, being responsible for lymphocyte migration to the central nervous system (CNS) (Irani and Griffin, 1996).

In humans, SINV infection may cause fever, rash, and arthritis (Laine et al., 2004). Viral arthritis is characterized by swelling, pain and leukocyte infiltrate into the joint (Fraser, 1986), all of which are associated to inflammatory alterations of the underlying endothelium. The mechanisms governing pathogenic outcome and extent of SINV replication in human cells are not sufficiently well characterized (Calabrese and Naides, 2005).

Bradykinin-related vasoactive peptides, collectively called kinins, are proteolytically derived sentinel mediators that are rapidly

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generated in pathophysiological conditions, including infection-associated inflammation. Recent studies revealed that platelet-derived polyphosphates may directly initiate proteolytic cascades dependent on Factor XII (FXII), such as the intrinsic pathway (contact system) of coagulation and the kallikrein–kinin system (KKS) (Van der Meijden and Heemsherk, 2010). The autocatalytic cleavage of FXII generates small amounts of FXIIa, which then converts prekallikrein into active plasma kallikrein, a serine protease that amplifies the contact system through the reciprocal activation of FXII into FXIIa. As the amplification loop progresses, plasma kallikrein releases bradykinin (BK) from an internal segment of high molecular weight kininogen. Once released in the bloodstream, the short-lived BK acts as paracrine hormone, inducing vasodilation in arterioles via production of nitric oxide (NO), through the stimulation of bradykinin B2 receptor (BK2R) constitutively expressed in the endothelium. BK may then be further metabolized in the bloodstream by carboxypeptidase N (kininase 1), thereby generating des-Arg-bradykinin (des-Arg-BK). As opposed to the BK2R agonist, des-Arg-BK triggers the kinin B1 receptor (BK1R), a GPCR subtype that is induced in inflamed tissues (Austin et al., 1997; Bhoola et al., 1992; Menke et al., 1994). In other settings, BK is known to trigger a broad spectrum of biological responses, including cellular proliferation and rescuing from apoptosis, observed after renal, cardiac and brain injuries (Bledsoe et al., 2006; Xia et al., 2004). Molecular analysis of the intracellular pathways controlled by BK2R revealed that BK can induce cAMP formation (Regoli et al., 1990), activation of PI3K-phospho-Akt pathway and p42/p44MAPK (Bovenzi et al., 2010; Greco et al., 2006), and upregulation of anti-apoptotic mediators in endothelial cells (Bovenzi et al., 2010).

Recent progress in microbial immunity provided clues to understand how activation of the kinin system may modulate inflammation and immunopathogenesis of numerous infectious diseases, including bacteria, protozoan and virus infections (Christiansen et al., 2008; Folkerts et al., 2000; Kaman et al., 2009; Monteiro et al., 2007, 2009). Respiratory syncytial virus (RSV), influenza, and other respiratory viruses increase local kinin levels and kinin receptor expression, these effects being associated to increased severity of clinical symptoms (Barnett et al., 1990; Bengtson et al., 2007; Naclerio et al., 1988). Although not directly related to KKS, there is evidence that HIV infections enhance Substance P expression in immune cells, which increases virus replication (Ho et al., 2002; Li et al., 2001), thus setting an interesting precedent for the studies involving the KKS.

It was previously reported that activation of BK2R by BK is involved in inflammatory processes related to arthropathies and that BK2R antagonist may inhibit carrageenan and lipopolysaccharide (LPS)-induced arthritis in rat by reducing joint pain, edema and neutrophil infiltration (Valenti et al., 2010). Also, kallikrein inhibitors, such as kallistatin are associated to a protective effect in rat arthritis model (Wang et al., 2005). Although Alphavirus infection causes alterations in vascular permeability and cell survival modulation, the possibility that kinins may influence pathogenic outcome was not the object of systematic studies.

Except for studies with non-replicating SINV vectors (Tseng et al., 2004; Wollmann et al., 2005) and few reports of infection of human macrophages or PBMCs (Assunção-Miranda et al., 2010; Dhanushkodi et al., 2011) little efforts were invested in characterizing the molecular mechanisms regulating SINV infection in human cells. Here, we analyzed if human endothelial cells were permissive to SINV infection and evaluated whether BK signaling would modulate virus replication and cellular apoptosis. We observed that SINV induced an upregulation of BKRs on human brain microvascular endothelial cells (HBMECs). In addition, we observed that signaling of BK2R increases HBMECs permissiveness to infection by promoting a delay in the virus-induced apoptosis. Extending these studies to the context of in vivo infection, we obtained evidences that it is possible to reduce acute viral load in the CNS through the local administration of HOE-140, a specific antagonist of BK2Rs.

Results

Human brain microvascular endothelial cells upregulate kinin receptors upon infection by Sindbis virus

The endothelium plays a central role in the pathogenesis of Sindbis virus infection. However, the direct interaction of endothelial cells with the virus was not evaluated before. We investigated whether endothelial cells would be a target for SINV infection in vitro. HBMECs were infected with SINV at an MOI of 0.1 and the supernatants were titrated by plaque assay after different time points. Virus titers released in the supernatants increased over time, suggesting a productive infection of these cells. The peak of virus detection was observed at 36 h p.i. and virus titers decreased thereafter (Fig. 1A). Consistent with these results, RT-PCR showed the presence of viral genome in the cell lysates and supernatants of HBMECs (Fig. 1B). The expression of viral proteins in infected cells was confirmed by immunofluorescence and flow cytometry analysis (Figs. 1C and D).

After showing that HBMECs are susceptible to SINV, we sought to determine whether the viral infection could influence endothelium sensitivity to vasoactive kinin. To this end, we compared the expression profile of BK2R and BK1R in normal and SINV-infected HBMECs by quantitative real-time PCR. Assays performed 24 h p.i. revealed that SINV infection induced an increase of 3.7 and 23.8 fold in BK2R and BK1R transcription, respectively, as compared to the non-infected HBMECs, which displayed low levels of such transcripts (Fig. 2A). FACS analysis showed that the transcriptional upregulation observed in SINV-infected HBMEC translated into a modest increase in the percentage BKR-expressing cells and in the magnitude of BKR expression (mean fluorescence intensity-MFI) (Fig. 2B). Control experiments showed that transcription or surface expression of both BKRs was not significantly modulated in HBMECs mock-infected or treated with heat-inactivated virus (Figs. 2A and B).

We then compared BKR surface expression between the subpopulations of infected and bystander cells (Fig. 2C). The results revealed that increased BK2R and BK1R expressions were mostly related to SINV-infected ($SINV^+$) cells. Fig. 2C demonstrated that 58.6% and 56% of all $SINV^+$ (5.39% and 7.32% total) express BK2R and BK1R, respectively. In contrast, only 22.17% and 21.9% (20.13% and 18.99% total) of $SINV^-$ cells express the receptors, similar to what was observed in noninfected cultures (mock). Inactivated virus did not affect BKR expression. We also observed a higher MFI of BKR surface expression in SINV-infected cells than in non-infected HBMECs (36.88 $SINV^+$ vs 9.60 $SINV^-$ for BK2R; 54.72 $SINV^+$ vs 11.59 $SINV^-$; data not shown). Taken together, mRNA and protein expression data support that SINV infection indeed upregulated BKR expression.

Bradykinin increases SINV replication in a BK2R-dependent way

Given evidences that endothelial cells infected by SINV upregulate BKRs, we then investigated the influence of BK-induced signaling on virus replication. To this end, HBMECs were exposed, or not, to physiological concentrations of BK (10 nM) and virus replication was analyzed. These assays were performed in the presence of the ACE inhibitor lisinopril (ACEi) in order to prevent BK degradation by this metallopeptidase, which is highly expressed in endothelial cells, such as HBMECs. Our results showed that addition of ACEi/BK simultaneously to SINV did not influence the progression of infection (Fig. 3A). Strikingly, however, the virus load was significantly increased when the HBMECs were treated with BK 24 h after viral exposure (Fig. 3A), i.e., coinciding with the time point when BKRs are upregulated. Additional experiments showed that BK stimulated viral replication in dose-dependent manner (Fig. 3B). Of note, addition of the ACEi alone did not significantly increase the viral load (Fig. 3C), suggesting that blockade of angiotensin I production per se is not sufficient to increase HBMEC susceptibility to SINV. These results suggested that the ACEi

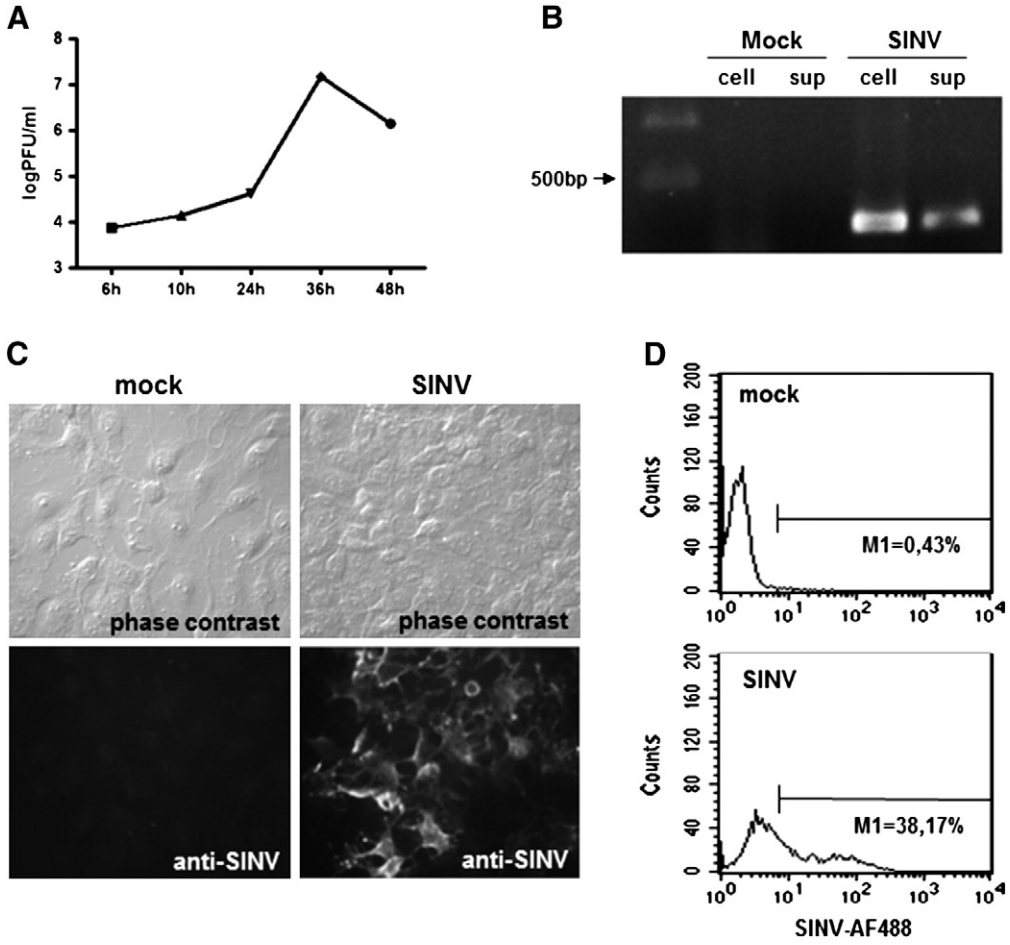

Fig. 1. Human brain microvascular endothelial cells are permissive to in vitro infection by SINV. HBMECs were cultured with SINV at a MOI 0.1 or with supernatant of non infected VERO cells (mock) and the infection was evaluated by different methods. A) Time course of HBMECs infection evaluated by plaque assay. B) After 48 h p.i. the cells and supernatants were harvested and viral RNA present in both fractions was amplified by RT-PCR. C) After 48 h p.i. the cells were fixed, permeabilized and stained with mouse anti-SINV antibody followed by anti-mouse IgG conjugated to AlexaFluor 546. The slides were then analyzed by fluorescence microscopy. D) After 48 h p.i., the cultured cells were harvested and stained as in (C) and were analyzed by flow cytometry. M1 indicates the percentage of $SINV^+$. The data are representative of two independent experiments.

lisinopril enhanced viral infection because it potentiates BK-induced signaling of BKRs (Fig. 3C). Therefore, we performed all subsequent experiments in the presence of ACEi.

As an extension of these studies, we checked whether BK-induced increase in viral load was neutralized by specific antagonists of BKRs. Indeed, we found that HOE-140, a selective inhibitor of BK2R, abrogated BK-induced increase of viral replication (Fig. 3D). In spite of the increased expression of BK1R, addition of its specific antagonist ([Leu8]-desArg9-BK) did not significantly reduce virus replication (Fig. 3E). Consistent with these data, the addition of des-Arg-BK (i.e., BK1R agonist) did not increase SINV titers (data not shown). We then repeated these assays using primary cultures of human umbilical vein endothelial cells (HUVECs). Similarly to HBMECs, BK addition enhanced SINV replication in HUVECs, whereas HOE-140 blocked these effects (Fig. 3F). Collectively, these results suggested that BK increased the permissiveness of endothelial cells to SINV through the signaling of the BK2R.

Bradykinin inhibits SINV-induced apoptosis

SINV infection has been reported to induce cell death in CNS murine cells and in human macrophages (Assunção-Miranda et al., 2010; Griffin, 2005; Irusta and Hardwick, 2004). Apoptosis is an event usually observed at a late time point after virus infection and is considered to be a mechanism to control virus dissemination (Lee et al., 2005; Teodoro and Branton, 1997). Given that BKR engagement is associated to cell signaling pathways involved in rescuing cells from apoptosis (Bledsoe et al., 2006; Xia et al., 2004), we reasoned that perhaps BK boosts viral replication because it prolongs survival of SINV-infected cells through the induction of anti-apoptotic pathways. In order to explore this issue, we first checked if SINV was also inducing cell death in HBMECs. Analysis by MTT assay (Fig. 4A) and neutral red staining (not shown) demonstrated a decrease in the proportion of live HBMECs after SINV infection, which was statistically significant at 36 h p.i. and thereafter. Of note, inactivated SINV failed to induce cell death (Fig. 4A). We then analyzed whether BK, added 24 h after the onset of infection, could rescue HBMECs from cell death. The results showed that addition of BK 24 h p.i. inhibited cell death, at least in the first 12 h (end point 36 h p.i.). These effects were no longer observed when we run MTT assays at 48 h p.i. (Fig. 4B). Also, inhibition of virus-induced cell death could be detected even when BK was added in the absence of ACEi, suggesting that the effect mediated by ACEi in some experiments resulted from inhibition of endogenous BK present in the cell culture. These results suggested that BK2R signaling within the narrow window (24 to 36 h) is sufficient to create a window of opportunity for viral growth in HBMECs.

In order to determine if the observed cell death could be attributed to apoptosis, we infected HBMECs and double stained them with propidium iodide (PI) and AlexaFluor488-Annexin V. Assessment of the percentage of live (AnexV − PI−), dead (AnexV − PI+) or apoptotic (either AnexV + PI or AnexV + PI+) cells revealed that there was a

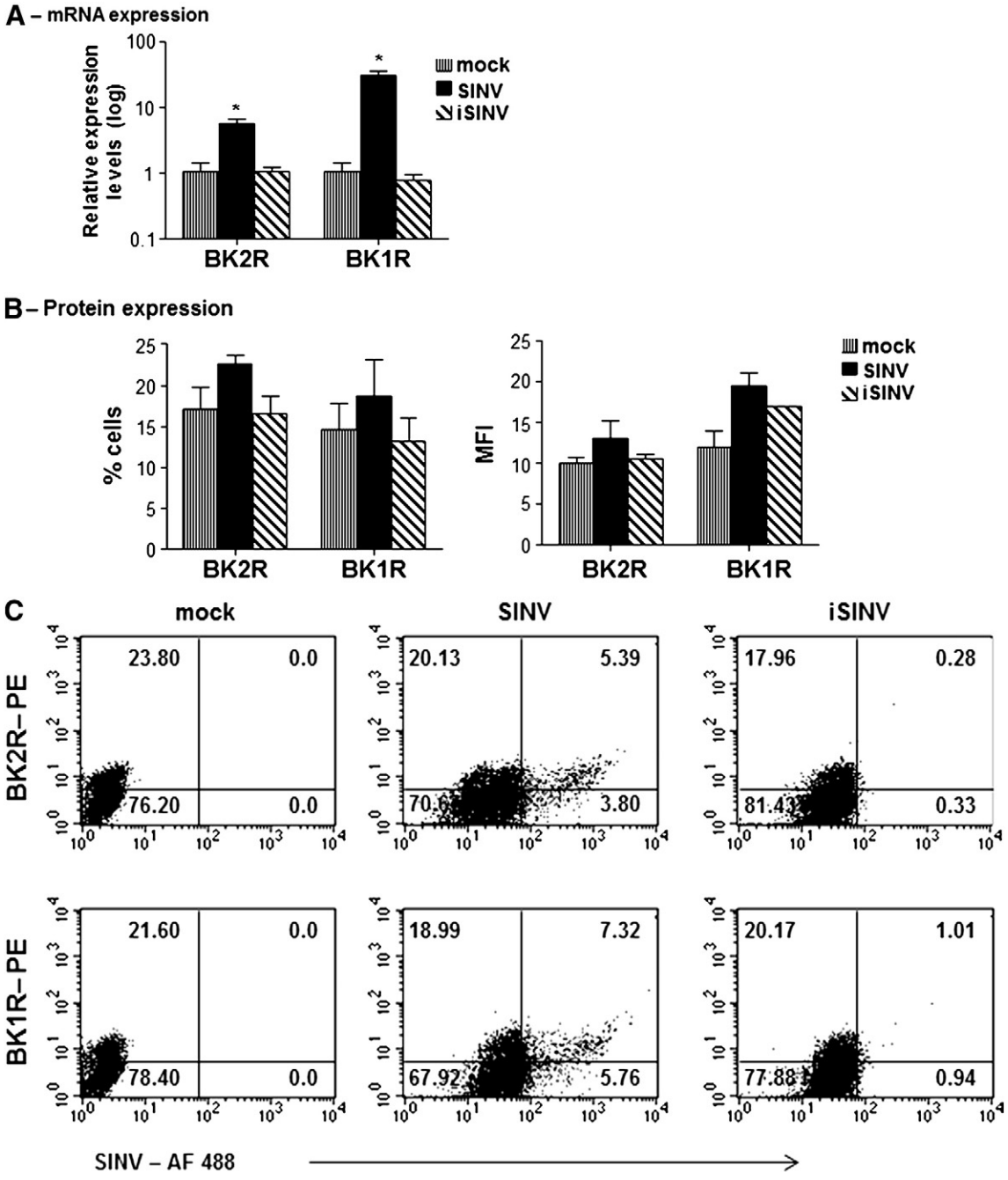

Fig. 2. SINV infection upregulates the expression of BK receptors. HBMECs were mock-infected or cultured with infective (SINV) or heat-inactivated SINV (iSINV) with an MOI of 0.1 and the expression of BK1R and BK2R was evaluated by different methods. A) Total cellular RNA was extracted 24 h p.i. and subjected to quantitative real-time PCR, using specific commercial assays for B1 or B2 receptors. B) After 24 h p.i., the cells were harvested and stained with anti-BK1R or anti-BK2R antibodies, followed by staining with anti-rabbit IgG conjugated to PE. The expression of B1 and B2 receptors in the cell surface was evaluated for flow cytometry. The results are expressed as the percentage of cells expressing the receptors (% cells) or as mean fluorescence intensity (MFI). C) After 24 h p.i., the cells were harvested, stained as in (B), and then permeabilized and incubated with anti-SINV antibody, followed by incubation with anti-mouse IgG antibody conjugated to AlexaFluor 488. The cells were analyzed by flow cytometry and the figure is a representative quadrant plot of SINV vs BK2R or BK1R staining. Insert numbers express the percentage of cells in each quadrant. The data are representative of three independent experiments and error bars indicate SD values between replicates in all experiments. * $p \leq 0.05$ in relation to the mock.

significant increase in the apoptotic cell frequency among infected cells at 36 h p.i., which was reverted upon addition of BK in preceding 12 h. We did not observe any statistical difference between BK-treated and untreated HBMECs infected cells at 48 h p.i. (Fig. 4C). To further test the hypothesis that BK could be inhibiting apoptosis, we performed FACS analysis of SINV-infected HBMECs at 36 h p.i. (12 h after BK stimulation), using a PI-containing hypothonic fluorochrome solution, which binds the cellular DNA, thereby allowing the analysis of cell cycle phases and the determination of the frequency of apoptotic cells. Again, we observed that HBMECs treated with BK (in the presence or absence of ACEi) underwent decreased apoptosis. Of note, addition of HOE-140 reverted the BK-induced rescuing of apoptosis (Fig. 4D), suggesting that delayed apoptosis may account for the enhanced virus replication observed in BK-treated HBMECs.

BK-driven modulation of viral replication and apoptosis is dependent on PI3K activation

Cell survival induced by BK2R engagement is usually related to activation of the PI3 kinase (PI3K) and phosphorylation of either Akt

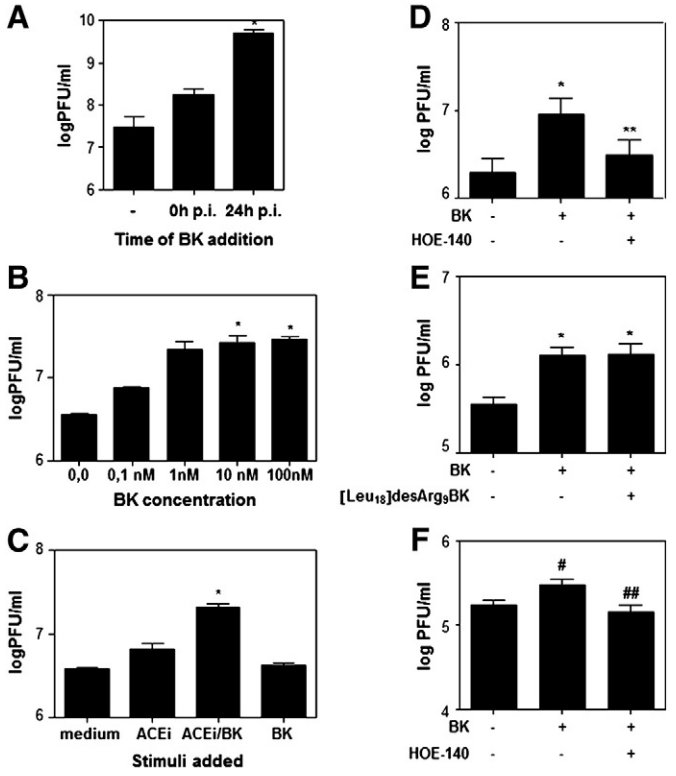

Fig. 3. BK increases viral replication in HBMECs infected cells in a BK2R-dependent pathway. A) HBMECs were infected with SINV (MOI = 0.1) and treated at the indicated time points with bradykinin (10 nM), after a pretreatment with the ACE inhibitor lisinopril (25 μM). After 48 h p.i., virus titers in the supernatants were evaluated by plaque assay. B) HBMECs were infected with SINV (MOI = 0.1) and, 24 h later, the cells were treated with ACEi/BK at the indicated concentrations. Virus titers in the supernatants were evaluated by plaque assay at 48 h p.i. C) HBMECs were infected with SINV (MOI = 0.1) and, 24 h later, the cells were treated with either BK or ACEi, or with both stimuli (ACEi/BK). After 24 h post stimuli (48 h p.i.), the culture supernatants were harvested and viral replication was evaluated by plaque assay. D–E) HBMECs were infected and treated with ACEi/BK (at 24 h p.i.), in the presence or absence of the B2 receptor antagonist — HOE-140 (100 nM) (D) or B1 receptor antagonist — [Leu18]desArg9BK (100 nM) (E). Viral replication was analyzed at 48 h p.i. by plaque assay. F) HUVECs were infected with SINV and treated as described in (D). Viral replication was evaluated at 48 h p.i. by plaque assay. The data are representative of five independent experiments and error bars indicate SD values between replicates in all experiments. * $p \leq 0.05$ and # $p \leq 0.1$ when comparing to SINV alone; ** $p \leq 0.05$ and ## $p \leq 0.1$ when comparing to BK-treated.

factor (protein kinase B-PKB) or ERK MAP kinase (Krieg et al., 2004; Prado et al., 2002; Penna et al., 2008). To investigate the role of these signaling pathways in the BK-induced modulation of virus replication and cell survival, HBMECs were infected and treated with BK, in the presence or absence of the PI3K inhibitor wortmannin (100 nM). Addition of wortmannin to BK-treated cultures abrogated both effects mediated by the BK peptide (Figs. 5A and B). These data indicate that BK2R signaling induces increased cell survival and virus replication through the activation of PI3K.

We also performed kinetics experiments to investigate the effect of SINV infection and BK treatment on the PI3K-driven activation responses. Infection of HBMECs resulted in a transitory increase of Akt, which peaked at 24 h p.i. and decayed thereafter. In addition, SINV induced an earlier phosphorylation of ERK (1 h p.i.), which decayed at 10 h p.i. We then examined the effects of BK added 24 h p.i. Our results showed that BK upregulated pERK, but not pAKT, at 36 h p.i. (12 h after BK addition) (Figs. 6A and B). Modulation of Akt phosphorylation was not observed even at earlier or later time points (data not shown), suggesting that activation of ERK MAPK may be involved in BK/PI3K-mediated cell survival and enhanced viral replication. Indeed, similar to HOE-140 and wortmannin, addition of the ERK inhibitor PD98059 abolished BK-mediated increased cell survival and virus replication in HBMECs (Figs. 7A and B).

Blocking BK2R inhibits SINV replication in the CNS in vivo

In order to investigate the in vivo relevance of the observations made in tissue culture conditions, BALB/c mice were infected i.c. with a suspension of SINV (5×10^4 PFU) containing, or not, the BK2R antagonist HOE-140. Analysis of the viral load in the brain (24 h p.i.) revealed that HOE-140 significantly reduced the virus titer in the CNS (Fig. 8). These results suggest that SINV infection may be enhanced as result of KKS activation in the in vivo settings.

Discussion

In the present work, we tested the hypothesis that BK, the short-lived vasoactive peptide generated by activation of the KKS, may influence the susceptibility of human endothelial cells to infection by Sindbis *Alphavirus*. The rationale to undertake this study was based on a precedent that kinins can induce vasodilatation, vascular permeability, cytokine secretion and inhibition of apoptosis of different cell types, including endothelial cells (Aliberti et al., 2003; Bhoola et al., 1992; Bovenzi et al., 2010; Prado et al., 2002; Yin et al., 2005), being therefore capable of contributing to the pathogenesis of infections caused by arbovirus, such as Sindbis. Knowledge about the pathogenetic mechanisms underlying SINV pathogenesis is still limited to studies in experimentally infected mice (Griffin et al., 1994; Kerr et al., 2002; Ryman et al., 2007). Pertinent to the present work, the encephalitis described in the SINV-infected mice is associated to extravasation through the BBB (Irani and Griffin, 1996). In addition, it has been reported that alphavirus-induced arthritis observed in humans is associated with leukocyte infiltrate into the joint (Fraser, 1986), which may be a consequence of altered vascular leakage and endothelial cell activation. Thus, there is substantial evidence that endothelium dysfunction contributes to SINV infection-associated vasculopathy, both in mice and humans.

Except for a few studies with glioma cell lines and primary macrophages and PBMCs (Assunção-Miranda et al., 2010; Dhanushkodi et al., 2011; Wollmann et al., 2005), the analysis of the outcome of SINV infection of human cells involved studies with nonreplicating vectors (Tseng et al., 2004; Wollmann et al., 2005). Despite the cited indications that SINV infection leads to endothelial cell dysfunction, there were no systematic attempts to determine if the formation of proinflammatory mediators may influence endothelial cell permissiveness to SINV infection.

In the present report, we demonstrated that human brain microvascular endothelial cells (and primary endothelial cells) were permissive to SINV infection in vitro. Our results showed that B1 and B2 bradykinin receptors were upregulated upon endothelial cell infection, increasing their sensitivity to signaling by BK. Notably, there was a significant BK2R-dependent increase in virus replication upon addition of BK 24 h p.i., coinciding with the period of maximal BK receptor expression.

Regulation of kinin generation or kinin receptor expression has been previously observed during RNA virus infections, however, in these studies these events were mainly associated to the inflammatory response (Barnett et al., 1990; Christiansen et al., 2008; Folkerts et al., 2000; Proud et al., 1990; Tripp et al., 2000). Human and bovine rhinovirus (hRSV and bRSV) infections resulted in increased local formation of kinins, the severity of clinical disease being correlated with the level of kinins detected in respiratory secretions (Naclerio et al., 1988; Proud et al., 1990). Interestingly, double strand RNA surrogates, like poli I:C, also upregulated the expression of BK1R and BK2R, therefore suggesting that activation of innate immune receptors, such as Toll like receptor 3 (TLR3) or RIG-I/MDA5 may upregulate BKR expression (Bengtson et al., 2007). In preliminary studies, we observed that infection of endothelial cells by flaviviruses also induced an upregulation of BK receptors, suggesting that it may represent a more

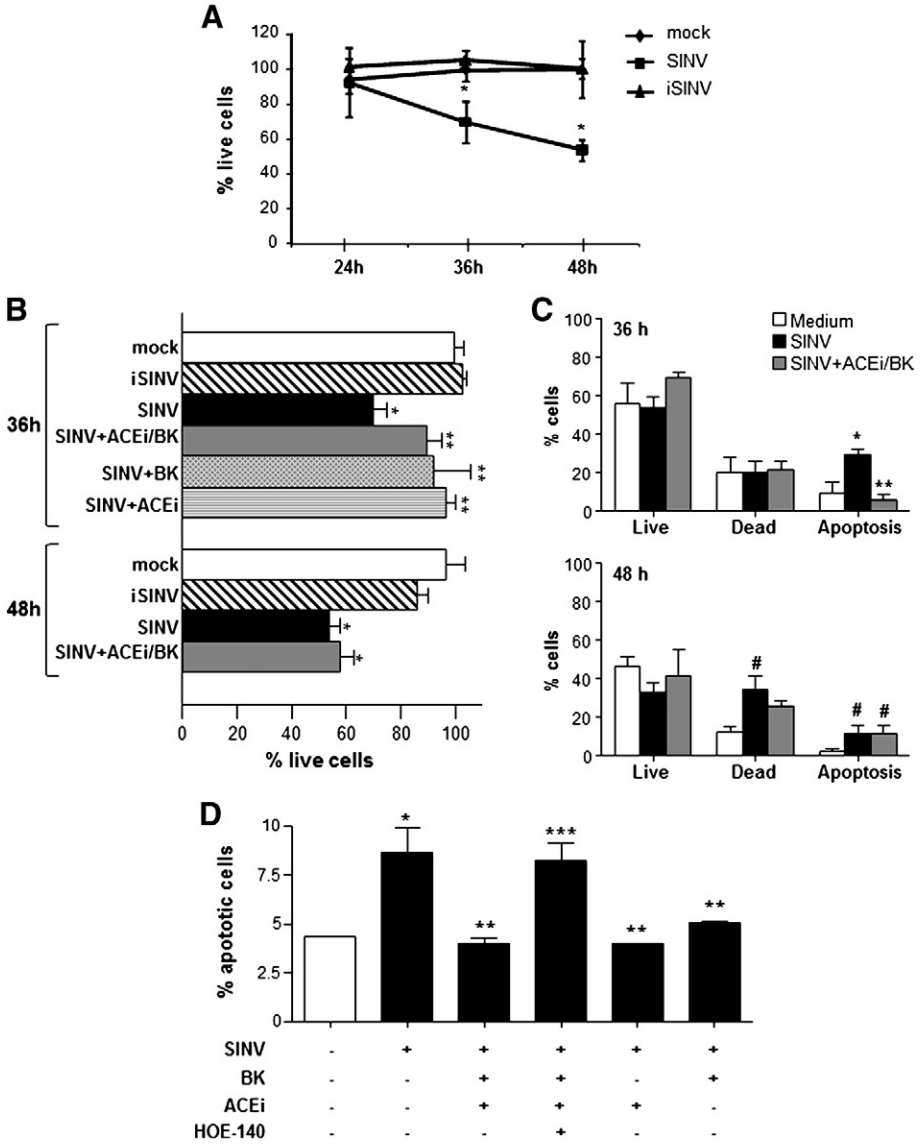

Fig. 4. BK inhibits SINV-induced cell death. A) HBMECs were mock-infected or cultured with infective (SINV) or heat-inactivated SINV (iSINV) with a MOI of 0.1 and cell viability was evaluated by MTT assay at different time points p.i. B) HBMECs were infected with SINV and, 24 h later, the cells were treated with BK, ACEi or both stimuli (ACEi/BK). After 36 and 48 h incubation, cell viability was evaluated by MTT assay. The data were normalized according to the results obtained with the cells cultured with culture medium only (adjusted to 100%). C) The cells were double stained with propidium iodide (PI) and AlexaFluor488-Annexin V. The percentage of live (PI−/Annex−), dead (PI+/Annex−) or apoptotic (PI−/Annex+, and PI+/Annex+) cells were analyzed by flow cytometry at 36 and 48 h p.i. D) HBMECs were cultured as in (B). After 36 h p.i. (12 h post stimuli), the cells were incubated with hypotonic fluorochrome solution and apoptosis was analyzed by flow cytometry. The results indicate the percentage of apoptotic cells. The data are representative of three independent experiments and error bars indicate SD values between replicates in all experiments. * $p \leq 0.05$ when comparing to mock-infected; ** $p \leq 0.05$ when comparing to SINV-infected; *** $p \leq 0.05$ when comparing to BK-treated; # $p \leq 0.1$ when comparing to mock-infected.

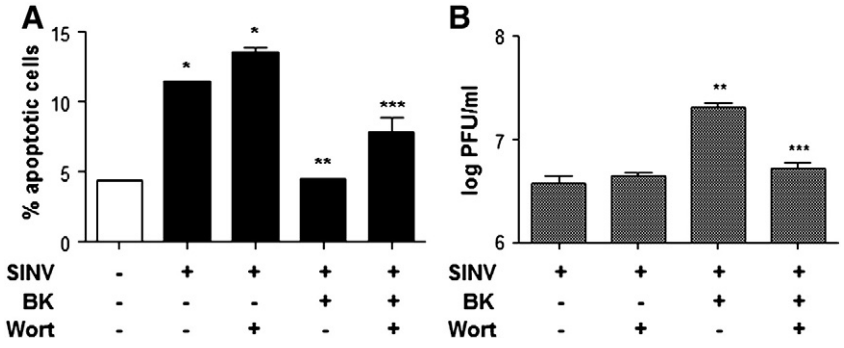

Fig. 5. Modulation of viral load and apoptosis mediated by BK is dependent on PI3K activation. HBMECs were infected with SINV (MOI = 0.1) and, after 24 h p.i., the cells were treated with BK (10 nM), in the presence or absence of the wortmannin (wort, 100 nM). A) 36 h p.i the cells were harvested and incubated with PI and AlexaFluor488-Annexin V. The percentage of apoptotic cells was evaluated by flow cytometry. B) 48 h p.i., the culture supernatants were collected and viral titers were evaluated by plaque assay. The data are representative of three independent experiments and error bars indicate SD values between replicates in all experiments. * $p \leq 0.05$ when comparing to mock-infected; ** $p \leq 0.05$ when comparing to SINV-infected; *** $p \leq 0.05$ when comparing to BK-treated.

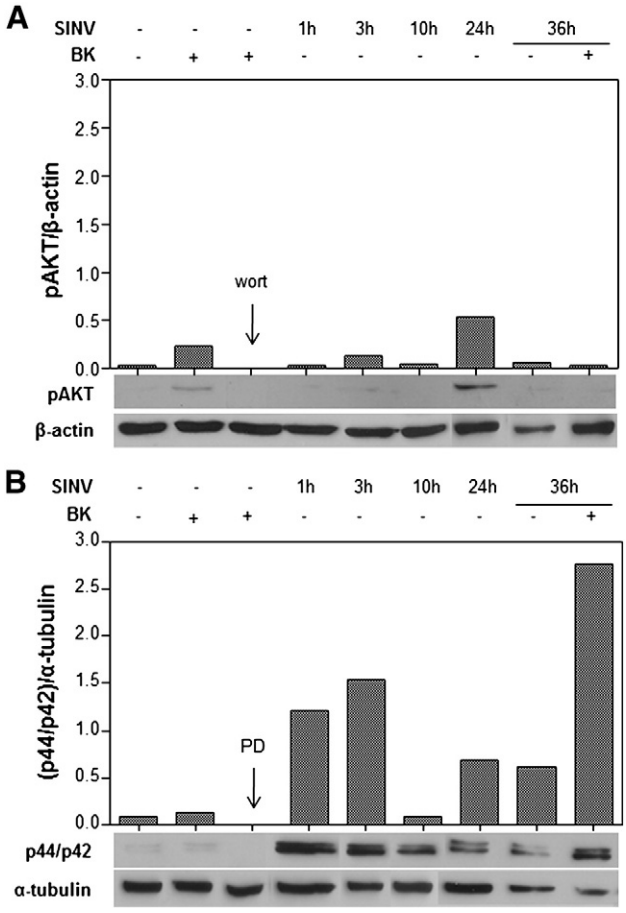

Fig. 6. Modulation of AKT and ERK phosphorylation mediated by SINV infection and BK treatment. HBMECs were infected with SINV (MOI = 0.1) or mock control for the indicated time points. At 24 h p.i. the cells were stimulated with BK, in the presence or absence of wortmannin (wort; 100 nM) or PD98059 (PD; 30 μM), as indicated. The cells were lysed at the indicated time points and whole-cell lysates were loaded onto SDS-PAGE gels. Blot were run and probed with anti-phospho Akt antibody (pAkt) (A) or with anti-phospho ERK antibody (p44/p42) (B). The blots were also probed with anti-b-actin or a-tubulin as a load control. Bar graphics show the ratio of phosphorylated kinases and constitutive proteins. The data are representative of three independent experiments.

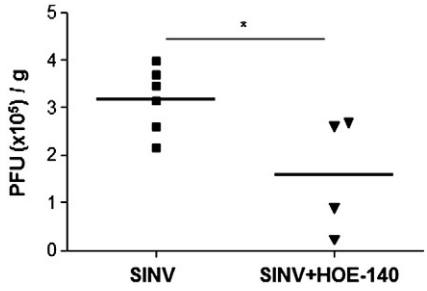

Fig. 8. BK2R blocking inhibits SINV replication in the CNS in vivo. BALB/c mice were pre-treated with captopril (ACE inhibitor, 100 mg/kg) for 1 h and then inoculated with SINV (5×104 PFU, i.c.), with or without HOE-140 (100 mg/kg). After 24 h of infection, the animals were sacrificed, the brains were harvested and the viral load was quantified by plaque assay. Results are shown as the number of PFU per g tissue. Dots represent individual animals from one of two independent experiments showing similar data.

general adaptive response to innate stimuli induced by RNA viruses (unpublished data).

Beyond the effects on BK-sensitivity of SINV-infected cells, there is a precedent that HIV infection enhances Substance P expression in immune cells (Ho et al., 2002), which then increases virus replication in latently infected human immune cells (Li et al., 2001). Moreover, bRSV produces a peptide (p27), which is released from infected cells and is further posttranslationally modified and converted into virokinin a member of the tachykinin family (Zimmer et al., 2003). Calves infection with a p27-deficient bRSV showed diminished virus titer in different tissues (Valarcher et al., 2006), demonstrating that kinin-mediated signals can enhance virus replication.

Cellular apoptosis following viral infection is considered as a host defense mechanism, since it limits virus production and prevents neighboring cells from being infected by progeny virions (Teodoro and Branton, 1997). Thus, most animal viruses have evolved strategies to evade or delay early apoptosis. This can be achieved by the generation of virus-coded anti-apoptotic proteins or, more often, by inducing or stimulating endogenous anti-apoptotic cellular pathways (Lee et al., 2005; Roulston et al., 1999). Conversely, several viruses actively induce apoptosis at late stages of infection, thus allowing the dissemination of progeny viruses, while avoiding host inflammatory and immune responses (Levine, 2002; Teodoro and Branton, 1997).

It was previously reported that flaviviruses induce the activation of the anti-apoptotic PI3K/Akt pathway early during infection; this process is inhibited at late stage of infection, allowing for cellular apoptosis and enhancing virus-induced cell death (Lee et al., 2005). This mechanism may also occur in SINV infection, since it was demonstrated that the infection of mosquito cells with recombinant SINV expressing proapoptotic genes turns a persistent infection into a lytic process. As a consequence of this manipulation, virus production was subsequently decreased compared to persistently infected cells, which continued to produce high levels of virus over the next several days (H. Wang et al., 2008).

BK2R signaling of muscle and endothelial cells results in increased nitric oxide production, cAMP upregulation, PKB/Akt phosphorylation,

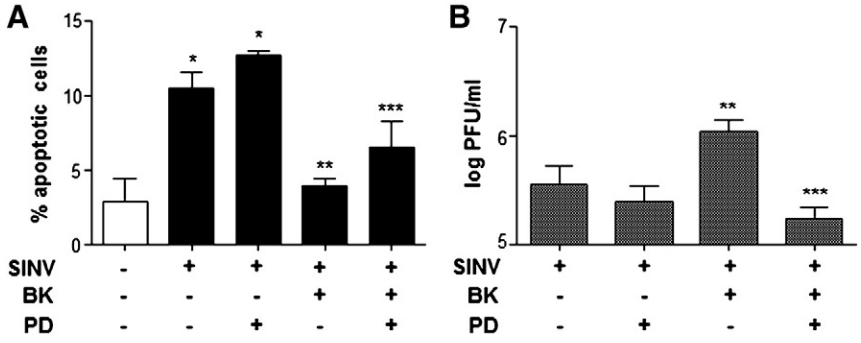

Fig. 7. Increased viral replication and diminished apoptosis mediated by BK is dependent on ERK activation. HBMECs were infected with SINV (MOI = 0.1) and 24 h later, the cells were treated with BK (10 nM), in the presence or absence of the ERK inhibitor PD98059 (PD; 30 μM). A) At 36 h p.i. the cells were harvested and incubated with PI and AlexaFluor488-AnnexinV and the frequency of apoptotic cells was evaluated by flow cytometry. B) At 48 h p.i. the culture supernatants were harvested and viral titers were measured by plaque assay. The data are representative of three independent experiments and error bars indicate SD values between replicates in all experiments.*$p \leq 0.05$ when comparing to mock-infected; ** $p \leq 0.05$ when comparing to SINV-infected; *** $p \leq 0.05$ when comparing to BK-treated.

and MAPK activation (Harris et al., 2001; Krieg et al., 2004; Prado et al., 2002). These effects are mainly dependent on PKA or PI3K activation and resulted in several phenotypic changes, such as increased cell survival and inhibition apoptosis (Bledsoe et al., 2006; S. Wang et al., 2008; Roberts et al., 2004; Yao et al., 2008; Yin et al., 2005). Based on our findings, we hypothesized that conditions leading to excessive activation of the KKS, perhaps associated to FXII-dependent activation of the contact system, may generate high levels of BK in the bloodstream of SINV-infected patients. If extrapolated to the in vivo settings, our data suggest that activation of BKR may sustain anti-apoptotic responses extended periods, keeping the cells alive, thus ultimately favoring viral replication. Interestingly, significant levels of death/apoptosis in non treated infected cultures started to be observed at the peak of virus replication (36 h p.i.). The number of dead/apoptotic cells increased further on, coinciding with a decrease in the viral load. Addition of BK after 24 h of infection resulted in a delay of cell death/apoptosis, which was only observed at 48 h, probably reflecting the enhanced virus replication observed at this time point. Conversely, we found that the addition of HOE-140 or wortmannin reversed the increase in cellular survival and virus replication induced by BK, suggesting that BK2R is the upstream signal transducer inducing PI3K activation.

PI3K activation may stimulate phosphorylation of Akt and ERK MAPK, and both pathways are involved in rescuing cells from apoptosis (Aksamitiene et al., 2011; Harris et al., 2001; Krieg et al., 2004; Prado et al., 2002; Zhang et al., 2011). We observed that SINV infection induced the phosphorylation of both Akt and ERK at early time points p.i. and this phosphorylation was decreased later on. Therefore, the addition of BK may prolong one or both signal pathways, allowing the endothelial cells to live longer. Infected cells treated with BK showed sustained pERK levels at 36 h p.i., but the same was not observed for Akt. Also, addition of a pERK inhibitor to the cultures reversed apoptosis inhibition and virus replication enhancement induced by BK, indicating that ERK may act further downstream from PI3K in our cellular system. A similar pathway was recently observed in other studies showing that activation of PI3K can result in ERK phosphorylation and increased cell survival/proliferation in a way independent on pAkt (Aksamitiene et al., 2011).

Virus infections are usually associated to vascular leakage that leads to the release of relatively high concentrations of several mediators that may be involved in vascular permeability, including histamine, prostanoids (Chatuverdi et al., 1991), and possibly bradykinin as well. In this context, it will be interesting to know whether SINV infection might lead to platelet activation, perhaps allowing for polyphosphate-driven generation of kinins in vivo. Although not directly addressed in our study, vascular leakage may contribute to the extravasation of cells and soluble mediators through BBB that are observed during SINV-mediated encephalitis in mice (Irani and Griffin, 1996). Also, even though CNS involvement is not a clinical feature of human SINV infection, it is conceivable that plasma extravasation plays an underlying role in the progression of arthritis and rash (Heitsch, 2000; Jaffar-Bandjee et al., 2009), which are common sequels of inflammation in the human SINV infection (Laine et al., 2004). Interestingly, a kallikrein inhibitor, kallistatin, has been described to inhibit arthritis in a rat model (Chai et al., 1993; Wang et al., 2005), suggesting that inhibition of bradykinin generation in extravascular tissues may likewise attenuate viral-induced arthritis.

In order to evaluate whether our in vitro results might have in vivo relevance, we inoculated mice with SINV in the presence of the BK2R inhibitor HOE-140. Our data showed that the prevention of BK2R signaling by the specific antagonist resulted in a diminished viral titer in the CNS. These data suggest that BK2R activation by endogenous BK also increases SINV replication in vivo. As already discussed, the protective effects mediated by the BK2R antagonist may result from blockade of anti-apoptotic pathways induced by BK, ultimately favoring the progression of virus infection in permissive endothelial cells. Alternatively, the BK2R blocker may have curbed BK-induced maturation of dendritic cells (Aliberti et al., 2003), perhaps hampering the link between inflammatory edema and BK2R-driven induction of type-1 effector T $CD4^+$ and T $CD8^+$ cells, as recently demonstrated in mice infected by two intracellular pathogens, the intracellular protozoan *Trypanosoma cruzi* and the Gram-negative periodontal bacterium *Porphyromonas gingivalis* (Monteiro et al., 2007, 2009). Admittedly, additional studies are required to determine if BK2R-driven induction of anti-viral immunity may enhance resistance to intracerebral SINV infection in this mouse model.

In conclusion, we have reported that BK-induced activation of human endothelial cells increase SINV viral load through the activation of BK2R-driven anti-apoptotic responses. The observation that SINV-infected endothelial cells upregulate BK2R, which in turn render the cells hypersensitive to BK, provides a mechanistic basis to investigate the potential influence of KKS regulation on the pathogenic outcome of infection-associated vasculopathy caused by a wide range of RNA virus.

Materials and methods

Cells and virus

Human brain microvascular endothelial cells (HBMEC), a generous gift from Dr. Dennis J. Grab (The Johns Hopkins University, MD, USA), have been previously described (Nikolskaia et al., 2006). This is a cell line derived from primary human BMECs and immortalized with a plasmid containing the simian virus 40 large-T antigen (Stins et al., 1997). The cells were cultured in medium 199 (M199) supplemented with 10% inactivated fetal calf serum (FCS — Invitrogen, Carlsbad, CA) and antibiotics (Sigma Chem Co; St Louis, MO) (complete medium).

VERO cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 5% inactivated FBS. Human umbilical vein endothelium cells (HUVEC, gently provided by Dr. Verônica Morandi, UERJ, RJ, Brazil) were cultured in M199 supplemented with 10% inactivated FCS and antibiotics.

The Malaysian MRE16 strain of Sindbis virus (SINV; Sammels et al., 1999) was a gift from Dr. Andréa Thompson DaPoian (IBqM, UFRJ, RJ, BR). SINV was propagated in VERO cells for 5 days, harvested and stored at −80 °C until use. Virus titer was determined by plaque assay using VERO cells. Virus inactivation was obtained after heating at 56° for 30 min and was confirmed by plaque assay and RT-PCR in VERO cells.

Cell infection and stimulation

The cells were incubated with SINV, at a multiplicity of infection (MOI) of 0.1, for 90 min at 37 °C in 5% CO_2 atmosphere. As a control, the cells were incubated with either supernatant of noninfected VERO cells (mock-infected) or heat-inactivated SINV (iSINV). Cells were, then, washed with phosphate buffer saline (PBS) and cultured with complete medium. In some experiments, the cells were also treated with 10 nM bradykinin (BK) (Calbiochem, Sigma, Saint Louis, MO) or des-Arg-bradykinin (des-Arg-BK) (Sigma, Saint Louis, MO), after a 30 minute preincubation with lisinopril (25 μM) (Sigma, Saint Louis, MO), an inhibitor of the angiotensin converting enzyme (ACE), herein referred as ACEi. The antagonists of BK2R, HOE-140 (Aventis Pharmaceuticals, NJ, EUA), or of BK1R, [Leu8]des-Arg9-BK (Sigma, Saint Louis, MO), and the PI3K inhibitor wortmannin (Sigma, Saint Louis, MO) were added at a concentration of 100 nM. The pERK inhibitor, PD98059 was used at a concentration of 30 μM. The antagonists and inhibitors were added 30 min before the addition of the BK or des-Arg-BK, when indicated.

The cells and supernatants were harvested 48 h after beginning of the culture and virus infection was analyzed by plaque-forming assay, immunofluorescence, flow cytometry or RT-PCR.

Virus detection by plaque assay

HBMECs or HUVECs were infected and treated or not with the peptides (BK or des-Arg-BK) at different time points after infection, as indicated. After 48 h of infection, the supernatants were harvested and serial dilutions were inoculated in VERO cells. After 60 min of incubation at 37 °C, the inoculum was substituted for 500 μl of carboxymethilcelulose 3%. The cells were incubated for seven days at 37 °C in 5% CO_2 atmosphere and, then, fixed with 10% formaldehyde for 2 h and stained with 0.04% violet crystal for 10 m. The number of plaque forming units (PFU) was determined and titers were expressed as PFU per milliliter.

Virus detection by RT-PCR

HBMECs cells (10^6 cells/well) were infected and treated as described. After 48 h p.i. the supernatants and cells were harvested and RNA extracted using Trizol Reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol. Total RNA (5 μg) was reversely transcribed to cDNA using High-Capacity cDNA Archive Kit (Applied Biosystems, CA, USA) using the following reaction parameters: 25 °C for 10 min, 50 °C for 50 min, 85 °C for 5 min and chilled on ice. PCR was carried out using the Gene Amp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). The reactions included 2 μl of cDNA, primers (20 μM each), TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and water added to a final volume of 25 μl. The sequences of SINV primers used were 5′tgt.aaa.cca.cca.gct.gac.ca3′, as the sense primer, and 5′cac.cac.gct.tcc.tca.gaa.at 3′ as antisense, flanking a 426 bp sequence located at *E1* region (Assunção-Miranda et al., 2010). Cycle conditions were as follows: after an initial 2-min hold at 95 °C, the samples were cycled 45 times at 95 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min.

Analysis of SINV infection by immunofluorescence and flow cytometry

HBMECs (10^6 cells/well) were infected with SINV for 48 h, as described. The cells were harvested, fixed with 4% paraformaldehyde for 10 min, and blocked with PBS containing 4% normal mouse serum and 0.1% saponin. The cells were then incubated with mouse anti-SINV (a kind gift from Dr. Andrea T. DaPoian) for 1 h, washed and stained with anti-mouse IgG conjugated to Alexa fluor-488 (Invitrogen, Carlsbad, CA) for 30 min. The percentage of $SINV^+$ cells were evaluated by flow cytometry using a FACScalibur cytometer (Becton Dickson Immunocytometry System). Events (10,000) were acquired and were analyzed using the CellQuest software. Alternatively, the cells were seeded onto coverslips, which were stained as described, and mounted onto glass slides using ProLong Antifade reagent (Molecular Probes, Eugene, OR). HBMECs infection was then analyzed by fluorescence microscopy using Axioplan II equipment (Zeiss, Germany).

Analysis of BKR expression by quantitative real-time PCR and flow cytometry

HBMECs (10^6 cells/well) were incubated with SINV (MOI of 0.1) in its native or inactivated forms, or with mock control. After 24 h of infection the cells were harvested and BK1R and BK2R transcription and expression were analyzed by quantitative real-time PCR (qPCR) and flow cytometry, respectively. Total RNA was extracted from HBMECs using the RNeasy mini Kit (Qiagen, Valencia, CA) and treated with DNAse (Qiagen, Valencia, CA), according to manufacturer's instructions. Total RNA (1 μg) was reversely transcribed to cDNA using SuperScript III First-strand Synthesis System (Invitrogen, Carlsbad, CA) and random hexamer primers (Invitrogen, Carlsbad, CA) using following parameters: 50 °C for 30 min, 85 °C for 5 min and chilled on ice. Afterwards, RNaseH (2U) (Invitrogen, Carlsbad, CA) was added and samples were incubated at 37 °C for 20 min. qPCR was carried out using the ABI PRISM 7500 device (Applied Biosystems, Foster City, CA). Human β-actin assay (Applied Biosystems, Foster City, CA) was used for normalization due to its constitutive production in chosen cells. The reactions were performed in triplicates and included 2 μl of cDNA, BK1R or BK2R gene expression assays (TaqMan® gene expression assays — Applied Biosystems, Foster City, CA, USA), TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) and water added to a final volume of 25 μl. Triplicates of non-template controls (NTC) were always included in the reactions. Cycle conditions were as follows: after an initial 2-min hold at 50 °C and 10 min at 95 °C, the samples were cycled 40 times at 95 °C for 15 s and 60 °C for 1 min. Baseline and threshold for cycle threshold (Ct) calculation were set automatically with Sequence Detection Software version 1.4 (Applied Biosystems, Foster City, CA). The efficiency of amplification (E) of a target molecule was calculated from the slope of the standard curve (plot of Ct vs the negative $\log_{10}$ concentration of the target) derived from the slopes ($E = [10^{(-1/Slope)}] - 1$). For relative calculation, the ΔΔCt method was used (Livak and Schmittgen 2001, Applied Biosystems 7500/7500 Fast Real-Time PCR Systems — User Bulletin) once all assays met the amplification efficiency criteria of 100% ± 10% (Applied Biosystems — Application Note 127AP05-02).

To analyze the expression of BKR protein in the cells surface, the cells were fixed with 4% paraformaldehyde for 10 min and blocked with PBS containing 2% normal mouse serum and 1% fetal calf serum for 30 min. Rabbit anti-BK1R or anti-BK2R antibody (Santa Cruz Biotechnology CA, EUA) were added and this treatment was followed by incubation with anti-rabbit IgG antibody conjugated to PE (Invitrogen, Carlsbad, CA) to detect BKRs. The cells were then permeabilized with 0.1% saponin and stained with mouse anti-SINV, followed by incubation with anti-mouse IgG antibody conjugated Alexa fluor-488 (Invitrogen, Carlsbad, CA). Samples were run in a FACSCalibur cytometer, and data analyses were performed using the CellQuest software (Becton Dickson Immunocytometry System).

Analysis of cell viability

Cell viability was evaluated by MTT metabolization measurement. HBMECs (1.25×10^4/well) were inoculated with SINV (MOI of 0.1), in its native or inactivated forms, or mock-infected. After different time points post infection, the cells were incubated with 0.5 mg/ml MTT for 2 h and the acid solution (0.04 M HCl in isopropanol) was added. The absorbance was measured at 570 nm.

Analysis of apoptosis

HBMECs cells (10^6) were inoculated with SINV (MOI of 0.1) or mock-infected. After 24 h p.i. the cells were stimulated with BK, in the presence of the ACEi, lisinopril. After 12–24 h post stimuli (36–48 h p.i.), the cells were washed with PBS, harvested in 100 μl annexin buffer, and incubated with Alexa fluor 488-annexin V and propide iodide (PI) (Annexin V-FITC Apoptosis Detection Kit, Becton Dickinson and Company, Franklin Lakes, NJ). After 15 min, the cells were harvested in 400 μl of annexin V buffer. The proportion of live ($AnnexinV^-/PI^-$), dead ($AnnexinV^-/PI^+$), or early and late apoptotic cells ($AnnexinV^+/PI^-$ and $AnnexinV^+/PI^+$) were evaluated by flow cytometry, using the FACScalibur equipment and CellQuest software (Becton Dickson Immunocytometry System).

Alternatively, the apoptosis was measured by PI staining only, after cell permeabilization. PI is a DNA intercalator and its staining indicates the content of DNA in a given cell. Therefore, the amount of sub-diploid apoptotic cells can be quantified by flow cytometry. HBMECs cells (1.25×10^4) were inoculated with SINV (MOI of 0.1) or mock-infected. After 24 h p.i. the cells were stimulated with ACEi/BK, in the presence or absence of HOE-140 (100 nM), wortmannin (100 nM) or PD 98059 (30 μM). After 12 h post stimuli (36 h p.i.) the cells were detached with trypsin and harvested in 200 μl of the

Hypotonic Fluorochrome solution (HFS — 0.1% sodium citrate, 0.1% triton X-100, 50 μg/ml of the propidium iodide). The cells were incubated for 1 h and the percentage of apoptotic cells was evaluated by flow cytometry.

Western blotting

HBMECs (10^6 cells/well) were inoculated with SINV (MOI of 0.1) or mock-infected for the indicated periods of time. After 24 h p.i. the cells were stimulated with BK, in the presence or absence of HOE-140, wortmannin, or PD98059. Cells were washed with PBS and lysed in lysis buffer (10 mM Tris–HCl pH 7.5; 5 mM de EDTA pH 8.0; 150 mM NaCl; 0.1% NP-40). Extracts were clarified by centrifugation, and protein concentration was determined by the Bradford assay (Bio-Rad D_C Protein Assay). Lysates containing equal amounts of protein were boiled with SDS sample buffer containing 2-mercato-ethanol. Proteins were separated by SDS-PAGE and then electro-transferred to nitrocellulose membranes (Thermo Fisher Scientific Inc., Rockford, IL, USA), which were blocked in 5% bovine serum albumin in Tris-buffered saline solution containing 0.1% Tween-20. Primary antibodies were diluted according to the manufacturer's instructions and membranes incubated overnight at 4 °C with pERK or pAKT (Cell Signalling, Danvers, MA, USA), and β-actin or α-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for normalization. After washing, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG antibodies (Santa Cruz Biotechnology) and proteins were visualized using ECL Western blotting detection systems (Super Signal® West Pico Chemiluminescent Substrate Thermo Scientific), according to the manufacturer's instructions. The ratio of phosphorylated kinases/constitutive proteins was determined using the ScionImage software.

In vivo infection

Male and female BALB/c mice with ages ranging from 4 to 6 weeks were obtained from the animal facility of the Instituto de Microbiologia Prof. Paulo de Góes from Federal University of Rio de Janeiro. The animals were bred and housed according to institutional policies for animal care and usage. All experimental protocols were submitted to the Ethical Committee from the Centro de Ciências da Saúde of Federal University of Rio de Janeiro. The mice were pretreated with the ACE inhibitor captopril at 100 mg/kg (i.p.) and then inoculated intracerebrally (i.c.) with 5×10^4 PFU of SINV, with or without HOE-140 (100 μg/kg). Control mice were inoculated with endotoxin-free medium (vehicle). The animals were sacrificed 24 h p.i. and the brain was removed and macerated in PBS. Brain extract was centrifuged and supernatant was stored in −70° for later use. Virus-containing brain suspensions were evaluated by plaque assay in VERO cells and normalized by brain weight.

Statistical analysis

The mean and SD were calculated for each experimental group. Differences between groups were analyzed by the *Student's t* test for unpaired samples using the PRISM statistical analysis software (GraphPadSoftware, Inc., San Diego, CA). Results with $p<0.05$ were considered significant.

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