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Influence of Kinin Peptides on Monocyte–Endothelial Cell Adhesion

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ABSTRACT

Adhesion of leukocytes to vascular endothelium in response to proinflammatory mediators is an important component of the overall inflammatory reaction. In the current work, we used a retinoic acid-differentiated human promonocytic cell line, U937 and a human microvascular endothelial cell line, HMEC-1 to analyze the effect of the potent pro-inflammatory bradykinin-related peptides (kinins) on cell adhesion. Bradykinin (BK) and kinin metabolites without the C-terminal arginine residue enhanced adhesion of the monocyte-like cells to fibronectin and to the HMEC-1 cells. Expression of adhesion proteins on the surface of both cell types was altered by the kinin peptides. In the monocyte-like cells, expression of CD11b, a subunit of Mac-1 integrin, was significantly increased whilst in the endothelial cells, a strong increase in the production of intercellular adhesion molecule 1 (ICAM-1) was observed. The positive bradykinin-induced effect on the cell–cell interaction was reversed by a carboxypeptidase inhibitor (MGTA), hence we suspected a significant role of the des-Arg kinin metabolites, which acted through the kinin receptor type 1. Indeed, the expression of this receptor was up-regulated not only by agonists but also by interferon-γ and bradykinin. Kinin peptides also regulated signal transducer and activator of transcription proteins (STATs) activated by cytokines. Taken together, the above observations support our hypothesis that kinins stimulate monocyte adhesion to the vessel wall, especially during pathological states of the circulatory system accompanied by proinflammatory cytokine release. J. Cell. Biochem. 115: 1985–1995, 2014.

KEY WORDS: MONOCYTE ADHESION; INFLAMMATION; KININS; KININ RECEPTORS; ADHESION PROTEINS

Bradykinin (BK) and kallidin (KD), two major representatives of a group of bioactive peptides called kinins, are generated from proteinaceous precursors, kininogens, by kallikreins, that is, specific serine proteases (for extensive reviews see Bhoola et al., 1992; Colman and Schmaier, 1997; Blais et al., 2000). These peptides contribute to a wide number of physiological processes such as control of blood pressure, local blood flow, and electrolyte and glucose transport; they also play important roles in diverse pathological states, including inflammation, infection, diabetes, and cancer [Maeda et al., 1999; Leeb-Lundberg et al., 2005]. They regulate vasodilatation, vascular permeability, cell migration, and pain at the site of injury. The local concentration of kinins in blood and tissues is tightly regulated in form of a balance between kinin generation by kallikreins and their simultaneous degradation by

Abbreviations: β-Act, β-actin; B1R, bradykinin receptor type 1; B2R, bradykinin receptor type 2; BK, bradykinin; CPM, carboxypeptidase M; DAA, dansyl-Ala-Arg-OH; DAKDes-Arg^{10}-KD; DABK, des-Arg^{9}-BK; DHOE-140, des-Arg^{10}-icabant acetate; EF-2, elongation factor 2; FBN, fibronectin; FBR, fibrinogen; FBS, foetal bovine serum; HOE, icabant acetate; ICAM-1, intercellular adhesion molecule 1; IL-6, interleukin 6; INF-γ, interferon-γ; KD, kallidin; MGTA, 2-mercaptomethyl-3-guanidinoethyl thiopropanoic acid; PBS, phosphate-buffered saline; PMN, polymorphonuclear; RA, retinoic acid; STAT, signal transducer and activator of transcription.

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numerous peptidases, collectively called kininases. The most important kininases are angiotensin-converting enzyme and neutral endopeptidase 24.11 [Bhoola et al., 1992; Blais et al., 2000; Joseph and Kaplan, 2005], which degrade kinins to biologically inactive peptides. There are also other kininases such as carboxypeptidase M (CPM) and carboxypeptidase N that remove the C-terminal arginine residue of kinins and generate des-Arg peptides, which possess alternative biological activities [Blais et al., 2000]. There are two types of cellular receptors belonging to the G protein-coupled receptor family that recognize kinin peptides, bradykinin receptor type 1 and type 2 (B1R and B2R, respectively) [Blais et al., 2000; Leeb-Lundberg et al., 2005]. The first type preferably binds des-Arg kinin peptides, whereas B2R primarily recognizes full-length kinins, BK and KD. The B2 receptor is ubiquitous in mammals, while B1R, which in general is not expressed constitutively, can be up-regulated by certain stimuli during inflammation, infection, and trauma [Marceau et al., 1998; Calixto et al., 2000; Leeb-Lundberg et al., 2005].

One of the earliest cellular events associated with inflammatory states in mammals is leukocyte adhesion to the vascular endothelium in the blood vessels. Endothelial cells, located at an anatomic interface between flowing blood and vascularized tissue, regulate access of leukocytes to the tissues. In case of inflammation, a successful response requires neutrophil recruitment, lymphocyte recirculation, and monocyte trafficking which occur in three steps, including rolling, adhesion, and transmigration of leukocytes [Ley et al., 2007]. The cells that participate in these processes, both leukocytes and endothelial cells, must be activated by cytokines to up-regulate the expression of specific adhesion molecules on their surfaces [Meager, 1999]. A wide number of membrane proteins, such as selectins and cell–adhesion molecules in the endothelium, and integrins in the leukocytes, play different roles during cell translocation [Ley et al., 2007]. Involvement of kinin receptors in chemotaxis, cell migration, and trafficking was proposed [Koyama et al., 2000; McLean et al., 2000; Sainz et al., 2004; Lu et al., 2010; Yang et al., 2010]. We have recently reported that the adhesion of polymorphonuclear leukocytes (PMN) can be enhanced by kinin peptides [Guevara-Lora et al., 2011a]. PMN adhesion to the endothelial cells was regulated by BK and, even more remarkably, by B1R agonists.

The goal of the current work is to focus on the effects of kinin peptides (B1R and B2R agonists) on the adhesion of mature cells with monocytic properties, derived from a human promonocytic cell line (U937), to human microvascular endothelial cell line (HMEC-1). We plan to elucidate the role of kinin receptors in this process, and to determine expression of selected adhesion molecules, such as Mac-1 integrin and intracellular adhesion molecule 1 (ICAM-1). Finally, we study a role of signal transducer and activator of transcription proteins (STATs) in the kinin-activated cell interactions since these are the molecules that regulate signaling in inflammation and are simultaneously associated with leukocyte adhesion.

METHODS

MATERIALS

BK, KD, des-Arg^9-BK (DABK), des-Arg^{10}-KD (DAKD), and dansyl-Ala-Arg-OH (DAA) were purchased from BaChem (Germany). Endothelial growth factor, fibronectin (FBN), fibrinogen (FBR), and interferon-γ (INF-γ) were supplied by BD Sciences and 2-mercaptomethyl-3-guanidinoethyl thiopropanoic acid (MGTA) by Calbiochem. Foetal bovine serum (FBS), RPMI and endothelial basal medium, phosphate-buffered saline (PBS), antibiotics and antimycotics were supplied by Cytogen (Austria). Captopril and o-phenanthroline were from Fluka (Switzerland). [α^{32}P]-dCTP was supplied by Hartmann Analytic (Germany). Cell Tracker Red CMTPX was from Molecular Probes. [^{3}H]-DAKD and Ultima Gold^{TM} scintillation fluid were supplied by Perkin-Elmer. M-MLV reverse transcriptase and total RNA isolation kit were purchased from Promega. Acrylamide, all-*trans*-retinoic acid (RA), bacitracin, hydrocortisone, icatibant acetate (HOE 140) and des-Arg^{10}-icabant acetate (DHOE-140), proteinase inhibitor cocktail, interleukin 6 (IL-6), SYBR Green Jump Start Taq Ready Mix, and other basic chemicals were purchased from Sigma. Mouse phycoerythrin-conjugated anti-human ICAM-1 antibody was purchased from BioLegend (Germany) and mouse FITC-conjugated anti-human CD11b and CD18 antibodies from BD Bioscience. Rabbit anti-human ICAM-1 antibody was purchased from Santa Cruz Biotechnology. Rabbit anti-human B1R antibody and rabbit anti-human β-actin were purchased from Abcam and horseradish peroxidase-conjugated goat antibody to rabbit IgG from Sigma.

CELL CULTURE

The U937 cells were cultured in HEPES-stabilized RPMI medium supplemented with 10% FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 2.5 μg/ml amphotericin B for 5 days in a humidified atmosphere containing 5% CO_2 at 37°C. To induce monocytic differentiation, the U937 cells were suspended in 2% FBS medium supplemented with 1 μM RA for 3 days. The differentiated cells were incubated for one more day in the medium without RA before the experiments were carried out.

HMEC-1 cells were cultured in Endothelial Basal Medium supplemented with an antibiotic/antimycotic mixture as described above and 5% FBS. The cell growth was induced by addition of hydrocortisone and endothelial growth factor at final concentrations of 10 μg/ml and 10 ng/ml, respectively. Before the experiments were carried out, the cells were cultured 1 day without growth activators.

CELL ADHESION ASSAYS

The RA-differentiated U937 cells (2.5×10^5) or a monolayer of HMEC-1 cells were stimulated with one of the kinins: 1 μM BK, 1 μM DABK, or 1 μM DAKD for 24 h in their respective medium supplemented with kininase inhibitors, 20 μM captopril and 500 μM bacitracin. After this time, stimulated monocyte-like cells were placed into wells of a microtiter plate previously coated with 10 μg/ml FBN or FBR and incubated for 2 h. Next, unbound cells were removed and the remaining cells were fixed and visualized with Crystal Violet dye according to a protocol described previously [Guevara-Lora et al., 2011a]. The study of monocyte adhesion to the endothelial cells was performed with RA-differentiated cells labeled with Cell Tracker CMTPX. The previously kinin-stimulated U937 cells were labeled according manufacturer's instruction and then placed on kinin-stimulated

monolayer of HMEC-1 cells. Detailed description of the cell adhesion analysis was provided elsewhere [Guevara-Lora et al., 2011a]. Control samples were incubated with 10 μM MGTA for 10 min before cell stimulation with BK.

ANALYSIS OF ADHESION PROTEIN EXPRESSION BY FLOW CYTOMETRY AND WESTERN BLOTTING

The RA-differentiated U937 cells (2×10^6) were stimulated with 1 μM BK or 1 μM DAKD in the presence of kininase inhibitors for 24 h. Stimulated and non-stimulated cells were washed twice with ice-cold PBS and then incubated with FITC-conjugated antibodies specific to human adhesion proteins CD11b and CD18 at 1:100 dilution for 20 min at 4°C. After washing and resuspension, the labeled cells were analyzed by flow cytometry (Cytometer LSRII, Becton Dickinson, Germany). In addition, the changes in ICAM-1 expression on the HMEC-1 cells under the influence of either just BK, DAKD at 1 μM final concentration or together with 10 ng/ml INF-γ were also analyzed by flow cytometry. Confluent HMEC-1 cells were incubated with the stimulants for 12 h, detached, and incubated with phycoerythrin-conjugated anti-human ICAM-1 antibody (at 1:100 dilution) in the way as described above for integrin determination. Nonspecific fluorescence was determined using isotype-matched human IgG.

Confluent HMEC-1 cells in 5 cm^2-dishes were incubated with BK and DAKD at final concentration of 1 μM. Some BK-induced samples were previously treated with 10 μM MGTA for carboxypeptidase activity inhibition. Positive control samples with 10 ng/ml INF-γ were performed. After 12 h, the cells were washed with PBS and lysed by sonication on ice in 50 μl of 50 mM Tris buffer, pH 8.0, supplemented with 65 mM CHAPS, 2 mM EDTA, 1 mM Na_3VO_4, 5 mM NaF, 1 mM dithiothreitol, 10% glycerol, and protease inhibitor cocktail. Total protein content was determined with BCA assay, then the extract proteins were separated by SDS–PAGE electrophoresis. The ICAM-1 protein contents in the cell extracts were analyzed by Western blotting with rabbit anti-human ICAM-1 and goat anti-rabbit IgG conjugated with horse peroxidase at 1:2,000 and 1:20,001: and 1:20,000 dilutions, respectively. Enhanced chemiluminescence reaction with Super Signal West Pico (Promega) was performed according to the manufacturer's instructions. The enhanced chemiluminescence signal was registered on Fusion Fx Chemiluminescence system (Vilber Lourmat). The control protein was β-actin (β-Act), and their intracellular amount was also determined by Western blotting with rabbit anti-human β-actin (dilution 1:5,000).

GENE EXPRESSION ANALYSIS BY REAL TIME PCR

The RA-differentiated U937 cells (2.5×10^6) were incubated with 1 μM BK, 1 μM DAKD, and 10 ng/ml INF-γ in RPMI medium supplemented with kininase inhibitors. Analogous experiments were performed with confluent HMEC-1 cells. After the incubation, total RNA was isolated and the mRNA expression of B1R, ICAM-1, and elongation factor 2 (EF-2) was studied by real time PCR. To this end, the isolated RNA samples were first transcripted to cDNA according to the manufacturer's instruction and then 2 μl of cDNA were amplified in a 15 μl reaction mixture with the appropriate primers. The primer pair sequences were: 5′ gcaactgaacgtggcagaa 3′ and 5′ gcccaagacaaacaccagatc 3′ for B1R, 5′ cgactggacgagagggattg 3′ and 5′ cccattatgactgcggctgcta 3′ for ICAM-1, and 5′ gcggtcagcacactggcata 3′ and 5′ gacatcaccaagggtgtgcag 3′ for the housekeeping gene EF-2 (all from Genomed, Poland). The PCR conditions included an initial denaturation step of 5 min at 95°C followed by 40 cycles (15 s denaturation at 95°C, 15 s at 58°C and 30 s at 72°C). The reaction was performed with the use of a RotorGene 3000® Thermocycler (Corbett® Life Science). Data analysis was undertaken using the ΔCt method, with EF-2 used as a reference gene.

WESTERN BLOTTING OF THE B1 RECEPTOR

The RA-differentiated cells (3.0×10^6) were incubated with 10 ng/ml INF-γ at 37°C for different time periods. After cell protein isolation, as described above, the protein extracts were analyzed for B1R by Western blotting with rabbit anti-human B1R antibodies at 1:500 dilution, followed by horseradish peroxidase-conjugated goat antibody specific to rabbit IgG 1:25,000 dilution [Guevara-Lora et al., 2011b]. The control of the protein isolation was also performed by determination of β-Act by Western blotting.

ANALYSIS OF CARBOXYPEPTIDASE ACTIVITY

The analysis of carboxypeptidase activity in the RA-differentiated U937 and HMEC-1 cells was performed by measurement of enzymatic degradation of the labeled peptide DAA [Tan et al., 1995]. To this end, the RA-differentiated U937 cells (3×10^6) or the HMEC-1 cells (monolayer in 10-cm dishes) were lysed with mild sonication in PBS supplemented with proteinase inhibitor cocktail. Next, 50 μl of lysates were incubated with 0.2 mM DAA in 0.1 M Hepes buffer, pH 7.0, supplemented with 0.2% Triton X-100 for 2 h at 37°C. The enzymatic reaction was stopped by addition of 150 μl 1 M citric acid and the product obtained was extracted with 1 ml of chloroform with vigorous vortexing. After centrifugation, the fluorescence of the organic phase was measured using an F-4500 fluorescence spectrophotometer (Hitachi, Japan). The control samples, in which carboxypeptidase activity was inhibited with 00 μM MGTA for 10 min before the incubation with the substrate, were also prepared. The carboxypeptidase activity was presented as the difference in fluorescence intensity between samples measured without or in the presence of 10 μM MGTA after 2-h incubation with the substrate.

DETERMINATION OF des-Arg PEPTIDES BY A COMPETITIVE RECEPTOR-BINDING ASSAY

In order to determine des-Arg-kinin production from exogenous BK, the RA-differentiated cells (3.0×10^6) were incubated with 10 ng/ml INF-γ in RPMI medium at 37°C for 3 h. After incubation, the supernatant was removed and the cells were incubated with 1 μM BK in PBS for up to 15 min at 37°C. Control samples were treated with 10 μM MGTA for 10 min prior to the incubation with the BK peptide. After the incubation, the samples were immediately placed in the ice bath. Next, the supernatants were separated from the cells by centrifugation and immediately analyzed for des-Arg kinin contents using a radiocompetitive receptor-binding assay, with B1R-over-expressing HEK-293 cells and [^{3}H]-DAKD as the competitive ligand to B1 receptors as previously described [Zubakova et al., 2007; Guevara-Lora et al., 2011b].

ELECTROPHORETIC MOBILITY-SHIFT ASSAY

The RA-differentiated U937 cells (3.0×10^6) and confluent HMEC-1 cells were stimulated with one of the following, 1 μM BK, 1 μM DAKD, 10 ng/ml INF-γ or 50 ng/ml IL-6 at 37°C for 30 min. In order to activate either STAT-1 or STAT-3, the cells were co-incubated with the kinins and either INF-γ or IL-6, respectively. The extent of STATs stimulation was determined by electrophoretic mobility-shift assay (EMSA). Some cells were treated with antagonists specific to either B1 (DHOE-140) or B2 receptor (HOE-140) first to validate the observed effects to be kinin mediated. The whole cell proteins were isolated according to Sadowski et al. [1993]. After stimulation, the cells were washed in cold PBS. Next, the cells were suspended in the lysis buffer (50 mM Hepes, pH 8.0, supplemented with 10 mM CHAPS, 2 mM EDTA, 1 mM NaF, 1 mM dithiothreitol and 10% glycerol). After 30-min incubation on ice, the mixture was centrifuged at 14,000*g* for 5 min and the supernatants were analyzed for the protein content by the BCA method. The remaining supernatant was kept at −80°C until use. A double-stranded, [α^{32}P]-dCTP-labeled oligonucleotide with the sequence 5′ agctcatttcccgtaaatc 3′ (SIEm67 element) was used for specific STAT-1/3 determination. Electrophoretic mobility shift assay was performed in a 4.5% acrylamide/bis-acrylamide gel. The measurement of the activation of STATs was further performed according to the procedure described earlier [Stalinska et al., 2005]. The dried gels were analyzed by autoradiography and the results were quantified with use of a scanning densitometer (Fluor S, Bio-Rad program Quantity One).

RESULTS

KININ PEPTIDES INCREASE MONOCYTE ADHESION TO FIBRONECTIN, FIBRINOGEN, AND TO HMEC-1 CELLS

First, we investigated adhesion of monocyte-like cells to either extracellular matrix or directly to the endothelial cells. The RA-differentiated U937 cells were cultured on plates coated with FBN, FBR or a confluent layer of HMEC-1 cells for a limited time, then their adhesion was measured. Overall, there was an enhanced adhesion of the monocytes to the extracellular matrix proteins. The adhesion of RA-differentiated U937 cells to FBN-coated surface was significantly enhanced after their incubation with BK and DAKD. In this case, the peptide-induced increase in monocyte-like cells adhesion was approximately 30% higher than that of non-treated cells (Fig. 1A). There was only an insignificant increase in the U937 cells adhesion to the FBR-coated surface following their stimulation with the selected kinins.

The effect of the analyzed kinin peptides on adhesive ability in the monocyte-like cells was more evident when the stimulated cells were cultured on top of the endothelial cells. Media supplementation with BK, DAKD, or DABK resulted in increased cellular adhesion by 50%, 33%, and 35%, respectively as compared to an untreated control (Fig. 1B). Specificity of the kinin mediated effect was verified with a specific inhibitor of carboxypeptidases, MGTA, which resulted in a reversal of BK positive impact on the monocyte adhesion to the endothelial cells (Fig. 1B).

The above experiments demonstrated a definite influence of bradykinin and selected kinin peptides on monocyte increased adhesion to the extracellular matrix or to even greater extent, to the endothelial cells.

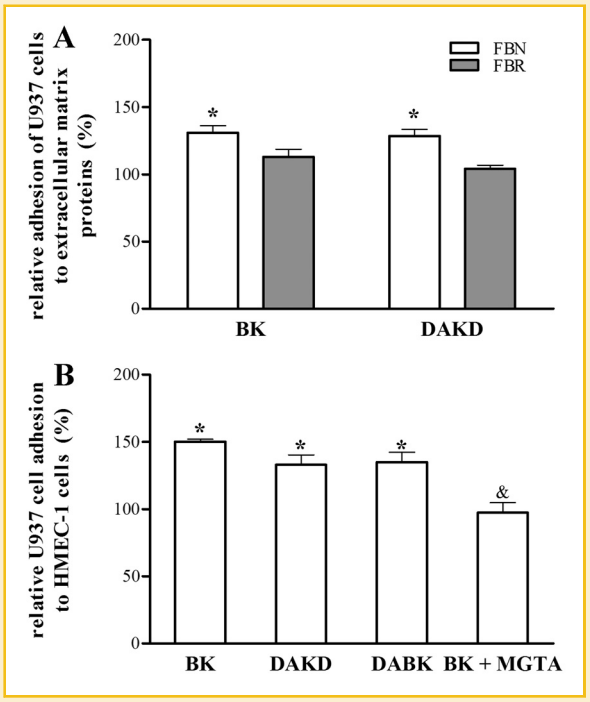

Fig. 1. The effect of kinin peptides on the adhesion of RA-differentiated human promonocyte cell line U937. After RA differentiation, the cells were incubated with 1 μM BK, 1 μM DABK, and 1 μM DAKD at 37°C for 24 h. Cell adhesion to extracellular proteins, either fibronectin (FBN) or fibrinogen (FBR) (A), or to confluent monolayer of HMEC-1 endothelial cells (B) was analyzed by cell adhesion assays (colorimetric and fluorometric, respectively). The monocyte-like cell adhesion to HMEC-1 cells was also analyzed in cells pretreated with 10 μM MGTA for 10 min before BK incubation. The results are presented as the percentage of cell adhesion after stimulation with kinin peptides relative to that of untreated cells, assumed to be 100%, and the values represent the means ± SD from at least three experiments. *Statistical significance was calculated by Student's *t*-test at $P < 0.05$ against the control sample (non-treated cells) and $^{\&}P < 0.5$ against sample incubated with BK.

KININ PEPTIDES INCREASE EXPRESSION OF THE ADHESION PROTEINS IN MONOCYTE-LIKE CELLS AND HMEC-1

The CD11b and CD18 subunits of Mac-1, an important adhesion molecule in leukocytes, and the ICAM-1 protein from endothelial cells were selected to further analyze the role of kinins in the cell–cell interaction. The monocyte-like cells showed a moderate amounts of the two Mac-1 subunits, out of which only CD11b slightly increased after treatment with the kinin peptides. We observed a more pronounced increase in mean fluorescence intensity of the FITC-coated CD11b antibody in the cells stimulated with BK and DAKD as compared to a non-stimulated control (Fig. 2A). The DAKD peptide showed a stronger, significant effect as compared to BK (the increase by 44% and 22%, respectively). The analysis of the second subunit of Mac-1 integrin, the CD18 protein, did not reveal any significant changes.

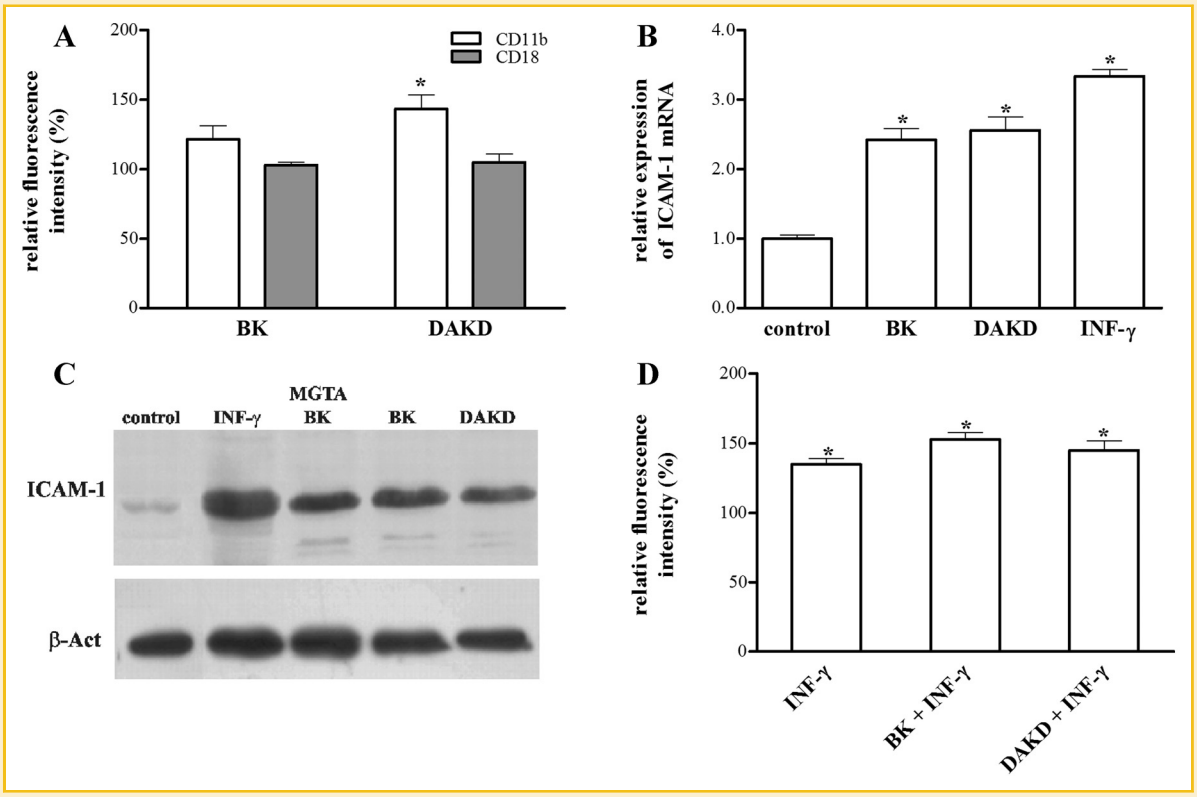

Fig. 2. The effect of kinin peptides on the expression of adhesion molecules in RA-differentiated U937 and HMEC-1 cells. Monocyte-like cells (2.5×10^5) were treated with 1 μM BK and 1 μM DAKD, and the expression of Mac-1 subunits was analyzed by flow cytometry (A). Confluent endothelial cells stimulated with 1 μM BK, 1 μM DAKD, and INF-γ were studied for the expression of ICAM-1 mRNA by real-time PCR (B). ICAM-1 protein determination was also performed in HMEC-1 cells stimulated like above or with peptides in the presence of INF-γ by Western blotting (C) or flow cytometry (D), respectively. All values represent the means ± SD from at least three experiments. *Statistical significance was calculated by Student's *t*-test at $P < 0.05$ against untreated cells.

The study of one of the most important adhesive molecule of the endothelial cells, ICAM-1, was performed at gene and protein levels. The expression of ICAM-1 mRNA was significantly augmented, more than twofold, by BK as well as DAKD in comparison with the untreated cells (Fig. 2B). The effect of INF-γ on the ICAM-1 mRNA expression was also studied. As expected, a significant increase in mRNA expression was observed in these samples (above threefold higher as compared to the non-stimulated cells) (Fig. 2B). The Western blot analysis of ICAM-1 production in the presence of kinins corroborated the above observed effect (Fig. 2C). Both BK and DAKD induced generation of high amounts of ICAM-1 protein, visualized as a band at molecular weight near 90 kDa. Densitometric analysis of ICAM-1/β-Act ratios showed a nine- and sevenfold augmentations of ICAM-1 expression after BK and DAKD stimulation, respectively. Interestingly, the presence of MGTA led to inhibition of the BK-induced effect, and the relative ICAM-1 content was reduced approximately by 20%. A major effect on ICAM-1 release by INF-γ was also observed (above tenfold). A strong positive effect of INF-γ was also demonstrated in the flow cytometry study (Fig. 2D). Presence of INF-γ alone increased ICAM-1 expression by 35%, while addition of BK or DAKD resulted in even stronger stimulation of the above adhesion molecule, by 53% and 45% respectively.

ENHANCED B1R EXPRESSION BY PRO-INFLAMMATORY MEDIATORS

The expression of the B1 receptor was analyzed by real time-PCR in the monocyte-like and endothelial cells after their stimulation with BK, DAKD, and INF-γ. The selected substances increased B1R mRNA expression in both cell types, however a much stronger effect was observed in the endothelial cells (Fig. 3A). The mRNA content was increased four- to eightfold in the stimulated cells as compared to the controls. DAKD induced the most significant changes, observed within 30 min, whereas the maximal effects of BK and INF-γ, although still lower than that of DAKD, were obtained after 4 h incubation. The response of the U937 cells to the selected pro-inflammatory substances was less remarkable. The B1R expression was increased about twofold, however it was achieved after a shorter, 1–2 h, incubation time.

The B1R protein expression analyzed by Western blotting confirmed the above observations. The monocyte-liked cells were stimulated with INF-γ for increasing time periods (0, 4, 6, and 8 h), which led to increasing amounts of B1R protein (Fig. 3B). Initially, a weak band with an apparent molecular weight of 40 kDa was observed in the untreated cells, while after 4 h of their incubation with the cytokine an additional band appeared near 82 kDa. We presumed that these bands corresponded to the different states of

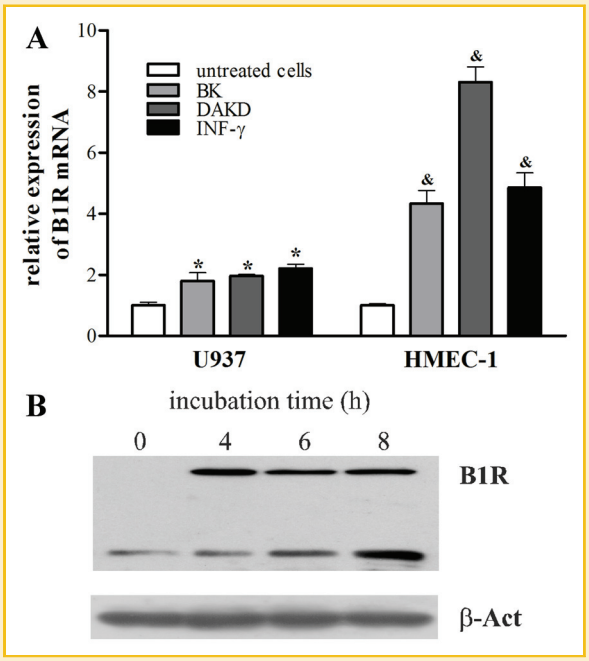

Fig. 3. The effect of pro-inflammatory mediators on the expression of B1 receptors. A: The maximal expression of B1R mRNA observed after incubation of U937 monocyte-like cells and HMEC-1 cells with 1 μM BK, 1 μM DAKD, and INF-γ during different incubation periods (described in the text) determined by real time PCR. Statistical significance was calculated by Student's *t*-test at $P < 0.05$ against untreated cells for experiments with monocyte-like cells (*) and at $P < 0.01$ against non-stimulated HMEC-1 cells (&). All values represent means ± SD from at least three experiments. B: Western blotting analysis of the B1R protein expression in monocyte-like cells after incubation with 10 ng/ml INF-γ for 4, 6, and 8 h.

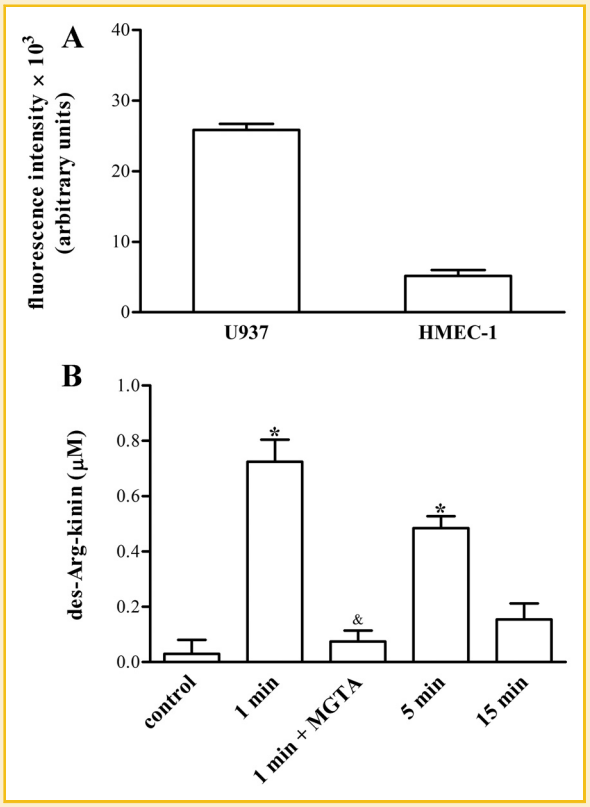

Fig. 4. The carboxypeptidase activity of the RA-differentiated U937 and HMEC-1 cell lines and the contribution of carboxypeptidases to des-Arg kinin formation by the RA-differentiated U937 cells. A: The enzymatic activity of CPM in the RA-differentiated U937 cells and HMEC-1 cells was studied by fluorometric measurements of the release of the dansyl-labeled alanine from DAA. B: The des-Arg kinin formation by monocyte-like cells after incubation with 1 μM BK at 37°C was observed by a radiocompetitive receptor-binding assay. The values represent the means ± SD from at least three experiments. Statistical significance was calculated by Student's *t*-test at $P < 0.05$ for samples incubated for different periods against the untreated cells (*) or for the samples first treated with MGTA before incubation with BK against the cells incubated with BK for 1 min (&).

glycosylation of the B1R protein as described elsewhere [Fortin et al., 2006]. The control of protein isolation showed a similar amount of β-actin protein with a molecular weight around 40 kDa in each sample.

CARBOXYPEPTIDASE ACTIVITY IN THE MONOCYTE-LIKE AND ENDOTHELIAL CELLS

The activities of carboxypeptidases in the RA-differentiated U937 and HMEC-1 cells were analyzed by measurement of enzymatic degradation of the dansyl-labeled synthetic peptide DAA. Both types of cells showed a high levels of enzyme activity (Fig. 4A). To confirm the carboxypeptidase activity against kinin peptides, we chose to monitor degradation of BK in the monocyte-like cells because of their higher CPM activity on the synthetic fluorogenic substrate (fivefold) as compared to the endothelial cells. The U937 cells cleaved BK quickly (Fig. 4B). The appearance of high amounts of des-Arg kinins in the supernatant was observed after 1-min incubation. The production of these peptides slowly decreased, and after 30 min the peptide concentration was undetectable. As a negative control, some samples were first treated with the carboxypeptidase inhibitor MGTA, which effectively inhibited the BK degradation (Fig. 4B).

KININ PEPTIDES MODULATE ACTIVATION OF STAT-1 AND STAT-3 IN THE RA-DIFFERENTIATED U937 AND HMEC-1 CELLS

The EMSA technique, used for analysis of transcription factor signaling, showed that both BK and DAKD modified activation of STATs in the RA-differentiated U937 cells, however only in the presence of the cytokines, INF-γ or IL-6 (Figs. 5A and C). Densitometric analysis of the gels allowed to demonstrate observed changes as percentages of the positive control, in which cells were stimulated with cytokine alone taken as 100%. There was a significant decrease in the INF-γ-induced STAT-1 activation after BK treatment (by 40%), while DAKD led to a significant increase of INF-γ induced STAT-1 under the same conditions (by 70%) (Fig. 5B). Both, BK and DAKD enhanced the IL-6-induced STAT-3 activation by 71% and 35%, respectively (Fig. 5D). The specificity of the observed effects was confirmed by incubating the cells with antagonists of the B2 and B1 receptors (HOE-140 and DHOE-140, respectively) before the kinin stimulation. In each case, the levels of STAT-1 and STAT-3

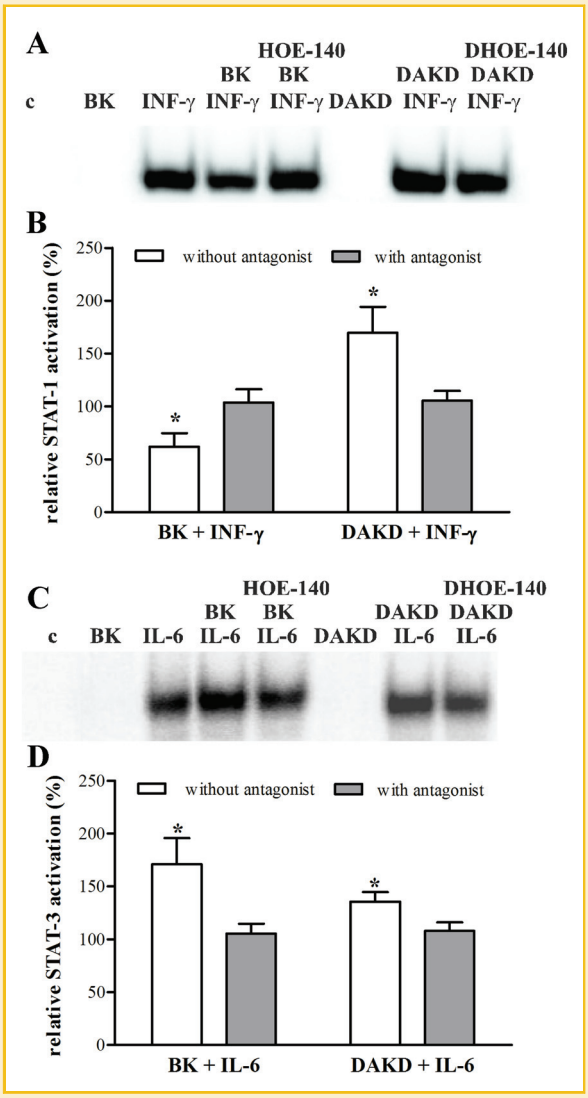

Fig. 5. The effect of BK and DAKD on the activation of STAT proteins in the RA-differentiated human monocyte cell line U937. The cells were incubated with 1 μM BK or 1 μM DAKD in the presence of 10 ng/ml INF-γ or 50 ng/ml IL-6 for 30 min. In some experiments, the incubation was performed with just BK or DAKD, or in the presence of kinin antagonists. In each experiment, a positive control sample was prepared by incubation with cytokine (INF-γ or IL-6) alone. After protein extraction, the samples were analyzed for STAT-1 (A) and STAT-3 (C) activation by EMSA. The STAT-1 (B) and STAT-3 (D) activation in samples induced by kinin + cytokine was calculated and presented relative to the positive control sample, assumed to be 100%. A,C: The EMSA images from representative experiments. All values in (B) and (D) are presented as means ± SD from at least three experiments. *Statistical significance was calculated by Student's *t*-test at $P < 0.05$ against the positive control.

activation returned to the level obtained for the positive control samples (Figs. 5B and D).

Similar experiments performed with the endothelial cells showed enhancement of STAT-3 activation after BK and DAKD stimulation (Fig. 6). The peptide effect was time-dependent. Incubation with BK and DAKD resulted in a weak activation after 15 min, while the maximal effect was observed after 30 min of stimulation (Fig. 6A). Similarly to the experiments with U937 cells, supplementation of BK-induction with a cytokine, IL-6, resulted in a significant increase of STAT-3 (by 30–35% as compared to the positive control) (Figs. 6B and C). There was no activation of STAT-3 in the cells incubated with DAKD + IL-6. The use of receptor antagonists caused loss of the BK-induced activation (Figs. 6B and C). STAT-1 was not activated by kinin peptides alone, while slight effects of BK and DAKD in the presence of INF-γ were observed, but they were insignificant (results not shown).

DISCUSSION

The most important functions of leukocytes, including phagocytosis, and chemotaxis of macrophages and granulocytes, depend on adhesion of these cells to the vessel wall [Gahmberg et al., 1999]. Although this research area has been extensively investigated, new concepts are being constantly introduced in order to extend the knowledge of leukocyte recruitment, especially during inflammatory processes [Ley et al., 2007]. In this study, we added new data to this field, demonstrating that the proinflammatory kinin peptides were able to improve monocyte adhesion, affecting both monocytes and endothelial cells. We observed that the kinin peptides, both B2R and B1R agonists, at concentrations at which these substances occurred in body fluids during numerous pathological states, caused an increased adhesion of monocyte-like cells to the extracellular matrix proteins and endothelial cells. We previously demonstrated similar effects in PMN [Guevara-Lora et al., 2011a]. In the current work, we further characterized roles of the kinins in the endothelial cells and their stimulation of the monocyte adhesion. Similarly as PMN, the kinin-stimulated monocyte-like cells showed increased Mac-1 expression in the membrane, especially of CD11b subunits. However, the effect of the des-Arg kinin metabolite was more significant, suggesting a strengthened mediation of B1R. Next, we chose Mac-1, an important integrin involved in intravascular crawling of leukocytes, and ICAM-1, one of the complementary adhesion molecules for this interaction on the endothelial cell surface [Ley et al., 2007] to study their expression in response to the selected kinin peptides. The effect of BK and DAKD on ICAM-1 mRNA (RT-PCR) and protein expression (WB) were evident and significant. As expected, the most significant increase in ICAM-1 protein production was achieved after INF-γ incubation, as shown by both Western blotting and flow cytometry. INF-γ represented a cytokine, which was produced by activated T lymphocytes; it played diverse roles, such as antiviral activity as well as a variety of immunomodulatory and inflammatory functions [Shinozawa et al., 2002; Harvey and Ramji, 2005; Zhang, 2007]. The regulation of ICAM-1 expression by INF-γ was widely demonstrated, indicating augmented cell interaction [Chang et al., 2002; Harvey and Ramji, 2005]. Surprisingly, the ICAM-1 protein expression visualized by Western blotting in the cells stimulated by BK and DAKD could not be confirmed in the flow cytometry. In the latter, only in the presence of INF-γ, augmentation of the protein expression on the cell membranes was observed. We suspected that ICAM-1 might have been degraded at the membrane site, and soluble ICAM-1 form (sICAM-1) was generated as it was

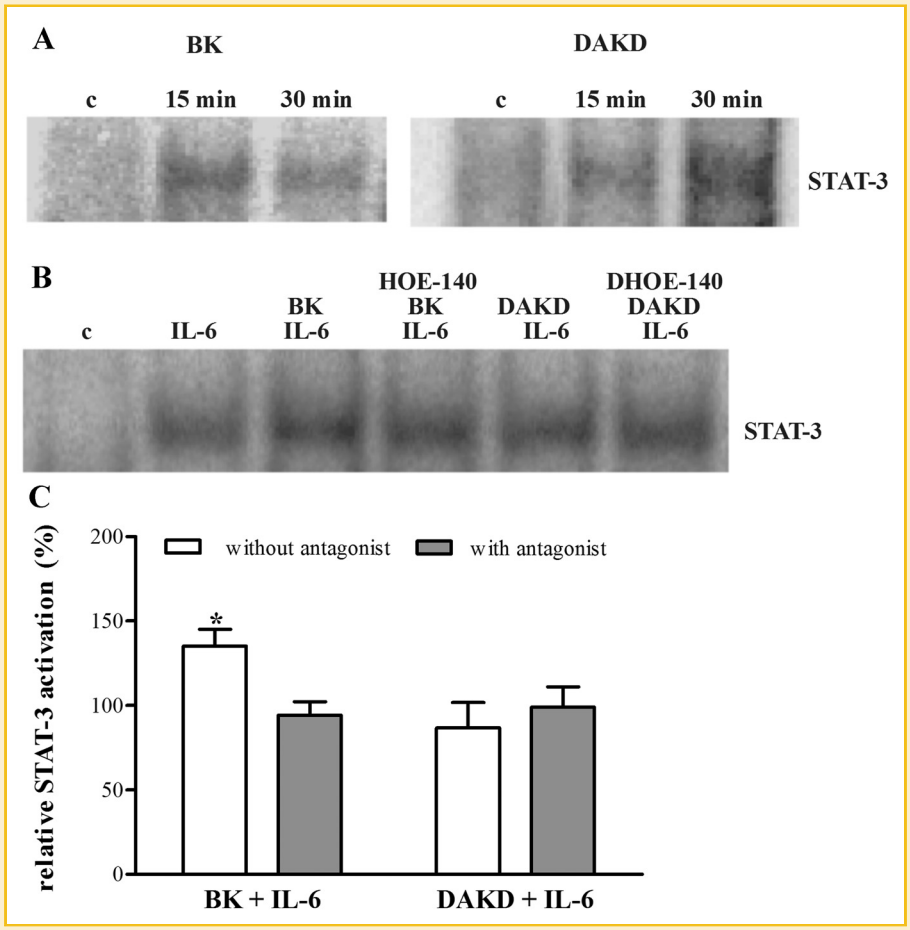

Fig. 6. The effect of BK and DKD on the activation of STAT proteins in the human cell line HMEC-1. A: The cells were incubated with 1 μM BK, 1 μM DAKD for 15 and 30 min. The protein extracts were analyzed for STAT-3 activation by EMSA. Additional samples were performed with 1 μM BK, 1 μM DAKD in the presence of 50 ng/ml IL-6. In each experiment, a positive control sample was prepared by incubation with cytokine. The figure depicts the EMSA image (B) and the percentage of STAT-3 activation (C) relative to the positive control sample (IL-6), assumed to be 100%. The values represent the means ± SD from at least three experiments. *Statistical significance calculated by Student's *t*-test at $P < 0.05$ against the positive control.

shown elsewhere [Rothlein et al., 1991; Leung, 1999]. The enzymatic lysis of this membrane protein seemed even more probable since bradykinin and kinin-related peptides were regarded as strong pro-inflammatory mediators. Notwithstanding, the increased ICAM-1 expression in HMEC-1 by kinin peptides in the presence of INF-γ allowed to assume that kinin peptides might cause an enhancement of the adhesive potential of these cells.

We observed that BK enhanced adhesion of the monocyte-like cells to the HMEC-1 monolayer, which was confirmed by inhibition of such stimulation by a specific inhibitor of carboxypeptidases, MGTA. The above observation together with detected weak inhibition of the BK-induced effect on ICAM-1 production suggested that adhesion processes might be in part attributed to the formation of des-Arg peptides, the B1R agonists. In order to verify this hypothesis, we tried to better characterize this particular receptor in the selected cell models. At first, we analyzed the effect of kinins and INF-γ on B1R in HMEC-1 and RA-differentiated U932 cells. All these substances, even BK, induced expression of this receptor in both cell types, with a stronger response in HMEC-1 cells. Interestingly, the stimulation of B1R mRNA expression by BK and INF-γ was delayed as compared to the immediate effect of DAKD. Such a time lag in response suggested autoregulation of kinin receptors by their agonists in the endothelial cells as we previously reported for U937 cells [Guevara-Lora et al., 2009]. In this study, we established that a similar regulation of B1R expression might occur in the endothelial cells. Additionally, here, we demonstrated that INF-γ stimulated generation of this receptor in HMEC-1 cells confirming an earlier report, in which an amplificatory effect of INF-γ in the presence of other cytokines on B1R expression was noted in the vascular cells [Koumbadinga et al., 2010]. Furthermore, we showed that the cell lines used as the models for this study possess the enzymatic ability to transform B2R agonist into B1R agonists. A previous study showed enhancement of adhesion of differentiated U937 cell by cathepsin X, another carboxypeptidase, through Mac-1 [Obermajer et al., 2006]. To sum up, the effect of BK on monocyte–endothelial cell interactions could result not only from its direct

action on the cells, but also from generation of des-Arg kinins, which as potent proinflammatory peptides could propagate inflammatory reactions.

Our discovery of kinin-induced cell adhesion obliged to suggest a possible convergence between signaling pathways of kinin receptors and those promoting the adhesion processes in cells, especially during inflammatory response. The agonist binding to the kinin receptors activated signal transduction through reaction cascades mediated by numerous proteins. The most important signaling pathways with participation of kinin receptors were reviewed elsewhere [Yu et al., 2002; Leeb-Lundberg et al., 2005]. Among the transcription factors activated by these receptors, the NF-κB and AP-1 were well studied, but exact mechanism of BK mediated via B2R activation of signal pathways associated with the Janus kinases and with STAT proteins was poorly understood. Until now, there was only a single report that showed association of kinin peptides with these transcriptional factors in bovine aortic endothelial cells [Ju et al., 2000]. There is an increasing number of reports demonstrating the role of STAT activation in cell adhesion [Xie et al., 2001; Zhu et al., 2003; Silver et al., 2004; Profita et al., 2008]. In our study, some changes in STAT-1 and STAT-3 activation by BK and DAKD were observed in endothelial and monocyte-like cells.

The monocyte-like cells were incubated simultaneously with kinin peptides and selected cytokines to stimulate either STAT-1 or STAT-3, because peptides alone did not cause any changes in STAT activation. In this study, the STAT-1 activation in monocyte-like cells was significantly decreased by BK. We hypothesized that this effect could be associated with the increased adhesion of RA-differentiated cells to the extracellular matrix proteins and to the endothelial cells. Our results and such assumption would confirm an earlier report, where the enhanced cell adhesion to the extracellular matrix proteins together with the diminution of STAT-1 activation were demonstrated, suggesting an interaction of this transcriptional factor with the integrin signaling pathways [Xie et al., 2001]. On the other hand, DAKD caused an opposite effect on STAT-1 activation with a significant adhesion to fibronectin and to the endothelial cells. An accurate interpretation of this effect might require additional studies. Here, we could only postulate an indirect role of this protein in the monocyte adhesion. One possible interpretation might rely on the ability of cytokine-stimulated U937 cells to produce ICAM-1 [Dustin et al., 1986] that could be turned into its soluble form, sICAM-1, by enzymes such as elastase [Lawson and Wolf, 2009]. Soluble ICAM-1 played a pro-inflammatory role through activation NF-κB, MAPK, and Erk transcriptional factors and release of cytokines, such as INF-γ or tumor necrosis factor-α [Lawson and Wolf, 2009]. This explanation would not be in accord with our results, because we did not observe any ICAM-1 expression in the monocyte-like cells. On the other hand, both BK and DAKD caused significant changes in STAT-3 activation. This protein mediates inflammatory responses through either extrinsic or intrinsic pathways [Levy and Darnell, 2002]. The expression of many cytokines, chemokines, and other mediators associated with inflammation are then induced. Moreover, the receptors for many of these mediators could further activate STAT-3. Hence, this protein could propagate the inflammatory state by autocrine and paracrine loops. In addition, the collaboration between STAT-3 and NFκB was broadly documented [Grivennikow and Karen, 2010], especially in the propagation of immune response. Therefore, taking into account that NFκB was a transcriptional factor promoting cell adhesion [Sokoloski et al., 1993], we anticipated that a crosstalk between these factors might contribute to the kinin-induced monocyte adhesion. In summary, both BK and DAKD promoted monocyte adhesion, both to the extracellular matrix proteins as well as to the endothelial cells, and the STAT signaling pathways seem to be involved in these processes.

The increased monocyte adhesion to the HMEC-1 cells could be also attributed to the BK and DAKD-mediated enhanced ICAM-1 production in the endothelial cells. ICAM-1 increased expression on the surface of the endothelial cells was associated with STAT-1 signaling [Roebuck and Finnegan, 1999]. However, in our study, kinin peptides were unable to activate this protein, neither alone nor in the presence of INF-γ in the HMEC-1 cells. Therefore, we hypothesized that kinins regulated ICAM-1 expression indirectly through NFκB or AP-1, since up-regulation of this adhesion protein by these signaling pathways was previously shown [Roebuck and Finnegan, 1999]. Then, the observed inhibition of BK effect on cell–cell interactions and ICAM production would be in agreement with the above proposed model, especially that B1R agonists was shown to strongly activate NFκB and AP-1 [Marceau et al., 1998; Calixto et al., 2004; Leeb-Lundberg et al., 2005]. In turn, BK and DAKD activated STAT-3, which in case of BK was even further enhanced in the presence of IL-6. We expected that STAT-3 regulated ICAM-1 expression on the cell surface, since the gene promoter of this protein included a specific sequence for STAT-3 binding [Caldenhoven et al., 1995]. Furthermore, a regulation of the STAT-1 functions by STAT-3 was previously proposed [Ho and Ivashkiv, 2006], which allowed to assume that the observed kinin-induced effects might be related to this type of interaction. Our results strongly indicated that the kinin-induced ICAM-1 expression might be regulated by this protein but it would require further studies on the role of STAT-3 in the stimulation of ICAM-1 expression by the kinins.

Taken together, we demonstrated the alteration of STAT activation by kinin peptides, both in the monocyte- and endothelial-cell models, and we suggested a possible role of these signaling molecules in the regulation of leukocyte–endothelial cell interactions through increased amounts of adhesion proteins on the cell surface. The effect of BK and DAKD on the activation of STATs was inhibited by selective antagonists of kinin receptors, suggesting their direct involvement. However, additional studies would be necessary to establish the exact role of kinin-activated STATs in the cell adhesion because of other potential signaling pathways.

In conclusion, we propose that the kinin-induced increase in the expression of membrane proteins, such as Mac-1 or ICAM-1, might contribute to leukocyte adhesion to the vessel wall, especially during inflammatory responses during which INF-γ is released. The function of the B1 receptors seems to be important for leukocyte–endothelial cell interactions, in which we propose a significant role of signaling molecules such as STAT. These observations might be helpful for the interpretation of the regulatory roles of kinin receptors in many diseases, especially in those presenting perturbation of leukocyte adhesion to the vessel walls.

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