Bioregulation of Kallikrein-related Peptidases 6, 10 and 11 by the Kinin B_1 Receptor in Breast Cancer Cells

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Abstract. *The sera of patients with breast cancer have higher levels of des[Arg^9]bradykinin, a kinin B_1 receptor (B_1R) agonist, than that from healthy individuals. Stimulation of breast cancer cells with the analog Lys-des[Arg^9]bradykinin causes release of metalloproteinases-2 and -9 and increases cell proliferation. We examined the possibility that breast cancer cells, in addition to B_1R, express the kinin-forming protease true tissue kallikrein (KLK1) and the endogenous proteins termed kininogens from which kinins are enzymatically released. Furthermore, we investigated whether stimulation of breast cancer cells with a B_1R agonist would modify the cellular levels of KLK6, KLK10 and KLK11, three kallikrein-related peptidases with a still poorly-understood biological role in breast cancer. We found that breast cancer cells expressed KLK1 and kininogens, and that stimulation of estrogen-sensitive breast cancer cells with the B_1R agonist produced down-regulation of KLK10 (a protease associated with growth suppression) but up-regulation of KLK11 and KLK6 (peptidases related to increased cell proliferation and invasiveness, respectively). Furthermore, we showed that the B_1R agonist acts as a functional stimulus for the secretion of KLK1 and KLK6, an event relevant for kinin production and cell invasion, respectively.*

Breast cancer is the most common malignancy in women and the principal mechanism for the treatment of estrogen-positive tumors is inhibition of estrogen receptor-α or estrogen production. However, a number of patients develop resistance to such anti-estrogen treatment. Variable or no response to therapy that targets estrogen receptors suggests the presence of alternate molecular mechanisms which in concert with the tumor microenvironment may contribute to breast cancer, independent of the estrogen signaling system, further compromising our ability to develop novel therapeutic strategies that could improve disease outcome.

In a comprehensive study Villanueva *et al*. (1) have reported that protease activities superimposed on the *ex vivo* coagulation and complement degradation pathways contribute to generation of not only cancer-specific, but also cancer type-specific serum peptides with a molecular mass below 3 kDa (serum peptidome). These authors describe that bradykinin and *des*[*Arg*^{9}]bradykinin, including their hydroxylated forms, are at higher levels in serum of patients with breast cancer when compared to that from healthy individuals. Subsequent studies confirmed that des[Arg9]bradykinin is present at increased levels in patients with breast cancer, supporting its potential use as diagnostic biomarker (2). The kinins *des*[Arg^9]bradykinin and Lys-*des*[Arg^9]bradykinin (LDBK) are two proinflammatory peptides generated by removal of the C-terminal arginine by carboxypeptidases M and N from bradykinin or Lys-bradykinin, respectively (3, 4). Both *des*[*Arg*^{9}]bradykinin and LDBK exert their biological actions by activating a G protein-coupled receptor known as kinin B1 receptor (B_1R), a receptor that is usually minimally expressed on cells, but is up-regulated in inflammation and cancer (3-5). Importantly, functional B_1Rs are expressed not only in malignant breast lesions, including ductal carcinoma *in situ* (DCIS) and invasive ductal carcinoma (IDC), but also in benign fibroadenomas (6).

The parental kinin molecules bradykinin and Lys-bradykinin are formed by the enzymatic action of two serine proteases, true tissue kallikrein (KLK1) and plasma kallikrein

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(KLKB1), acting on circulating or locally-synthesized substrates called high (H-kininogen) and low (L-kininogen) molecular weight kininogens. KLK1 belongs to the human tissue kallikrein family that comprises of a closely related multigene group of 15 serine endopeptidases, designated as *KLK1* to *KLK15* (kallikrein-like tissue kallikreins), located in a tightly clustered locus of over 320 kilobases on chromosome 19q13.3-13.4, which represents the largest contiguous group of proteases within the human genome (7-11). KLK1 is considered as the true or classical kallikrein (5), and KLK2 to KLK15 form a group of proteases of tryptic or chymotryptic activity known as kallikrein-related peptidases. All kallikrein-related peptidases are steroid hormone-regulated and variably expressed in hormone-dependent cancer. Whereas KLK-3, -4 and -7 are up-regulated by androgens, KLK5 is modulated by estrogen and progestins (12). In normal breast tissue, all 15 KLKs mRNA/protein have been identified, whereas in breast cancer, the expression of all KLKs except KLK2 has been reported (13). Although protein expression of several KLKs in breast cancer tissue has not yet been assessed, four of them (KLK5, -7, -10 and -14) have been associated with an unfavorable poor prognosis, whereas other members of the family (KLK3, -9, -12, -13 and -15) predict a favorable prognosis (13). Furthermore, high levels of *KLK3* mRNA have been found in tissue samples of breast cancer at early stage, and low levels or no expression in advanced stage, an issue related to methylation of the promotor region in the *KLK3* gene; for this reason, *KLK3* is considered a tumor-suppressor candidate. On the other hand, KLK6 has both cancer-inhibiting and cancer-promoting activities depending on the tissue and tumor microenvironment. KLK6 has been implicated in the tumorogenesis of colonic, squamous cell carcinoma and primary breast cancer (9, 13). Expression of KLK11 has been detected in both human breast cancer tissue and in non-cancerous mammary glands, but significantly higher KLK11 expression has been observed in histological grade I/II than in grade III breast tumors, an issue that could be related to the proliferative rate (13).

We therefore designed experiments to investigate whether levels of KLK6, -10 and -11 are modified after stimulation of estrogen-sensitive breast cancer cells with a B_1R agonist. Furthermore, we examined the possibility that breast cancer cells, in addition to B_1R, express the kinin-forming protease KLK1 and kininogens, substrates from which kinins are released enzymatically, and finally we evaluated the capacity of KLK6 to modulate the invasion of estrogen-sensitive breast cancer cells.

Materials and Methods

Human tissue samples. Subsequent to ethical approval (Human Research Ethics Committee of Sir Charles Gairdner Hospital, Nedlands, Perth, Western Australia; and Universidad Austral de Chile, and Hospital Base, Valdivia, Chile. Approval numbers ORD043 and CI280606), breast cancer samples that were estrogen receptor-positive and HER2-negative were selected and obtained from the Department of Tissue Pathology, PathWest, Nedlands, Perth, Western Australia, and from the Servicio de Patologia, Hospital Base, Valdivia, Chile. Ethics Committees accepted the archival nature of the breast cancer tissue included in our study and waived the need for written informed consent from the participants. Collection of human samples included 11 of DCIS and 22 of IDC that were characterized according to hormonal status and expression of the HER2 receptor. All cases were found to be HER2-negative (range 0 to +1) and estrogen receptor-positive tumors (range 20 to 90% for DCIS and 20 to 100% for IDC), whereas progesterone receptors were absent in 36% of IDC. IDCs were subclassified histologically into three grades according to Elston and Ellis (14).

Breast cancer cell lines and culture conditions. The cell lines used in the current study were obtained from the American Type Culture Collection (Manassas, VA, USA). MCF-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM), ZR-75-1 cells in RPMI-1640, and T47D and MDA-MB-231 cells in DMEM-F12 medium. All culture media were supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 25 ug/ml amphotericin B. Cells were cultured at 37˚C in a humidified incubator with 5% CO_2 and 95% air. We principally used the MCF-7 cell line because it is useful for *in vitro* breast cancer studies, and it has retained several ideal characteristics peculiar to the mammary epithelium. These include the ability of MCF-7 cells to process estrogen, in the form of estradiol, via estrogen receptors in the cell cytoplasm. This makes the MCF-7 cell line an estrogen receptor-positive control cell line. When grown *in vitro*, the cell line is capable of forming domes and the epithelial-like cells grow in monolayers.

Immunohistochemistry. Human tissues (DCIS and IDC) were fixed in 10% buffered formalin, embedded in paraffin wax, cut into 5 μm sections and adhered to poly-L-lysine-coated slides. Tissue sections were dewaxed and rehydrated before antigen retrieval was performed on all sections by boiling the slides in EDTA buffer (pH 8.0). Sections were treated with absolute methanol/3% H_2O_2 for 5 min to quench endogenous pseudoperoxidase activity, rinsed in distilled water and then in Tris-HCl buffer. After blocking non-specific binding for 5 min with Dako protein block (Dako, Carpinteria, CA, USA), tissue sections were incubated with each of the following antisera: i) monoclonal or polyclonal antibodies to human KLK1 to -15 (R&D Systems, Minneapolis, MN, USA and Abcam, Cambridge, MA, USA); ii) anti-B1R directed to the *C*-terminal 16 amino acids ISSSHRKEIFQLFWRN of human B_1R (6, 15); iii) anti-human kininogen, an antibody that recognizes both high and low molecular weight kininogens (16). All antibodies were reconstituted and diluted in 0.01 M Phosphate Buffer Saline (PBS) containing 1% IgG-free bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA) and 0.2% NaN_3. Bound antibodies were detected with LSAB+ biotin/streptavidin-peroxidase kit (Dako) and peroxidase activity developed with 3,3'diaminobenzidine (Dako).

Breast cancer cell lines were fixed with periodate-lysine-p-formaldehyde and washed thoroughly with PBS and immunostained as described above. Immunostained breast tissue and cells were contrasted with hematoxylin and mounted with Depex or Mowiol™ (Polysciences, Inc, Warringthon, PA, USA), respectively. Salivary gland and normal breast tissue sections were used as positive controls

for KLK identification. Negative controls included omission of primary antibody, its replacement by non-immune immunoglobulins (Dako), or incubation with a mixture of B_1R antibody and an excess of the same peptide used for immunization.
The intensity and percentage of tumor cells that stained positively with each antibody were evaluated by two independent observers.

Confocal microscopy. MCF-7 cells were grown on 22×22 mm coverslips and after 48 h, the medium was replaced by phenol red-free DMEM without serum. Cells were incubated for a further 48 h, washed with Dulbecco's phosphate-buffered saline (DPBS), fixed with methanol at −20˚C for 45 min, air dried and stored at −80˚C until used. For immunolabeling, coverslips were treated with DPBS-3% BSA at room temperature for 30 min and then incubated overnight with each primary antibody (1:250), washed and then incubated with the corresponding F(ab')2 fragments conjugated with Alexa-488 (1:1000; Invitrogen, Grand Island, NY, USA). Antibodies were diluted in DPBS-1% BSA. The DNA marker dye, 4',6-diamidino-2-phenylindole (DAPI; 0.5 μg/ml; Invitrogen) was used to label the nuclei. Immunolabeling was evaluated using a confocal microscope (Fluoview FV1000; Olympus, Tokyo, Japan) at 488 nm argon-krypton (for Alexa-488) and 405 nm diode (for DAPI) lasers at appropriate excitation values. Optical sections were digitalized at 0.8 μm, and the images were processed using the free software Image J (NIH, Bethesda, MD, USA).

Reverse transcriptase-polymerase chain reaction (RT-PCR). Expression of B_1R, L- and H-kininogens, *KLK1* to *KLK15*, and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) mRNAs was assessed by extracting total RNA (Promega, Madison, WI, USA). cDNAs were synthesized using random hexamer primers (2.5 mM), ImProm-II™ reverse transcriptase (Promega) and 2 μg of total RNA in a 20 μl reaction mixture. Amplification of cDNAs was performed using GoTaqR Green Master Mix (Promega). PCRs were performed using 0.1 μM specific primers (Biosource International, Camarillo, CA, USA), 2 μl of cDNA and an Eppendorf thermocycler (Hamburg, Germany). Primers and PCR conditions are shown in Table I.

Because it had been assumed previously that kininogens may be endocytosed from the culture medium, cells were maintained for 48 h without fetal bovine serum. Detection of transcripts for both kininogens demonstrates that breast cancer cells have the capacity to synthesize these kinin precursors. Semiquantitative PCR was performed to compare the expression of each KLK gene of interest with respect to the expression of *GAPDH* (reference gene) and because differences were evident, quantitative PCR was not performed. Differences in expression were confirmed at the protein level.

Western blotting. Cells were homogenized with cold RIPA buffer supplemented with enzymatic inhibitors (20). Proteins contained in culture media were precipitated using a ProteoExtract™ Protein Precipitation Kit (Calbiochem, LaJolla, CA, USA). Proteins present in cell homogenates or culture media were separated by electrophoresis and transferred onto Immobilon membranes (Millipore, Billerica, MA, USA). Membranes were incubated with primary antibodies for 2 h and then with the corresponding peroxidase-labeled secondary antibody for 30 min. Peroxidase activity was developed using a commercial chemiluminescence kit (Pierce, Rockford, IL, USA). The antibodies used for the first immunodetection procedure were stripped off as previously described (15) and β-actin (Sigma-Aldrich) was then detected as a control for protein loading.

Transfection of B1R and KLK6 siRNAs. The protocol for silencing B_1R, had been developed previously (15). Briefly, 1×10^6 MCF-7 cells were placed in DMEM without antibiotics/antimycotics and incubated with siRNA–Lipofectamine complexes prepared according to the manufacturer's instructions (Invitrogen). Transfection of B_1R and KLK6 siRNAs was performed for 24 to 48 h using 100 pmoles of each siRNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or control siRNA (Block-it fluorescent oligo, an unrelated siRNA useful to control efficiency of transfection; Invitrogen). Efficiency of transfection was checked 24 h and 48 h after transfection by visualizing the incorporated Block-it fluorescent oligo. Success of *KLK6* siRNA was assessed by detecting the expression levels of KLK6 under basal conditions and after stimulation with the B_1R agonist.

Enzyme linked immunoassays (ELISA). ELISA was performed to determine the functional effect of LDBK on KLK6 in MCF-7 cells. The MCF-7 cells were cultured in DMEM with phenol red, and then maintained in basal medium without phenol red and fetal bovine serum for 48 h prior to stimulation with 10 nM LDBK for 3 h. To quantify KLK6 secretion, the culture fluid from the basal (unstimulated) and stimulated MCF-7 cells was individually harvested and reduced by centrifugation at 3000 × *g* in an Ultracel-3K column for 30 min and KLK6 levels were determined by ELISA (RayBiotech, Inc, Norcross, GA, USA).

Invasion assays. MCF-7 cells were grown to 80% confluence, starved of serum overnight and then harvested using 0.5 mol/l EDTA (pH 6.8). The cells were plated at a density of 50×10^4 per well were seeded in six-well BD BioCoat transwell chambers (Becton Dickinson, Franklin Lakes, NJ, USA) with 8.0-μm pore size polycarbonate membrane and matrigel and grown for 24 h. After this period, conditioned medium obtained (approximately 2 ml, previously concentrated from 10 ml) from unstimulated or LDBK-stimulated (10 nM for 3 h) MCF-7 cells was added to the upper chamber; each medium was concentrated by centrifugation as described previously. Positive controls were performed by adding 10 ng/ml of epidermal growth factor (EGF) to the upper chamber instead of the conditioned medium. Simultaneously, DMEM containing 20% fetal bovine serum was added to the lower chamber as a chemotactic stimulus. Invasion assays without serum in the lower chamber were used as negative controls (not shown). After 24 h at 37˚C with 5% CO_2, the experiment was stopped by wiping the cells from the upper chamber with a cotton swab and fixing filters with methanol cooled at −20˚C. Finally, filters were stained with Harris hematoxylin for 20 min and invasion was quantified by counting 10 microscopic fields at ×400 for two independent observers. Each experiment was performed in triplicate and results were expressed as mean±SE.

Data analysis. Intensity of the immunoblotted protein bands was quantified using an automated image digitizing system as described previously (15). Mann-Whitney *U*-test and Student's *t*-test were used to analyze differences between groups (GraphPad InStat^R, San Diego, CA, USA). Values are expressed as the mean±SE and significance was considered acceptable at the 5% level ($p<0.05$).

Table I. *List of primers, sequences, their product size, and annealing temperature to amplify the various kallikrein-related peptidases*

	Primers (F: forward, R: reverse)	Product size (bp)	Annealing temperature
B1R:	F 5'-CTCGACCTTCCAGGCTTAA-3' R 5'-CTTTCCTATGGGATGAAGATAT-3'	214	55°C
H-Kininogen:	F 5'- CGATATTCAGCTACGAATTGC-3' R 5'-TGAATCCCGCTCTTCATCTTG-3'	553	57°C
L-Kininogen:	F 5'-CGATATTCAGCTACGAATTGC-3' R 5'- CTTGTACTCGCAGGACCTTAG-3'	526	56°C
GAPDH	F 5'- GCACCGTCAAGGCTGAGAAC-3' R 5'-ATGGTGGTGAAGACGCCAGT-3'	142	61°C
KLK1	F: 5'-CTCCTGGAGAACCACACCCGCC-3' R: 5'-GCGACAGAAGGCTTATTGGGGG-3'	419	60°C
KLK2	F: 5'-GGCAGGTGGCTGTGTACAGTC-3' R: 5'-CAACATGAACTCTGTCACCTTCTC-3'	479	51°C
KLK3	F: 5'-CCCACTGCATCAGGAACAAAAGCG-3' R: 5'-GGTGCTCAGGGGTTGGCCAC-3'	601	60°C
KLK4	F: 5'-GCGGCACTGGTCATGGAAAACG-3' R: 5'-AACATGCTGGGGTGGTACAGCGG-3'	437	60°C
KLK5	F: 5'-GTCACCAGTTTATGAATCTGGGC-3' R: 5'-GGCGCAGAACATGGTGTCATC-3'	328	52°C
KLK6	F: 5'-GAAGCTGATGGTGGTGCTGAGTCTG-3' R: 5'-GTCAGGGAAATCACCATCTGCTGTC-3'	454	55°C
KLK7	F: 5'-CCGCCCACTGCAAGATGAATGAG-3' R: 5'-AGCGCACAGCATGGAATTTTCC-3'	371	57°C
KLK8	F: 5'-GCCTTGTTCCAGGGCCAGC-3' R: 5'-GCATCCTCACACTTCTTCTGGG-3'	416	60°C
KLK9	F: 5'-TCTTCCCCCACCCTGGCTTCAAC-3' R: 5'-CGGGGTCTGGAGCAGGGCTCAG-3'	409	57°C
KLK10	F: 5'-GGAAACAAGCCACTGTGGGC-3' R: 5'-GAGGATGCCTTGGAGGGTCTC-3'	468	55°C
KLK11	F: 5'-CTCTGGCAACAGGGCTTGTAGGG-3' R: 5'-GCATCGCAAGGTGTGAGGCAGG-3'	461	60°C
KLK12	F: 5'-TTGACCACAGGTGGGTCCTCA-3' R: 5'-GTGTAGACTCCAGGGATGCCA-3'	542	58°C
KLK13	F: 5'-GGAGAAGCCCCACCCACCTG-3' R: 5'-CACGGATCCACAGGACGTATCTTG-3'	441	60°C
KLK14	F: 5'-CACTGCGGCCGCCCGATC-3' R: 5'-GGCAGGGCGCAGCGCTCC-3'	485	58°C
KLK15	F: 5'-CTACGGACCACGTCTCGGGTC-3' R: 5'-GACACCAGGCTTGGTGGTGTTG-3'	459	60°C

Results

Breast cancer cell lines and human breast cancer tissue express all components of the kinin pathway (KLK1, kininogens and kinin B_1R). Since evidence suggested that high levels of B_1R agonist, *des*[Arg^9]bradykinin circulate in patients with breast cancer (1), we investigated the presence of the kinin cascade proteins in both breast carcinomas and breast cancer cell lines (estrogen-sensitive: MCF-7, ZR-75-1 and T47D, and estrogen-insensitive: MDA-MB-231). Immunolabeling for KLK1, kininogens and B_1R was visualized in the four breast cancer cell lines (Figure 1 and 2B) and in breast tumor samples (Figure 2C). On laser scanning confocal microscopy, performed on permeabilized MCF-7 cells, KLK1 was distinctly visualized on the plasma membrane and in the cytoplasm (Figure 1C and D). Furthermore, when non-permeabilized, live cells were immunostained, the fluorescent label was observed in a punctate pattern on the cell membrane (Figure 1E). Kininogens and kinin B_1R were visualized as cytoplasmic and also as membrane-localized immunolabeling, respectively (Figure 1F and G).

To investigate the molecular expression of KLK1, H- and L-kininogens and B_1R in the four breast cancer cell lines, they were cultured and processed for RT-PCR and western blotting. In the RT-PCR experiments, mRNA transcripts appeared as distinct bands for *KLK1* (419 bp), H-kininogen (553 bp), L-kininogen (526 bp) and B_1R (214 bp) (Figure

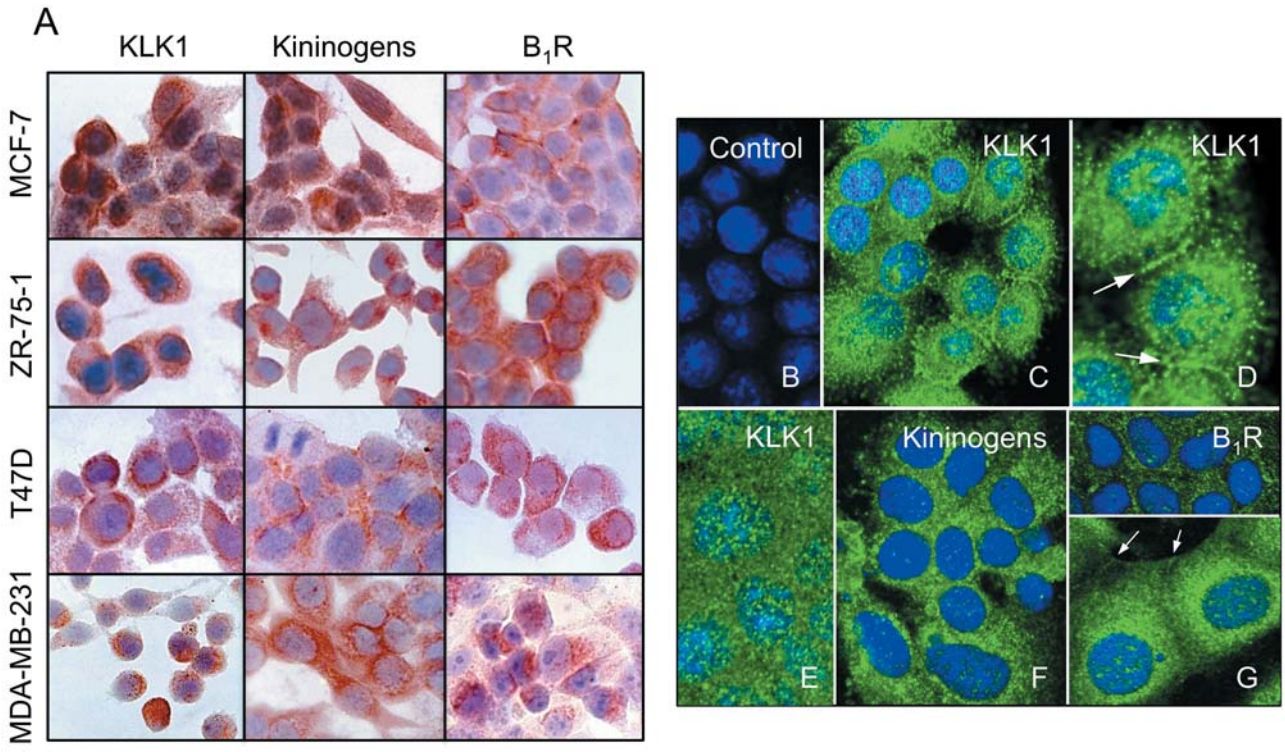


Figure 1. *Identification of kinin pathway components in estrogen-sensitive (MCF-7, T47D, ZR-75-1) and estrogen-insensitive (MDA-MB-231) breast cancer cells. A: Visualization of KLK1 (kinin-forming enzyme), kininogens (kinin-releasing substrates) and kinin B_1 receptor (B_1R) by immunocytochemistry. Cells were incubated overnight with primary antibodies and immunostained using the biotin-streptavidin method and visualizing peroxidase activity by diaminobenzidine tetrahydrochloride as chromogen. Nuclei were counterstained with hematoxylin. Original magnification: ×250. B-G: Confocal microscopy using methanol fixed, permeabilized (B-D; F, G) and live, non-permeabilized cells (E). The anti-kininogen antibody recognizes both H- and L-kininogens. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Arrows point out membrane localization of KLK1 or B_1R. Images representative of two independent experiments are shown. Original magnification, ×450.*

2A). The western blots gave a value for the relative molecular mass of each protein component of the kinin pathway (Figure 2B), which were in agreement with previous reports on other cell types and in plasma. Coincidently, tissue sections obtained from both DCIS and IDC expressed all components of the kinin pathway (Figure 2C). As previously described by us (6), kinin B_1R immunoreactivity was observed in both the cytoplasm and the cell membrane of neoplastic cells (Figure 2C). Omission of primary antibodies or co-incubation of sections with anti-B_1R antiserum and an excess of the same peptide used for immunization resulted in absence of immunoreactivity (Figure 2C, control).

Breast cancer cell lines and breast tumors express kallikrein-related peptidases. A variable expression of RT-PCR transcripts was shown in MCF-7 cells for the remaining kallikrein-related peptidases (Figure 3A) except for KLK7, -9 and -15, which were not detected even after using identical experimental conditions and the primers shown in the Table I. Omission of transcriptase reverse in the cDNA stage and amplification of the housekeeping gene, *GAPDH*, confirmed the quality of the cDNAs and lack of genomic contamination. In parallel experiments, KLKs were visualized by immunocytochemistry (Figure 3B) or western blotting (Figure 4A). In most cells, kallikrein-related peptidases stained strongly mainly in the cytoplasm, displaying a granular pattern (Figure 3B). Protein expression of various KLKs was also observed by western blotting at their corresponding relative molecular masses in two other breast cancer cell lines apart from MCF-7 cells. Figure 4A shows protein expression for KLK6, -10 and -11 in both estrogen-sensitive (MCF-7 and ZR-75-1) and -insensitive breast cancer cells (MDA-MB-231).

Immunostaining of tissue sections obtained from estrogen receptor-positive human breast tumors (DICS and IDC) confirmed the expression of KLKs, validating our results achieved using breast cancer cell lines. More than 80% (range=77-90%) of breast tumor cells stained for each KLK, independently of the stage of neoplasia or type of tumor (*i.e.* DCIS or IDC). Immunoreactivity for KLKs was mainly present in the cytoplasm of breast cancer cells and was more

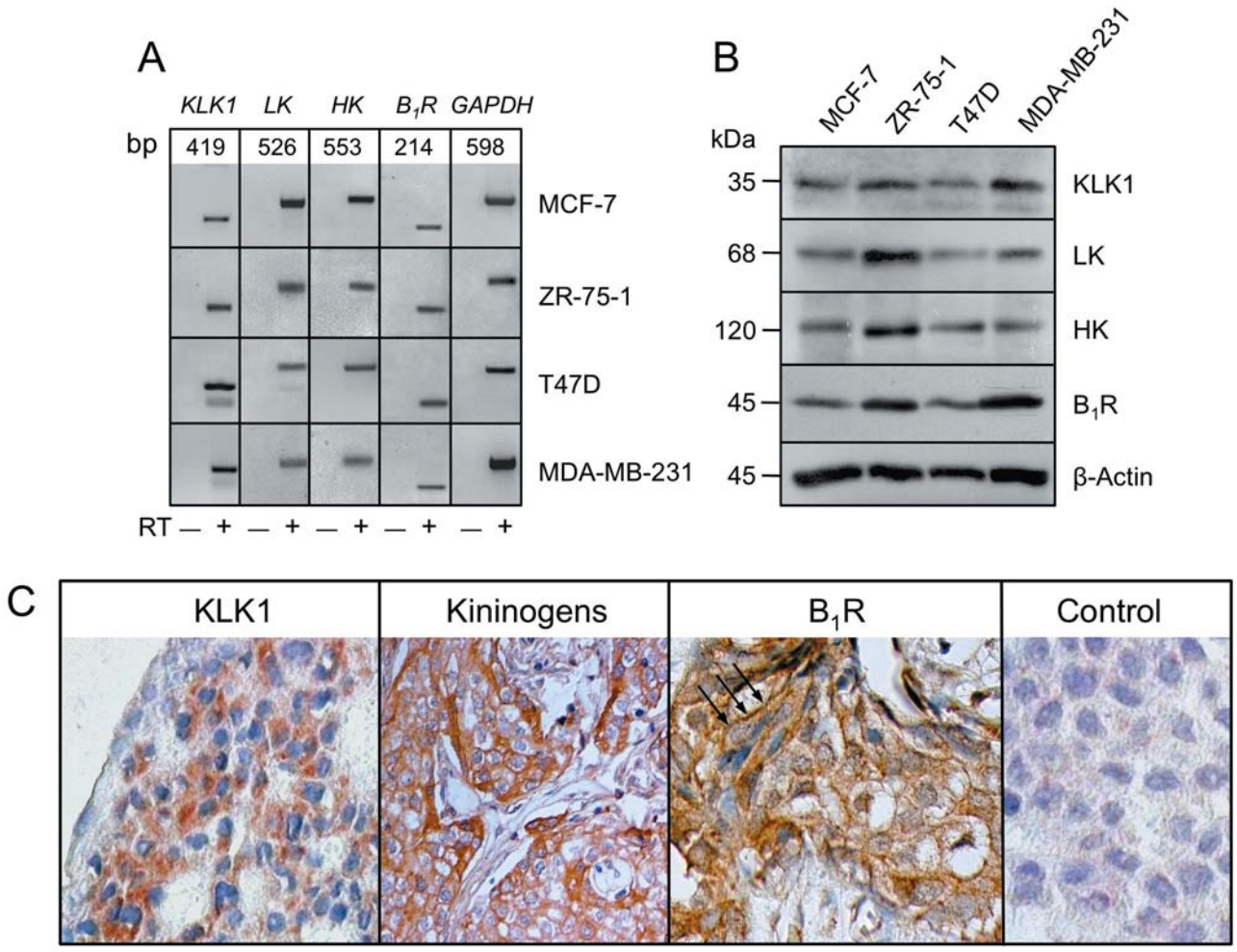


Figure 2. *Expression of true kallikrein (KLK1), kininogens and kinin B_1 receptor (B_1R) mRNA/protein in breast cancer cells and in human breast tumors. A: Transcripts for all components of the kinin pathway are expressed in estrogen-sensitive (MCF-7, T47D, ZR-75-1) and estrogen-insensitive (MDA-MB-231) breast cancer cells. After polymerase chain reaction (PCR) was performed, samples were analyzed by agarose gel electrophoresis. bp, base pairs; (–), samples in which reverse transcriptase (RT) was omitted; LK, L-kininogen; HK, H-kininogen. B: The corresponding protein expression levels were analyzed by western blotting. Total cell lysates transferred to Immobilon membranes were incubated with primary antibodies followed by the appropriated peroxidase-labeled secondary antibody. C: Immunohistochemical analysis of kinin pathway proteins in sections from paraffin included invasive ductal carcinomas. Tissue sections were incubated overnight with primary antibodies and immunostained using the biotin-streptavidin method, as described in Figure 1. Original magnification, ×400. Arrows point out membrane localization of B_1R. The images shown are representative of three independent experiments.*

intense in DCIS than in IDC. Antibody to kallikrein-related peptidases also immunolabeled nuclei of some tumor cells, but the percentage of stained nuclei was low and staining was less intense (data not shown). In Figure 4B, we show protein expression for KLK6, -10 and -11 in three cases of IDC.

B_1R signals expression of KLK6, -10 and -11 from estrogen-sensitive breast cancer cells. Expression of both mRNA and protein of various components of the kallikrein pathway in breast cancer cells strongly supports the view that kinin peptides may be formed in the tumor microenvironment and therefore may autoregulate functionality of tumor cells. To test this concept, experiments were designed to determine the levels of expression of *KLK6*, *-10* and *-11*, selected based on their carcinogenic biology, after stimulation of B_1R. MCF-7 cells stimulated with 10 nM LDBK for 2 h showed an increased relative expression of *KLK6* and *KLK11* transcripts when compared with untreated cells (Figure 5). By comparison, relative expression of *KLK10* transcripts diminished considerably (Figure 5).

Next, we examined whether these changes were reflected at the protein level. B_1R stimulation in MCF-7 or TD47

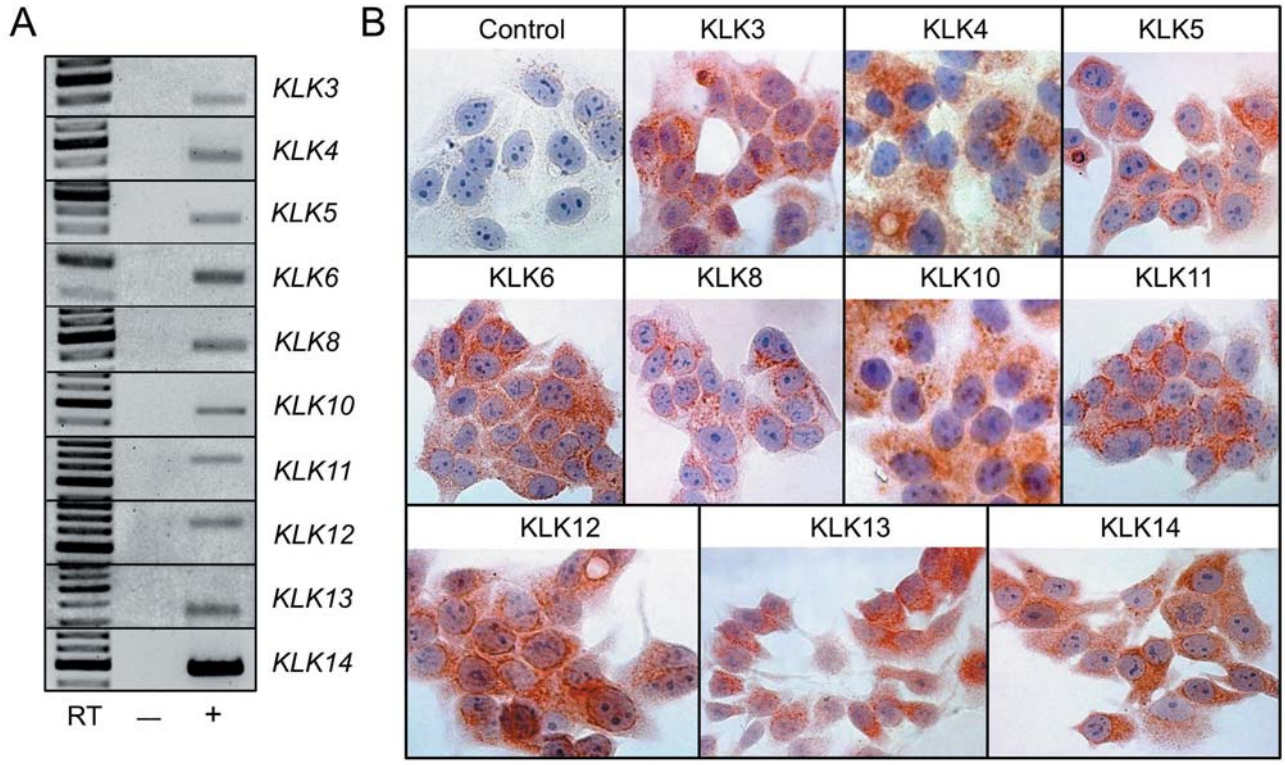
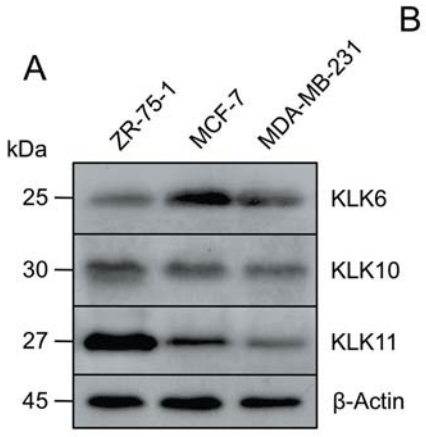
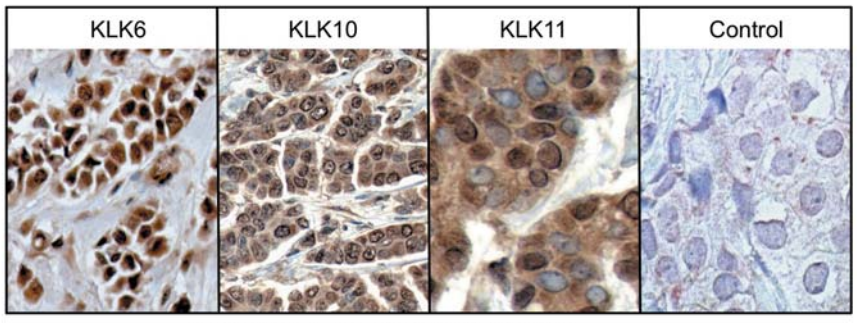


Figure 3. *Expression of kallikrein-related peptidases (KLK) mRNA/protein in MCF-7 breast cancer cells. A: Expression of various KLK mRNAs was determined by reverse transcriptase-polymerase chain reaction (RT-PCR). After PCR was performed, samples were analyzed by agarose gel electrophoresis. bp, base pairs; (–), samples in which reverse transcriptase was omitted. B: Visualization of each KLK by immunocytochemistry using the biotin/streptavidin-peroxidase method and visualizing peroxidase activity by diaminobenzidine tetrahydrochloride as chromogen. Nuclei were counterstained with hematoxylin. Original magnification, ×600. Representative images of three independent experiments are shown.*





Percentage of cytoplasmic staining

Breast tumor	KLK6	KLK10	KLK11
DCIS	81 + 0.01	85 + 0.05	85 + 0.03
IDC	83 + 0.06	84 + 0.06	81 + 0.07

Figure 4. *Identification of kallikrein-related peptidases (KLK) 6, 10 and 11 in estrogen-sensitive and -insensitive breast cancer cells and in human breast tumors. A: Proteins present in total cell lysates were separated by polyacrylamide gel electrophoresis and transferred onto Immobilon membranes that were incubated with primary antibodies followed by the appropriate peroxidase-labeled secondary antibody and a chemiluminescence kit. B: Representative images of estrogen-positive invasive ductal carcinoma (IDC) immunostained for KLK6, KLK10 and KLK11 are shown. Tissue sections were incubated overnight with primary antibodies and immunostained using the biotin/streptavidin-peroxidase method as described in Figure 1. DCIS, Ductal carcinoma in situ. Original magnifications KLK6 and KLK10, ×200; KLK11 and control, ×500.*

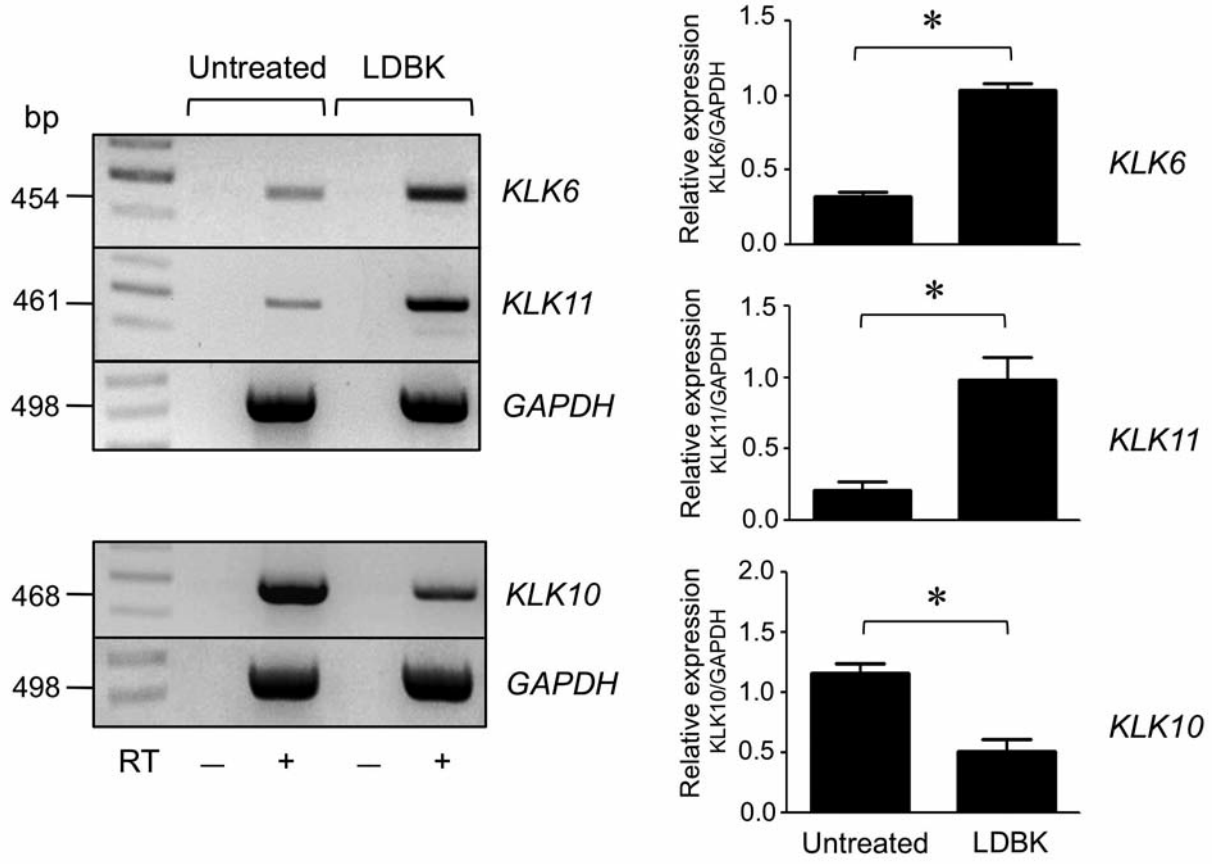


Figure 5. *The kinin B_1 receptor (B_1R) agonist modulates the expression of kallikrein-related peptidases (KLK) 6, 10 and 11 transcripts in estrogen-sensitive breast cancer cells. MCF-7 cells were stimulated with 10 nM LDBK for 2 h and total RNA was extracted. The cDNAs were amplified by PCR and transcripts were analyzed by agarose gel electrophoresis. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; bp, base pairs; (–), samples in which reverse transcriptase (RT) was omitted. Values represent the mean±SE (n=3). *p<0.05 between untreated and stimulated cells.*

breast cancer cells increased protein levels of both KLK6 and KLK11 in a period of 4 to 8 h (Figures 6 and 8). Transfection of cells with a *KLK6* siRNA abolished the effect produced by LDBK and at the same time reduced the basal level of KLK6 (Figure 9C). In contrast, in the same cells, B_1R stimulation produced a decrease in the expression of KLK10 at concentrations as low as 1 nM LDBK (Figure 7). These effects are clearly the result of activation of B_1R since a specific *B_1R* siRNA completely reversed the effect produced by LDBK (Figures 6B and 7B). Out of these three kallikreins, we only performed a functional evaluation of KLK6, using a Matrigel assay to analyze the invasive capacity of MCF-7 cells.

B_1R activates secretion of KLK1 and KLK6 and matrigel invasion by MCF-7 cells. The question whether kinin B_1R agonists cause the secretion of KLK1 (kinin-forming enzyme) and KLK6 proteases from cultured breast cancer cells was tested experimentally. When MCF-7 cells were stimulated by LDBK, a significant amount of KLK1 was secreted into the culture medium and similarly, there was secretion of KLK6, when assessed by western blotting (Figure 9A). Additionally, the secretion of KLK6 by MCF-7 cells was confirmed using a specific ELISA (Figure 9A). Conditioned media of unstimulated and LDBK-stimulated MCF-7 cells were used to analyze the invasive capacity of these cells on inserts covered with Matrigel (Figure 9B). Conditioned medium of LDBK-stimulated cells, added to the upper chamber, increased the number of invading MCF-7 cells when compared to cells challenged with conditioned medium of unstimulated cells or MCF-7 cells that had been previously treated with a *KLK6* siRNA. MCF-7 cells treated with the *KLK6* siRNA showed reduced basal levels of KLK6 and did not increase KLK6 levels in response to stimulation with LDBK (Figure 9C). The number of invading cells produced by conditioned medium of LDBK-stimulated MCF-7 cells was higher than that observed in MCF-7 cells that were directly stimulated with 20 ng/ml EGF (Figure 9B).

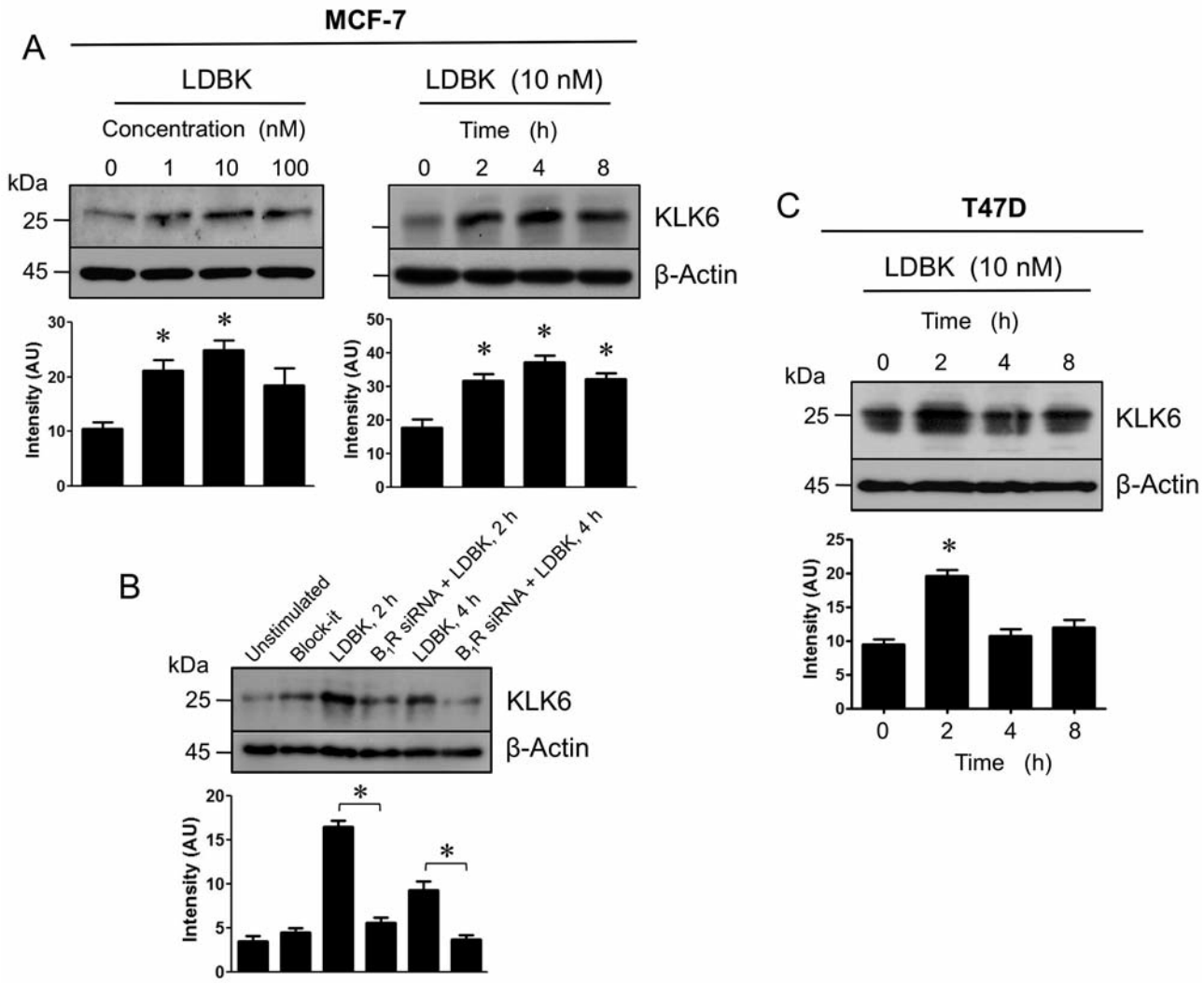


Figure 6. *The kinin B_1 receptor (B_1R) agonist increases the expression of kallikrein-related peptidase 6 (KLK6) protein in estrogen-sensitive breast cancer cells in a dose- and time-dependent manner. A, C: MCF-7 and T47D cells were stimulated for 2 h with different concentrations of the B_1R agonist Lys-des[Arg^9]bradykinin (LDBK) or for different periods of time with 10 nM LDBK. Proteins were separated by polyacrylamide gel electrophoresis and transferred onto Immobilon membranes and then immunoprinted for KLK6. Representative western blots of three independent experiments (n=3) are shown. *p<0.05 Between unstimulated and stimulated cells. B: The changes in expression of KLK6 protein were reversed by a B_1R siRNA. Transfected and non-transfected cells were directly stimulated with 10 nM LDBK for 2 h or 4 h and immunoreactivity to KLK6 protein was determined by western blotting. Block-it is a commercial unrelated fluorescent siRNA. Results are shown as mean±SE (n=3). *p<0.05 between transfected and non-transfected cells.*

Discussion

Expression of all members of the kinin cascade in breast cancer cell lines and breast tumor tissue strongly suggests that kinins may be generated in the tumor microenvironment. The occurrence of KLK1 (kinin-forming enzyme) and kininogens at mRNA and protein levels in several breast cancer cell lines gives credence to the possibility that breast cancer cells secrete the kinin-forming kininogenases and kininogen substrates to form kinins extracellularly, which upon autocrine activation of B_1R would induce their proliferation, release of metalloproteases (MMPs) and invasion (6,15). Our previous experiments had shown that activation of B_1R enhances proliferation of MCF-7 and ZR-75-1, two estrogen-sensitive breast cancer cells, and activates the extracellular signal-regulated kinases 1/2 signaling pathway (6) causing the release of MMP2 and MMP9, essential for extracellular matrix degradation (15). Relevant to this situation is the presence of binding sites for B_1R agonists in breast tumors and the fact that the B_1R agonist

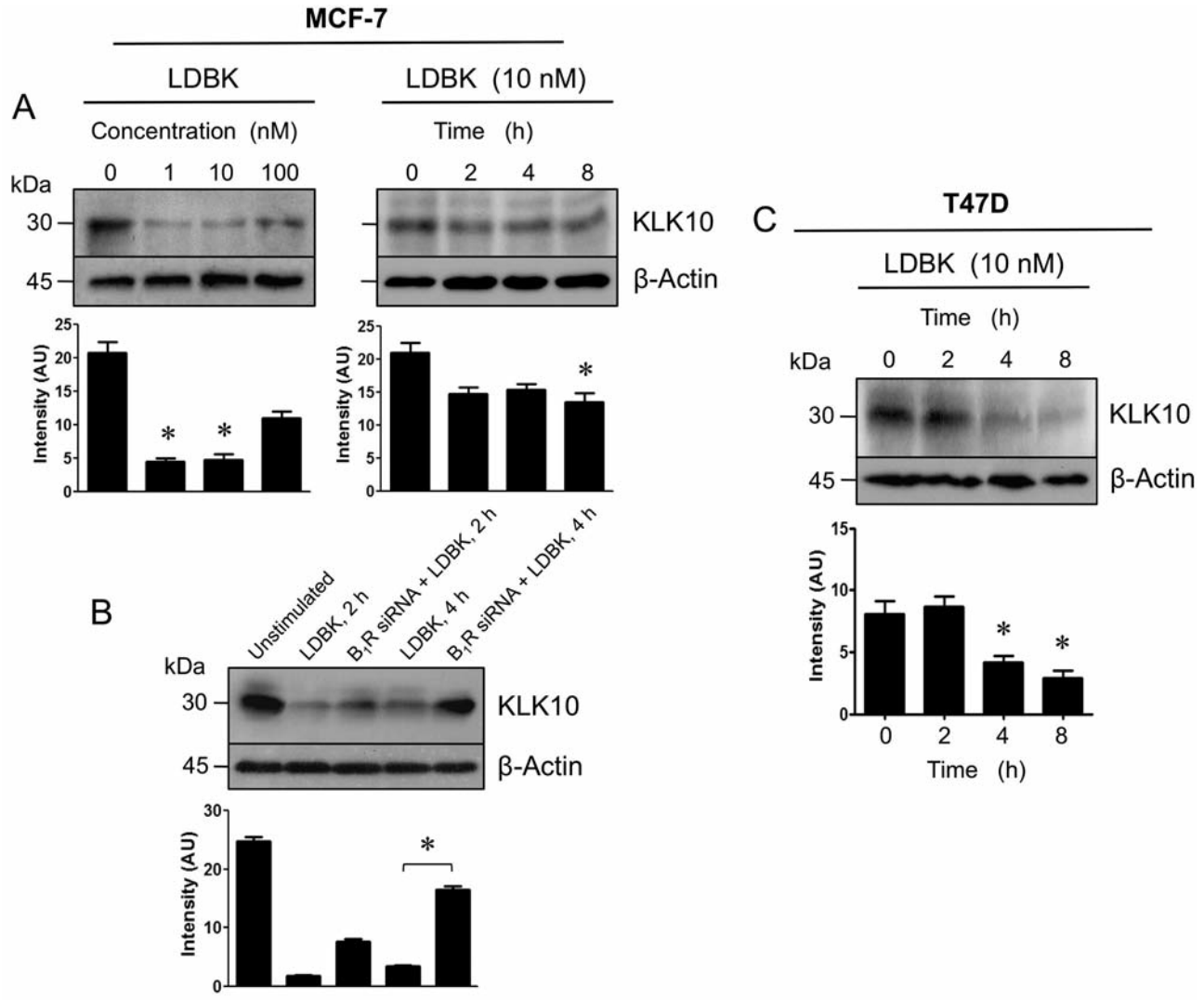


Figure 7. *The kinin B_1 receptor (B_1R) agonist reduces the expression of kallikrein-related peptidase 10 (KLK10) protein in estrogen-sensitive breast cancer cells. A, C: MCF-7 and T47D cells were stimulated for 2 h with different concentrations of Lys-des[Arg^9]bradykinin (LDBK) or for different times with 10 nM of the agonist. Levels of KLK10 protein were determined by western blotting as described in the Materials and Methods. Representative images of three independent experiments are shown. Values represent the mean±SE (n=3). *p<0.05 between unstimulated and stimulated cells. B: The changes in expression of KLK10 protein were reversed by a B_1R siRNA. Transfected and non-transfected cells were directly stimulated with 10 nM LDBK for 2 h or 4 h and immunoreactivity to KLK10 protein was determined. Representative western blots of two independent experiments (n=2) are shown. Values correspond to mean±SE (n=3). *p<0.05 between transfected and non-transfected cells.*

des-[Arg^9]bradykinin has been reported to circulate at higher levels in the serum of patients with breast cancer when compared to healthy individuals (1, 6). Herein, we have shown that the kinin-generating enzyme KLK1 and its substrates, kininogens, are also expressed in breast cancer cells. Moreover, the augmented secretion of KLK1 and the presence of kininogens in these cells may contribute to explain the generation of high levels of B_1R agonist reported in patients with breast cancer. Formation of kinins in the breast tumor microenvironment is even more likely due to the presence of the kinin cascade proteins on functional cells such as neutrophils, fibroblasts and endothelial cells, thereby supporting a role as modulators of breast cell carcinogenesis (5, 17). Furthermore, the KLK1 inhibitor FE999024 has been shown to reduce invasiveness of KLK1-secreting MDA-MB-231 cells through Matrigel, and of the lung interstitium in an animal model inoculated with these cells (18). The current study confirms that most of the kallikrein-related peptidases are expressed in human breast tumors (not shown) and shows that they are also expressed in cell lines arising from those

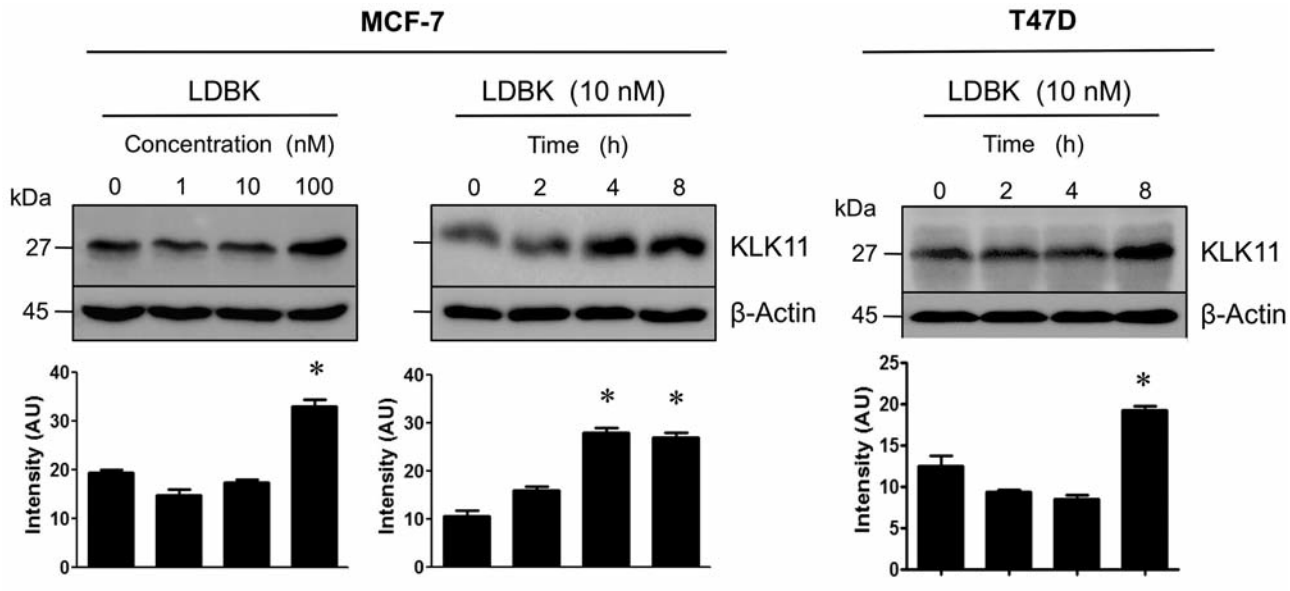


Figure 8. *The kinin B_1 receptor (B_1R) agonist increases the expression of kallikrein-related peptidase (KLK11) protein in estrogen-sensitive breast cancer cells. MCF-7 and T47D cells were stimulated for 2 h with different concentrations of Lys-des[Arg^9]bradykinin (LDBK) or different periods of time using 10 nM of the peptide. Levels of KLK11 protein were determined by western blotting as described in the Materials and Methods. Representative images of three independent experiments are shown. Values represent the mean±SE (n=3). *p<0.05 between unstimulated and stimulated cells.*

tumors. Functional dynamics of kallikrein-related peptidases in cancer cells involves degradation of extracellular matrix proteins in order to enhance invasion of normal tissue. Furthermore, some of these enzymes (KLK3 and KLK4) initiate epithelial–mesenchymal transition of prostate cancer cells, a process that permits epithelial cancer cells to undergo transformation to mesenchymal-like phenotypes, thereby enhancing invasiveness and metastasis (19, 20).

Since the B_1R has been shown to modulate functionality of breast cancer cells (6, 15, 17) and circulating levels of B_1R agonist are increased in patients suffering from this type of neoplasia, we explored possible functional changes in the levels of KLK6 (associated with the invasive capacity of cancer cells lines), KLK10 (considered a tumor suppressor) and KLK11 (associated with increased cell proliferation) in estrogen-sensitive breast cancer cell lines following stimulation by a B_1R agonist.

The protein encoded by the *KLK6* gene is a highly active trypsin-like serine protease that selectively cleaves at arginine residues (21-23). Even when *KLK6* was proposed as a tumor-suppressor gene because its re-expression in MDA-MB-231 cells significantly reduced the ability of these cells to form tumors in nude mice (21, 24), this effect was not observed in a subset of human breast tumors which overexpressed KLK6 (24). In fact, when expressed, KLK6 degrades most extracellular matrix proteins (22, 23), thereby enhancing migration of cancer cells, probably at the beginning of cancer progression. Our experiments show that stimulation of two estrogen-sensitive breast cancer cell lines with the B_1R agonist LDBK produces an increase in cellular levels of KLK6. Furthermore, LDBK-stimulated MCF-7 cells secrete more KLK6 into the incubation medium than non-stimulated cells. Secretion of KLK6 may favor a more invasive phenotype of breast cancer cells as shown by our invasion assay using LDBK-conditioned media and transwell chambers covered with matrigel. Participation of KLK6 in this process was highlighted by a *KLK6* siRNA that simultaneously reduced the cellular levels of KLK6 and the number of invading cells when compared with non-transfected cells. The presence of KLK6 in conditioned medium from other breast cancer cell lines such as BT474 (non-invasive) and MDA-MB-468 (metastatic origin) has been reported (25).

Evidence suggests that KLK10 works as a tumor-suppressor protein because it is expressed in normal breast epithelial cells, but is reduced in breast cancer cell lines and during cancer progression (26). Evaluation of epigenetic hypermethylation indicated that KLK10 was a prognostic marker for early-stage malignancy in patients with breast cancer (26-28). Alternate splicing of this gene results in multiple transcript variants encoding the same protein. Evidence suggests that the CpG island sites in exon 3 of *KLK10* become progressively methylated as cancer progresses causing down-regulation of the gene with the

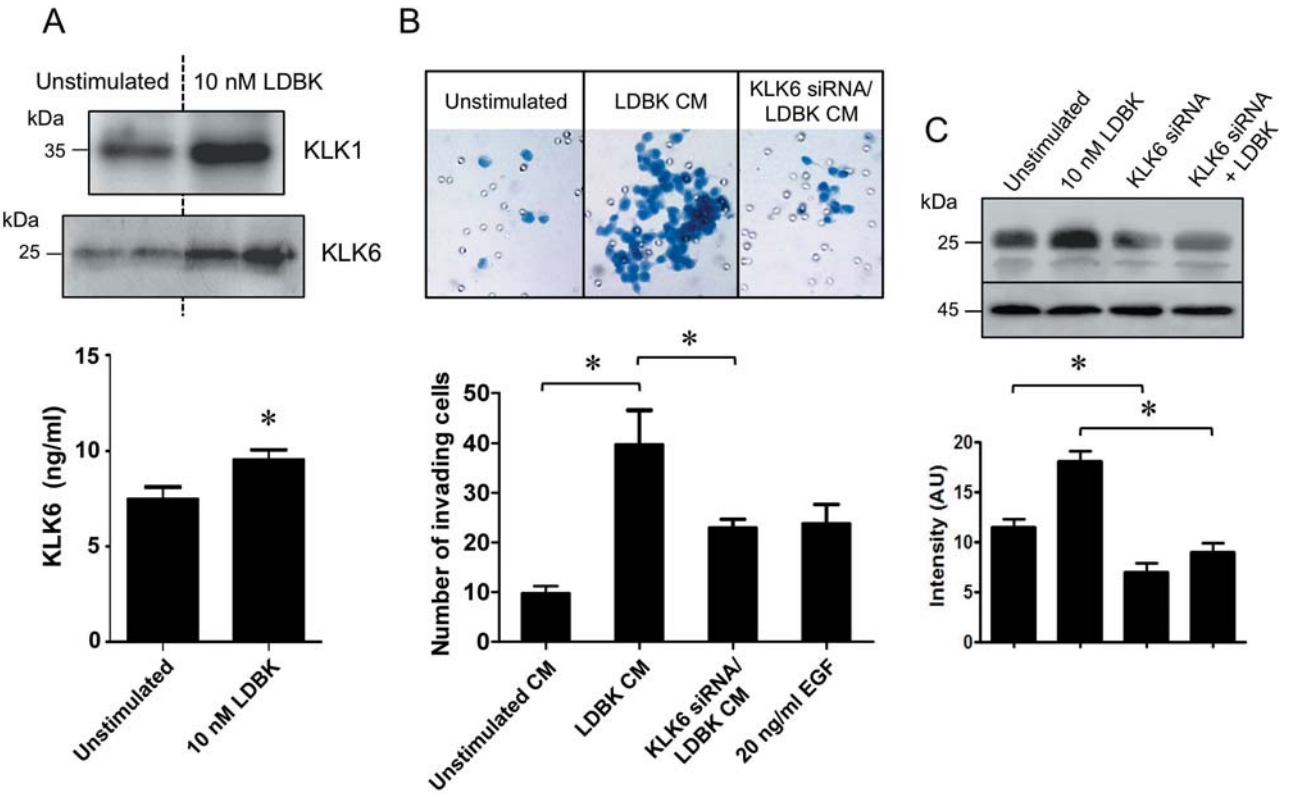


Figure 9. *The kinin B_1 receptor (B_1R) agonist enhances the secretion of true tissue kallikrein (KLK1) and kallikrein-related peptidase 6 (KLK6) into the culture medium and favors the invasive capacity of estrogen-sensitive breast cancer cells. A: MCF-7 cells were cultured and then synchronized in the absence of bovine serum for 48 h prior to stimulation with 10 nM Lys-des[Arg^9]bradykinin (LDBK) for 3 h. Subsequently, conditioned media (CM) were concentrated and KLK1 and KLK6 proteins visualized by western blot or a specific KLK6 enzyme linked immunoassay. B: To assess invasive capacity, MCF-7 cells were seeded on top of Matrigel-coated inserts and allowed to invade toward 20% fetal bovine serum (chemoattractant). The CM obtained from unstimulated or LDBK-stimulated (10 nM for 3 h) MCF-7 cells was added to the upper chamber. After 24-48 h, the experiment was stopped and the number of cells was determined. Controls were performed by adding 20 ng/ml epidermal growth factor (EGF) to the upper chamber instead of CM (positive control), or by using cells transfected with a commercial KLK6 siRNA. C: The effect of KLK6 siRNA on KLK6 protein levels was confirmed by western blotting. Results correspond to mean±SE of three independent experiments (n=3). *$p<0.05$.*

consequent increase of tumorigenesis. Because of promoter methylation, *KLK10* is frequently rendered ineffective and undetectable in some IDCs (26-28). Moreover, transfection of *KLK10* into KLK10-negative breast cancer cells reduces their tumorigenicity (28). Stimulation of breast cancer cells with LDBK reduced the expression of *KLK10* transcripts and protein, in agreement with the already described effects of this B_1R agonist, supporting the malignant conduct of breast cancer cells (6, 15).

Expression of KLK11 has been detected in both breast cancer tissue and in non-cancerous mammary glands, but significantly higher expression is observed in histological grade I/II and even greater in grade III breast tumors (29). As observed for KLK6, stimulation of estrogen-sensitive breast cancer cells with the B_1R agonist increased the expression of KLK11, an effect that may increase cell proliferation and survival, since KLK11 induces the release of insulin-like growth factor type I by degrading insulin-like growth factor binding protein-3 (29). The increased proliferative rate of estrogen-sensitive breast cancer cells stimulated with the B_1R agonist seen previously by us (6) may be associated with the action of this kallikrein-related peptidase.

In summary, we showed that the B_1R agonist reduces KLK10, enhances expression of KLK6 and KLK11, and augments the secretion of KLK1 and KLK6 from estrogen-sensitive breast cancer cells. An augmented secretion of KLK1 and the presence of kininogens in these cells could contribute to explain the generation of high levels of B_1R agonist reported in patients with breast cancer (Figure 10). Our study highlights the importance of B_1R agonists in the progression of breast cancer and opens a new door for the search of more effective antagonists that in conjunction to traditional therapeutic procedures may be used to target breast cancer.

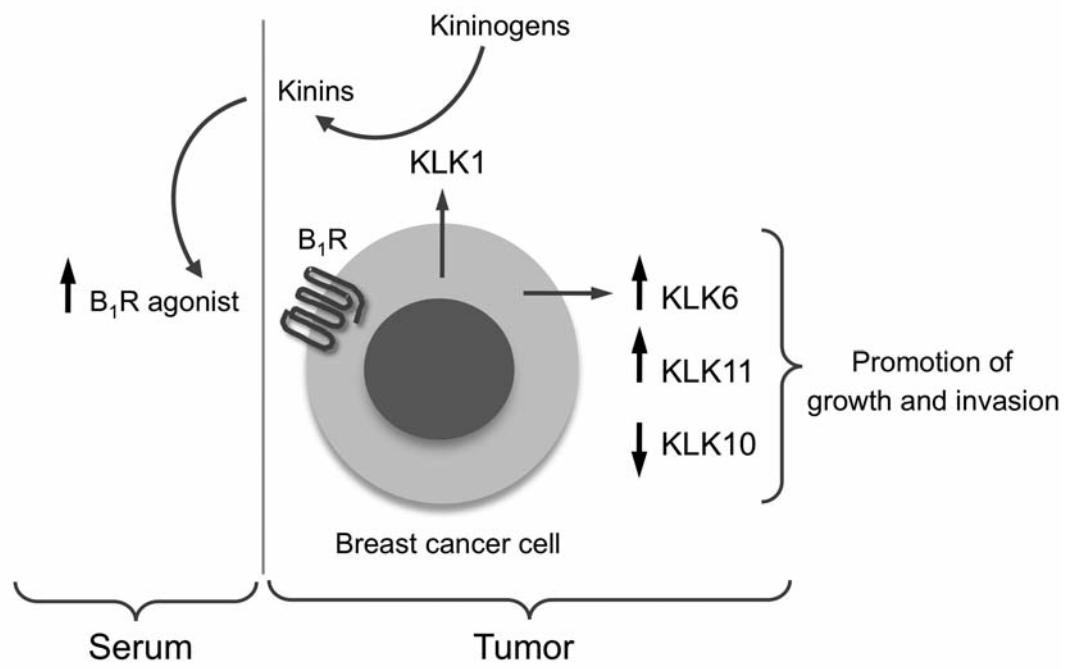


Figure 10. *Schematic representation of the kinin B_1 receptor (B_1R) agonist-related mechanism potentially leading to modulation of kallikrein-related peptidase (KLK) -6, -10 and -11 in estrogen-sensitive breast cancer cells. Because breast cancer cells express all components of the kinin system, kininogenase activity of true tissue kallikrein (KLK1), released to the tumor microenvironment, on kininogens will result in kinin formation that may in turn increase level of B_1R agonists. By stimulating B_1R, these agonists may modify the levels of kallikrein-related peptidases (i.e. reduce KLK10 and increase KLK6 and KLK11) in breast cancer cells, favoring growth and tumor invasion.*

Declaration of Interest

The Authors involved in this study have no potential conflicts of interest to disclose and they have received no payment in preparation of this article.

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