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Activation of kinin B_1 receptor increases the release of metalloproteases-2 and -9 from both estrogen-sensitive and -insensitive breast cancer cells

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ABSTRACT

The kinin B_1 receptor (B_1R) agonist Lys-des[Arg^9]-bradykinin (LDBK) increases proliferation of estrogen-sensitive breast cancer cells by a process involving activation of the epidermal growth factor receptor (EGFR) and downstream signaling via the ERK1/2 mitogen-activated protein kinase pathway. Here, we investigated whether B_1R stimulation induced release of the extracellular matrix metalloproteases MMP-2 and MMP-9 via ERK-dependent pathway in both estrogen-sensitive MCF-7 and -insensitive MDA-MB-231 breast cancer cells. Cells were stimulated with 1–100 nM of the B_1R agonist for variable time-points. Western blotting and gelatin zymography were used to evaluate the presence of MMP-2 and MMP-9 in the extracellular medium. Stimulation of B_1R with as little as 1 nM LDBK induced the accumulation of these metalloproteases in the medium within 5–30 min of stimulation. In parallel, immunocytochemistry revealed that metalloprotease levels in the breast cancer cells declined after stimulation. This effect was blocked either by pre-treating the cells with a B_1R antagonist or by transfecting with B_1R-specific siRNA. Activation of the ERK1/2 pathway and EGFR transactivation was required for release of metalloproteases because both the MEK1 inhibitor, PD98059, and AG1478, an inhibitor of the EGFR-tyrosine kinase activity, blocked this event. The importance of EGFR-dependent signaling was additionally confirmed since transfection of cells with the dominant negative EGFR mutant HERCD533 blocked the release of metalloproteases. Thus, activation of B_1R is likely to enhance breast cancer cells invasiveness by releasing enzymes that degrade the extracellular matrix and thereby favor metastasis.

1. Introduction

Kinins are bioactive peptides released by the enzymatic action of plasma kallikrein (KLKB1) or tissue kallikrein (hK1, *KLK1*) on circulating or locally synthesized high molecular weight substrates known as kininogens, and therefore these two proteases are referred to as kininogenases [1–3]. The human tissue kallikrein gene family (*KLK1* to *KLK15*) encodes a group of 15 proteases (hK1 to hK15),

Abbreviations: MMPs, metalloproteases; B_1R, Bradykinin B_1 receptor; B_2R, Bradykinin B_2 receptor; EGF, Epidermal growth factor; EGFR, Epidermal growth factor receptor; LDBK, Lys-des[Arg^9]-bradykinin; MAPK, Mitogen-activated protein kinases; ERK1/2, Extracellular-regulated kinases 1/2.

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several of which have been implicated in cancer-related processes [3]. Nonetheless, of the 15 members of the kallikrein-related peptidase family, only hK1 tissue kallikrein has the ability to release the kinin moiety from kininogen substrates [2,3]. Previous studies have demonstrated that the kinin-forming enzyme, hK1 tissue kallikrein, is expressed in the breast and is also present in milk and breast cyst fluids [4]. So far, the presence of immunoreactive hK1 tissue kallikrein has been described in a few cases of ductal breast carcinomas [5,6].

Kinins bind to two types of G protein-coupled rhodopsin-like receptors known as B_1 (B_1R) and B_2 (B_2R) receptors which upon stimulation trigger intracellular signaling events that include activation of phospholipase C, generation of inositol 3-phosphate, Ca^{2+} mobilization and arachidonic acid release [1–3]. Whereas the B_2R is constitutively expressed in many cells of the human body, the B_1R is generally latent but up-regulated during inflammation or by members of the cytokine family such as IL-1β and TNF-α [7]. The B_2R is activated by bradykinin, its natural ligand, whereas the B_1R is switched on by analogues that lack the Arg^9 present at the carboxy terminus of the bradykinin or Lys-bradykinin molecules [1].

So far, only a few studies have evaluated the role of kinin receptors as well as the underlying molecular mechanisms that trigger their activation in breast cancer cells. B_2R stimulation increases the proliferation of normal and tumoral breast cells through signaling pathways that include PKC activation, phosphorylation of extracellular signal-regulated kinases 1/2 (ERK1/2) of the mitogen-activated protein kinases (MAPK) family, and partial epidermal growth factor receptor (EGFR) transactivation [8–10]. We together with other researchers have reported on the expression and activation of the B_1R in breast tumors and in two breast cancer cell lines [11–13]. B_1R binding sites are detected not only in invasive ductal carcinomas but also in ductal carcinomas *in situ* and in benign lesions of the breast like fibroadenomas [11,13]. Similarly, two estrogen-sensitive breast cancer cell lines, MCF-7 and ZR-75, express both B_1R mRNA and protein. Once stimulated, B_1R triggers cell proliferation at nanomolar concentrations of the ligand [13]. Interestingly, experiments using signal transduction inhibitors revealed that the proliferative effects depend on EGFR activity and subsequent ERK1/2 MAPK phosphorylation [13]. So far, there are no reports on the behavior of the kinin B_1R in other types of breast cancer cells.

Matrix metalloproteases (MMPs) are an important group of proteolytic enzymes that are involved in carcinogenesis, namely tumor invasion, metastasis, and angiogenesis [14,15]. Among the existing human MMPs, MMP-2 (gelatinase A/Mr 72,000, type IV collagenase) and MMP-9 (gelatinase B/Mr 92,000, type IV collagenase) are extensively glycosylated enzymes that are abundantly expressed in various malignant tumors [16]. MMP-2 plays an important role in breast cancer and has been detected in early stages of breast carcinoma but not in normal resting breast tissue [17]. In addition, analysis of gene expression in human tumors has linked presence of MMP-9 to poor prognosis in breast cancer [18]. Both MMPs are considered to be key enzymes for tumor invasion and metastasis, because they degrade type IV collagen, the major protein component of basement membranes that provides the molecular network support for all epithelial tissues [19]. Destruction of basement membrane is a prerequisite for breast cancer cells to invade the connective tissue that surrounds the tumoral breast epithelia. Gelatinases can also hydrolyze other components of the extracellular matrix such as elastin, vitronectin, and aggrecan [20]. In fact, the presence in cancerous tissues of active MMP-2 is indicative of an increased risk of metastasis and recent studies suggest that MMP-2 should be considered as a new predictive and prognostic biomarker of breast carcinoma [21]. Furthermore, the activation rate of pro-MMP-2 and the formation of active MMP-2 are being used as indicators of cancer metastasis [22]. Functional regulation of the MMPs occurs at multiple levels. Expression of the different MMP mRNAs is under tight, cell type-dependent control that is associated with specific responses. MMP transcripts are generally present at low levels, but these rise rapidly when the tissues undergo remodeling, such as during wound healing, inflammation or cancer. The activation of transcription factors that control the expression of the different MMPs, is mediated by three classes of MAPKs [14,23]. The initially produced latent form of the enzyme is then activated by either another MMP or by serine-proteases that degrade the pro-domain [24].

In the current study, we investigated whether activation of B_1R by its natural agonist, caused the release of MMP-2 and MMP-9 from estrogen-sensitive MCF-7 and estrogen-insensitive MDA-MB-231 breast cancer cell lines. Furthermore, the possibility that release of MMP-2 and MMP-9 involved transactivation of EGFR and then activation of the ERK1/2 MAPK signaling pathway was also evaluated.

2. Material and methods

The experiments delineating the cellular and molecular mechanisms of Lys-des[Arg^9]-bradykinin (LDBK) function utilized either MCF-7 breast cancer cells cultured in semiconfluent monolayers or multicellular spheroids. This 3D culture model will in the future allow us to investigate the interaction of breast cancer cells with other cellular components of the tumor microenvironment. Similar experiments were performed on the estrogen-insensitive MDA-MB-231 breast cancer cell line using semiconfluent monolayers.

2.1. RT-PCR analysis

Expression of B_1R mRNA in MDA-MB-231 breast cancer cells was assessed by extracting total RNA (Promega, USA). Then cDNAs were synthesized using random hexamer primers and ThermoScript reverse transcriptase (Promega, USA) in a 20 μl reaction mixture containing reaction buffer (250 mM Tris–acetate pH 8.4, 375 mM potassium acetate, 40 mM magnesium acetate and 5 mM di-thiothreitol), 0.5 mM dNTP, 2.5 μM random hexamer primers and 2 μg of total RNA. The duration of the reaction was 1 h at 52 °C. Amplification of cDNA by PCR was performed using

25 μl of reaction mixture containing PCR buffer (20 mM Tris–HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$), 0.2 mM dNTP, 1 U of *Taq* polymerase (Invitrogen, USA), 0.1 μM specific primers (Biosource International, Camarillo, CA, USA) and 2 μl of cDNA in a GeneAmp 2400 thermocycler (Perkin Elmer, Boston MA, USA). The primers used for the B_1R 5′-TTCTTATTCCAGGTGCAAGCAG-3′ (sense) and 5′-CTTTCCTA-TGGGATGAAGATAT-3′ (antisense) generated a fragment of 214 bp. The house keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was amplified using the primers: 5′-GCAGGGGGGAGCCAAAAGGG-3′ (sense) and 5′-TGCCAGCCCCAGCGTCAAAG-3′ (antisense) to yield a fragment of 566 bp. PCR conditions were: 95 °C for 5 min, *Start Hot* at 80 °C for 2 min, followed by denaturation at 95 °C for 30 s, annealing at 55 °C for 40 s, and extension at 72 °C for 30 s during 35 cycles. PCR products were analyzed after separation by 1% agarose gels containing 20 μg/ml ethidium bromide for UV detection.

Changes in MMPs expression were evaluated by real time PCR of cDNAs from MCF-7 cells and normalized with respect to the housekeeping β-actin gene that showed the lowest variability. PCR was performed on a Mx3000P® (Stratagene, USA) using Brilliant II SYBR Green qPCR Master Mix (Stratagene, USA) according to the manufacturer's instructions and using the following primer pairs: MMP2, 5′-CCTCGTATACCGCATCAATCT-3′ (reverse) and 5′-CCGT-GGTGAGATCTTCTTCT-3′ (forward); MMP-9, 5′-CTTGCTG-CTGCTAAAGTTCG-3′ (reverse) and 5′-TTCATCTTCCAAGG-CCAATC-3′ (forward); β-actin, 5′-TCAAACATGATCTGGGT-CAT-3′ (reverse) *and* 5′-CCCAGGCACCAGGGCGTGAT-3′ (forward). The general amplification protocol (45 cycles) was set as follows: initial denaturation for 10 min at 95 °C; denaturation for 30 s at 95 °C; specific primer annealing at 54 °C for 30 s and amplification at 72 °C for 30 s. The settings for the melting curve protocol were as follows: denaturation at 95 °C; cooling to 60 °C (5 °C above the primer annealing temperature) and heating to 95 °C (speed 0.1 °C/s). The fluorescence emitted by double-stranded DNA-bound SYBR-Green was measured once at the end of each additional heating step, and then continuously during the melting curve program. Product specificity was confirmed by melting curve analysis and product size. Efficiency for MMP-2, MMP-9 and β-actin was 103%, 103% and 98% respectively. The results obtained were normalized respect to β-actin expression considering the efficiency recorded for each gene and expressed as fold changes respect to the unstimulated group.

2.2. Cell culture and construction of multicellular spheroids

The estrogen-sensitive cell line MCF-7 was cultured in phenol red-free Dulbecco's modified Eagle's medium (DMEM) and MDA-MB-231 cells in DMEM-F12 containing 10% fetal bovine serum. Then cells were cultivated in the absence of fetal bovine serum for 24 h prior to stimulation with the B_1R agonist LDBK (Sigma Aldrich, USA). Confluent monolayers of MCF-7 and MDA-MB-231 cells were treated with 0.25% trypsin/1 mM EDTA (Invitrogen) and viability was assayed by trypan blue exclusion. To prepare defined size/cell number spheroids, MCF-7 cells were suspended in phenol red -free DMEM containing 20% (w/v) methocel (Sigma) and seeded at 5×10^5 cells/well in non-adherent round bottom 96-well plates (Becton Dickinson, USA) [25]. Under these conditions all suspended cells contribute to the formation of a single spheroid per well. The spheroids were cultured for 2 days and thereafter they were stored in DMEM/methocel for 12 h prior to each experiment. The MDA-MB-231 cells were used only as semiconfluent monolayers.

2.3. Western blotting

Partially purified cell membranes were prepared and used to estimate the expression and molecular characteristics of the B_1R protein in MDA-MB-231 breast cancer cells as previously described [13]. Stimulation of breast cancer cells with the B_1R agonist was stopped by homogenization with cold RIPA buffer (50 mM Tris–HCl pH 7.4 containing 150 mM NaCl, 0.25% deoxycholic acid, 1% Nonidet P40, 1 mM EDTA, 1 mM Na_3VO_4, 250 μg/ml *p*-nitrophenyl phosphate, 1 mM phenylmethane-sulphonyl fluoride, 1 mM NaF, 1 μg/ml leupeptin, 1 μg/ml pepstatin and 1 μg/ml aprotinin). Proteins were separated by electrophoresis and then transferred onto immobilon P membranes

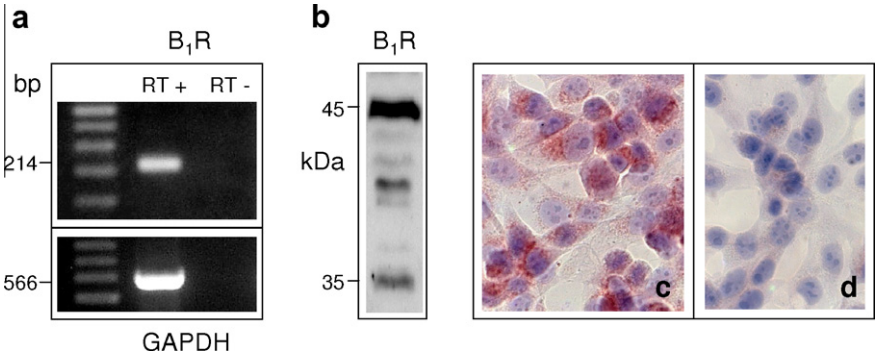

Fig. 1. MDA-MB-231 breast cancer cells express the bradykinin B_1R. (a) PCR was carried out and samples were analyzed by agarose gel electrophoresis. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; bp, base pairs. (b) Detection by Western blotting of the B_1R in crude membranes preparation. (c) Detection of B_1R by immunocytochemistry using the same anti-peptide antibody and the biotin/streptavidin-peroxidase method. (d) Cells were incubated with non-immune serum of same origen and at the same dilution.

(Millipore, USA). Filters were incubated with anti-MMP-2, anti-MMP-9 or anti-phospho ERK1/2 MAPK antibodies (Cell Signaling Technology Inc., USA) and with a peroxidase-labeled secondary antibody. Bound antibodies were detected using a chemiluminescence kit (Pierce, USA). The antibodies used for the first immunodetection procedure were removed as previously described [26] and actin (Sigma) was then detected as a control for loading in each lane.

2.4. Immunocytochemistry

Breast cancer cells were grown and stimulated on permanox tissue culture chambers (Nunc Brand Products, USA). Expression of B_1R protein in MDA-MB-231 cells was determined using an antiserum raised against a peptide corresponding to the C-terminal 16 amino acids (ISSSHRKEIFQLFWRN) of the B_1R [13]. MMPs levels in MCF-7 and MDA-MB-231 breast cancer cells were

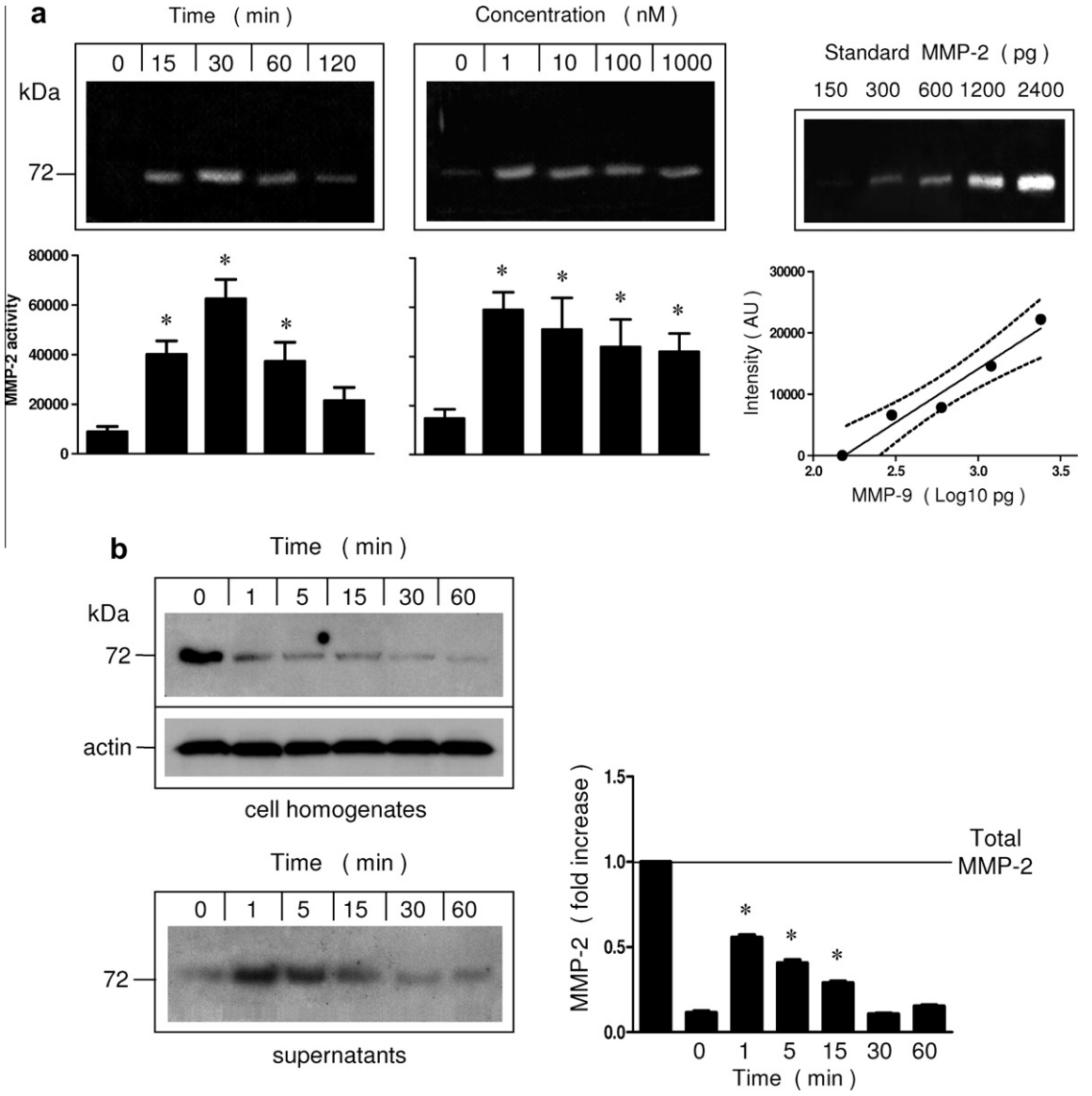

Fig. 2. The B_1R agonist, LDBK induces the release of MMP-2 from multicellular spheroids of MCF-7 cells. (a) Spheroids were stimulated for variable periods of time with 10 nM LDBK or stimulated for 15 min with various concentrations of LDBK. MMP-2 release was determined by gelatin zymography of cell-free supernatants. Gelatinase activity was estimated by densitometry and compared with the activity of different concentrations of a commercially available preparation of recombinant MMP-2 standard. (b) The MMP-2 protein levels released into the media at different intervals were estimated by Western blotting and compared with the immunoreactive MMP-2 protein present in homogenates prepared from the same cells. The protein visualized at time zero represents the total amount of immunoreactive MMP-2 present in the unstimulated MCF-7 cells. The levels of immunoreactive MMP-2 present in the supernatants from 1 to 60 min after stimulation are shown in the graph. Representative zymograms and Western blots are shown. Values represent the mean ± SEM ($n = 3$). $^*P < 0.05$ between unstimulated and stimulated cells.

evaluated in unstimulated cells and after stimulation with the B_1R agonist. Cells were fixed with 4% formalin-PBS for 30 min and then incubated overnight with a monoclonal antiserum raised against MMP-2 (Calbiochem, USA) or a polyclonal antibody directed to MMP-9 (Cell signaling). Bound immunoglobulins were detected with the LSAB + biotin/streptavidin-peroxidase kit (Dako, USA) and peroxidase was visualized with 3-amino-9-ethylcarbazol. Cells were counterstained with hematoxylin and mounted with Mowiol™ (Polysciences, USA). Controls included omission of primary antibody or its replacement by non-immune serum of the same origin at the same dilution [26,27].

2.5. Measuring MMP-9 and MMP-2 activities by gelatin zymography

Zymography was performed as described previously [26]. Briefly, non-reducing Laemmli's buffer was added directly to the cell-free supernatants obtained from unstimulated and stimulated adherent semiconfluent monolayers or multicellular spheroids. In another set of experiments, the gelatinases were concentrated by incubating the supernatants with agarose beads coated with gelatin (Sigma). After overnight incubation at 4 °C, the beads were recovered by centrifugation, and samples eluted with sample buffer [28]. Proteins were separated immediately on a 7.5% SDS–PAGE gel containing 1 mg/ml gelatin. After electrophoresis, the gel was washed for 1 h in a buffer containing 2.5% Triton X-100 and then incubated overnight in a digestion buffer containing calcium and zinc at 37 °C with constant stirring for 24 h. After staining the gel with Coomassie brilliant blue R-250, the appearance of clear bands was indicative of gelatinase activity. Gels were visualized using a transilluminator, scanned and the bands quantified by densitometry as described [26]. Within a certain range, the area of substrate digestion is proportional to the amount of enzyme present in the supernatant. To deter-

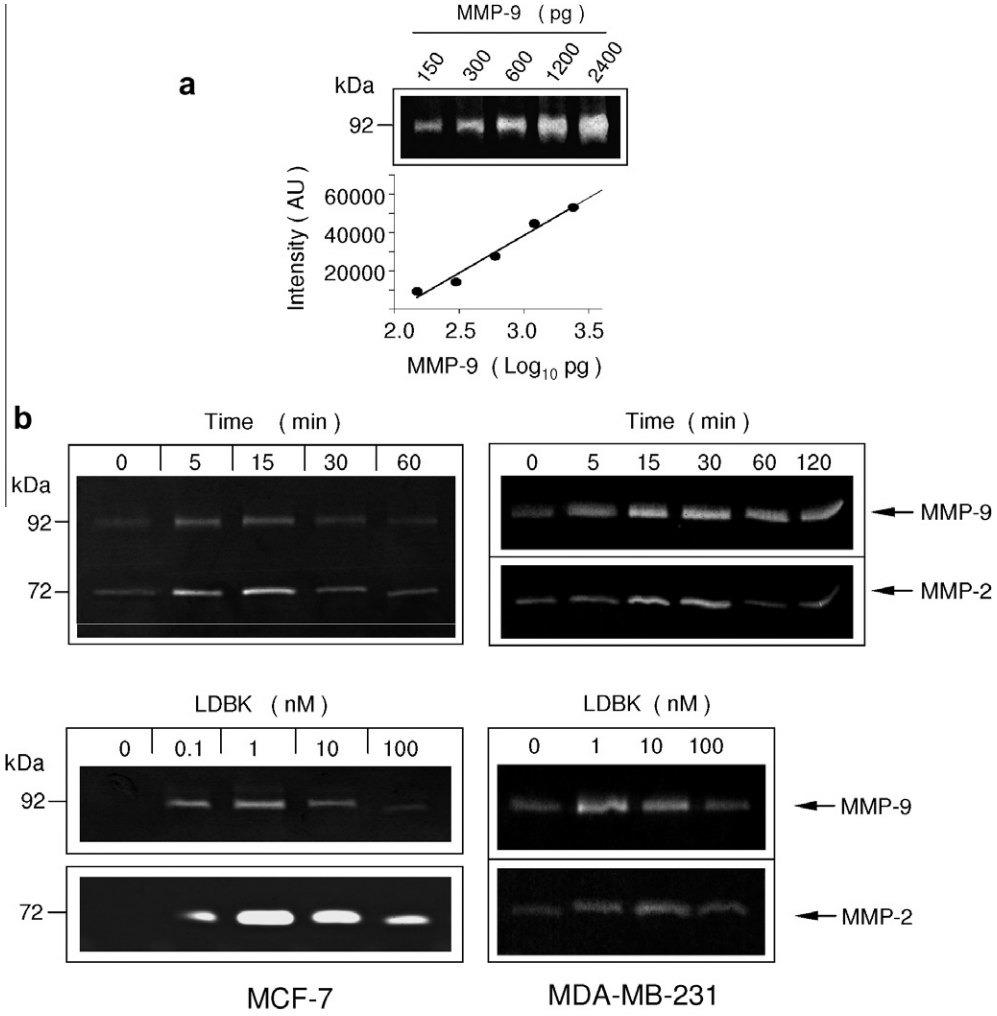

Fig. 3. The B_1R agonist induces the release of MMP-2 and MMP-9 from MCF7 and MDA-MB-231 cells. (a) Standard curve using 150–2400 pg of a commercially available recombinant MMP-9 preparation. (b) Spheroids of MCF-7 cells (left hand side) or monolayers of MDA-MB-231 cells (right hand side) were stimulated with 10 nM LDBK for different periods of time or with nanomolar concentrations of LDBK for 15 min. The MMPs released into the media were enriched with the aid of gelatin-coated agarose beads. Representative zymograms of three independent experiments are shown.

mine these values, we used known concentrations of commercially available MMP-2 and MMP-9 preparations to establish the respective calibration curves (Figs. 2 and 3).

2.6. Transfection of B_1R siRNA and the EGFR dominant negative mutant HERCD533

All cells were washed three times with phenol-red-free DMEM at the beginning of each experiment. Transfection conditions were as follow: spheroids or adherent monolayers (1×10^6 cells) were placed in DMEM without antibiotics or antimicotics. Next, the cells were incubated with siRNA-lipofectamine complexes prepared according to the Lipofectamine'2000 protocol (Invitrogen). Transfection was performed with 100 pmol of B_1R siRNA (Sta Cruz Biotechnology, USA) or siRNA control (Block-it fluorescent oligo, Invitrogen) for 24 h in the same medium and then cells were left for an additional period of 24 h in the same medium, but with antibiotics and antimicotics prior to stimulation with the B_1R agonist LDBK. Efficiency of transfection was controlled 24 h and 48 h after transfection by detecting the incorporated block-it fluorescent oligo by fluorescence microscopy. Silencing of the B_1R was confirmed by determining the level of expression of B_1R mRNA (Fig. 6) and the phosphorylation of ERK 1/2 MAPK in the transfected cells stimulated with the B_1R agonist (not shown). Results obtained using the B_1R siRNA were compared with those obtained by pre-treating the cells for 30 min with an excess of a B_1R antagonist (des-[Arg^9]-Leu^8-BK or Lys-des-[Arg^9]-Leu^8-BK, Bachem) before stimulation.

Inhibition of EGFR function was achieved by over-expressing the EGFR dominant negative mutant HERCD533 that was generously provided by Dr. Sylvain Meloche (University of Montreal, Quebec, Canada). For this purpose 4–6 μg of the EGFR dominant negative mutant HERCD533 were transfected using Lipofectamine. Efficiency of transfection was controlled 24 h and 48 h after transfection, by detecting the expression of green fluorescent protein (GFP) by fluorescence microscopy.

2.7. Quantification of results and statistical analysis

The intensity of immunoreactive protein bands, visualized after immunoblotting, was quantified using an automated image digitizing system (Un-Scan-It, Silk Scientific Inc., Orem, UT, USA) as described previously [26]. The Mann–Whitney U test and the student's *t*-test were used to analyze differences between groups (GraphPad $InStat^R$,

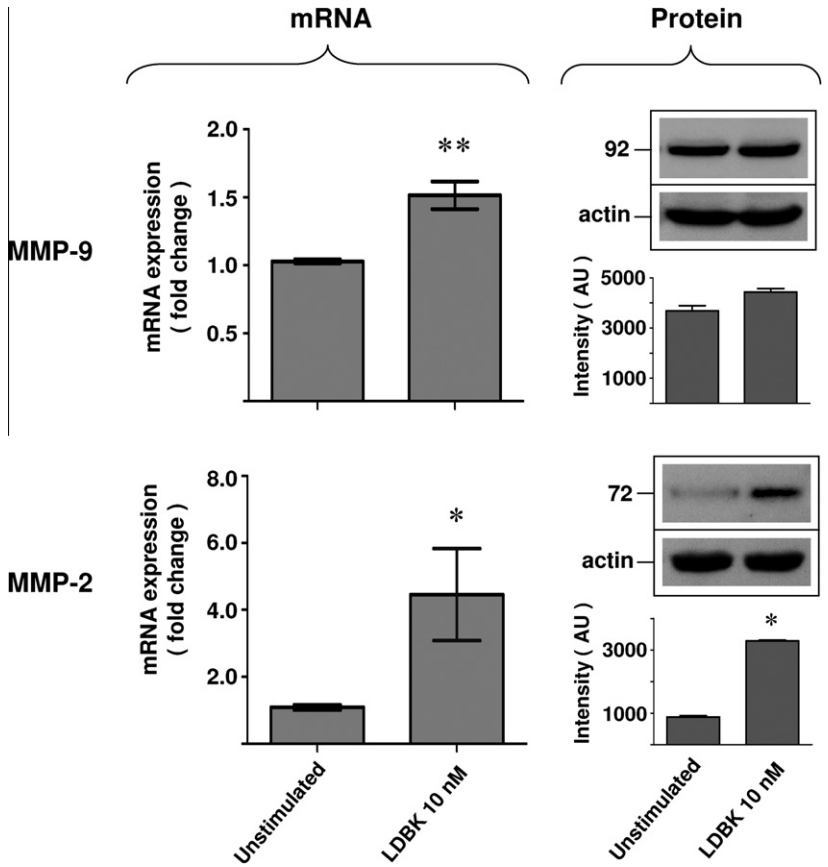

Fig. 4. B_1R stimulation increases the expression of MMP-2 and MMP-9. Monolayers of MCF-7 breast cancer cells were stimulated with 10 nM LDBK for 2 h and total RNA was extracted. The cDNAs were amplified by quantitative PCR as described (Section 2). Protein levels were quantified by western blotting after stimulation with 10 nM LDBK for 3 h. Values represent the mean ± SEM. $^*P < 0.01$ ($n = 6$); $^{**}P < 0.005$ ($n = 5$) between unstimulated and LDBK treated cells (Mann–Whitney U test).

San Diego, CA, USA). Values are expressed as mean ± SEM and significance was considered acceptable at the 5% level ($P < 0.05$).

3. Results

3.1. Expression of B_1R in MDA-MB-231 breast cancer cells

First we employed three different methods to demonstrate the presence of kinin B_1R since MDA-MB-231 cells had not been characterized previously in this respect. Using RT-PCR a band of the expected size (214 bp) was detected, whereas for controls in which reverse transcriptase was omitted no such band was observed (Fig. 1a). In addition, an antibody raised against a 16 amino acid peptide from the carboxyterminus of the human receptor identified a prevalent immunoreactive band of 45 kDa by Western blotting of cell homogenates (Fig. 1b). Using the same antibody, the receptor was also detected by immunocytochemistry in these cells (Fig. 1c). Pre-immune serum applied under the same conditions failed to yield significant staining (Fig. 1d).

3.2. The B_1R agonist induces the release of MMP-2 and MMP-9

Supernatants obtained after stimulation of MCF-7 spheroids with the B_1R agonist, LDBK were directly assayed to identify the presence of metalloproteases (Fig. 2). Gelatin zymography revealed that concentrations of LDBK as low as 1 nM induced significant release of MMP-2 observed as gelatinase activity at 72 kDa. MMP-2 release was maximal between 15 and 30 min following stimulation with 1–10 nM LDBK (Fig. 2a). Analysis of same supernatnats by Western blotting and using an antibody to MMP-2 also identified an immunoreactive band of 72 kDa, confirming that gelatinase activity corresponded to MMP-2 (Fig. 2b). To determine the amount of MMP-2 released with respect to the total enzyme present in these cells, we also analyzed homogenates of unstimulated cells. This approach revealed that stimulation of breast cancer cells with the B_1R agonist released approximately 50% of the MMP-2 contained in these cells (Fig. 2b).

Similar experiments were done to evaluate the presence of MMP-9. By comparison, MMP-9 levels detected in supernatants of spheroids or monolayers of MCF-7 cells stimulated with LDBK were smaller than those observed for MMP-2 (not shown). Nevertheless, when the MMPs released into the medium were enriched by incubating each supernatant with agarose particles coated with gelatin, MMP-9 was clearly detectable in each supernatant (Fig. 3b). This procedure showed that when monolayers of MCF-7 cells were stimulated with 10 nM LDBK, both MMP-2 and MMP-9 were released into the incubation medium, whereby the levels detected peaked within the 5 to 15 min of agonist stimulation (Fig. 3b). When breast cancer cells were stimulated with different concentrations of the B_1R agonist LDBK, a dose-dependent response was observed (Fig. 3b). Release of MMP-2 and MMP-9 was detected using concentrations of LDBK as low as 0.1 nM, but maximal responses were detected with 1–10 nM of LDBK after 15 min (Fig. 3b). Stimulation of MDA-MB-231 cell monolayers with 10 nM LDBK resulted in the release of both MMP-9 and MMP-2 to the incubation media (Fig. 3b). In this cell line, gelatinase activity due to MMP-9 was higher than that attributed to MMP-2 and reached a maximum 15–30 min after stimulation (Fig. 3b). Incubation of cells with the solution used to solubilize the B_1R agonist (0.9% NaCl), did not induce the release of the MMPs (not shown).

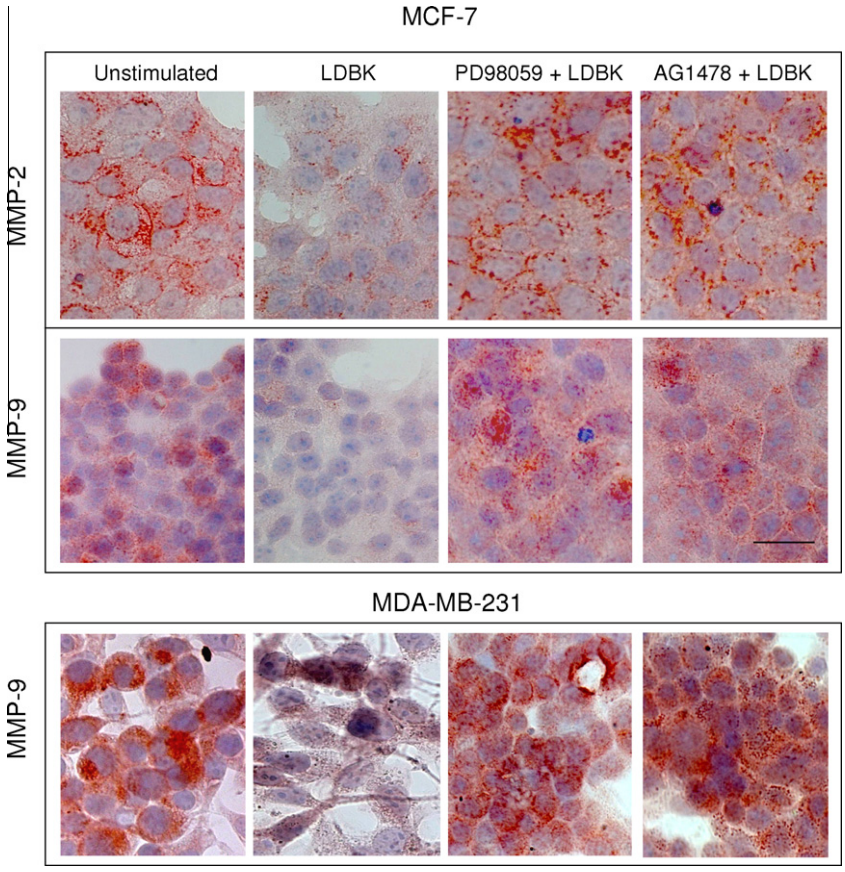

Fig. 5. Visualization of MMP-2 and MMP-9 in stimulated and unstimulated MCF-7 and MDA-MB-231 cells. The cells were directly stimulated with 10 nM LDBK for 15 min or pre-incubated with 10 μM of the MEK1 inhibitor PD98059 or 1 μM of the EGFR-tyrosine kinase inhibitor AG1478 for 30 min before stimulation with the B_1R agonist. Then, cells were fixed with 4% formalin-PBS, permeabilized and processed for immunocytochemistry according to the biotin/streptavidin-peroxidase technique. Representative images are shown. *Scale bar* = 20 μm.

By quantitative PCR, expression of both MMP-2 and MMP-9 was detected in monolayers of unstimulated MCF-7 cells. However, upon stimulation with 10 nM of the B_1R ligand, LDBK, for 2 h both MMP-2 and MMP-9 mRNA levels increased significantly, although the increase was greater for MMP-2. At the protein level, only MMP-2 increased significantly following stimulation with LDBK for a 3 h period (Fig. 4).

3.3. Release of MMP-2 and MMP-9 depends on B_1R activation

The presence of immunoreactive MMP-2 and MMP-9 in the cytoplasm of non-stimulated and stimulated MCF-7 and MDA-MB-231 breast cancer cells was confirmed by using immunocytochemistry (Fig. 5). Also by this method, unstimulated MCF-7 breast cancer cells appeared to contain higher amounts of immunoreactive MMP-2 protein than MMP-9 (Fig. 5). As anticipated incubation of both MCF-7 and MDA-MB-231 cells with LDBK resulted in loss of immunostaining due to MMPs. Absence of staining occurred when the primary antibody was omitted or replaced by a non-immune mouse/rabbit IgG, at the same concentrations (not shown). Interestingly, loss of MMP-2 and MMP-9 specific immunostaining upon addition of LDBK was efficiently blocked by either the MEK1 inhibitor PD98059 or the EGFR kinase inhibitor AG1478.

To further investigate the relevance of B_1R, MCF-7 and MD-MB-231 breast cancer cells, were transfected with a commercially available B_1R-specific siRNA and then stimulated with 10 nM LDBK. Both for MCF-7 (Figs. 6a and b) and MDA-MB-231 (Fig. 6d) prior transfection with siRNA reduced significantly LDBK-induced MMP activity. Specificity of the siRNA used was demonstrated in parallel experiments in which the levels of B_1R transcripts were determined by RT-PCR 24 and 48 h post-transfection. As expected, the levels of B_1R mRNAs decreased significantly after siRNA transfection (Fig. 6c and e). Furthermore, when transfected cells were stimulated with the B_1R agonist LDBK, activation of ERK1/2 MAPK signaling pathway was reduced (data not shown). Also, MMP2 protein levels in supernatants of siRNA transfected MCF-7 cells decreased following LDBK stimulation (Fig. 7a). Additional control experiments, in which cells were pre-incubated with an excess amount of a B_1R antagonist, prior to stimulation with LDBK under the same conditions, corroborated that LDBK stimulated the release of MMPs by activating the kinin B_1R (Fig. 7b).

3.4. Release of MMPs is linked to EGFR transactivation and the ERK1/2 MAPK pathway

In a previous study, using inhibitors, we showed that the proliferative effect produced by the B_1R agonist on estrogen-sensitive MCF-7 and ZR-75 breast cancer cells depended on the activity of the EGFR and subsequent phosphorylation of ERK1/2 MAPK [13]. Thus, we asked whether the release of MMP-2 and MMP-9 depended on the activation of this pathway. For this purpose, MCF-7 and MDA-MB-231 breast cancer cells were pre-incubated with either inhibitors or the vehicle controls before stimulation with the B_1R agonist. Gelatinase activity in the supernatants of MCF-7 and MDA-MB-231 was significantly reduced by PD98059, a MEK1 inhibitor that precludes ERK1/2 MAPK phosphorylation and activation (Figs. 8a and 9c), and by AG1478, an inhibitor of the EGFR-tyrosine kinase (Figs. 8a and 9c). Inhibition of MMPs release by PD98059 and AG1478 was previously determined by immunocytochemistry (Fig. 5). Then, we wished to determine whether stimulation of the B_1R in these multicellular structures produced similar effects to those observed previously for MCF-7 monolayers. Indeed, these experiments using inhibitors on spheroids, revealed that phosphorylation of ERK1/2 MAPK also

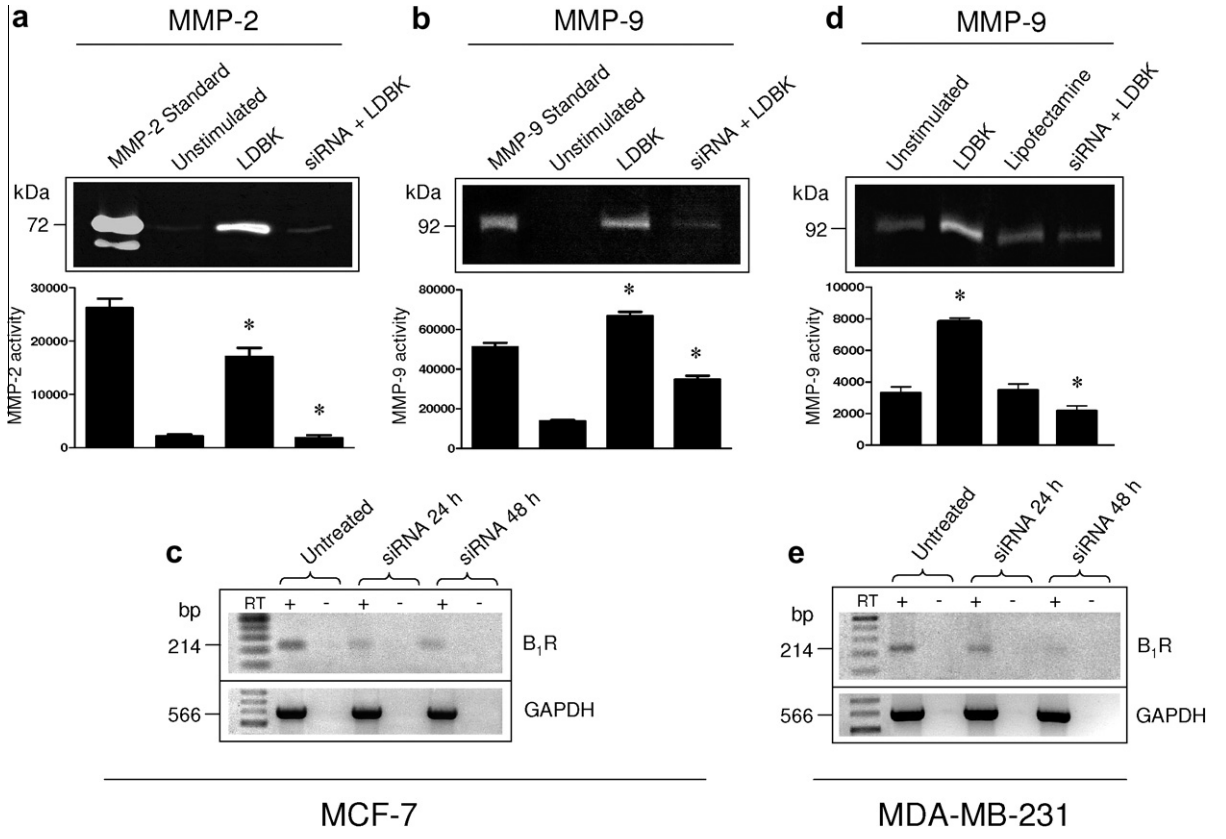

Fig. 6. Release of MMP-2 and MMP-9 is blocked after silencing of the B_1R with a siRNA. (a, b, and d) Transfected and non-transfected cells were directly stimulated with 10 nM LDBK for 15 min and MMP-2 and MMP-9 activity was determined by gelatin zymography of cell-free supernatants. The gelatinase activity was estimated by densitometry as previously described. (c and e) Effect of siRNA on B_1R mRNA levels in MCF-7 and MDA-MB-231 cells, 24 h and 48 h after transfection. Values represent the mean ± SEM ($n = 3$). $^*P < 0.05$ between unstimulated and LDBK stimulated cells and between non-transfected and transfected cells stimulated with LDBK.

depended on EGFR transactivation in this situation (Fig. 8b). Incubation of MCF-7 cells with dimethylsulfoxide diluted with phenol red-free DMEM to the same extent as in the experiments described above (final concentration of 0.001% dimethylsulfoxide), neither affected the release of MMPs or the phosphorylation of ERK1/2 MAPK. Identical results were obtained using the estrogen-insensitive breast cancer cell line MDA-MB-231 (Fig. 9b, right panel). Stimulation of these cells with 10 nM LDBK resulted in phosphorylation of ERK1/2 MAPK that reached a maximum 5 to 15 min following agonist stimulation (Fig. 9a, left panel). Furthermore, a dose-dependent response was observed when cells were stimulated with 1 to 100 nM LDBK (Fig. 9a, middle panel). This response was blocked by preincubation of cells with a kinin B_1R antagonist, but not by a B_2R antagonist (Fig. 9a, right panel). Release of MMPs, exemplified by MMP-9, was also dependent on EGFR transactivation and activation of the ERK1/2 MAPK pathway since its accumulation in the incubation media was reduced by pretreatment of cells with AG1478 and PD98059 (Fig. 9c). This finding confirmed that previously obtained by immunocytochemistry (Fig. 5). As expected, phosphorylation of ERK1/2 MAPK in MDA-MB-231 cells was blocked either by AG1478 or PD98059 (Fig. 9b). Finally, we used breast cancer cells over-expressing the EGFR dominant negative mutant HERCD533 to confirm that release of MMPs and ERK1/2 phosphorylation observed after stimulation with the B_1R agonist, involved activation of the EGFR. The MCF-7 cells (Fig. 8c) and MDA-MB-231 cells (Fig. 9c, right panel) over-expressing HERCD533 blocked both the phosphorylation of ERK1/2 MAPK (Fig. 8c) and the release of MMP-9 (Fig. 9c), respectively suggesting that both events were dependent on transactivation of the EGFR.

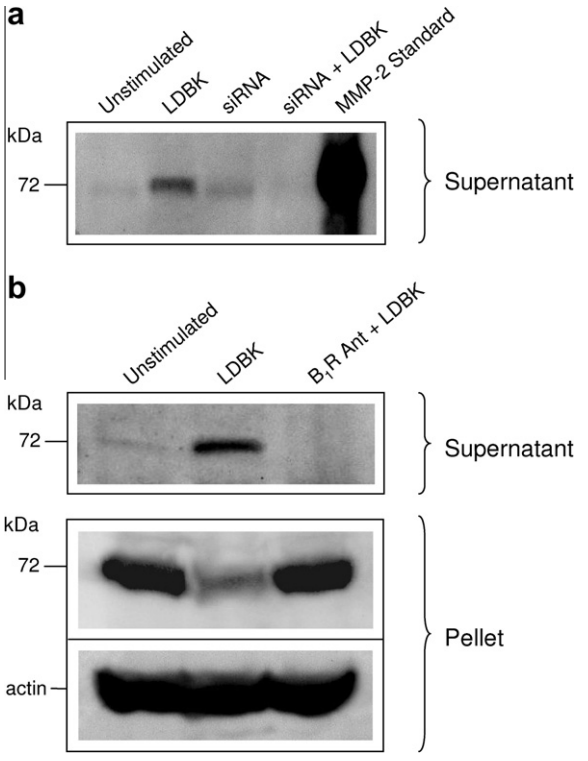

Fig. 7. Release of MMP-2 is blocked by a siRNA and by preincubation of MCF-7 cells with a B_1R antagonist. (a) Transfected and non-transfected cells were directly stimulated with 10 nM LDBK for 15 min and the MMP-2 immunoreactive protein released into the supernatants was determined by Western blotting. (b) The cells were directly stimulated with 10 nM LDBK for 15 min or pre-incubated with an excess (1 μM) of the B_1R antagonist for 30 min before stimulation with LDBK. The immunoreactive MMP-2 protein present in the supernatants and in the cellular pellets was determined by Western blotting. Western blots are representative of two independent experiments.

4. Discussion

Neoplastic cells fulfill their metastatic potential after acquiring those advantageous characteristics, which allow them to leave the primary tumor, migrate and after invading surrounding tissues, travel to secondary sites, establish metastatic foci and proliferate again [29–32]. Several studies highlight the relevance of MMPs levels in this context and suggest their levels are closely associated with increased tumor invasion, systemic migration and metastasis. In all these processes, MMP-2 and MMP-9 are thought to play a critical role [24,32–35].

In this study, we have characterized, for the first time, the bradykinin B_1R in the estrogen-insensitive MDA-MB-231 breast cancer cell line and show that agonist-dependent stimulation in this cell type and in estrogen-sensitive MCF-7 cells enhances the release of MMP-2 and MMP-9. Furthermore, stimulation of MCF-7 cells with the B_1R agonist increased MMP-2 and MMP-9 mRNA levels. At the protein level MMP-2 increased more than MMP-9. Our results are similar to those reported by Das et al. [36] who showed that the mRNA and protein levels of MMP-2 and MT1-MMP were up-regulated when MCF-7 cells were stimulated with fibronectin. Although no differences in mRNA levels of MMP-9, between fibronectin-exposed cells and controls, were observed, pro-MMP-9 activity in zymograms increased in the fibronectin-exposed cells. Similar to our findings Esparza et al. [37] indicate that fibronectin up-regulates MMP-9 and induces coordinated expression of MMP-2 and its activator MT1-MMP (MMP-14) in human T lymphocyte cell lines. Alternatively, Cortes-Reinosa et al. [38] showed that stimulation of MCF-7 cells with collagen-IV does not increase MMP-9 expression.

Our results also demonstrate that supernatants obtained from cell monolayers and multicellular spheroids stimulated with the B_1R agonist contain higher levels of MMP-2 and MMP-9 than their corresponding unstimulated controls. The release of both MMPs occurs rapidly at nanomolar concentrations of the B_1R agonist and activity is higher for MMP-2 than for MMP-9 in the MCF-7 cells and higher for MMP-9 than for MMP-2 in MDA-MB-231 cells. The release of MMPs into the incubation medium was confirmed when immunoreactivity to MMPs present in the non-stimulated and stimulated cells was estimated by immunocytochemistry. Further, the low level of MMP-9 recorded in MCF-7 cells coincided with weak immunoreactivity visualized by immunocytochemistry in unstimulated MCF-7 cells. In fact, the presence of MMP-9 in the supernatants of these cells stimulated with the B_1R agonist was clearly detected only when the MMPs released into the media were captured with the aid of gelatin-coated beads.

Total MMP-2 protein levels were not significantly altered in either MCF-7 and MDA-MB-231 cells, indicating that in tumor cells the proteolytic balance shifts towards increased enzymatic activity. This may occur either by increasing the expression of pro-enzymes and/or by decreasing inhibitor levels [39–42]. Stimulation with the B_1R agonist increased MMP-9 protein levels in MCF-7 cells but not in MDA-MB-231 cells. Nevertheless, the same agonist augmented the release of MMP-9 in MDA-MB-231

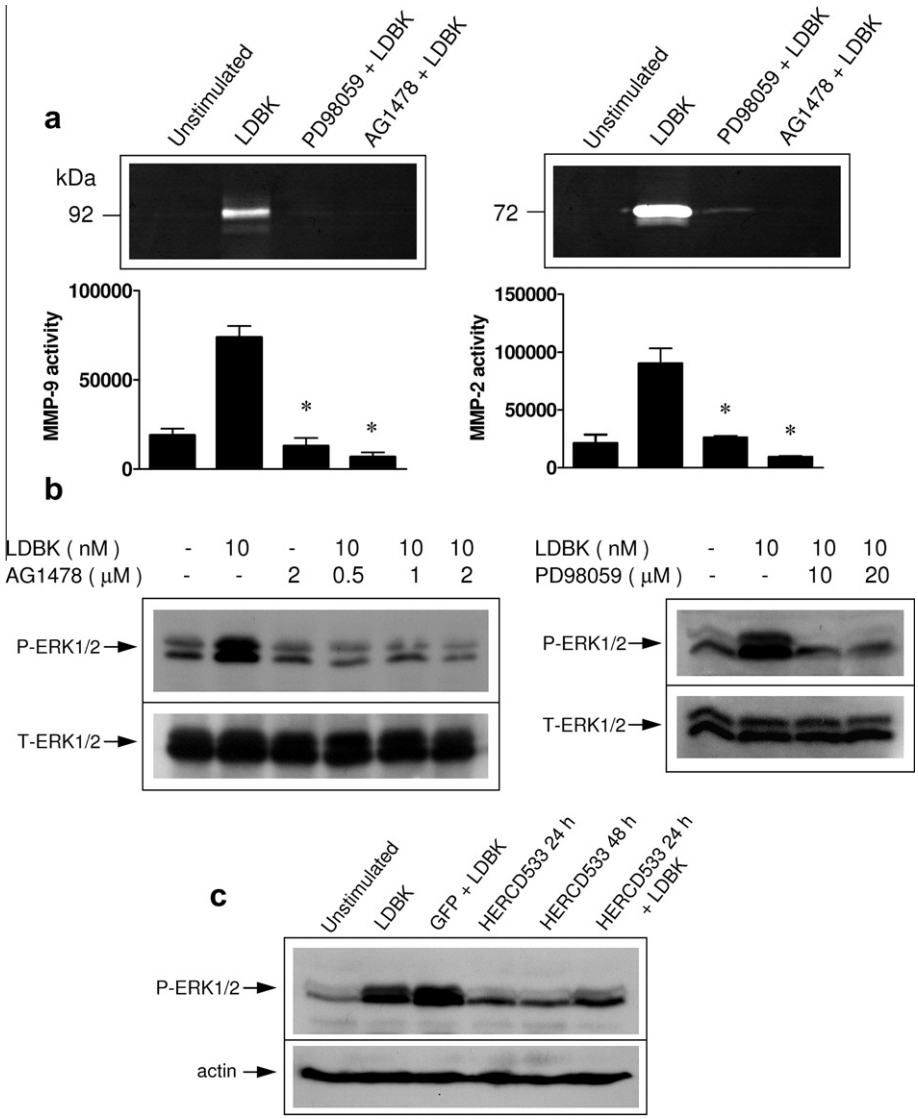

Fig. 8. Release of MMP-2 and MMP-9 from MCF-7 cells is inhibited by blocking of the ERK1/2 MAPK pathway or the EGFR. (a) Spheroids were directly stimulated with 10 nM LDBK for 15 min or pre-incubated with 10 μM of the MEK1 inhibitor PD98059 or 1 μM of the EGFR-tyrosine kinase inhibitor AG1478 for 30 min before stimulation with the B_1R agonist. MMPs released into the media were enriched with the aid of gelatin-coated agarose beads and their activity was determined by gelatin zymography as described. Representative zymograms of three independent experiments are shown. (b) Spheroids were treated as in (a) and phosphorylation of ERK1/2 MAPK (P-ERK1/2) in the absence or presence of different concentrations of both inhibitors was determined by Western blotting. T-ERK1/2, phosphorylated and non-phosphorylated ERK1/2 MAPK. (c) Cells were transfected with the dominant negative EGFR mutant HERCD533 and stimulated with LDBK 24 h post-transfection. Values represent the mean ± SEM ($n = 3$). $^*P < 0.05$ between LDBK stimulated cells and those pretreated with inhibitors before LDBK stimulation.

cells supporting the role of this MMP in invasiveness and emphasizing the importance of B_1R to modulate the expression or release of MMPs in breast cancer cells.

Increased expression of EGFR or its EGF-like ligands and hyperactivation of subsequent MAPK and protein kinase B (AKT) signaling pathways have been linked to anti-hormone resistance growth, metastatic progression, especially of breast and ovary tumors, where it correlates with a poor patient prognosis [43–47]. In a previous study we showed that ERK1/2 MAPK phosphorylation produced by B_1R stimulation in MCF-7 and ZR-75 cells is the result of EGFR transactivation, an effect that can be blocked by the EGFR-tyrosine kinase inhibitor, AG1478 [13]. Our present results indicate that EGFR transactivation occurs irrespective of whether cell monolayers or multicellular spheroids are used or whether estrogen-sensitive or estrogen-insensitive breast cancer cells are analyzed. Moreover, gelatinase activity detected in the supernatants of stimulated monolayers or spheroids was significantly reduced by PD98059 and by AG1478. The dependence of MMP-2 and MMP-9 release on indemnity of ERK1/2 MAPK signaling pathway and EGFR transactivation, respectively was confirmed when the cell content of these enzymes was estimated by immunocytochemistry or when the cells were

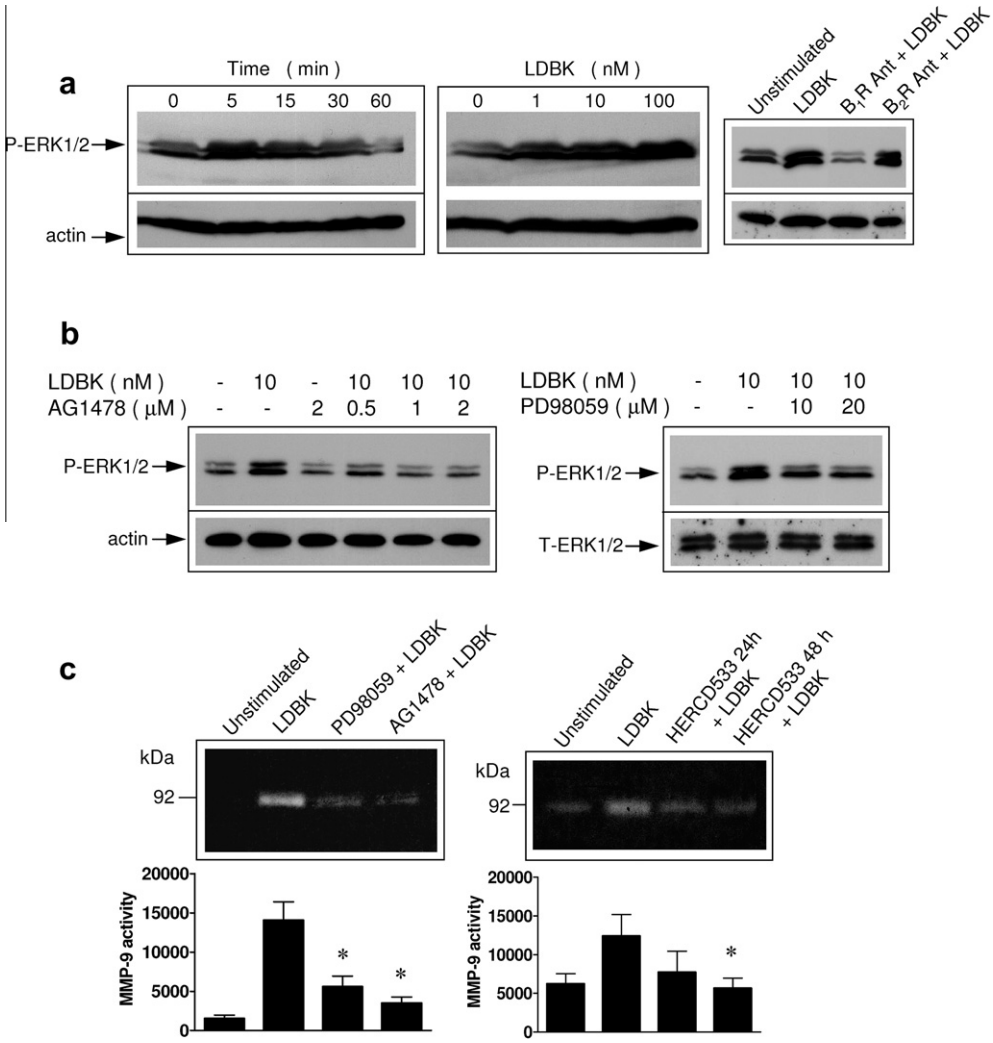

Fig. 9. Release of MMP-9 from MDA-MB-231 cells is inhibited by blocking of the ERK1/2 MAPK pathway or the EGFR. (a) Cell monolayers were stimulated with 10 nM LDBK for different periods (left hand side) or with nanomolar concentrations of LDBK for 5 min (center) and phosphorylation of ERK1/2 MAPK (P-ERK1/2) was estimated by Western blotting. The phosphorylation of ERK1/2 MAPK was dependent on B_1R stimulation because it was blocked by pretreatment of cells with a B_1R antagonist but not by a B_2R antagonist (right hand side). (b) Monolayers of MDA-MB-231 cells were directly stimulated with 10 nM LDBK for 5 min or pre-incubated with the EGFR-tyrosine kinase inhibitor AG1478 or the MEK1 inhibitor PD98059 for 30 min before stimulation with the B_1R agonist. (c) Cells were stimulated with 10 nM LDBK for 5 min or pre-incubated with 10 μM of PD98059 or 1 μM of AG1478 for 30 min before stimulation with the B_1R agonist (left hand side). MMPs released into the media were enriched with the aid of gelatin-coated agarose beads and their activity was determined by gelatin zymography as described. In another set of experiments, cells were transfected with the dominant negative EGFR mutant HERCD533 before stimulation with 10 nM LDBK (right hand side). Representative zymograms of three independent experiments are shown. Values represent the mean ± SEM ($n = 3$). $^*P < 0.05$ LDBK stimulated cells and those pretreated with inhibitors or transfected with the dominant negative EGFR mutant HERCD533 before stimulation.

transfected with a negative dominant mutant to EGFR. A previous study using the MEK1 inhibitor PD98059 showed that inhibition of the ERK1/2 MAPK signaling pathway produced only a partial inhibition of MMP-2 activation, and no changes in the expression of MMP-2 or MMP-9 [37]. Although the ERK1/2 MAPK pathway and EGFR transactivation are essential for the release of MMPs after B_1R stimulation, selective secretion of MMP-2 or MMP-9 seems to depend on the type of breast cancer cells i.e. estrogen-sensitive versus -insensitive cells. Activation of B_1R in both types of breast cancer cells is an important observation considering breast tumor heterogeneity, functional differences between MMPs and the concentration finally available in the microenvironment of these tumors. We have previously shown that activation of B_1R in human neutrophils induces the release of MMP-9 [26] emphasizing the importance of this receptor for tumor microenvironment. It is worth noting that in both neutrophils and breast cancer cells, B_1R is constitutively expressed and levels may be enhanced by the network of cytokines present in tumor microenvironment. The use of 3D culture models like the one employed in this study may be an appropriated

approach to evaluate, in the near future, the interaction of breast cancer cells with other cellular components of tumor microenvironment such as leukocytes, fibroblasts or endothelial cells.

MMPs can promote the growth of tumor cells through several mechanisms: cleavage of proteins, which by binding growth factors keep them inactive, or liberating cell surface factors such as TGF-α, which can then interact with its receptor, to induce growth and survival of tumoral cells [33,48–50]. MMPs can also modulate the proliferative capacity of cellular components of the extracellular matrix. Further, these proteases can strip death receptors sited on the cell surface or release survival factors and components of the extracellular matrix that can interact with cellular integrins, which then trigger anti-apoptotic signals [33,48]. In this respect, the importance of MMP-9 to enhance breast cancer cell migration and metastatic capacity through activation of αvβ3 integrin has been reported [51]. Furthermore, αvβ6 integrin has been shown to promote invasion of squamous cell carcinoma cells through the up-regulation of MMP-9 [52]. Similarly, MMP-2 binds to integrin αvβ3 and αvβ3-mediated surface presentation of active MMP-2, which contributes to cellular invasion [53]. Connected to this event, available evidence indicates that tumor-associated MMPs can also stimulate processes associated with epithelial–mesenchymal transition, a developmental change that is activated in tumor cells during cell invasion and metastasis [54]. Interestingly, MMP-2 and MMP-9 are among the MMPs directly related to the regulation of angiogenesis. These proteases cleave extracellular matrix components such as collagen type I, IV and fibrin, which interact with integrins and promote the formation of new blood vessels. In fact, MMP-2 and MMP-9 can release VEGF from extracellular matrix stores to increase angiogenesis, decrease cancer-cell apoptosis and favor the bioavailability of VEGF [55,56].

The importance of MMPs in breast cancer has led to their use as biomarkers by measuring levels in body fluids. Until now, MMP-2 and MMP-9 concentrations and activities have been analyzed by ELISA and gelatin zymography, respectively, in blood and urine samples of breast cancer patients. Despite some divergent data, many of these studies have linked circulating MMPs to breast cancer presence, disease status, lymph node metastasis or other clinicopathological parameters suggesting their potential use in breast cancer screening and follow-up studies assessing the risk of metastasis [57].

The experimental evidence presented in the current study on estrogen-sensitive and estrogen-insensitive breast cancer cells indicates that the kinin B_1R could participate in two key events of breast cancer development: cell proliferation [13] and expression/release of MMP-2 and MMP-9 (this study). Therefore, B_1R may represent an attractive therapeutic target to decrease growth and invasiveness of breast cancer cells.

Disclosure statement

The authors of this study have no conflict of interest or any financial disclosures to make.

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