Inflammation Research (2019) 68:845–855
https://doi.org/10.1007/s00011-019-01264-6

Inflammation Research

ORIGINAL RESEARCH PAPER

Interactions between carboxypeptidase M and kinin B1 receptor in endothelial cells

Paola Bianchi Guimarães[1] · Rafael Filippelli da Silva[1] · Carolina Caldas Hoff[2] · Liliam Fernandes[3] · Clovis Ryuichi Nakaie[1] · Jair Ribeiro Chagas[1] · Adriana Karaoglanovic Carmona[1] · Michael Bader[4] · João Bosco Pesquero[1]

Received: 4 November 2018 / Revised: 3 May 2019 / Accepted: 13 June 2019 / Published online: 19 June 2019

Abstract

Introduction Carboxypeptidase M (CPM) is a glycosylphosphatidylinositol anchored enzyme that plays an important role in the kallikrein–kinin system (KKS). CPM catalytic domain hydrolyzes Arg from C-terminal peptides (i.e., bradykinin and kallidin), generating des-Arg-kinins, the agonists of B_1 receptor (B_1R). It is known that CPM and kinin B_1R are co-localized in the plasma membrane microdomains, where they interact with each other, facilitating receptor signaling.

Aims We hypothesized here that this CPM-B_1R interaction could also affect the activity of the enzyme.

Methods Thus, in this work, we evaluated the impact of B_1R presence or absence on CPM activity and expression, using primary culture of microvascular endothelial cells from wild-type, kinin B_1R knockout mice ($B_1^{-/-}$), and transgenic rats overexpressing B_1 receptor exclusively in the endothelium. In addition, HEK293T cells, as wells as $B_1^{-/-}$ primary culture of endothelial cells, both transfected with B_1R, were also used.

Results CPM expression and activity were downregulated in cells of knockout mice compared to control and this reduction was rescued after B_1R transfection. Cells overexpressing B_1R presented higher levels of CPM mRNA, protein, and activity. This profile was reverted by pre-incubation with the B_1R antagonist, R715, in highly expressing receptor cells.

Conclusions Our data show that kinin B_1R positively modulates both CPM expression and activity, suggesting that CPM-B_1R interaction in membrane microdomains might affect enzyme activity, beyond interfering in receptors signaling. This work highlights the interactions among different components of KKS and contributes to a better understanding of its pathophysiological role.

Keywords Carboxypeptidase M · B_1 receptor · Kinins · Microdomains · Enzymatic activity

Introduction

The plasma membrane is a complex of dynamically distributed proteins and lipids that are arranged in membrane microdomains, such as caveolae and lipid rafts. These structures are lateral assemblies of specific lipids, in particular, cholesterol and glycosphingolipids [1–4], where glycosylphosphatidylinositol (GPI)-anchored proteins are also localized [5]. They possess biological characteristics that are essential for cellular processes such as proliferation, cell movement, signaling, viability, and protein trafficking [6, 7].

Carboxypeptidase M (CPM) is a GPI-anchored metallopeptidase that plays an important role in the kallikrein–kinin system (KKS) and is localized in lipid-rafts microdomains. CPM is widely distributed in the body, being present in kidney, intestine, lung and placental

Responsible Editor: Mauro Teixeira.

✉ João Bosco Pesquero
jbpesquero@unifesp.br

[1] Departamento de Biofísica, Universidade Federal de São Paulo, Rua Pedro de Toledo 669, 9° andar fundos, São Paulo, SP 04039-032, Brazil

[2] Departamento de Biociências, Universidade Federal de São Paulo, Santos, SP, Brazil

[3] Instituto de Ciências Ambientais, Químicas e Farmacêuticas (ICAQF), Universidade Federal de São Paulo, Diadema, SP, Brazil

[4] Max-Delbrück-Center for Molecular Medicine, Berlin, Germany

microvilli, brain, blood vessels, and peripheral nerves [8–10]. Human CPM is a monomeric protein of 443 amino acid residues, composed of two domains, whereas N-terminal is the catalytic one, requiring Zn^{2+} ion to activate a bound water molecule for substrate hydrolysis [11]. Since both domains are located on plasma membrane outer surface, CPM is considered an ectoenzyme [12]. CPM catalytic domain hydrolyzes Lys or Arg from C-terminal peptides (i.e., bradykinin and kallidin), generating B_1 receptor (B_1R) agonists, as des-Arg^9-BK (DBK) [13]. Its C-terminal domain attaches the enzyme to GPI anchor [13, 14] and contains the residues implicated with B_1R interaction [15]. Since CPM modulates anaphylatoxins and kinins activity, in addition to be present on differentiated immune cells [16], this enzyme is probably linked to inflammatory processes. In addition to its constitutive expression in several tissues and cell types, CPM presents a markedly increase in expression in lung and aorta endothelial cells during inflammation [17], a similar pattern to B_1R expression.

Kinin receptor belongs to G-protein-coupled receptors (GPCRs) class, specifically to family A (Rhodopsin like receptors) [18]. Receptor activation causes IP_3 formation and Ca^{2+} influx. On endothelial cells, Ca^{2+} participates in secondary messengers cascade, leading to nitric oxide synthesis by eNOS, inducing vascular relaxation [19–22].

B_2 receptor is constitutively expressed in several tissues, and can be desensitized [23, 24]. On the other hand, B_1R expression in healthy tissues is absent or very low expressed, but is induced under pathological conditions, such as inflammation [25]. B_1R is constitutively activated, maintains Ca^{2+} increase after stimulation [26], is not desensitized [27], is translocated from membrane, and undergoes aggregation [28].

CPM and B_1R are co-localized in caveolin-rich microdomains, interacting with each other. In this situation and in the presence of B_2R agonists, CPM enhances B_1R signaling [29]. This interaction, mediated by enzyme C-terminal region, which conformational modifications are propagated to receptor, is important for an efficient signaling through B_1R in response to B_2R agonists [10]. Another positive modulation of CPM in B_1R signaling, arising from this interaction, is an increase in endothelial function regarding higher nitric oxide release through activation of iNOS [10, 30].

Membrane microdomains facilitate protein interaction, forming heterodimers [31, 32]. This interaction interferes both in receptor signaling and in enzyme activity [33, 34]. However, enzyme–receptor interactions in membrane microdomains are not well understood, especially from the enzymatic point of view. In this context, this study becomes crucial, considering the role of ectoenzymes such as CPM in KKS. Therefore, we hypothesized that CPM–B_1R interaction might also affect enzyme activity. Thus, the aim of the present work was to evaluate CPM expression and activity in the presence of B_1R in endothelial cell models.

Materials and methods

Materials

If not otherwise indicated, all reagents were purchased from Sigma (St. Louis, MO, USA). Cell-culture media and supplements were purchased from Life Technologies (Carlsbad, CA, USA). Dansyl-Ala-Arg-OH substrate was purchased from Bachem (Bubendorf, Switzerland). All other chemicals were of analytical grade and commercially available.

Animals

Kinin B_1 receptor knockout mice ($B_1^{-/-}$) [35] and C57Bl/6 control mice; transgenic rats TGR (Tie_2B_1), overexpressing the B_1 receptor exclusively in the endothelium [36], and their Sprague–Dawley controls were obtained from Centro de Desenvolvimento de Modelos Experimentais para Medicina e Biologia (CEDEME/UNIFESP). Animals were maintained on standard chow at 22 °C on a 12-h light–dark cycle, and allowed ad libitum access to food and water. All procedures were approved and performed in accordance with guidelines of Ethics Committee of UNIFESP (Protocol No. 0100/08), conformed to Guide for the Care and Use of Laboratory Animals published by US National Institutes of Health (NIH Publication No. 85-23, revised in 1996).

Cellular models

Primary culture of endothelial cells from lung microvasculature

Microvascular endothelial cells from pulmonary beds were extracted from $B_1^{-/-}$ and C57/Bl6 mice and from TGR(Tie_2B_1) and control Sprague–Dawley rats.

Cell cultures were established according to procedures previously described elsewhere [37]. Animals were anaesthetized (ketamine/xylazine association) and euthanized by cervical decaptation, lungs were excised, washed with phosphate buffered saline (PBS), cut into $1 \times 1 \times 1$ mm^3 pieces, and placed in six-well (35 mm) dishes. Tissues were recovered with Dulbecco's modified Eagle's medium (DMEM low glucose) supplemented with fetal bovine serum (FBS, 20%) and gentamicin (40 mg/l), pH 7.4, and placed in a CO_2 incubator (Sheldon Mfg. Inc., USA). Lung explants were discarded after 60 h. All cells were incubated at 37 °C in a humidified atmosphere of 95% O_2 and 5% CO_2. Medium was changed every 2–3 days, and cells were subcultured between days 6 and 8 by harvesting with trypsin–EDTA.

Cells with 80–90% confluence were used in all experimental procedures.

B_1R transiently transfected cells

For expression in mammalian cells, the human B_1R cDNA was cloned into pCDH-CMV-MCS-EF1-Puro (System Biosciences—Palo Alto, CA, EUA) at *Nhe*I e *Bam*HI restriction sites. All PCR fragments used were amplified using high fidelity DNA polymerase *AccuPrime*™ *Pfx SuperMix* (LifeTechnologies—Carlsbad, CA, USA). Constructs were verified by DNA sequencing.

Microvascular endothelial cells from $B_1^{-/-}$ mice and human embryonic kidney 293T cells (HEK293T) were transiently transfected with a plasmid carrying the human B_1R coding region, with Attractene Transfection Reagent (Qiagen—Hilden, Germany) containing 1 µg of DNA, according to manufacturer's instructions.

$B_1^{-/-}$ cells were cultured as described above, whereas HEK293T cells were cultured in DMEM high glucose medium supplemented with 10% FBS, 100 units/ml penicillin, and 100 µg/ml streptomycin. Transfected HEK293T cells were named HEK-B_1 and non-transfected cells, used as control, HEK-ctrl.

All cells were incubated at 37 °C in a humidified atmosphere of 95% O_2 and 5% CO_2. Medium was changed every 2–3 days, and cells were subcultured between days 6 and 8 by harvesting with trypsin–EDTA. Cells with 80–90% confluence were used in all experimental procedures 48 h after transiently transfection.

Analysis of CPM and B_1R expression by qPCR

CPM and B_1R mRNA expressions in cells were assessed by SYBR Green or TaqMan® real-time PCR, depending on case, using the following set of primers independently: hCPM (Fwd: 5′-TACCACCGCCAGGAAGGG-3′; Rev: 5′-CCTTTGGAAACCGCCCC- 3′); mCPM (Fwd: 5′-AAGCTTAACCCCGGACGAT-3; Rev: 5′-ATTTACAGC ACGACAGCTCCA-3′); rCPM (Fwd: 5′-TGCCTGGGT CCTACGTGATA-3′; Rev: 5′-ACTGAAGGGCTGGGA TTTCG-3′); hB_1R (Fwd: 5′-TGGCAGCCTCTGATCTGG TG -3′; Rev: 5′- CCACCACCAGGAAGATGCTG -3′); mB_1R TaqMan® Gene Expression Assay Mm04207315_s1 (Life Technologies); rB_1R (Fwd: 5′-CCATACAAAACC CCAGCTGAA-3′; Rev: 5′-CTTTGGTTAGAAGGCTGT AGCTTCA-3′; h18S (Fwd: 5′-GGCCCTGTAATTGGA ATGAGTC-3′; Rev: 5′-CCAAGATCCAACTACGAG CTT-3′); mβ-actin (Fwd: 5′-CTGGCCTCACTGTCC ACCTT-3′; Rev: 5′-CGGACTCATCGTACTCCTGCTT-3′); rβ-actin (Fwd: 5′-CTGGCCTCACTGTCCACCTT-3′; Rev: 5′-CGGACTCATCGTACTCCTGCTT-3′); mβ-actin TaqMan®Gene Expression Assay Mm99999915_g1 (Life Technologies—Carlsbad, CA, USA). All primers were purchased from Exxtend Biotechnology (Campinas, São Paulo, Brazil).

For this procedure, culture medium was siphoned off and total RNA was isolated using TRIzol® reagent (Invitrogen— Carlsbad, CA, USA) according to manufacturer's instructions. After purification, the presence of intact RNA was verified on a Sybr®Green-stained (Invitrogen—Carlsbad, CA, USA) agarose gel and the total RNA (1 µg) was reverse-transcribed to cDNA using Moloney murine leukemia virus reverse transcriptase enzyme, according to manufacturer's instructions (Life Techonologies—Carlsbad, CA, USA). The reaction product was amplified by real-time PCR on 7500 PCR Detection System (ABI Prism, Applied Biosystems—Foster City, CA, USA) using SYBR Green or TaqMan Universal PCR Master Mix, depending on case (Applied Biosystems—Foster City, CA, USA). Thermal cycling conditions were composed by an initial denaturation step of 95 °C for 10 min, followed by 50 cycles at 95 °C for 15 s, and 60 °C for 1 min. Standard and melting curves were performed in parallel to check reaction efficiency and specificity, respectively. Experiments were performed in triplicate for each data point. CPM and B_1R mRNA abundance was quantified as a relative value compared to an endogenous control, whose abundance was assumed not to change between varying experimental conditions. CPM and B_1R mRNA expressions were obtained from cycle threshold (Ct) associated with exponential growth of PCR products. Relative quantitation for CPM and B_1R mRNA expression was obtained by $2^{-\Delta Ct}$ parameter, in which ΔCt represents the subtraction of endogenous control Ct values from CPM or B_1R values [38].

Analysis of CPM protein expression

Protein expression was determined by Western blotting analysis [39]. Cell extracts were separated by SDS-PAGE (7.5%) and transferred to a polyvinylidene fluoride membrane. Membranes were blocked overnight at 4 °C in Odyssey Blocking Buffer (LI-COR®—Lincoln, NE, USA). Immunoblot was performed by incubation with an anti-CPM primary antibody (Sigma—St. Louis, MO, USA) at 1:1000 dilution in block buffer for 1 h at room temperature, followed by membrane incubation with a secondary antibody fluorophore-conjugated (*IRDye*® secondary antibodies—LI-COR®—Lincoln, NE, USA—1:10.000 dilution in block buffer) for 1 h at room temperature. Anti-actin antibody (C4 clone, Merck Millipore—Darmstadt, Germany) was used as endogenous control. Immunoreactive bands were visualized in Odyssey Classic infrared imaging system (LI-COR®—Lincoln, NE, USA), and quantified by the ImageJ software (NIH—Bethesda, MD, USA).

Measurement of CPM activity on cells monolayer

CPM activity was measured using Dansyl-Ala-Arg-OH substrate as described elsewhere [29], with modifications for live and adherent cells. Briefly, endothelial cells were plated in 12-well plates at 10^5 cells per well, following incubation at 37 °C in 200 µl of HBSS buffer (140 mmol/l NaCl, 5 mmol/l KCl, 0.1 mmol/l $CaCl_2$, 0.63 mmol/l $MgSO_4$, 1 mmol/l Na_2HPO_4, 6.1 mmol/l glucose, and 10 µmol/l $ZnCl_2$, pH 7.4), containing 200 µmol/l of Dansyl-Ala-Arg substrate. Culture supernatant was collected at different times (0–30 min), and the reaction was stopped with 150 µl of 1.0 mol/l citrate, pH 3.0, followed by extraction of fluorescent product with 1.0 ml of chloroform. Organic layer, corresponding to chloroform phase, was transferred to black 96-well plates (Life Techonologies—Carlsbad, CA, USA) and fluorescence was measured ($\lambda_{ex} = 340$ nm; $\lambda_{em} = 495$ nm) in a microplate spectrofluorometer (Biotek—Winooski, VT, USA). Arbitrary fluorescence units (AFU) were converted into µmol/l of hydrolyzed substrate based on a calibration curve obtained using a standard solution of complete hydrolyzed substrate. Enzyme activity was inhibited by 20 µmol/l of B-type regulatory carboxypeptidases inhibitor DL-2-mercaptomethyl-3-guanidino-ethylthiopropanoic acid (MGTA) used as control in assays. To verify the interference of kinin B_1R in CPM activity, kinetic experiments were carried out in the presence of 1 µmol/l B_1R antagonist, R715, for 30 min. Protein content of each well was determined by Bradford method [40], using bovine serum albumin as standard. Measurements were performed in triplicate and CPM activity values were reported as µmol/l of substrate hydrolyzed per min per mg protein (µmol/l min^{-1} mg^{-1}).

Measurement of CPM activity on cell-culture supernatant

In this assay, endothelial cells were plated in 12-well plates at 10^5 cells per well, following incubation at 37 °C for 1 h in 500 µl of HBSS buffer (140 mmol/l NaCl, 5 mmol/l KCl, 0.1 mmol/l $CaCl_2$, 0.63 mmol/l $MgSO_4$, 1 mmol/l Na_2HPO_4, 6.1 mmol/l glucose, and 10 µmol/l $ZnCl_2$, pH 7.4). Afterward, cell supernatants were collected and 200 µmol/l of Dansyl-Ala-Arg-OH substrate were added in, according to kinetic points (0–60 min at 37 °C), as described in the previous section.

Statistics

All data are shown as mean ± SE (n), number of experiments. Statistical analyses were performed using Student t test. For statistical comparison, 95% confidence intervals were used. All statistical analyses were performed using the GraphPad-Prism 3.0 Program (GraphPad Software).

Results

B_1R ablation cellular model

Initially, to test the hypothesis that kinin B_1R could influence CPM expression and activity, microvascular endothelial cells from control C57Bl/6 and kinin B_1 receptor knockout mice were used. Genetic deletion of B_1R reduced by half mRNA levels of CPM (Fig. 1a). As expected, no expression of B_1R mRNA could be detected in cells from the kinin B_1 receptor knockout mice and a small expression in the cells from C57Bl/6 (data not shown). A small, but significant decrease in CPM expression was also observed at protein level, where the absence of B_1R caused around ~ 1.3-fold reduction in protein expression (Fig. 1b). In this B_1 receptor ablation model, CPM activity decreased by half when compared to control mice cells (Fig. 1c). The assay specificity was confirmed by pre-incubation with 20 µmol/l of MGTA (Fig. 1c).

In addition, we also asked the question whether the pharmacological blockade of the kinin B_1R receptor could interfere with CPM expression. However, after treating the cells from wild-type mice with the kinin B_1R receptor antagonist R715, no alteration in CPM activity was observed (Fig. 2a). To verify if the decrease on CPM activity in endothelial cells from $B_1^{-/-}$ mice group was due to differential release of CPM from membrane (shedding), we also analyzed CPM activity in cell-culture supernatant. In this case, supernatant from both groups, WT and $B_1^{-/-}$, showed a similar and 100 times lower CPM activity when compared to activity in the cell membrane (Fig. 2b).

B_1R overexpression cellular model

To evaluate CPM expression and activity in an opposite situation from B_1 receptor ablation, we used microvascular endothelial cells from pulmonary beds from control Sprague–Dawley and transgenic rats overexpressing B_1 receptor exclusively in the endothelium. In this overexpressing B_1R model, CPM mRNA expression was increased eightfold when compared to control group (Fig. 3a). B_1R mRNA was seven times more expressed in TGR (Tie_2B_1) cells' group in relation to control (data not shown). In accordance with mRNA measurement, Western blot analysis revealed a two fold higher expression of CPM protein in transgenic rat cells (Fig. 3b). In addition, CPM activity increased almost twice in the cells, where B_1R are overexpressed, in comparison with control (Fig. 3c). Assay specificity was confirmed by CPM activity inhibition with MGTA (Fig. 3c). Pre-incubation of cells with kinin B_1 antagonist R715 reduced significantly CPM activity in

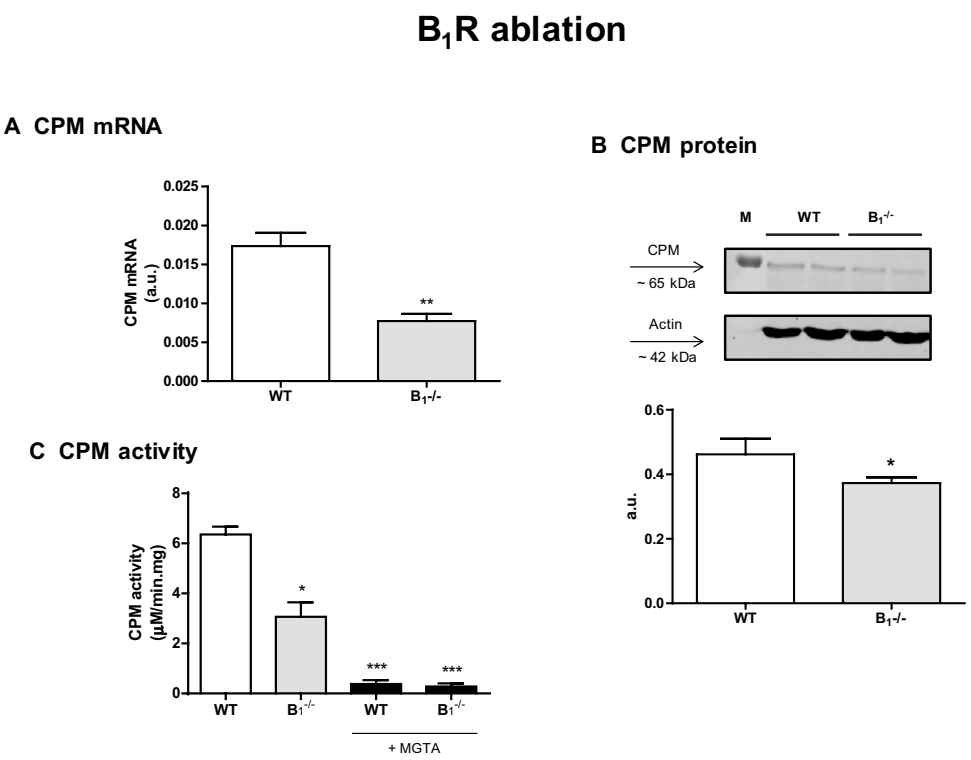

Fig. 1 CPM expression and activity on endothelial cells from WT and $B_1^{-/-}$ mice. **a** Total RNA was extracted from endothelial cells obtained from WT and $B_1^{-/-}$ mice. qPCR was performed using primers for CPM and β-actin cDNAs. Data are mean ± SEM of $2^{-\Delta Ct}$ parameter from cells of four animals and represent the relative expression between CPM and β-actin mRNA; $^{**}p < 0.01$ vs WT. **b** Western blot analysis of CPM in primary endothelial cells obtained from WT and $B_1^{-/-}$ mice. Total protein (50 µg) was extracted from both group of cells, blotted in SDS-PAGE (7.5% gel), and quantified in relation to actin expression. Data are mean ± SEM ($n = 2$, triplicate); $^{*}p < 0.05$ vs WT. **c** CPM activity was measured by the cleavage of Dansyl-Ala-Arg-OH substrate (200 µmol/l) in WT and $B_1^{-/-}$ cells. MGTA (20 µmol/l)—DL-2-mercaptomethyl-3-guanidino-ethylthiopropanoic acid—specific inhibitor of B-type regulatory carboxypeptidases. Data are mean ± SEM ($n = 4$, triplicate); $^{*}p < 0.05$ vs WT, $^{***}p < 0.001$ vs WT and $B_1^{-/-}$

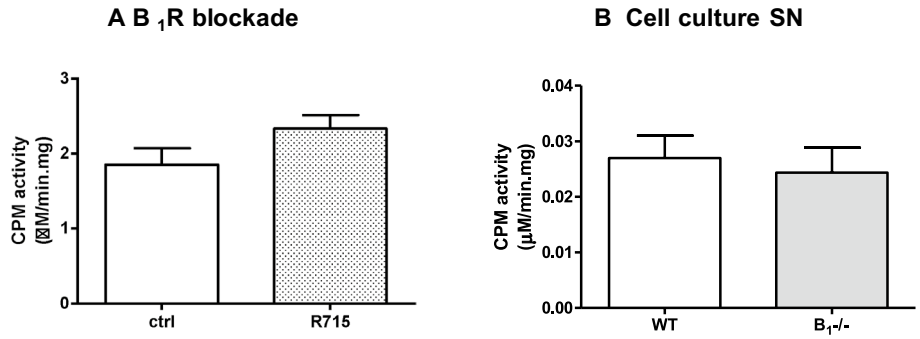

Fig. 2 CPM activity after B_1R blockade and in supernatant of endothelial cells from WT and $B_1^{-/-}$ mice. **a** CPM activity was measured by the cleavage of Dansyl-Ala-Arg-OH substrate (200 µmol/l) in cells from WT mice incubated with 1 µmol/l R715 (B_1R antagonist). Data are mean ± SEM ($n = 4$, triplicate). **b** Shedding of CPM from endothelial cells membrane. CPM activity was measured in the supernatant of mice endothelial cell culture, by the cleavage of Dansyl-Ala-Arg-OH (200 µmol/l). Data are mean ± SEM ($n = 3$, duplicate)

TGR ($Tie2B_1$) cells, but had no effect in control group (Fig. 4a). CPM activity was also evaluated in cell-culture supernatant, but no difference between transgenic TGR (Tie_2B_1) and control rat was observed (Fig. 4b).

B_1R transfection cellular model

To confirm B_1R influence on carboxypeptidase M expression and activity, we developed an artificial in vitro model,

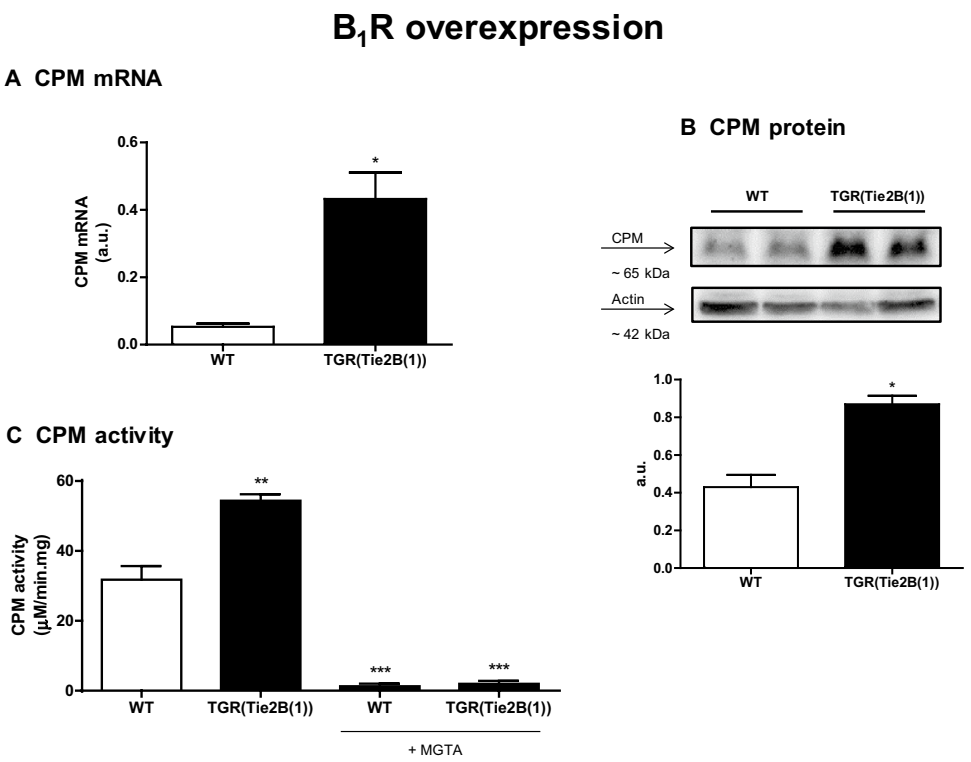

Fig. 3 CPM expression and activity on endothelial cells from WT and overexpressing B_1R transgenic rats. **a** Total RNA was extracted from endothelial cells obtained from WT and TGR ($Tie2B_1$) rats and qPCR was performed using primers for CPM and β-actin cDNAs. Data are mean ± SEM of $2^{-\Delta Ct}$ parameter from cells of four animals and represent the relative expression between CPM and β-actin mRNA; $^*p < 0.05$ vs WT. **b** Western blot analysis of CPM in primary endothelial cells from rats. Total protein (50 µg) was extracted from both group of cells, blotted in SDS-PAGE (7.5% gel), and quantified in relation to actin expression. Data are mean ± SEM ($n = 2$, triplicate). $^*p < 0.05$ vs WT. **c** CPM activity in primary endothelial cells from rats overexpressing B_1R exclusively in endothelium [TGR ($Tie2B_1$)] and WT was measured by the cleavage of Dansyl-Ala-Arg-OH substrate (200 µmol/l). MGTA (20 µmol/l)—DL-2-mercaptomethyl-3-guanidino-ethylthiopropanoic acid—specific inhibitor of B-type regulatory carboxypeptidases. Data are mean ± SEM ($n = 3$, duplicate); $^{**}p < 0.01$ vs WT

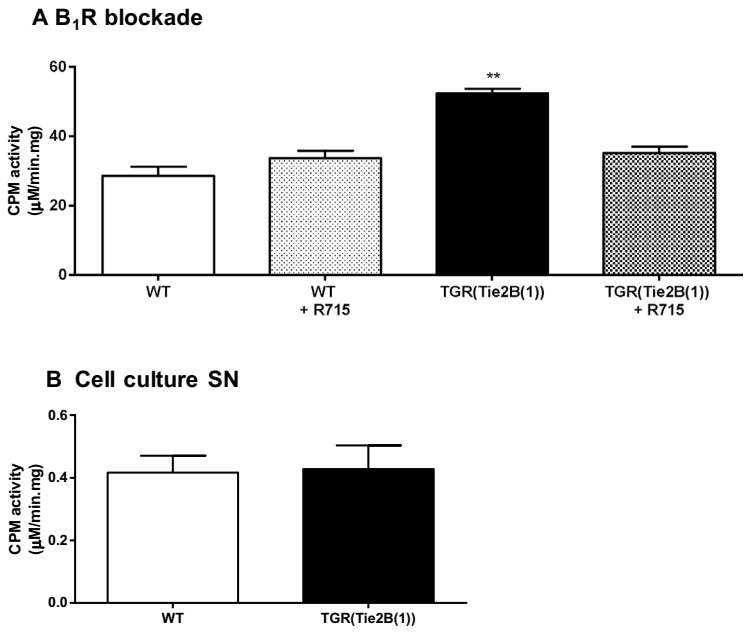

Fig. 4 CPM activity after B_1R blockade and in the supernatant of endothelial cells from WT and TGR ($Tie2B_1$) rats. **a** CPM activity was measured by the cleavage of Dansyl-Ala-Arg-OH substrate (200 µmol/l) in cells from WT and TGR ($Tie2B_1$) rats treated with 1 µmol/l R715 (B_1R antagonist). Data are mean ± SEM ($n = 3$, triplicate). $^{**}p < 0.01$ vs WT and TGR ($Tie2B_1$) + R715. **b** Shedding of CPM from the endothelial cell membrane. CPM activity was measured in the supernatant of rat endothelial cell culture, by the cleavage of Dansyl-Ala-Arg-OH (200 µmol/l). Data are mean ± SEM ($n = 3$, duplicate)

by transfecting HEK293T cells with a plasmid carrying the human B_1R coding region. Control cells had no detectable B_1R mRNA, whereas transfected cells presented a high expression of B_1R mRNA (data not shown). CPM mRNA expression was three times higher in HEK-B_1 when compared to control cells (Fig. 5a). In agreement with mRNA measurement, CPM protein expression was also two times increased in HEK-B_1 cells (Fig. 5b). Following CPM expression levels, CPM activity measured in monolayer cells was 1.3-fold higher in cells expressing B_1R receptor when compared to non-transfected cells (Fig. 5c) and this effect was blocked by incubation of cells with R715 (Fig. 5c). MGTA could inhibit Dansyl-Ala-Arg-OH substrate cleavage by CPM, demonstrating assays' specificity (Fig. 5c).

B_1R phenotype rescued model

Finally, we rescued the wild phenotype, by transfecting $B_1^{-/-}$ microvascular endothelial cells with a B_1R expressing plasmid, to confirm whether CPM expression and activity decrease in these cells were really due to B_1R absence. Interestingly, B_1R transfection in $B_1^{-/-}$ cells caused an increase in CPM expression and activity, dependent on B_1R transfected amount. CPM protein expression levels augmented around 3.5 times (Fig. 6a) in this model. In agreement, CPM activity was almost three times increased in this model and the effect could specifically be reduced by R715 (Fig. 6b) and inhibited by MGTA (data not shown).

Discussion

In this work, we intended to prove the hypothesis that the interaction between the carboxypeptidase M and the kinin B1 receptor, in addition to change the pharmacology of the receptor, could also modulate the enzyme activity. Therefore, using different cell models, we could demonstrate that higher CPM expression and activity was observed in cell models, where B1 receptors were upregulated, such as microvascular endothelial cells from pulmonary beds of transgenic rats overexpressing the B1 receptor exclusively in the endothelium and B1R transfected HEK293T cells. Conversely, using microvascular endothelial cells obtained from pulmonary beds of transgenic mice knockout for kinin B1 receptor, we observed a marked reduction in CPM activity, suggesting that in B1R absence, CPM activity is downregulated. To

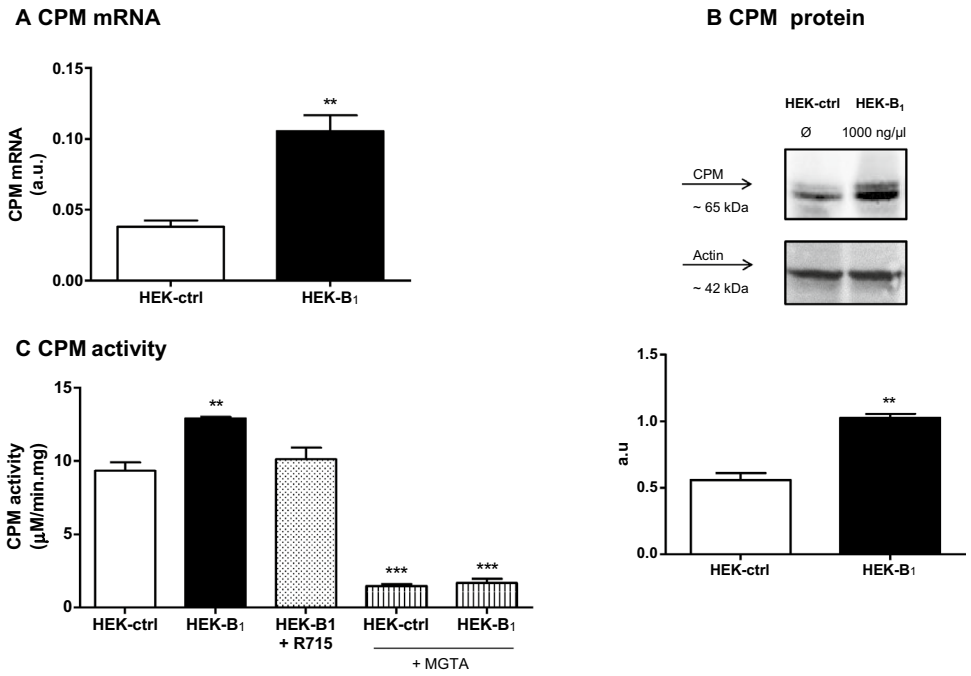

Fig. 5 CPM expression and activity on control HEK cells and transfected with B_1R. **a** Total RNA was extracted from HEK-ctrl and HEK-B_1 cells and qPCR was performed using primers for CPM and β-actin cDNAs. Data are mean ± SEM of $2^{-\Delta Ct}$ parameter from cells of four animals and represent the relative expression between CPM and β-actin mRNA; $^{**}p < 0.05$ vs WT. **b** Western blot analysis of CPM in HEK-ctrl and HEK-B_1 cells. Total protein (50 µg) was extracted from both group of cells, blotted in SDS-PAGE (7.5% gel), and quantified in relation to actin expression. Data are mean ± SEM ($n = 3$, duplicate); $^{**}p < 0.05$ vs HEK-ctrl, $^{***}p < 0.001$ vs HEK-ctrl and HEK-B_1. **c** CPM activity was measured by the cleavage of Dansyl-Ala-Arg-OH substrate (200 µmol/l) in HEK-B_1 cells. MGTA (20 µmol/l)—DL-2-mercaptomethyl-3-guanidino-ethylthiopropanoic acid—specific inhibitor of B-type regulatory carboxypeptidases. Data are mean ± SEM ($n = 2$, duplicate). $^{**}p < 0{,}01$ vs HEK-ctrl

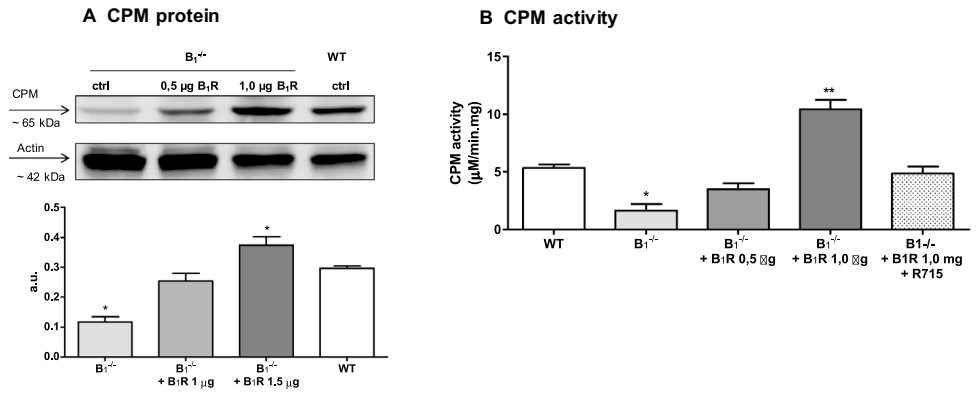

Fig. 6 CPM activity and expression on endothelial cells from WT mice, $B_1^{-/-}$ and $B_1^{-/-}$ transfected with B_1R. **a** Western blot analysis of CPM in cells from WT mice, $B_1^{-/-}$ and $B_1^{-/-}$ transfected with B_1R. Total protein (50 µg) was extracted from cells, blotted in SDS-PAGE (7.5% gel), and quantified in relation to actin expression. Data are mean ± SEM ($n = 2$, duplicate). $^{**}p < 0.05$ vs WT. **b** CPM activity was measured by the cleavage of Dansyl-Ala-Arg-OH substrate (200 µmol/l). MGTA (20 µmol/l)—DL-2-mercaptomethyl-3-guanidino-ethylthiopropanoic acid—specific inhibitor of B-type regulatory carboxypeptidases. Data are mean ± SEM ($n = 3$, duplicate); $^{*}p < 0.05$ vs WT, $^{**}p < 0.01$ vs WT, $B_1^{-/-}$ and $B_1^{-/-} + B_1R$ 1 µg

further confirm these findings, rescuing B1 receptor expression in these knockout cells significantly increased the activity of the carboxypeptidase. These data show for the first time in the literature that a clear modulation of CPM expression is promoted by the kinin B1 receptor.

Kallikrein–kinin system regulates several important physiological pathways, such as blood pressure balance, nociception, pain, and inflammation. The physiological effects are attributed to the action of the nonapeptide BK and its bioactive fragment, DBK, in two different GPCRs. BK acts through the constitutively expressed B_2R, whereas DBK acts through B_1R, low expressed, but highly induced under pathological conditions [19, 25, 41]. Kinins cleavage and regulation are tissue specific and can be modulated by pathological conditions; however, CPM remains as the main pathway for kinin B_1 receptor agonists generation [42, 43].

Carboxypeptidase M, a metallopeptidase GPI-anchored ectoenzyme, is widely expressed in several tissues, being localized mainly in membrane lipid-rafts microdomains [12–14], where B_1R is also co-localized, thus enabling interaction with each other. These membrane sections are essential for interactions between proteins in cell-surface membrane, enhancing not only receptors' signaling, but also enzymes' function [31–34].

Direct interaction between several G-protein-coupled receptors has been demonstrated by a variety of approaches such as biochemical, physiological, and pharmacological [44, 45]. Kinin B_2–B_1 receptors' heterodimers' formation has already been described in the literature with conflicting results. It has been shown to contribute to an adaptive initial response of B_2R to a prolonged action of B_1R in chronic injuries [46] and a downregulation of B_1 receptor function in this same B_2R–B_1R heterodimerization complex [28]. This interaction has also been described for kinin receptors with different receptor systems, such as B_2R-AT_1 angiotensin receptor [47–49] and B_2R-Mas angiotensin 1–7 receptor [50].

In addition to GPCRs interactions, the presence of receptors and enzymes' heterocomplexes on plasma membrane is crucial in mediating and regulating receptors' function, transmitting extracellular signals to intracellular compartment [51]. The membrane-coupled peptidases play an essential role in signaling regulation and peptide function, owing to their active site located in membrane extracellular portion. In this milieu, peptidases cleave the agonists, regulating both circulating levels and availability nearby to respective receptors. However, few studies have explored these interactions with the focus on the enzyme.

Sabatini et al. [34] demonstrated an allosteric regulation of angiotensin-I converting enzyme activity at the cell membrane by B_2R, without alteration on the expression levels of ACE. In this context, the analysis of ectoenzymes activity in more complex situations, and considering their interactions with other molecules, is determinant to better understanding its role in local and global systems, shedding light into molecular mechanisms that could be aimed for new treatments of disease states, such as inflammation. Therefore, herein, we analyzed the interaction between CPM and kinin B_1 receptor, evaluating the influence of the receptor on enzyme activity and expression in different cell models.

First, we used the kinin B_1 receptor absence model. For this purpose, we analyzed CPM expression and activity in endothelial cells from kinin B_1R knockout mice. Interestingly, cells from this animal model showed a marked reduction in CPM expression and activity, suggesting that, in B_1R absence, CPM activity is strongly down regulated.

On the other hand, CPM activity could be rescued in vitro by transiently transfecting these cells with the B_1 receptor, and this effect was shown to be dependent on B_1R amount in cell membrane, reinforcing the concept that CPM activity and expression are regulated by B_1R. Although transgenic knockout animals are important tools to improve our knowledge on gene function and pathway, to confirm these observations, phenotype rescue remains one of the most reliable approaches [52].

The same interaction pattern was observed when we evaluated CPM expression and activity in an opposite model, i.e., a system, where B_1R expression is increased. Therefore, we used microvascular endothelial cells from pulmonary beds of TGR (Tie_2B_1) rats, a model of high expression of the kinin B_1 receptor [36]. Consistently with the rational that co-expression of these two molecules leads to modulation in CPM, both expression and activity of this kininase were significantly increased in these cells. In addition, the findings observed in these natural cell models were supported by an artificial maneuver in which HEK293T cells were transiently transfected with B_1R. Similar to the findings observed in kinin transgenic overexpressing endothelial cells, the presence of B_1R in HEK293T cells leads to a significantly higher CPM activity, strongly indicating a positively modulation in CPM activity by the B_1R. Similar to our data, Zhang et al. [30] also demonstrated a positive modulation of iNOS by B_1R through an increased nitric oxide release in endothelial cells.

Recently, our group showed that ACE/B_2R interaction modulates ACE activity, and this effect could be blocked by icatibant, a kinin B_2 receptor antagonist [34]. Similarly, the results herein show that R715, B_1R antagonist, was able to normalize the increased CPM activity in highly expressing B_1R cell models. These findings suggest that R715 is able to bind to the heteroduplex CPM-B_1R and block the interaction between both molecules. Interestingly, the antagonist was not able to modify CPM activity in the cells of both wild-type mice and rats, probably due to the absence or low expression of the receptor in these cells.

GPI membrane anchored proteins, such as CPM, are susceptible to shedding from the membrane by proteases and phospholipases action [53]. CPM undergoes shedding [54]; however, until now, little is known about the mechanism involved in this process. It has been demonstrated that CPM is released from membrane by ACE [55] and this soluble CPM could be involved in peptide metabolism of extracellular fluids, such as urine, seminal plasma [14, 54]. Therefore, based on this assumption and on our findings that kinin B_1 receptor expression modulates CPM activity in plasma membrane, we investigated whether CPM shedding could be modulated by the B_1 receptor. However, our data showed that CPM shedding was not different in the both cell models tested, indicating that neither B_1R absence nor overexpression alters this event.

Taking this data together, we could infer that in B_1R absence, both CPM activity and expression are downregulated, a finding that could be confirmed by rescuing $B_1^{-/-}$ phenotype with transiently B_1R transfection. On the other hand, the model of high expression of the B_1R showed an opposite effect. Moreover, transfecting increasing amounts of B_1R in the cells revealed a dose effect of the receptor on CPM expression and activity. Thus, we could clearly demonstrate transcriptional and translational effects of kinin B_1 receptor on CPM.

Even though all cellular models studied herein pointed to a B_1R/CPM interaction that leads to modulation of CPM expression and this regulation might have important physiological outcomes, there are some limitations that must be considered. For instance, we did not show the direct interaction and dimerization of these two proteins by the use of specific methods like co-immunoprecipitation, cross-linking or fluorescence resonance energy transfer analysis, as shown by others [29].

In summary, our work shows for the first time that the interaction between CPM and kinin B_1 receptor alters not only B_1R pharmacology, but also modulates enzyme expression and activity. Since the genes responsible for the expression of these molecules are under control of inflammatory stimuli [41, 56], our results suggest that this interaction at the plasma membrane could lead to a differential phenotype in several metabolic conditions, where both components are expressed. Considering the putative role of CPM and B_1R in different patho-physiological processes such as inflammation, sepsis, macrophage differentiation, and cardiovascular processes, we believe that this work brings original data regarding ectoenzymes and receptor interactions and highlights the blockade of these molecules as a key target for new therapeutic approaches.

Acknowledgements We thank Eduardo Spitti Resende for technical assistance.

Funding This work was supported by grants from the São Paulo State Research Foundation—FAPESP (Grant Nos. 2014/03790-5 and 2014/27198-8) and research fellows from the Brazilian National Research Council (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) (Grant No. 374/12), and Deutsche Akademische Austauchdienst Probral.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Inder KL, Davis M, Hill MM. Ripples in the pond—using a systems approach to decipher the cellular functions of membrane microdomains. Mol BioSyst. 2013;9(3):330–8.
2. Rossy J, Ma Y, Gaus K. The organisation of the cell membrane: do proteins rule lipids? Curr Opin Chem Biol. 2014;20C:54–9.
3. Bissig C, Gruenberg J. Lipid sorting and multivesicular endosome biogenesis. Cold Spring Harb Perspect Biol. 2013;5:a016816.
4. Simons K, Ikonen E. Functional rafts in cell membranes. Nature. 1987;387:569–72.
5. Mukherjee S, Maxfield FR. Membrane domains. Annu Rev Cell Dev Biol. 2004;20:839–66.
6. Simons K, Toomre D. Lipid rafts and signal transduction. Nat Rev Mol Cell Biol. 2000;1(1):31–9.
7. Smart EJ, Graf GA, McNiven MA, Sessa WC, Engelman JA, Scherer PE, Okamoto T, Lisanti MP. Caveolins, liquid-ordered domains, and signal transduction. Mol Cell Biol. 1999;19:7289–304.
8. Zhang X, Skidgel R. Carboxypeptidase M. Handbook of proteolytic enzyme. 3rd ed. Oxford: Academic Press; 2013. p. 1357–66.
9. Deiteren K, Hendriks D, Scharpé S, Lambeir AM. Carboxypeptidase M multiple alliances and unknown partners. Clin Chim Acta. 2009;399:24–39.
10. Skidgel RA. Basic carboxypeptidases: regulators of peptide hormone activity. Trends Pharmacol Sci. 1988;9:299–304.
11. Reverter D, Maskos K, Tan F, Skidgel RA, Bode W. Crystal structure of human carboxypeptidase M, a membrane-bound enzyme that regulates peptide hormone activity. J Mol Biol. 2004;338(2):257–69.
12. Goding JW, Howard MC. Ecto-enzymes of lymphoid cells. Immunol Rev. 1998;161:5–10.
13. Skidgel RA, Davis RM, Tan F. Human carboxypeptidase M: purification and characterization of a membrane-bound carboxypeptidase that cleaves peptide hormones. J Biol Chem. 1989;264:2236–41.
14. Tan F, Balsitis S, Black JK, Blöchl A, Mao JF, Becker RP, Schacht D, Skidgel RA. Effect of mutation of two critical glutamic acid residues on the activity and stability of human carboxypeptidase M and characterization of its signal for glycosylphosphatidylinositol anchoring. Biochem J. 2003;370(Pt 2):567–78.
15. Zhang X, Tan F, Brovkovych V, Zhang Y, Skidgel RA. Crosstalk between carboxypeptidase M and the kinin B1 receptor mediates a new mode of G protein-coupled receptor signaling. J Biol Chem. 2011;286:18547–61.
16. Denis CJ, Lambeir AM. The potential of carboxypeptidase M as a therapeutic target in cancer. Expert Opin Ther Targets. 2013;17(3):265–79.
17. Hadkar V, Sangsree S, Vogel SM, Brovkovych V, Skidgel RA. Carboxypeptidase-mediated enhancement of nitric oxide production in rat lungs and microvascular endothelial cells. Am J Physiol Lung Cell Mol Physiol. 2004;287:L35–45.
18. Vroling B, Sanders M, Baakman C, Borrmann A, Verhoeven S, Klomp J, Oliveira L, de Vlieg J, Vriend G. GPCRDB: information system for G protein-coupled receptors. Nucleic Acids Res. 2011;39:D309–19.
19. Leeb-Lundberg LM, Marceau F, Muller-Esterl W, Pettibone DJ, Zuraw BL. International union of pharmacology. XLV. Classification of the kinin receptor family: from molecular mechanisms to pathophysiological consequences. Pharmacol Rev. 2005;57:27–77.
20. Mombouli JV, Vanhoutte PM. Kinins and endothelial control of vascular smooth muscle. Annu Rev Pharmacol Toxicol. 1995;35:679–705.
21. Field JL, Butt SK, Morton IK, Hall JM. Bradykinin B2 receptors and coupling mechanisms in the smooth muscle of the guinea-pig taenia caeci. Br J Pharmacol. 1994;113(2):607–13.
22. Oliveira L, Paiva AC, Sander C, Vriend G. A common step for signal transduction in G protein-coupled receptors. Trends Pharmacol Sci. 1994;15(6):170–2.
23. Marceau F. Kinin B1 receptors: a review. Immunopharmacology. 1995;30:1–26.
24. Steranka LR, Manning DC, DeHaas CJ, Ferkany JW, Borosky SA, Connor JR, Vavrek RJ, Stewart JM, Snyder SH. Bradykinin as a pain mediator: receptors are localized to sensory neurons, and antagonists have analgesic actions. PNAS. 1988;85(9):3245–9.
25. Marceau F, Bachvarov DR. Kinin receptors. Clin Rev Allergy Immunol. 1998;16(4):385–401.
26. Leeb-Lundberg LM, Kang DS, Lamb ME, Fathy DB. The human B1 bradykinin receptor exhibits high ligand-independent, constitutive activity. Roles of residues in the fourth intracellular and third transmembrane domains. J Biol Chem. 2001;276(12):8785–92.
27. Faussner A, Bathon JM, Proud D. Comparison of the responses of B1 and B2 kinin receptors to agonist stimulation. Immunopharmacology. 1999;45(1–3):13–20.
28. Kang DS, Gustafsson C, Mörgelin M, Leeb-Lundberg LM. B1 bradykinin receptor homo-oligomers in receptor cell surface expression and signaling: effects of receptor fragments. Mol Pharmacol. 2005;67(1):309–18.
29. Zhang X, Tan F, Zhang Y, Skidgel RA. Carboxypeptidase M and kinin B1 receptors interact to facilitate efficient B1 signaling from B2 agonists. J Biol Chem. 2008;283(12):7994–8000.
30. Zhang Y, Brovkovych V, Brovkovych S, Tan F, Lee BS, Sharma T, Skidgel RA. Dynamic receptor-dependent activation of inducible nitric-oxide synthase by ERK-mediated phosphorylation of Ser745. J Biol Chem. 2007;282(44):32453–61.
31. Marcic B, Deddish PA, Jackman HL, Erdos EG, Tan F. Effects of the N-terminal sequence of ACE on the properties of its C-domain. Hypertension. 2000;36:116–21.
32. Chen Z, Deddish PA, Minshall RD, Becker RP, Erdös EG, Tan F. Human ACE and bradykinin B2 receptors form a complex at the plasma membrane. FASEB J. 2006;20(13):2261–70.
33. Erdös EG, Deddish PA. The kinin system: suggestions to broaden some prevailing concepts. Int Immunopharmacol. 2002;2(13–14):1741–6.
34. Sabatini RA, Guimarães PB, Fernandes L, et al. ACE activity is modulated by kinin B2 receptor. Hypertension. 2008;51(3):689–95.
35. Pesquero JB, Araujo RC, Heppenstall PA, Stucky CL, Silva JA Jr, Walther T, Oliveira SM, Pesquero JL, Paiva AC, Calixto JB, Lewin GR, Bader M. Hypoalgesia and altered inflammatory responses in mice lacking kinin B1 receptors. PNAS. 2000;97(14):8140–5.
36. Merino VF, Todiras M, Campos LA, Saul V, Popova E, Baltatu OC, Pesquero JB, Bader M. Increased susceptibility to endotoxic shock in transgenic rats with endothelial overexpression of kinin B(1) receptors. J Mol Med. 2008;86(7):791–8.
37. Chen SF, Fei X, Li SH. A new simple method for isolation of microvascular endothelial cells avoiding both chemical and mechanical injuries. Microvasc Res. 1995;50:119–28.
38. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods. 2001;25(4):402–8.
39. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970;227:680–5.
40. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72:248–54.
41. Bhoola KD, Figueroa C, Worthy K. Bioregulation of kinins: kallikreins, kininogens and kininases. Pharmacol. 1992;44:1–79.

42. Erdös EG, Skidgel RA. Metabolism of bradykinin by peptidases in health and disease. In: Farmer SG, editor. The Kinin system. London: Academic Press; 1997. p. 111–41.
43. Erdös EG. Kinins, the long march–a personal view. Cardiovasc Res. 2002;54(3):485–91.
44. Bouvier M. Oligomerization of G-protein-coupled transmitter receptors. Nat Rev Neurosci. 2001;2(4):274–86.
45. Devi LA. Heterodimerization of G-protein-coupled receptors: pharmacology, signaling and trafficking. Trends Pharmacol Sci. 2001;22(10):532–7.
46. Kang DS, Ryberg K, Mörgelin M, Leeb-Lundberg LM. Spontaneous formation of a proteolytic B1 and B2 bradykinin receptor complex with enhanced signaling capacity. J Biol Chem. 2004;279(21):22102–7.
47. Quitterer U, AbdAlla S. Vasopressor meets vasodepressor: the AT1-B2 receptor heterodimer. Biochem Pharmacol. 2014;88(3):284–90.
48. Abadir PM, Periasamy A, Carey RM, Siragy HM. Angiotensin II type 2 receptor-bradykinin B2 receptor functional heterodimerization. Hypertension. 2006;48(2):316–22.
49. AbdAlla S, Lother H, Quitterer U. AT1-receptor heterodimers show enhanced G-protein activation and altered receptor sequestration. Nature. 2000;407(6800):94–8.
50. Silva LS, Peruchetti DB, Silva CTF, Ferreira-DaSilva AT, Perales J, Caruso-Neves C, Pinheiro AAS. Interaction between bradykinin B2 and Ang-(1–7) Mas receptors regulates erythrocyte invasion by *Plasmodium falciparum*. Biochem Biophys Acta. 2016;1860(11PtA):2438–44.
51. Scott JD, Pawson T. Cell signaling in space and time: where proteins come together and when they're apart. Science. 2009;326(5957):1220–4.
52. Eisener-Dorman AF, Lawrence DA, Bolivar VJ. Cautionary Insights on Knockout Mouse Studies: the Gene or Not the Gene? Brain Behav Immun. 2009;23(3):318–24.
53. Low MG. Glycosyl-phosphatidylinositol: a versatile anchor for cell surface proteins. FASEB J. 1989;3(5):1600–8.
54. Skidgel RA. Structure and function of mammalian zinc carboxypeptidases. Zinc metalloproteases in health and disease. London: Taylor and Francis Ltd.; 1996. p. 241–83.
55. Sun X, Wiesner B, Lorenz D, Papsdorf G, Pankow K, Wang P, Dietrich N, Siems WE, Maul B. Interaction of angiotensin-converting enzyme (ACE) with membrane-bound carboxypeptidase M (CPM)—a new function of ACE. Biol Chem. 2008;389(12):1477–85.
56. Proud D, Kaplan AP. Kinin formation: mechanisms and role in inflammatory disorders. Annu Rev Immunol. 1988;6:49–83.