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Evidence that kinin B_2 receptor expression is upregulated by endothelial overexpression of B_1 receptors

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ABSTRACT

Bradykinin (BK) and des-Arg^9-bradykinin (DBK) of kallikrein-kinin system exert its effects mediated by the B_2 (B_2R) and B_1 (B_1R) receptors, respectively. It was already shown that the deletion of kinin B_1R or of B_2R induces upregulation of the remaining receptor subtype [10,12,16,28,36]. However studies on overexpression of B_1R or B_2R in transgenic animals have supported the importance of the overexpressed receptor but the expression of another receptor subtype has not been determined [17,19,33]. Previous study described a marked vasodilatation and increased susceptibility to endotoxic shock which was associated with increased mortality in response to DBK in thoracic aorta from transgenic rat overexpressing the kinin B_1R (TGR(Tie$_2$B$_1$)) exclusively in the endothelium. In another study, mice overexpressing B_1R in multiple tissues were shown to present high susceptibility to inflammation and to lipopolysaccharide-induced endotoxic shock. Therefore the role of B_2R was investigated in the thoracic aorta isolated from TGR(Tie$_2$B$_1$) rats overexpressing the B_1R exclusively in the vascular endothelium. Our findings provided evidence for highly increased expression level of the B_2R in the transgenic rats. It was reported that under endotoxic shock, these rats exhibited exaggerated hypotension, bradycardia and mortality. It can be suggested that the high mortality during the pathogenesis of endotoxic shock provoked in the transgenic TGR(Tie$_2$B$_1$) rats could be due to the enhanced expression of B_2R associated with the overexpression of the B_1R.

1. Introduction

Kinins are potent inflammatory mediators and induce contraction and relaxation in several vascular and non-vascular smooth muscles [4,24]. The kinins belong to the kallikrein-kinin system (KKS) involved in the renal and cardiovascular regulation [4,13]. Bradykinin (BK: Arg^1-Pro^2-Pro^3-Gly^4-Phe^5-Ser^6-Pro^7-Phe^8-Arg^9), is a nonapeptide hormone which mediates the action of the constitutively expressed kinin B_2 receptor (B_2R). On the other hand, the kinin B_1 receptor (B_1R) is an induced receptor and its effect is mediated by des-Arg^9-BK (DBK), a 1–8 fragment of BK [25]. The expression level of B_1R is very low in healthy tissues but high in inflammatory conditions or after time-dependent incubation [13,15,25].

Genetically engineered animals have been inbred to allow a better understanding of the function of kinins and the role of each subtype of receptors. Therefore several transgenic animals have been created, such as mice deficient in the kinin B_1R [21,28], in the kinin B_2R [5,9,10,12] and also in both kinin B_1R and B_2R [8], as well as mice overexpressing the B_1R [17,19] or the B_2R [34]. It has been shown that the lack of kinin receptors may affect the reactivity of the vascular smooth muscle preparations or cause changes in the expression level of some receptors of kallikrein kinin system (KKS) and renin angiotensin system (RAS) in the same tissue [26].

A cross-talk between the RAS and KKS is based on the effect of angiotensin I (AngI) converting enzyme (ACE), which is responsible for the cleavage of AngI into the potent vasoconstrictor angiotensin II (AngII) and of the vasodilator BK into non-active peptide fragments [4,6,37]. The ACE enzyme is mostly expressed in the endothelial vascular smooth muscle, mainly in the pulmonary arteries [4].

Several studies have demonstrated interactions between kinin receptors and AngII type 1 receptor (AT_1R). Therefore it was reported that spontaneous interaction of B_1R and B_2R increases the ability of B_1R but not of B_2R to be stimulated by its agonist [11]; heterodimerization between B_2R and AT_1R causes increased activation of G protein signaling triggered by AT_1R but not by B_2R [1]; AngII may regulate the expression of B_2R mRNA [32], that B_2R gene is a

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downstream target of AngII AT_1R [29]; the activity of angiotensin converting enzyme (ACE) is enhanced in kinin B_1R knockout mice (B_1KO) [20] and by an interaction between ACE and kinin B_2R [27].

These data from the literature about cross-talk between RAS and KKS and the evidence for expression of AngII AT_1R protein and mRNA in endothelial cells [18,22,23,31,35] provide rationale for studying the interactions between AngII and BK receptors in addition to the assessment about vascular reactivity of the kinin as well as the expression level of B_2R in the aorta isolated from transgenic (TGR(Tie_2B_1)) rats.

2. Material and methods

2.1. Animals

Experiments were carried out using 300–350 g Sprague-Dawley rats as control (WT) and overexpressing B_1R (TGR(Tie_2B_1)), [17] from the "Centro de Desenvolvimento de Modelos Experimentais" (CEDEME) of the Universidade Federal de São Paulo (UNIFESP). The animals were maintained on standard rat chow at 21–23 °C and kept on 12 h light: 12 h dark cycle and allowed *ad libitum* access to food and water. The protocols used in this study were in accordance with current guidelines for the care of laboratory animals and ethical guidelines for investigations approved by the Animal Care Committee of UNIFESP.

2.2. Isometric relaxation and contraction recordings

Thoracic aorta were isolated from rat, cleared of connective tissue and mounted as ring preparations into 5 ml organ baths. The rings of aorta were bathed in carboxygenated (95% O_2/5% CO_2), and modified Krebs-Ringer solution: 144 mM NaCl, 5 mM KCl, 1.1 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.1 mM NaH_2PO_4, 1.25 mM CaCl and 5.5 mM glucose at 37 °C (pH 7.4). Resting tension was maintained at 0.5 g and the tissues were left to equilibrate for 90 min, with frequent changing of bathing solution. The tissue viability was assessed with a priming dose of 80 mM KCl and 1 μM norepinephrine (NE), as described previously by [30]. Following a 90 min washout and recovery period, changes in tension produced by the stimulants were measured with an isometric transducer TRI201 (Panlab s.l., Cornella, Barcelona, Spain) through an amplifier Powerlab 4/30 and software Labchart Pro V7 (ADInstruments, Colorado Springs, CO, USA).

Cumulative concentration–response curves were constructed for BK applying increasing concentrations (0.1 nM to 1 μM) of the agonist. On the other hand non-cumulative concentration–response curves were obtained for AngI and Ang II to avoid desensitization, as described previously by [3]. The presence of the endothelium in the thoracic aorta was confirmed pharmacologically by the acetylcholine-induced relaxing effect on aortic rings pre-contracted with NE 1 μM. Some curves were obtained in the presence and absence of lisinopril (1 μM), an ACE inhibitor, pre-incubated for 20 min and in the presence of R-715, specific antagonist of B_1R, since the tissue was isolated from the animal. The effect of specific blockers of B_2R, HOE-140 (1 μM), the nitric oxide synthase inhibitor, L-NAME (1 mM) and the cyclooxygenase inhibitor, indomethacin (1 μM) pre-incubated for 20 min were tested on the maximal response induced by BK. Curve-fitting analyses (GraphPad-Prism software, San Diego, California, USA) were used to determine the apparent affinity of agonists in terms of pD_2, which is the negative logarithm of the concentration of agonist that produces 50% of the maximal effect) and the maximal effect (E_{max}) was calculated in relation to the effect induced by 1 μM NE, which was considered 100%.

2.3. Real-time PCR

Animals of each group were sacrificed and their aorta isolated, dissected and immediately frozen in liquid nitrogen. Total RNA was isolated using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA). After purification, the presence of intact RNA was verified on an ethidium bromide-stained agarose gel. The total RNA was submitted to reverse transcription in the presence of 2.5 ng/μL of random hexanucleotides and 2.5 μM of oligo$(dT)_{20}$, 200 μM of dNTP, 10 mM of $MgCl_2$ and 2 units of SuperScript™ III First-Strand reverse transcriptase (Invitrogen, Carlsbad, CA, USA).

To determine the expression levels of kinin B_2 and AT_1 receptors, and ACE, real-time PCR was performed using 5 μl of samples containing 1:10 diluted cDNA. Each reaction was carried out with 10 μL of TaqMan Universal PCR Master Mix 2× (Applied Biosystems, Foster City, CA, USA), 1 μL of each pair of specific primers and a probe linked with a TAMRA dye and a FAM quencher. The used primers were for B_2R (reverse primer 5′-CACCACGCGGCACAG-3′, forward primer 5′-ATCACCATCGCCAATAACTTCGA-3′ and probe 5′-6FAM-CACCTCTCCGAACAGC-TAMRA-3′), for ACE (reverse primer 5′-CCTGCTGTGGTTCCAGGTACA-3′, forward primer 5′-AACACGGCTCGTGCAGAAG-3′ and probe, 5′-6FAM-CCTCCCAGAGTCCAGTCGCGTCA-TAMRA-3′) and for AT_1 receptor (reverse primer 5′-CAGTGTCCACGATGTCAGAAATTTT-3′, forward primer 5′-ACTTTCCTGGATGTGCTGATTCAG-3′ and probe 5′-6FAM-CTGGGCGTCATCCAT-TAMRA-3′) and beta-actin endogenous control (reverse primer 5′-GCCTGGATGGCTACGTACATG-3′, forward primer 5′-GGCCAACCGTGAAAAGATGAC-3′ and probe 5′-6FAM-CAGATCATGTTTGAGACCTT-TAMRA-3′) and Mili-Q water (Milipore Corporation) to 20 μL.

The real-time PCRs were performed with an ABI PRISM® 7000 sequence detection system (Applied) and cycle conditions were: 50 °C for 2 min, 95 °C for 10 min, followed by 50 cycles of 95 °C for 15 s (melting step), 60 °C for 1 min (anneal/extend step). Increases in the amount of reporter dye fluorescence during the 50 amplification cycles were monitored using Sequence Detector software (SDS version 1.6, Applied Biosystems). Quantification of the target amount was performed by measuring the threshold cycle, C_T, which is defined as the fractional cycle number at which the fluorescence encounters a fixed threshold. A normalized value to evaluate the mRNA expression was calculated as the difference in the threshold cycle: the C_T values of receptor minus C_T of internal standard (β-actin), resulting in ΔC_T. Since it is uncommon to use ΔC_T parameter as a relative expression parameter due to this logarithmic characteristic, the $2^{-\Delta C_T}$ parameter was used to express the relative gene expression data [14]. Final data are expressed as the ratio of the fold-change in the target gene in the transgenic rat over the fold-change in the target gene of control rat.

2.4. ACE activity

Thoracic aorta isolated from rat were quickly harvested, rinsed, blotted, frozed and homogenized in Tris–HCl buffer, pH 7.0, containing 50 mM NaCl and Tween 20, 0.2%. Subsequently, the samples were centrifuged at 1000 *g* for 10 min and the supernatant was frozen at −20 °C. The protein contents of the samples were measured by the method of Bradford using bovine serum albumin as standard. The ACE activity was determined using Abz-FRK(Dnp)P-OH (Abz = *ortho*-amino benzoic acid; Dnp = ethylenediamine) as substrates following the methodology previously described [7]. The increase in the fluorescence was continuously measured in a Hitachi F-7000 fluorimeter set at λ_{em} = 420 nm and λ_{ex} = 320 nm and the assays carried out in 96-well plates (final volume in each well = 0.2 mL). The evaluation of thoracic aorta ACE activity was performed at 37 °C in 0.1 M Tris–HCl buffer, pH 7.0, containing 0.05 M NaCl, 10 μM $ZnCl_2$, and inhibitors of the hydrolytic activities that

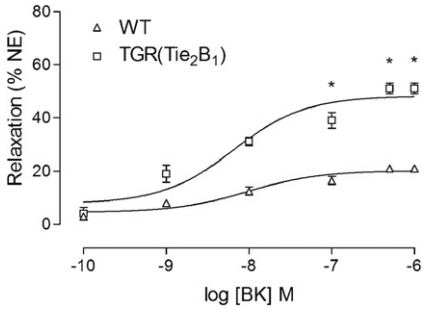

Fig. 1. Effect of the overexpression of B_1 receptors on the relaxation induced by BK in rat aorta. Cumulative concentration–response curves for bradykinin (BK) were determined in thoracic aortic rings isolated from WT and transgenic rat overexpressing the B_1 receptor specifically in the vascular endothelium (TGR(Tie_2B_1)). The isometric relaxing responses were calculated in relation to the increased tonus of the aorta induced by 1 μM norepinephrine (NE), considered 100%. Data are expressed as means ± SD of 5 experiments. *Significantly different from control WT animals ($P < 0.05$).

we want to suppress (10 μM E64, 1 μM pepstatin, 1 mM PMSF, 100 μM TLCK, and 100 μM TPCK). Before starting the reaction by the addition of 10 μM of Abz-FRK(Dnp)P-OH, the tissues homogenates were pre incubated for 5 min in the assay buffer, at 37 °C. To define the specificity for ACE, the assays were also performed in the presence of the cocktail of inhibitors plus 1 μM of the lisinopril. The slope was converted into nM of substrate hydrolyzed/min. The measurements were performed in triplicate and the results are expressed as means ± SD.

2.5. Drugs and primers

BK, AngI, AngII, lisinopril, L-NAME, indomethacin and HOE-140 were purchased from Sigma Chemical Co. (Dorset, U.K). R-715 was a gift from D. Regoli, Université de Sherbrooke, Quebec, Canada.

Concentrated solutions of peptides and other agents were prepared in water and kept at 20 °C until they were used. The stock solutions were serially diluted with Krebs–Ringer solution. Oligonucleotide primer and fluorogenic probe sets for Taqman™ Real-Time PCR were designed for kinin receptors and beta-actin using Assays-by-Design Service (Applied Biosystems).

2.6. Statistical analysis

Values are expressed as means ± SD and (n). The Student t-test was used to determine the statistical differences, with the level of significance set as $P < 0.05$.

3. Results

3.1. Effect of kinin BK on the aortic rings isolated from wild type and transgenic rats overexpressing the B_1R

Recordings of the relaxant effect induced by BK were obtained by cumulative increasing concentrations of the agonist into thoracic aortic preparations isolated from WT and rat overexpressing the B_1R specifically in the endothelium, TGR(Tie_2B_1). Non-cumulative concentration–response curves induced by BK were not different from the cumulative concentration curves. Fig. 1 shows the concentration-dependent relaxation to BK in the aortic rings isolated from WT and TGR(Tie_2B_1) rats. The maximal responses (%) were 21 ± 2 (4) for WT and 50 ± 5 (5) for TGR(Tie_2B_1) rats. The pD_2 (-log EC_{50}, concentration of the agonist that induces 50% of the maximal response) values were 8.0 ± 0.3 (4) for WT and 8.1 ± 0.3 (5) for TGR(Tie_2B_1).

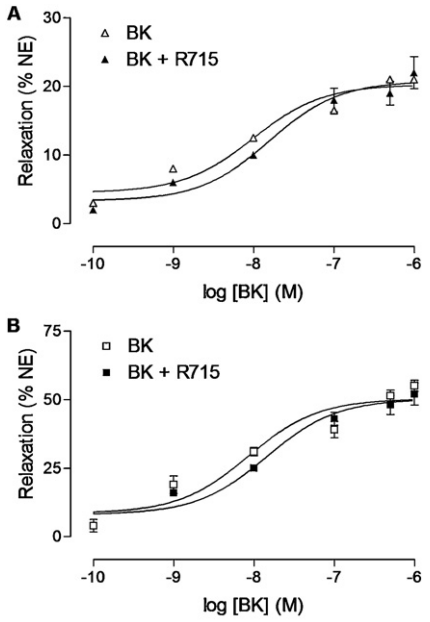

Fig. 2. Effect of specific kinin B_1 receptor antagonist, R-715, on the relaxant response induced by bradykinin (BK) in rat thoracic aorta. Effect of R-715 (1 μM) on BK induced response in aorta isolated from (A) WT and (B) transgenic rat with endothelial overexpression of kinin B_1 receptor (TGR(Tie_2B_1)). The responses were calculated in relation to the effect induced by 1 μM norepinephrine (NE), which was considered 100%. Data are means ± SD of 3 experiments.

3.2. Effect of kinin receptor antagonists on the relaxant responses induced by BK in thoracic aorta isolated from TGR(Tie_2B_1) rats

To evaluate whether the enhanced relaxant responses induced by BK were partly due to the activation of B_1R, the rings of thoracic aorta isolated from Fig. 2A, WT and Fig. 2B, rat overexpressing the B_1R specifically in the vascular endothelium (TGR(Tie_2B_1)) were preincubated with 1 μM of R-715, specific inhibitor of B_1R. As can be seen in Fig. 2, concentration–response curves for BK in the rat thoracic aorta were similar between WT and TGR(Tie_2B_1). The pD_2 values for BK in the presence of antagonist were 7.8 ± 0.1 (3) for WT and 7.8 ± 0.2 (3) for TGR(Tie_2B_1), whereas in preparations without the presence of the antagonist were 8.0 ± 0.3 (4) for WT and 8.1 ± 0.3 (5) for TGR(Tie_2B_1). The maximal response (%) to BK in the presence of 1 μM R-715 was 21 ± 1 (3) for WT and 50 ± 3 (3) for TGR(Tie_2B_1) and in non-treated preparations the values were 21 ± 2 (4) for WT and 50 ± 5 (5) for TGR(Tie_2B_1). On the other hand when 1 μM HOE-140 was pre-incubated, BK (100 nM) induced response

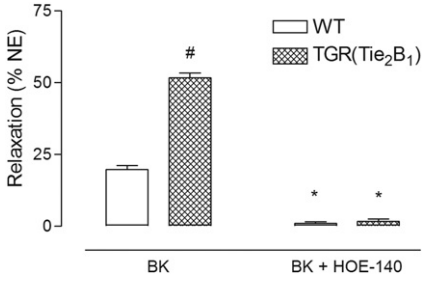

Fig. 3. Effect of specific kinin B_2 receptor antagonist on the relaxant responses induced by bradykinin (BK) in rat thoracic aorta. Effect of HOE-140 (1 μM) on the relaxant responses induced by 100 nM BK in rat aorta overexpressing the B_1 receptor exclusively in the endothelium (TGR(Tie_2B_1)) in comparison to the WT rat. The responses were calculated in relation to the effect induced by 1 μM norepinephrine (NE) which was considered 100%. *Significantly different ($P < 0.05$) from control (without antagonist) and #different from WT.

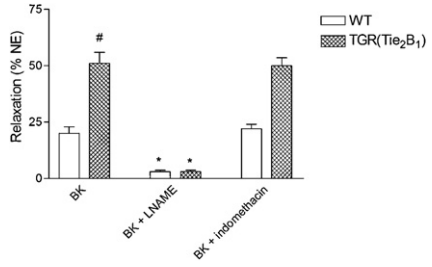

Fig. 4. Effect of L-NAME and indomethacin on bradykinin induced response in rat aorta. Relaxant responses induced by bradykinin (BK, 1 μM) in the presence or in the absence of 1 μM L-NAME or 1 μM indomethacin, pre-incubated for 20 min in rings of thoracic aorta isolated from WT rat and transgenic rat overexpressing the B_1 receptor exclusively in the endothelium (TGR(Tie_2B_1)). The relaxant responses were calculated in relation to the effect induced by 1 μM norepinephrine (NE). The data shown are the mean ± S.D. of 3 experiments. *Significantly different ($P < 0.05$) from the control (without inhibitors) and #different from WT.

was totally inhibited in rat aorta isolated from WT and TGR(Tie_2B_1) as shown in Fig. 3.

3.3. Effect of L-NAME and indomethacin on the bradykinin-induced relaxation

To verify if the BK-induced relaxation was mediated by NO, the inhibitor of NO synthase activity was tested. Pre-incubation with 1 mM L-NAME for 20 min completely blocked the maximal relaxation induced by BK in thoracic rings with endothelium-intact isolated from WT rat and TGR(Tie_2B_1). On the other hand, as shown in Fig. 4, the responses induced by BK in both preparations were not blocked by pre-incubation for 20 min with cyclooxygenase inhibitor indomethacin (1 μM).

3.4. Pharmacological determination of ACE activity in thoracic rings aorta isolated from transgenic rats overexpressing the B_1R

The finding that the reactivity to BK was enhanced in the transgenic kinin B_1R knockout mice [20] and that ACE activity can be influenced by B_2R and B_1R [2,27], led us to test the responsiveness of the thoracic aorta to AngI and to BK in the presence of lisinopril to evaluate a possible change in the ACE activity in TGR(Tie_2B_1) rats.

The role of ACE was tested on the relaxing responses to BK using lisinopril (1 μM) pre-incubated for 30 min. Under this condition, the curves concentration–responses to BK were obtained in the thoracic aorta of WT and TGR(Tie_2B_1) rats. Fig. 5 shows that the sigmoidal dose response curves were similar in both preparations (WT, Fig. 5A and TGR(Tie_2B_1), Fig. 5B), in comparison to those obtained in the absence of the inhibitor, indicating no significant difference in the sensitivity of BK to ACE activity in the TGR(Tie_2B_1) rats. Moreover, to determine the activity of ACE in TGR(Tie_2B_1) rats, on the conversion of AngI to AngII, contractile responses induced by AngI pre-incubated for 30 min with lisinopril were tested. Concentration–response curves were obtained incubating non-cumulative concentrations of AngI to avoid desensitization. In the presence of ACE inhibitor there was similar inhibition of the responses throughout all tested concentrations of the agonist in both strains of the rats (WT, 5C and TGR(Tie_2B_1), 5D). The pD_2 values expressing the potency and the maximal response (E_{max}) values are presented in Table 1.

3.5. Determination of ACE activity with Abz-FRK(Dnp)P-OH hydrolysis

The ACE activity was also determined using a selective fluorescence substrate assay for ACE with Abz-FRK(Dnp)P-OH as substrate.

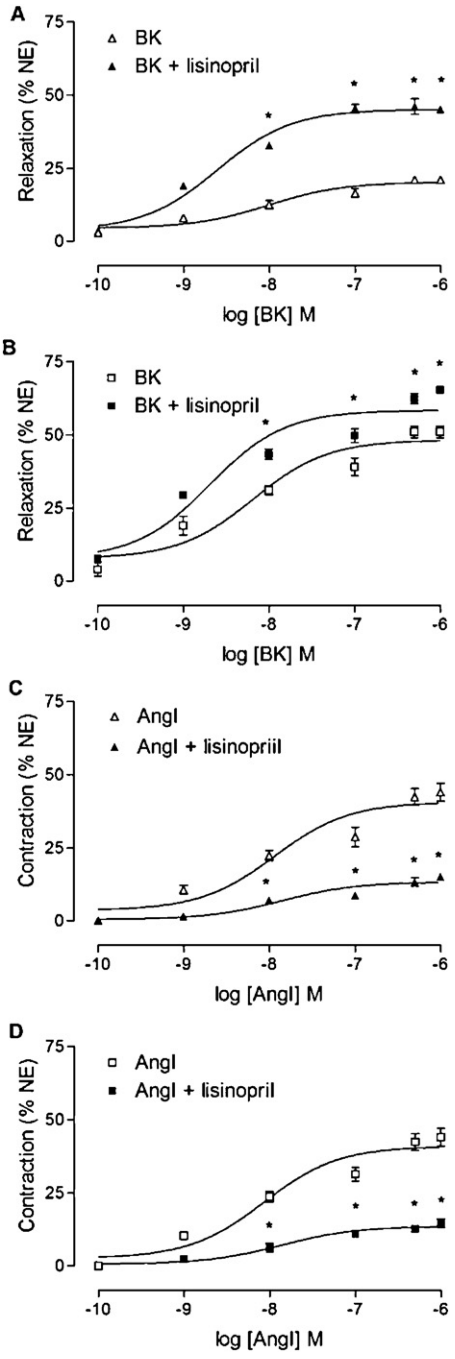

Fig. 5. Pharmacological determination of ACE activity in thoracic aorta. Cumulative concentration–relaxant response curves for bradykinin (BK) in thoracic aorta isolated from (A), WT and (B), from transgenic rat overexpressing the B_1 receptor specifically in the endothelium (TGR(Tie_2B_1)). Non-cumulative concentration–contractile response curves for angiotensin I (Ang I) in (C), WT and in (D), TGR(Tie_2B_1) rats. The curves were determined in the presence or absence of ACE inhibitor lisinopril (1 μM), pre-incubated for 20 min. The isometric relaxant responses to BK and contractile responses to AngI are expressed as percentage of the effect of 1 μM norepinephrine (NE), considered 100%. Data are means ± SD of 3 experiments. *Significantly different from the control WT animals ($P < 0.05$).

These results showed that the hydrolysis of the substrate was not different between WT and transgenic rat overexpressing the B_1R (Fig. 6). It was found that the cleavage of this substrate was completely abolished by 0.5 μM lisinopril.

Table 1
Effect of lisinopril on the apparent affinity and maximal responses induced by BK and AngI in rat thoracic aorta.

	BK		BK + lisinopril		Ang I		Ang I + lisinopril	
	pD_2	E_{max}	pD_2	E_{max}	pD_2	E_{max}	pD_2	E_{max}
WT	8.0 ± 0.3(4)	21 ± 2(4)	8.6 ± 0.2(3)	45 ± 3(3)*	7.9 ± 0.3(4)	41 ± 8(4)	7.8 ± 0.2(3)	13 ± 0.8(3)*
TGR(Tie_2B_1)	8.1 ± 0.3(5)	50 ± 5 (5)#	8.7 ± 0.4(3)	58 ± 5(3)*	8.0 ± 0.3(4)	41 ± 3(4)	7.8 ± 0.3(3)	14 ± 1(3)*

BK induced relaxant responses and contractile responses induced by AngI in the presence or in the absence of ACE inhibitor lisinopril (1 μM) in thoracic aorta isolated from WT rat and transgenic rat overexpressing the B_1 receptor specifically in the endothelium (TGR(Tie_2B_1)). The pD_2 values (negative logarithm of the agonist concentration (M) that induces 50% of the maximal response) were extracted from the concentration–response sigmoid logistic curves. The maximal response (E_{max}) was calculated in relation to 1 μM of norepinephrine, which was considered 100%. Data are means ± S.D. and (n) number of experiments.

* Different ($P < 0.05$) from BK or Ang I (without inhibitor).

\# Different from WT.

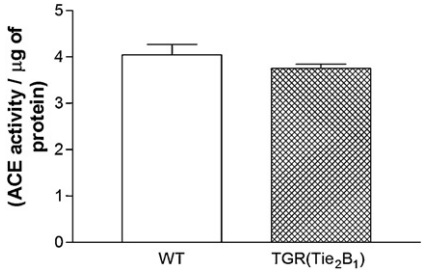

Fig. 6. Fluorescence assay of ACE activity. ACE activity assay was performed using a selective fluorescent substrate for ACE, Abz-FRK(Dnp)P-OH (*ortho*-aminobenzoic acid-FRK-(2,4-dinitrophenyl)P-OH) in the thoracic aorta isolated from WT rat and transgenic rat overexpressing the B_1 receptor specifically in the vascular endothelium (TGR)Tie_2B_1)). Data are means ± SD of 3 experiments.

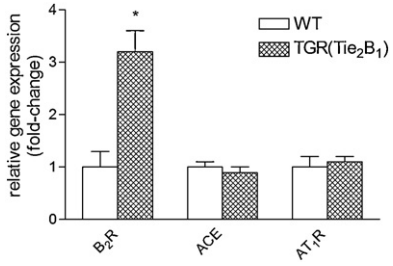

Fig. 7. Relative gene expression of kinin B_2 receptor, ACE and angiotensin II type I receptor. The expression levels of B_2 receptor, ACE and angiotensin II type 1 (AT_1 receptor) were determined in thoracic aorta isolated from wild-type (WT) rat and from endothelial overexpressed kinin B_1 receptor (TGR(Tie_2B_1)). The values of mRNA expression levels were quantified by real-time PCR. The values of the relative gene expression were calculated as $2^{-\Delta C_T}$ parameter, which is obtained by subtracting the C_T (threshold cycle) of gene target from the C_T of internal standard. Final data are expressed as the ratio of fold change of cDNA in the transgenic rat (TGR(Tie_2B_1)) target gene *versus* the fold change of cDNA in control. The data are means ± S.D. of 3–5 experiments. *Significantly different from the control value ($P < 0.05$).

3.6. Expression level of kinin B_2 receptors and of ACE

The expression levels of B_2R were determined by real time PCR relative quantification, since the maximum effect induced by BK in the transgenic TGR(Tie_2B_1) rats was higher than in the WT rats. Furthermore, expression level of ACE was evaluated. Fig. 7 shows the results about the levels of their expression, which was calculated by fold-up change of the transgenic rat over the control group. The expression level of B_2R increased about three folds in the TGR(Tie_2B_1) rat whereas that of ACE mRNA expression was not significantly different from the control WT rats.

3.7. Vascular reactivity to angiotensin II and expression level of AT_1 receptor in aorta isolated from TGR(Tie_2B_1) rat

Responsiveness of the thoracic aortic rings to angiotensin II (AngII) induced contractile response was assessed to evaluate

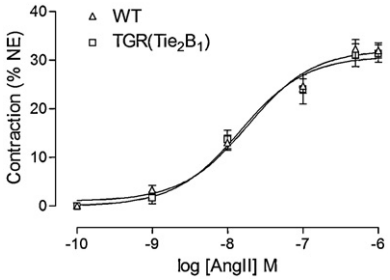

Fig. 8. Effect of endothelial overexpression of kinin B_1 receptors on the contraction induced by AngII in rat aorta. Non-cumulative concentration–response curves for angiotensin II (AngII) were determined in the thoracic aorta isolated from WT rat and transgenic rat overexpressing the B_1 receptor specifically in the endothelium (TGR(Tie_2B_1)). The isometric contractile responses are expressed as percentage of the effect of 1 μM norepinephrine (NE), considered 100%. Data are means ± SD of 5 experiments. *Significantly different from the control WT animals ($P < 0.05$).

any cross-talking between kinin and AT_1 receptors under conditions where the expression level of B_2R was shown to be increased in TGR(Tie_2B_1) rat. The concentration-responses curves were obtained using non-cumulative manner for stimulations to avoid desensitization. The data show that the vascular reactivity to AngII (Fig. 8) in the aortic rings from TGR(Tie_2B_1) rats was not altered when compared to that of WT rat. The maximal response values (%) were 31 ± 4 (4) for WT and 30 ± 2 (5) for TGR(Tie_2B_1) and the pD_2 values were 7.8 ± 0.2 (4) for WT and 7.9 ± 0.2 (5) for TGR(Tie_2B_1). In addition, the determination of AT_1 receptor mRNA expression revealed that in aorta overexpressing the B_1 and B_2 receptors, there was no significant difference from the control WT rats (Fig. 7).

4. Discussion and conclusion

The present study showed that the vascular reactivity to BK as well as the expression level of B_2R mRNA were increased in rats overexpressing the kinin B_1R (TGR(Tie_2B_1)) exclusively in the endothelium. The relaxation of aortic rings induced by BK was significantly greater in this transgenic rat than the control, which was completely abolished by B_2R antagonist HOE-140. The finding that the antagonist R-715 could not affect the responses to BK, indicated that no fragment of the peptide it was mediating the relaxant responses to BK. Furthermore the complete blockade of the responses induced by BK by its antagonist HOE-140 showed that only B_2R activation was responsible for the enhanced responses induced by BK in overexpressing endothelial aorta isolated from TGR(Tie_2B_1). It was also found that HOE-140 had no effect on DBK-induced relaxation, confirming what was reported by [17] that the increased response induced by DBK in TGR(Tie_2B_1) was inhibited specifically by the antagonist of B_1R. These authors reported that the responses to DBK were completely blocked by L-NAME in the isolated aorta from TGR(Tie_2B_1) rats which is in agreement with our study wherein a complete inhibition of BK induced effect by

L-NAME was found, indicating that relaxant responses induced by the kinins in the rat aorta are highly dependent on NO generation.

It was reported that mice overexpressing B_1R in multiple tissues induced hypertensive response to B_1R agonist, exacerbated paw and edema induced by carrageenan and high susceptibility to endotoxic shock induced by lipopolysaccharide [19]. The present study showed that B_2R was surprisingly overexpressed in the endothelium of thoracic aorta from TGR(Tie_2B_1) rat. This finding was unexpected since a downregulation should occur as a counter regulatory mechanism for overexpression of B_1R. It has been reported that the lack of one kinin receptor is compensated by the up-regulation of the other subtype, as shown in the case of deletion of B_2R [10,12,36] and of B_1R [16,28].

In another study [28], lipopolysaccharide treatment caused enhanced B_2R mRNA which was further increased in B_1KO mice with increased mortality. Although some studies have been reported about overexpression of B_1R [17,19] or B_2R [33] assessing the importance of the overexpressed receptor, the expression of the other remaining receptor subtype has not been determined.

The enhanced B_2R mRNA expression in TGR(Tie_2B_1) rat was correlated with the increased responsiveness of rat aorta to its agonist BK. The finding that the ability of ACE to convert AngI to AngII was not reduced neither the ACE mRNA was altered, provided evidence that the increase in the BK reactivity was not modulated by ACE activity due to the high expression of the B_2R. This conclusion could not confirm an effect of ACE/kinin B_2R interaction modulating ACE activity as previously described [20,27].

It is noteworthy that was found no evidence for increased activation of AT_1R since the vascular reactivity to AngII was maintained in the aorta isolated from (TGR(Tie_2B_1)) rats. Therefore the hypothesis that a spontaneous heterodimerization of AngII and BK receptors could trigger the AT_1R activation was not confirmed in contrast to that previously reported [1].

In conclusion, transgenic rats overexpressing kinin B_1R exclusively in the endothelium of TGR(Tie_2B_1) rats were shown to overexpress the kinin B_2R and to cause increased responsiveness to BK. It was reported that after lipopolysaccharide treatment, these transgenic rats presented a more pronounced hypotensive response and marked bradycardia associated with increased mortality when compared to non-transgenic control rats [17]. It was already reported that B_1R and B_2R were upregulated by endotoxins and that B_2R mRNA was further increased in B_1KO during the acute phase of endotoxin shock involving increased mortality [28]. Therefore the mechanism by which B_2R mRNA expression is increased in rats overexpressing kinin B_1R needs further investigation. Our finding supports an important role of B_1 and B_2 receptors during the pathogenesis of endotoxic shock. From this study it can be suggested that overexpression and increased activation of kinin B_2R could be involved in the high mortality during the pathogenesis of endotoxic shock, wherein B_1R expression is highly induced.

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References

[1] AbdAlla S, Lother H, Quitterer U. AT1-receptor heterodimers show enhanced G-protein activation and altered receptor sequestration. Nature 2000;407:94–8.
[2] Alves MF, Araujo MC, Juliano MA, Oliveira EM, Krieger JE, Casarini DE, et al. A continuous fluorescent assay for the determination of plasma and tissue angiotensin I-converting enzyme activity. Braz J Med Biol Res 2005;38:861–8.
[3] Barbosa AM, Felipe SA, Pesquero JB, Paiva AC, Shimuta SI. Disruption of the kinin B1 receptor gene affects potentiating effect of captopril on BK-induced contraction in mice stomach fundus. Peptides 2006;27:3377–82.
[4] Bhoola KD, Figueroa CD, Worthy K. Bioregulation of kinins: kallikreins, kininogens, and kininases. Pharmacol Rev 1992;44:1–80.
[5] Borkowski JA, Hess JF. Targeted disruption of the mouse B2 bradykinin receptor in embryonic stem cells. Can J Physiol Pharmacol 1995;73:773–9.
[6] Campbell DJ. Towards understanding the kallikrein-kinin system: insights from measurement of kinin peptides. Braz J Med Biol Res 2000;33:665–77.
[7] Carmona AK, Schwager SL, Juliano MA, Juliano L, Sturrock ED. A continuous fluorescence resonance energy transfer angiotensin I-converting enzyme assay. Nat Protoc 2006;1:1971–6.
[8] Cayla C, Todiras M, Iliescu R, Saul VV, Gross V, Pilz B, et al. Mice deficient for both kinin receptors are normotensive and protected from endotoxin-induced hypotension. Faseb J 2007;21:1689–98.
[9] Duka I, Duka A, Kintsurashvili E, Johns C, Gavras I, Gavras H. Mechanisms mediating the vasoactive effects of the B1 receptors of bradykinin. Hypertension 2003;42:1021–5.
[10] Duka I, Kintsurashvili E, Gavras I, Johns C, Bresnahan M, Gavras H. Vasoactive potential of the b(1) bradykinin receptor in normotension and hypertension. Circ Res 2001;88:275–81.
[11] Kang DS, Ryberg K, Morgelin M, Leeb-Lundberg LM. Spontaneous formation of a proteolytic B1 and B2 bradykinin receptor complex with enhanced signaling capacity. J Biol Chem 2004;279:22102–7.
[12] Kintsurashvili E, Duka I, Gavras I, Johns C, Farmakiotis D, Gavras H. Effects of ANG II on bradykinin receptor gene expression in cardiomyocytes and vascular smooth muscle cells. Am J Physiol Heart Circ Physiol 2001;281: H1778–83.
[13] 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.
[14] 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;25:402–8.
[15] Marceau F, Hess JF, Bachvarov DR. The B1 receptors for kinins. Pharmacol Rev 1998;50:357–86.
[16] Marcon R, Claudino RF, Dutra RC, Bento AF, Schmidt EC, Bouzon ZL, et al. Exacerbation of DSS-induced colitis in mice lacking kinin B1 receptor through compensation of up-regulation of kinin B2 receptors: the role of tight junctions and intestinal homeostasis. Br J Pharmacol 2013;168:389–402.
[17] Merino VF, Todiras M, Campos LA, Saul V, Popova E, Baltatu OC, et al. Increased susceptibility to endotoxic shock in transgenic rats with endothelial overexpression of kinin B(1) receptors. J Mol Med (Berl) 2008;86:791–8.
[18] Nakashima H, Suzuki H, Ohtsu H, Chao JY, Utsunomiya H, Frank GD, et al. Angiotensin II regulates vascular and endothelial dysfunction: recent topics of Angiotensin II type-1 receptor signaling in the vasculature. Curr Vasc Pharmacol 2006;4:67–78.
[19] Ni A, Yin H, Agata J, Yang Z, Chao L, Chao J. Overexpression of kinin B1 receptors induces hypertensive response to des-Arg9-bradykinin and susceptibility to inflammation. J Biol Chem 2003;278:219–25.
[20] Oliveira CR, Paredes-Gamero EJ, Barbosa CM, Nascimento FD, Batista EC, Reis FC, et al. Myelopoiesis modulation by ACE hyperfunction in kinin B(1) receptor knockout mice: relationship with AcSDKP levels. Chem Biol Interact 2010;184:388–95.
[21] Pesquero JB, Araujo RC, Heppenstall PA, Stucky CL, Silva Jr JA, Walther T, et al. Hypoalgesia and altered inflammatory responses in mice lacking kinin B1 receptors. Proc Natl Acad Sci USA 2000;97:8140–5.
[22] Pueyo ME, Arnal JF, Rami J, Michel JB. Angiotensin II stimulates the production of NO and peroxynitrite in endothelial cells. Am J Physiol 1998;274:C214–20.
[23] Pueyo ME, Michel JB. Angiotensin II receptors in endothelial cells. Gen Pharmacol 1997;29:691–6.
[24] Regoli D, Barabe J. Pharmacology of bradykinin and related kinins. Pharmacol Rev 1980;32:1–46.
[25] Regoli D, Barabe J, Park WK. Receptors for bradykinin in rabbit aortae. Can J Physiol Pharmacol 1977;55:855–67.
[26] Rodrigues ES, Martin RP, Felipe SA, Bader M, Oliveira SM, Shimuta SI. Cross talk between kinin and angiotensin II receptors in mouse abdominal aorta: molecular and functional aspects. Biol Chem 2009;390:907–13.
[27] Sabatini RA, Guimaraes PB, Fernandes L, Reis FC, Bersanetti PA, Mori MA, et al. ACE activity is modulated by kinin B2 receptor. Hypertension 2008;51: 689–95.
[28] Seguin T, Buleon M, Destrube M, Ranera MT, Couture R, Girolami JP, et al. Hemodynamic and renal involvement of B1 and B2 kinin receptors during the acute phase of endotoxin shock in mice. Int Immunopharmacol 2008;8:217–21.
[29] Shen B, Harrison-Bernard LM, Fuller AJ, Vanderpool V, Saifudeen Z, El-Dahr SS. The Bradykinin B2 receptor gene is a target of angiotensin II type 1 receptor signaling. J Am Soc Nephrol 2007;18:1140–9.
[30] Shimuta SI, Barbosa AM, Borges AC, Paiva TB. Pharmacological characterization of RMP-7, a novel bradykinin agonist in smooth muscle. Immunopharmacology 1999;45:63–7.
[31] Siddiqui AH, Hussain T. Enhanced AT1 receptor-mediated vasocontractile response to ANG II in endothelium-denuded aorta of obese Zucker rats. Am J Physiol Heart Circ Physiol 2007;292:H1722–7.
[32] Tan Y, Hutchison FN, Jaffa AA. Mechanisms of angiotensin II-induced expression of B2 kinin receptors. Am J Physiol Heart Circ Physiol 2004;286:H926–32.

[33] Wang D, Yoshida H, Song Q, Chao L, Chao J. Enhanced renal function in bradykinin B(2) receptor transgenic mice. Am J Physiol Renal Physiol 2000;278:F484–91.
[34] Wang DZ, Chao L, Chao J. Hypotension in transgenic mice overexpressing human bradykinin B2 receptor. Hypertension 1997;29:488–93.
[35] Williams B, Baker AQ, Gallacher B, Lodwick D, Angiotensin II. increases vascular permeability factor gene expression by human vascular smooth muscle cells. Hypertension 1995;25:913–7.
[36] Xu J, Carretero OA, Zhu L, Shesely EG, Rhaleb NE, Dai X, et al. The protective role of AT2 and B1 receptors in Kinin B2 receptor knockout mice with myocardial infarction. Clin Sci (Lond) 2013;124:87–96.
[37] Zacest R, Oparil S, Talamo RC. studies of plasma bradykininases using radiolabelled substrates. Aust J Exp Biol Med Sci 1974;52:601–6.