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Functional expression of kinin B_1 and B_2 receptors in mouse abdominal aorta

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Abstract

Previous studies have shown that the vascular reactivity of the mouse aorta differs substantially from that of the rat aorta in response to several agonists such as angiotensin II, endothelin-1 and isoproterenol. However, no information is available about the agonists bradykinin (BK) and DesArg^{9}BK (DBK). Our aim was to determine the potential expression of kinin B_1 and B_2 receptors in the abdominal mouse aorta isolated from C57BL/6 mice. Contraction and relaxation responses to BK and DBK were investigated using isometric recordings. The kinins were unable to induce relaxation but concentration-contraction response curves were obtained by applying increasing concentrations of the agonists BK and DBK. These effects were blocked by the antagonists Icatibant and R-715, respectively. The potency (pD_2) calculated from the curves was 7.0 ± 0.1 for BK and 7.3 ± 0.2 for DBK. The efficacy was 51 ± 2% for BK and 30 ± 1% for DBK when compared to 1 µM norepinephrine. The concentration-dependent responses of BK and DBK were markedly inhibited by the arachidonic acid inhibitor indomethacin (1 µM), suggesting a mediation by the cyclooxygenase pathway. These contractile responses were not potentiated in the presence of the NOS inhibitor L-NAME (1 mM) or endothelium-denuded aorta, indicating that the NO pathway is not involved. We conclude that the mouse aorta constitutively contains B_1 and B_2 subtypes of kinin receptors and that stimulation with BK and DBK induces contractile effect mediated by endothelium-independent vasoconstrictor prostanoids.

Key words
- Kinin B_1 and B_2 receptors
- Bradykinin
- DesArg^{9}bradykinin
- Indomethacin
- L-NAME

Introduction

Kinins are proinflammatory peptides that act as local hormones and activate the release of endothelium-derived relaxing factors such as nitric oxide (NO) and prostaglandins, increase vascular permeability, relax or contract smooth muscle, and provoke pain (for a review, see Ref. 1). Most of these effects of kinins are mediated by the activation of the B_2 receptor, which belongs to a family of peptide hormone receptors linked to G proteins, via the agonist bradykinin (BK).

Another kinin receptor, the B_1 receptor, is activated by the agonist DesArg^{9}BK (DBK) and is generally absent or expressed at very low levels in healthy tissues but is induced in

response to pathological insults and mediates cardiovascular and nociceptive responses in these conditions (2). It has been shown that the mouse stomach fundus constitutively expresses kinin B_1 and B_2 receptors (3) but no data regarding mouse vascular reactivity to kinins have been reported.

Therefore, our aim was to characterize pharmacologically the expression of B_1 and B_2 subtypes of kinin receptors in the mouse aorta through isometric contractions and using antagonists for these receptors. In this study we demonstrate that BK and DBK did not relax but induced endothelium-independent contractions of mouse abdominal aorta in a concentration-dependent fashion. These contractile responses to the kinins in rings of abdominal aorta were associated with the production of eicosanoids, whereas NO involvement was not demonstrable.

Material and Methods

Animals and organ preparations

The protocols used in the present study were in accordance with current guidelines for the care of laboratory animals and ethical guidelines for investigations, and were approved by the Animal Care Committee of the Federal University of São Paulo. Animals were maintained on standard mouse chow at 21-23ºC and kept on a 12-h light:dark cycle, with free access to food and water. After 12-16 weeks of age, the animals, weighing 26-30 g, were killed by cervical dislocation and exsanguination. The thoracic and abdominal aortas were removed, cleared of connective tissue and mounted as ring preparations using 40-mm tungsten wire, into 5-mL organ baths. Aortic rings were bathed in carboxygenated (95% O_2/5% CO_2) 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), pH 7.35, at 37ºC. Resting tension was maintained at 0.5 g. Tissues were left to equilibrate for 1 h, with frequent changes of bathing solution. Tissue viability was assessed by priming with 80 mM KCl and 1 µM norepinephrine (NE). Following a 90-min washout and recovery period, cumulative or non-cumulative concentration-response curves to the agonists were determined. Changes in tension produced by the stimulants were measured with an isometric transducer (FTA-10) through an amplifier (E805, Hewlett-Packard, Palo Alto, CA, USA) and a potentiometric recorder (RB-102, ECB, São Paulo, SP, Brazil), as described previously (4). Concentration-response curves were constructed for BK and DBK by applying from 0.1 nM to 1 µM agonist concentrations. In some experiments, these curves were obtained in the absence and in the presence of specific inhibitors. To determine the apparent affinity of agonists in terms of pD_2 (the negative logarithm of the concentration of agonist that produces 50% of the maximal effect) the curve-fitting analysis of the dose-response curves was carried out using the GraphPad-Prism software (San Diego, CA, USA). Data are reported as means ± SD.

Analysis of gene expression

Analysis of gene expression for kinin B_1 and B_2 receptors was carried out using real-time quantitative PCR. Five mice were killed and their aortas were isolated, dissected, frozen in liquid nitrogen and stored at -80ºC. Frozen tissue was homogenized in 1 mL TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and total RNA was isolated according to manufacturer instructions. Samples were submitted to a 20-µL reaction using the TaqMan® amplification system with an ABI PRISM® 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed with a 1:10 dilution sample of cDNA obtained from total RNA with the SuperScricpt™ III first-strand synthesis SuperMix for qRT-PCR (Invitrogen) for kinin B_1 and B_2 receptors and ß-actin was used as an internal standard.

Oligonucleotide primer and fluorogenic probe sets for Taqman™ Real-time PCR were designed for kinin receptors and ß-actin using the Assays-by-Design Service (Applied Biosystems) to meet all TaqMan® design guidelines. Probes were synthesized with the reporter dye 6-carboxyfluorescein covalently linked at the 5' end and the quencher dye 6-carboxy-tetramethyl-rhodamine was linked to the 3' end of the probe. Each reaction was carried out with 10 µL of Master Mix (Applied Biosystems), 1 µL of a mix containing two primers (18 µM each) and a probe (5 µM) specific for mRNA of the kinin B_1 receptor (probe 5'-CACAGGAACCCCAGACAGA-3', forward primer 5'-CTCCATACAAAACCCCAGCTGAA-3', reverse primer 5'-CTTTGGTTAGAAGGCTGTAGCTTCA-3'), kinin B_2 receptor (probe 5'-CTTTGGCATCGAAATGT-3', forward primer 5'-GGTTTCTGTCGGTGCATGAG-3', reverse primer 5'-GGACTTGTGTGGTGACGTTGA-3') or ß-actin (probe 5'-CAGATCATGTTTGAGACCTT-3', forward primer 5'-GGCCAACCGTGAAAAGATGAC-3', reverse primer 5'-GCCTGGATGGCTACGTACATG-3'), 5 µL of cDNA samples and Milli-Q water (Millipore Corporation, Billerica, MA, USA) to 20 µL. The cycle conditions were: 50ºC for 2 min, 95ºC for 10 min, followed by 50 cycles of 95ºC for 15 s (melting step), 60ºC 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). The PCR cycle when a given fluorescence threshold is crossed by the amplification curve is considered to be the first parameter analyzing mRNA expression and is named C_T. The larger the initial amount of copies, the lower the C_T number. A normalized value is obtained by subtracting C_T of kinin B_1 or B_2 receptor from C_T of ß-actin, resulting in ΔC_T. Since it is uncommon to use ΔC_T 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.

Drugs

BK and DBK were obtained from Bachem (Torrance, CA, USA); Icatibant (Aventis, Frankfurt, Germany) and R-715 were a gift from D. Regoli, Université de Sherbrooke, Quebec (Canada). DesArg^{9}Leu^{8}BK was synthesized in the Department of Biophysics, Federal University of São Paulo, São Paulo, SP, Brazil. Other reagents were purchased from Sigma Chemical Co. (Dorset, UK). TRIzol reagent and SuperScricpt™ III First-Strand Synthesis SuperMix for qRT-PCR were purchased from Invitrogen; TaqMan® Real-Time PCR primers and Master Mix for TaqMan® Real-Time PCR were from Applied Biosystems. Concentrated solutions of peptides (1 mg/mL) and other agents were prepared in water and kept at -20ºC until used. The stock solutions were serially diluted with Krebs-Ringer solution. Indomethacin solutions were prepared just before use by dissolving the drug in a small volume of saturated aqueous sodium carbonate, diluting to the desired volume with Krebs-Ringer solution. The vehicle used for the administration of indomethacin had no effect on the responses of the isolated tissue to agonists. All solutions were adjusted to pH 7.35 before use.

Statistical analysis

Values are reported as means ± SD. The Student *t*-test or analysis of variance (ANOVA) with multiple comparisons was used to determine the statistical differences, with the level of significance set as $P < 0.05$.

Results

BK and DBK were able to promote contractile responses in the aortic rings. The pD_2 values of BK and DBK calculated from concentration-response curves (Figure 1) indi-

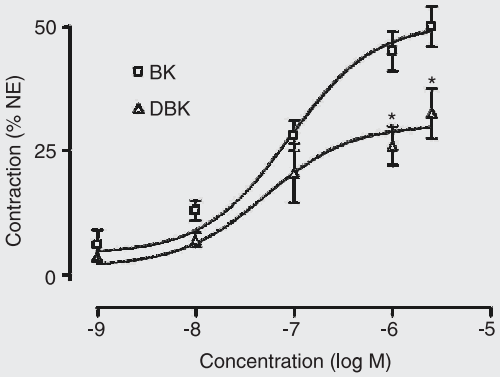

Figure 1. Cumulative concentration-response curves for the effects of bradykinin (BK) and Des-Arg^{9}BK (DBK), agonists of kinin B_2 and B_1 receptors, respectively, on rings of abdominal aorta isolated from mice. The isometric contraction responses were calculated in relation to the maximal effect of 1 µM norepinephrine (NE) which was considered to be 100%. Data are reported as the means ± SD of 5 experiments. *P < 0.05 compared to BK (ANOVA).

Table 1. Effect of indomethacin on the potency and maximal effect induced by kinins on mouse abdominal aorta rings.

Agonist	pD_2 (nM)	E_{max} (% NE)
BK	7.1 ± 0.1 (15)	51 ± 2 (15)
BK + indomethacin	ND	12.5 ± 0.7$^+$ (3)
DBK	7.3 ± 0.2 (8)	30 ± 1* (8)
DBK + indomethacin	ND	6 ± 2$^+$ (3)

Bradykinin (BK)- and DesArg^{9}BK (DBK)-induced isometric contractile responses were assessed in the absence and in the presence of 1 µM indomethacin, a cyclooxygenase inhibitor, preincubated for 20 min. The responses to the agonists were calculated in relation to the effect of 1 µM norepinephrine (NE) and the values of pD_2 (-log EC_{50}, concentration of the agonist inducing 50% of the maximal response) and the E_{max} (maximal effect) were obtained. Data are reported as means ± SD with the number of experiments in parentheses. ND = not determined. $^+$P < 0.05 compared to the BK-induced effect; *P < 0.05 compared to control (*t*-test).

cated that the potency was similar but the efficacy of BK was significantly higher than that of DBK (Table 1). These effects of BK and DBK were shown to be independent of the presence of the endothelium. The removal of the endothelium was confirmed by the lack of acetylcholine-induced relaxation of abdominal aorta precontracted with 1 µM NE.

In the presence of 1 µM Icatibant preincubated for 30 min, BK-induced contraction of abdominal aorta was drastically inhibited, whereas the DBK-induced effect was blocked by 1 µM R-715. A peptide antagonist of the B_1 receptor, 1 µM DesArg^{9}Leu^{8}BK preincubated for 30 min, had a similar effect (data not shown). Most results were obtained with R-715 since its effect was rapidly reversible. The results obtained with Icatibant and R-715 are shown in Figure 2.

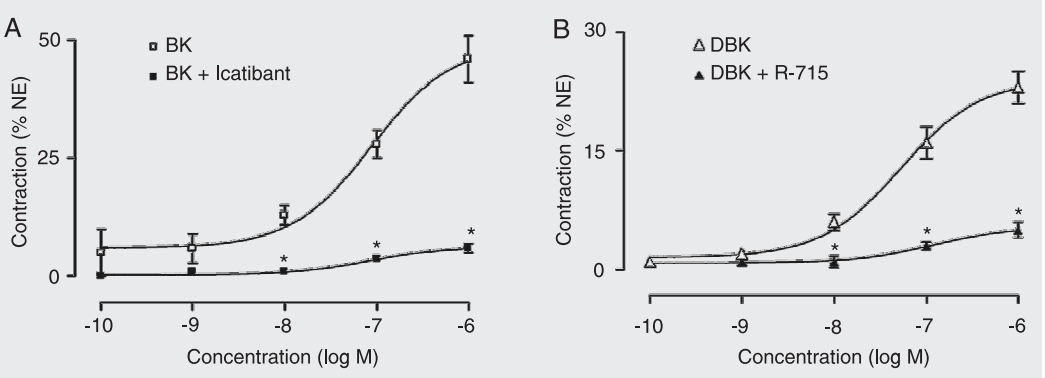

Figure 2. Effect of antagonists of the kinin B_2 receptor, 1 µM Icatibant and of the B_1 receptor, 1 µM R-715, on abdominal aortic rings isolated from mice. Cumulative concentration-response curves for bradykinin (BK) (A) and Des-Arg^{9}BK (DBK) (B) were determined in the presence and absence of the respective antagonists. The isometric contraction responses were calculated in relation to the maximal effect of 1 µM norepinephrine (NE) which was considered to be 100%. Data are reported as the means ± SD of 5 experiments. *P < 0.05 compared to control (ANOVA).

In order to evaluate the gene expression of kinin B_1 and B_2 receptors in the mouse aorta the amount of mRNA for both receptors was analyzed using a quantitative RNA assay. Kinin B_1 receptor mRNA showed detectable expression. This determination revealed that the level of expression of mRNA for the B_1 receptor was lower than that for the B_2 receptor (Figure 3).

B_2 receptors have been shown to mediate endothelium-dependent vasodilation as well as vasoconstriction (5,6). Therefore, the BK-induced relaxing response of mouse aortic rings was investigated. BK did not cause any relaxation in the resting aortic preparations with intact endothelium or on NE-induced contraction. Instead of relaxation, the administration of BK to NE-precontracted rings produced further dose-dependent contraction, above the increased tension produced by 1 µM NE (data not shown).

To investigate a possible role of NO release in the BK-induced contraction, an inhibitor of NO synthase activity was examined. Pre-incubation with 1 mM L-NAME for 20 min did not enhance the contractions induced by BK and DBK in aortic rings with an intact endothelium. The presence of the endothelium was confirmed pharmacologically by the acetylcholine-induced relaxing effect on aortic rings precontracted with NE.

The contractions induced by BK in mouse aortic rings were blocked by preincubation with 1 µM indomethacin for 20 min (Figure 4A). This effect was observed in endothelium-intact as well as in endothelium-denuded abdominal aorta. Similar results were observed for the effect of DBK (Figure 4B).

Stimulation of mouse aorta with exogenous NO and sodium nitroprusside (SNP) completely relaxed the NE-increased tone of vascular tissue. SNP did not induce relaxation of the aorta under resting basal tonus. The concentration-response curves for SNP are shown in Figure 5 and the pD_2 value was calculated (7.7 ± 0.2).

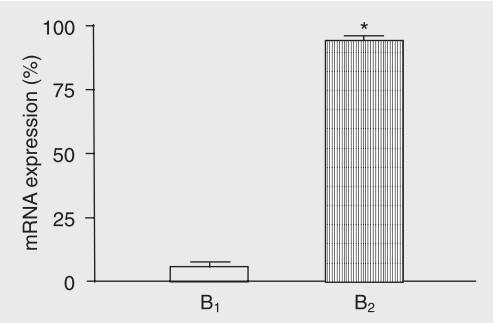

Figure 3. Expression of mRNA for kinin B_1 and B_2 receptors. Gene expression of kinin B_1 and B_2 receptors was determined by real-time quantitative PCR. ß-Actin was used as an internal standard. The $2^{-\Delta C_T}$ parameter was used to express the relative gene expression data. *P < 0.05 compared to B_1 (*t*-test).

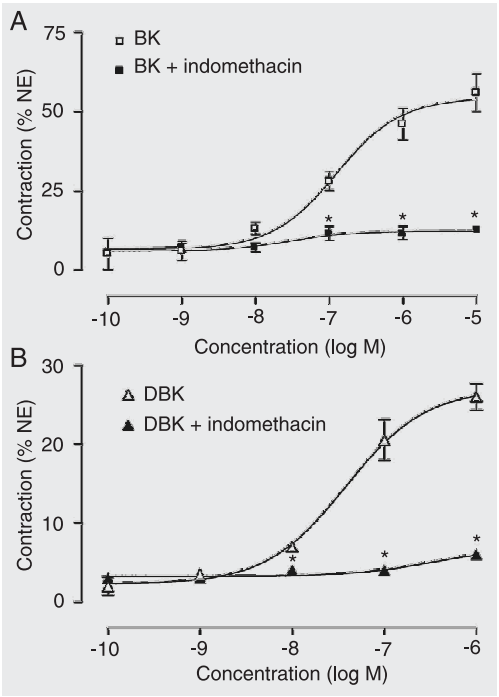

Figure 4. Effect of indomethacin on the effect of bradykinin (BK) and Des-Arg^{9}BK (DBK) in preparations of aortic rings. Cumulative concentration-response curves for BK (A) and DBK (B) were obtained in the absence and in the presence of 1 µM indomethacin, a cyclooxygenase inhibitor. Percent contraction was calculated from the steady-state tension induced by norepinephrine (NE). Data are reported as the means ± SD of 4 experiments. *P < 0.05 compared to control (ANOVA).

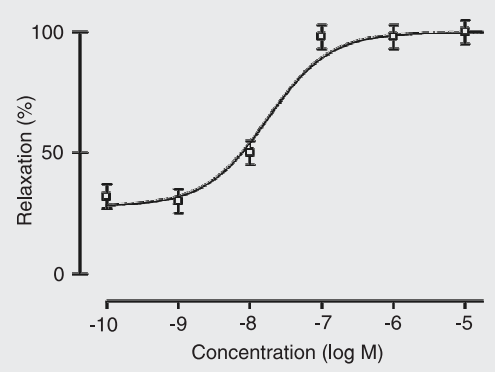

Figure 5. Sodium nitroprusside-induced relaxation in norepinephrine-precontracted rings of mouse aorta. Percent relaxation was calculated from the steady-state tension induced by norepinephrine. Data are reported as means ± SD of 5 independent determinations.

Discussion

We assessed the role of B_1 and B_2 receptors in mouse abdominal aorta using isometric force measurements. Our preliminary studies had revealed a low responsiveness of the thoracic aorta to the agonists BK and DBK. The poor response to BK by the thoracic aorta in comparison with the response of abdominal aorta was also observed in the contractions induced by angiotensin II and endothelin-1 in this tissue (6).

The present study showed that BK induced a potent contractile response in the abdominal aorta (51% of that induced by 1 µM NE) that was significantly inhibited by the B_2 receptor antagonist Icatibant, indicating that this agonist depends specifically on the activation of this receptor. On the other hand, DBK-induced contraction, whose efficacy was lower than that of BK (30% of NE), was shown to be specifically mediated by the B_1 receptor which was antagonized by the R-715 and DesArg^{9}Leu^{8}BK. By analyzing the gene expression of B_1 and B_2 receptors we could demonstrate B_1 receptor mRNA expression in freshly isolated mouse aorta which indicated that DBK-induced contractile responses were due to activation of the constitutive B_1 receptor rather than to its up-regulation. The lower expression of B_1 receptor than B_2 receptor mRNA was correlated with the lower efficacy of DBK compared to BK.

The contractile responses induced by BK and DBK in the mouse aorta were similar to those reported by Allogho et al. (3), who showed that the mouse stomach contains functional B_1 and B_2 receptors and that the efficacy of BK was greater than that of DBK.

In contrast, the rat aorta has been reported to be insensitive to DBK *in vitro*, even after bacterial lipopolysaccharide pretreatment (7). More recent studies have shown that rat aortic smooth muscle cells bind the tritiated B_1 receptor agonist ^{3}H-DBK and that B_1 receptor mRNA was detected (8). According to these observations, rat aortic smooth muscle cells have the potential to express B_1 receptors but the expression level may not be high enough to induce detectable contractile responses in the isolated rat aorta.

Our finding that BK-induced contractions were not affected by endothelium removal indicates that, in contrast to the vasodilator effect (9,10), the vasoconstriction induced by kinins results from a direct action on isolated mouse aortic smooth muscle. In agreement with this result, BK-induced contraction was not altered when the abdominal aorta was treated with L-NAME. This result suggests that NO-dependent vasodilation, which would antagonize part of the vasoconstrictive effect of BK, can be ruled out. It is noteworthy that endothelin-1-induced contraction of the same preparation was significantly reduced when L-NAME was absent from the medium (11), whereas this effect was not observed with angiotensin II-induced contraction (12).

The finding that the contractile responses to BK and DBK were significantly reduced by indomethacin in mouse endothelium-denuded abdominal aorta indicates that these responses are mostly mediated by vasoconstrictor prostanoids. In this regard, it is interesting to note that, as previously reported, the prostanoids derived from the arachidonic acid-cyclooxygenase pathway can be produced within the vascular smooth muscle (13). Since the BK- and DBK-induced effect was not completely blocked by the cyclooxygenase inhibitor, the remaining response could be ascribed to a direct rather than a mediated effect of the peptides. However, a possible involvement of other signaling pathways in this contractile effect cannot be ruled out.

In contrast to the mouse abdominal aorta, where kinins were unable to induce relaxation of NE-pre-contracted preparations, BK induced concentration-dependent relaxation in phenylephrine-pre-contracted rings of rat

aorta, which was inhibited by Icatibant. This vasorelaxation of endothelium-denuded rat aorta was ascribed to the generation of cyclooxygenase products through activation of B_2 receptors on smooth muscle cells (14). These results indicate that the effect of kinins on vessels may differ considerably among species.

The present study showed for the first time that mouse aortic tissues express functional constitutive kinin B_1 and B_2 receptors localized within the vascular wall and that BK and DBK induce contractile responses which are mostly mediated by prostanoids.

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