Abstract  Bradykinin binds to its receptor at target organs and exerts a wide spectrum of biological activities including vasodilation, smooth muscle contraction and relaxation, pain, and inflammation. To gain a better insight into the physiological function of this potent vasoactive peptide, we created transgenic mice that harbor the human bradykinin B2 receptor transgene under the control of the Rous sarcoma virus 3'-LTR promoter (RSV-cHBKR). Expression of HBKK in these transgenic mice was identified in the aorta, brain, heart, lung, liver, kidney, uterus, and prostate gland by reverse transcription-polymerase chain reaction (Southern blot analysis). Two transgenic mouse lines expressing the human B2 receptor resulted in a significant reduction of blood pressure (84 ± 0.6 mm Hg, n = 28, 76 ± 0.8 mm Hg, n = 24, P < 0.001) compared with the control littermates (96 ± 0.4 mm Hg, n = 52). Administration of Hoe 140, a bradykinin B2 receptor antagonist, restored the blood pressure of the transgenic mice to normal levels within 1 hour, and the effect diminished within 4 hours. The transgenic mice displayed enhanced blood pressure lowering effect induced by a bolus intravenous injection of kallikrein and showed increased response in kallikrein-induced uterine smooth muscle contractility compared with control littermates. These studies show that overexpression of human bradykinin B2 receptor causes a sustained reduction of blood pressure in transgenic mice. They also suggest that the B2 receptor-mediated signal transduction pathway plays a role in blood pressure regulation.

Key Words  • human bradykinin B2 receptor  • transgenic mice  • hypotension  • Hoe 140  • uterine contraction

Hypotension in Transgenic Mice Overexpressing Human Bradykinin B2 Receptor

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Tissue kallikrein is a serine protease which is capable of cleaving kinogen to release bioactive kinin peptides. On the binding of kinins to BK receptors, a broad spectrum of biological effects are generated. These include vasodilation, vasoconstriction, smooth muscle contraction and relaxation, ion transport, and glucose metabolism. There are two distinct BK receptor subtypes, namely, B1 and B2. The B2 receptor is widely distributed throughout mammalian tissues and has a greater affinity for intact BK and kallidin (LDK). While the B1 receptor has a greater affinity for kinin metabolites, Des-Arg9-BK or Des-Arg9-LBK. The B1 receptor appears in certain pathological processes. Most of the kinins' actions are mediated by B2 receptors.

BK is a potent vasoactive peptide. Intravascular injection of BK can cause an immediate dilatation of the arterial vessels with a concomitant fall in total peripheral vascular resistance and systemic blood pressure. The tissue kallikrein-kinin system has long been implicated in blood pressure regulation. Urinary excretion of tissue kallikrein was reported to be significantly reduced in essential hypertensive patients and genetically hypertensive rats. Polymorphisms in the kallikrein gene have been shown to cosegregate with high blood pressure in the offspring of spontaneously hypertensive rats and normotensive Brown Norway rats. Recently, we have established a direct link between blood pressure regulation and tissue kallikrein gene expression by transgenic animal models and somatic gene delivery strategies. We further demonstrated that the administration of Hoe 140, a specific B2 receptor antagonist, could restore the blood pressure of these transgenic mice to normal levels, indicating the hypotensive phenotype was mediated through BK B2 receptor in this transgenic animal model.

The human BK B2 receptor cDNA was cloned and sequenced from a human lung fibroblast cDNA library. It encodes a 364-amino acid protein with characteristics of the G-protein-coupled receptor superfamily. The genes encoding the human, mouse, and rat B2 receptors have also been cloned and sequenced. Molecular cloning of the B2 receptor gene and elucidation of the gene structure have played a crucial role in analyzing the potential function of the B2 receptor in blood pressure homeostasis by using transgenic and antisense approaches. A BK B2 receptor-deficient mouse line was generated by homologous recombination. However, these "knockout" mice failed to show any altered blood pressure phenotype. To determine the role of BK B2 receptor in blood pressure regulation, we created a transgenic mouse model carrying the human BK B2 receptor cDNA under control of the RSV 3'-LTR promoter.

Methods

Transgene Construction and DNA Preparation

The human BK B2 receptor cDNA (1.3 kb) containing the entire coding region was amplified from the total RNA of human renal proximal tubular cells by RT-PCR. The sense primer used was 5'-CCATGCCGCTTGCTCCG-3', and the antisense primer was 5'-GGAATGCCAAGGAGACA-3'. It was then cloned into pREP8 vector (Invitrogen) at the HindIII site downstream from the RSV-LTR enhancer/promoter. The final construct pREP8-cHBKR was confirmed by restriction mapping and DNA sequencing.

The plasmid pREP8-cHBKR was extracted by the alkaline lysis method and purified by cesium chloride ethidium bromide gradient centrifugation. The purified plasmid DNA was digested with Sal I to release a 2.4-kb transgene fragment, which contains the RSV-LTR enhancer/promoter, human BK B2 receptor cDNA, and an SV40 polyA segment. This fragment was separated from the vector fragment by sucrose gradient fractionation and dialyzed against the injection buffer (10 mmol/L...
Tissue-HCl/0 25 mmol/L EDTA, pH 7.5) The DNA concentration was measured by absorbance at 260 nm and confirmed by comparing with λ DNA after agarose gel electrophoresis. It was diluted to 4 μg/mL with the injection buffer and centrifuged at 10,000g for 1 hour to remove dust particles before injection.

**Generation and Identification of Transgenic Mice**

The purified RSV-chHBKR-SV40 polyA DNA was microinjected into F2 embryos (C57BL/6×DBA/2), which were allowed to develop in utero in the uterus of pseudopregnant females. Offspring were screened by Southern blot analysis to identify transgenic mice. Mouse genomic DNA was extracted from the tail biopsies and digested by Apa I followed by Southern blot analysis using an [α-32P]dATP nick-translated human B2 receptor cDNA probe. Two transgenic founders, No 8905 and No 9830, were identified. These founder mice were bred separately with C57BL/6 mice to establish two independent transgenic lines. The control mice were nontransgenic littermates from the mating. All experimental protocols were approved by the Institutional Animal Research Committee of the Medical University of South Carolina and were carried out according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

**Expression of Human BK B2 Receptor Transgene**

Human B2 receptor mRNA was identified in transgenic mice by RT-PCR Southern blot analysis. Fresh tissues (aorta, brain, heart, lung, liver, kidney, and uterus or prostate) of male and female transgenic and control mice were homogenized in guanidine thiocyanate buffer, and total RNA was purified by cesium chloride gradient centrifugation. One microgram of total RNA was subjected to RT-PCR Southern blot analysis as previously described using the transgene-specific primers and an internal probe. The sequence of the 5' primer is 5'-TTCTCTGGATACCGCTGACCG-3', the 3' primer, 5'-CTCATGATATTGAGTACGCTGCATCG-3', and the internal probe, 5'-CTCAGGTATCCTTGTGG-3'. The blotted membrane was washed twice in 6X SSC at 55°C and exposed to Kodak X-OMAT film at -80°C.

**Transient Transfection of Human Embryonic Kidney 293 Cells**

A human embryonic kidney 293 cell line was transfected with 20 μg of plasmid DNA of RSV-chHBKR by electroporation using a BTX-600 electric cell manipulator (BTX). At 72 hours after transfection, the cells were harvested and lysed in lysis buffer (5 mmol/L Tris-HCl, pH 7.4, with 5 mmol/L EDTA and 5 mmol/L EGTA) and microcentrifuged at 16,000g for 4°C for 10 minutes. The pellet was resuspended in binding assay buffer and ligand-binding assays were performed in duplicate.

**Preparation of Iodinated Ligand and Binding Assay**

Tyr5-BK (Sigma), the synthetic analog of BK, was used as radioligand for a BK binding assay. It was iodinated by using the chloramine-T method and purified by high-pressure liquid chromatography. The specific radioactivity was 167 35 Ci/mmole Crude membrane protein was prepared, and ligand binding assays were performed following the procedure described previously. The concentration of membrane protein was determined by the Lowry assay. For saturation studies, [125I]-Tyr5-BK was incubated in duplicate with 100 μg of membrane protein at 25°C in a volume of 250 μL for 60 minutes. Using a Millipore filter (Hoefer Scientific), the assay was terminated by filtration over a Whatman GF/C glass fiber filter presoaked with 0.1% aqueous polyethyleneimine for 3 hours. The tubes and filters were rinsed three times with 4 mL of ice-cold 25 mmol/L TES buffer, pH 6.8, and the filters were counted in a 1261 Multigamma counter (Pharmacia). Saturable binding was calculated by subtracting the nonspecific binding in the presence of 10 μmol/L unlabeled BK.

**Blood Pressure Measurement by the Tail-Cuff Method**

Systolic blood pressure was measured with the tail-cuff method as previously described. Five readings were taken for each animal. The control group was carried out by injection of PBS into transgenic mice or by injection of Hoe 140 into nontransgenic control mice.

**Blood Pressure Measurement by Arterial Cannulation and BK Injection**

Mice were anesthetized by intraperitoneal injection of 2,2,2-trifluoroethanol in tert-amyl alcohol (Avertin, 20 mg/mL, 0.4 mL/25 g body weight). The right femoral artery and the left carotid artery were cannulated with PE-10 catheters (Clay Adams) for direct arterial pressure recording and drug administration. The distal end of the cannula was connected to a physiological pressure transducer (Statham Laboratories) coupled with a model 7E polygraph (Grass Instrument Co). Blood pressure of the anesthetized mice was measured directly via the intra-arterial route. The mice were given a bolus intra-arterial injection of BK (Sigma) and blood pressure was measured at 1-minute intervals for 10 minutes after stimulation by BK.

**Statistical Analysis**

Group data are reported as mean±SEM. Comparisons of parameters between control and transgenic mouse groups were performed by two-way ANOVA. Differences were considered to be significant at a value of P<0.05.

**Results**

**Generation of Transgenic Mice**

Fig 1 shows the scheme for preparing the RSV-chHBKR gene fusion construct. The transgene contains the RSV-LTR enhancer-promoter element, the full-length human B2 receptor cDNA, and a portion of the SV40 intron. The heterogeneous RSV enhancer-promoter element directs a high level of expression in a wide variety of tissues and reduces the negative feedback control at the transcriptional level. The SV40 intron functions as a heterologous 3' untranslated sequence to increase the stability of the transcripts.

Two transgenic founders, No 8905 (female) and No 9830 (female), were identified from the 64 male progeny by Southern blot analysis. The human transgene was identified in these mice by Southern blot analysis. The human transgene was successfully detected in the genomic DNA of the transgenic mice as a 1.1-kb band in addition to the background of a 3.3-kb mouse endogenous B2 receptor gene band.
FIG 1. Diagram of the human BK B2 receptor transgene DNA construct. The solid bar represents a full-length human BK B2 receptor cDNA (1.3 kb). pRSV denotes the Rous sarcoma virus 3'-LTR promoter and SV40 pA represents the polyadenylation signal of the SV40 gene. The human BK B2 receptor cDNA was cloned into pREP8 at a HindIII restriction site and the transgene was released from pREP8 at a Sal I restriction site, as indicated.

Expression and Tissue Distribution of the Human BK B2 Receptor Transgene

Expression of the functional B2 receptor was analyzed in human 293 cells transfected with the RSV-cHBKR plasmid DNA. The results of the BK binding assay show the presence of specific B2 receptor binding sites in the membrane protein preparation from the transfected cells. The saturation curves demonstrated that the transfected cells, but not the nontransfected cells, have a high density of binding sites (Fig 2). Scatchard plot analysis revealed that the transfected cell membrane contained specific BK binding site with a KD of 0.93 nmol/L and Bmax of 1.01 pmol/mg protein as determined by analyzing saturation data with the equilibrium binding data analysis program.29

FIG 3. Expression of human BK B2 receptor mRNA in transgenic mice. RT-PCR Southern blot shows the expression of human BK B2 receptor cDNA in transgenic mice. RT-PCR was performed using 1 µg of total RNA. A pair of oligonucleotide primers specific to the human B2 receptor transgene were used. The RT-PCR products were hybridized with a nested probe specific to the transgene in the Southern blot analysis.

Transient Transfection of Human Embryonic Kidney 293 Cell Line

Expression of the functional B2 receptor was analyzed in human 293 cells transfected with the RSV-cHBKR plasmid DNA. The results of the BK binding assay show the presence of specific B2 receptor binding sites in the membrane protein preparation from the transfected cells. The saturation curves demonstrated that the transfected cells, but not the nontransfected cells, have a high density of binding sites (Fig 2). Scatchard plot analysis revealed that the transfected cell membrane contained specific BK binding site with a KD of 0.93 nmol/L and Bmax of 1.01 pmol/mg protein as determined by analyzing saturation data with the equilibrium binding data analysis program.29

BK Binding Assay

To analyze the transgene expression at the protein level, membranes were prepared from the kidney, uterus, and brain of both transgenic and control mice (n=6). The density of BK binding sites in the kidney of transgenic mice was approximately five times higher than that of the control mice (Fig 4). The density of BK binding sites in the uterus and brain of transgenic mice was 1.8-fold and 1.4-fold higher, respectively, than those in the control mice (Fig 4).

Blood Pressure Analysis of Transgenic Mice

Both lines of transgenic mice showed significant reductions in blood pressure compared with normal controls. The systolic blood pressure measured by the tail-cuff method was 84.2±0.6 mm Hg for line No. 8905 (mean±SEM, n=28) and 76.9±0.8 mm Hg for line No. 9830 (mean±SEM, n=24). The value for the negative siblings was 96.9±0.4 mm Hg (mean±SEM, n=52) with P<.001 (Fig 5). The values of blood pressure obtained from the
indirect tail-cuff method and direct intra-arterial cannulation were compared for accuracy.

To determine if B₁ receptor is responsible for the hypotensive effect, Hoe 140, a specific BK B₁ receptor antagonist, was injected intraperitoneally at a dose of 2 μg per mouse (n=3). Fig 6 shows that Hoe 140 administration restored the blood pressure of the transgenic mice to a level close to that of the control mice in 1 hour and the effect of Hoe 140 diminished within 4 hours while vehicle buffer (PBS) had no effect on the blood pressure of transgenic mice. Administration of Hoe 140 had no effect on the blood pressure of control littermates (data not shown).

Bolus intra-aortic injections of BK resulted in a transient decrease of mean blood pressure in both transgenic and nontransgenic control mice in a dose-dependent manner (n=8) (Fig 7). The sensitivity to BK-induced blood pressure reduction between the transgenic and control mice differs significantly. The vasodepressor effect of BK is more pronounced in transgenic mice than in control mice when the same dose is applied (P<.05).

**Utterine Contraction Assay**

To directly assess the effect of B₁ receptor overexpression on myometrium inotropy, we determined isometric tension induced by BK in the isolated tissue bath. In the RSV-cHBKR transgenic mice, the uterine smooth muscle contraction induced by 0.5 μmol/L BK was threefold higher than that of the control mice (n=3, P<.05) (Fig 8). When KCl (25 mmol/L) was applied in the tissue bath after stimulation with BK, no significant difference between the transgenic and control groups on the KCl-induced contraction was observed (Fig 8). There was no significant difference in uterine weight between transgenic and control mice.

**Discussion**

This study shows that transgenic mice overexpressing human BK B₁ receptor have a hypotensive phenotype. Two mouse lines carrying the human B₁ receptor transgene were independently isolated and characterized. The average blood pressure of one line was 12.7 mm Hg lower and the second line was 20.0 mm Hg lower than their nontrans-
Results of this study show that the blood pressure was significantly reduced in mice overproducing the B2 receptor. An interpretation for this finding is that both kinins and B2 receptors are not utilized efficiently in vivo. Kinins have a very short half-life, and B2 receptor may not capture kinins effectively before its degradation by kininases.3 In the kalikrein transgenic mice, a high level of kinins may enhance the chances of their binding to the BK B2 receptor. Alternatively, a high level of BK B2 receptor in the B2 transgenic mice may enable the receptor to capture the free kinins more rapidly. This may in part explain the findings that overexpression of either tissue kallikrein or B2 receptor results in a hypotensive phenotype.

It is also possible that a small fraction of the total B2 receptor pool exists in the activated conformation even in the absence of its agonist, thus the endogenous G-protein-coupled receptors exhibit spontaneous activity in their natural environment in the absence of agonist occupancy.32,33 When receptor expression is high, a small fraction of the receptors in the activated conformation could be sufficient to activate its intracellular effectors to conduct its signal transduction pathways. Overexpression of B2 receptor may therefore cause a significant increase in the number of spontaneously activated B2 receptors in the transgenic mice in the absence of BK and thus produce an increase in cellular concentration of second messengers to maintain an enhanced physiological response. An example in support of this possibility is provided by the studies employing transgenic mice overexpressing β2-adrenergic receptor.34

The transgenic animal model overexpressing BK B2 receptor could be useful for studying the physiological function of the B2 receptor in blood pressure regulation and inflammation. Recently, another animal model was generated in which the mouse BK B2 receptor gene was disrupted by homologous recombination.31 It is interesting to note that the B2 receptor-deficient mice did not show an altered blood pressure phenotype, but the contractility of uterine smooth muscles in response to BK was eliminated in these mice.31 These findings demonstrate the unique features and usefulness of each animal model in studying receptor function. It is likely that both the transgenic and knockout animal models could be useful for dissecting the functional roles of BK B2 receptor in blood pressure regulation.

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