Overexpression of Kinin B1 Receptors Induces Hypertensive Response to Des-Arg⁹-bradykinin and Susceptibility to Inflammation*

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We demonstrated that rat kinin B1 receptors displayed a ligand-independent constitutive activity, assessed through inositol phosphate production in transiently or stably transfected human embryonic kidney 293A cells. Substitution of Ala for Asn₁³⁰ in the third transmembrane domain resulted in additional constitutive activation of the B1 receptor. The constitutively active mutant N130A receptor could be further activated by the B1 receptor agonist des-Arg⁹-bradykinin. To gain insights into the physiological function of the B1 receptor, we have generated transgenic mice overexpressing wild-type and constitutively active mutant receptors under the control of human cytomegalovirus immediately early gene enhancer/promoter. The rat B1 receptor transgene expression was detected in the aorta, brain, heart, lung, kidney, uterus, and prostate of transgenic mice by reverse transcription-polymerase chain reaction/Southern blot analysis. Transgenic mice were fertile and normotensive. Overexpression of B1 receptors exacerbated paw edema induced by carrageenan and rendered transgenic mice more susceptible to lipopolysaccharide-induced endotoxic shock. Interestingly, the hemodynamic response to kinins was altered in transgenic mice, with des-Arg⁹-bradykinin inducing blood pressure increase when intravenously administered. Our study supports an important role for B1 receptors in modulating inflammatory responses and for the first time demonstrates that B1 receptors mediate a hypertensive response to des-Arg⁹-bradykinin.

Kinin peptides are released from kininogen precursors by the action of kallikreins in response to tissue injury (1). Kinins induce smooth muscle contraction, vasodilation, increased vascular permeability, and pain (1). Kinins exert their effects through selective activation of two seven-transmembrane domain (TMD) G protein-coupled receptors (GPCRs), B₁ and B₂ (2–4). The B₂ receptor is constitutively expressed, mediating the actions of intact kinins, bradykinin (BK) in rodents and Lys-BK or kallidin in humans (2). In contrast, the B₁ receptor is expressed at very low levels in normal tissues in most animal species but is induced under the influence of inflammation or exposure of tissues to noxious stimuli, mediating the effects of the carboxypeptidase metabolites of intact kinins, des-Arg⁹-BK (DABK), and des-Arg⁹-kallidin (2). The cellular responses of kinin receptors to agonists are transduced primarily via coupling to either Gq protein, which in turn activates phospholipase C to stimulate inositol phosphate production, or the Gi protein, acting through phospholipase A₂ to stimulate arachidonic acid pathway (5, 6).

Over the past years, transgenic and gene-targeting technologies associated with molecular biology tools have provided important knowledge concerning the role of kinin receptors in vivo. Transgenic mice expressing the human B₂ receptor under the control of the Rous sarcoma virus 3’-long terminal repeat promoter were hypotensive compared with control littermates (7). Administration of the B₂ receptor antagonist Hoe-140 blunted the blood pressure-lowering effect of the transgene, whereas intra-arterial bolus injection of BK produced more pronounced blood pressure reduction (7). In contrast, deletion of the B₁ receptor in mice produced an unaltered blood pressure phenotype (8) but led to salt-sensitive hypertension and altered nociception (9, 10). Using specific antagonists, the B₁ receptor has been implicated in toxic shock, inflammation, and nociception (9, 10). Studies of mice lacking the B₁ receptor provided support to these observations. B₁ receptor knockout animals were healthy, fertile, and normotensive and exhibited hypogesia and reduced inflammatory response (12).

Although much has been learned about the physiological role of the B₁ receptor, most studies are about the lipopolysaccharide (LPS)-induced B₁ receptors, because the B₁ receptor is expressed at very low levels, if at all, in normal tissue. In such experimental set-ups, the animals are under systemic inflammation conditions, which preclude the direct study of the function of the B₁ receptor. Therefore, the precise physiological and pathophysiological roles of the B₁ receptor remain elusive. To extend our understanding of the physiological function of the B₁ receptor, we have created a constitutively active mutant of the rat B₁ receptor and generated transgenic mice that overexpress the wild-type B₁ receptor and the mutant receptor under the control of human cytomegalovirus immediately early gene enhancer/promoter. The transgenic mice were characterized, and the findings are reported here.

EXPERIMENTAL PROCEDURES

Materials—Human embryonic kidney 293A cells were obtained from Quantum Biotechnologies. LPS (Salmonella enteritidis, LD₅₀ = 7.20 mg/kg) was from Difco Laboratories. myo-[³H]inositol was from PerkinElmer Life Sciences. LipofectAMINE, culture medium, restriction enzymes, and fetal calf serum were bought from Invitrogen. Puro-
mycin were from Clontech Lab. Hoe-140 was obtained from Hoechst. Sar-Tyr-εAhx-Lys-des-Arg^9-bradykinin and Sar-Tyr-εAhx-Lys-[β^3]Na^1,Le^3]-des-Arg^9-bradykinin were gifts from Dr. D. Regoli (13). All other chemicals were purchased from Sigma unless stated otherwise.

**Site-directed Mutagenesis**—The mutations were created by using the QuickChange site-directed mutagenesis kit (Stratagene), and the previously cloned wild-type rat B1 cDNA in pcDNA3 (Invitrogen) was used as a template (14). The following oligonucleotides were used as forward primers: 5′-GGC TTC TGG GGC GCC CTT TTA GTG TGG TC-3′ for the preparation of clone N54A (nucleotide changes underlined), 5′-GTC TTC AAG GCC GCC CTT GTG TTC AG-3′ for clone N130A, 5′-GCT ATC ATC AGT CAG TCA CAG TAC AGG CTC-3′ for clone D144Q, and 5′-GCT ATC ATG ATG CAC GCC TAC AGG CTC-3′ for clone D144T. The downstream primers were complementary to the forward primers described. All of the mutations were confirmed by DNA sequencing. The NruI/NorI fragments in the resultant plasmids were released and inserted at NruINorI sites in pIRESpuro (Clontech Lab), leading to vectors for stable transfection.

**Transfection and Selection**—For transient transfection, 293A cells were seeded into 12-well trays and left to adhere overnight. pcDNA-derived expression vectors were transfected with LipofectAMINE as previously described (14). Transfection mixtures were left on cells for 5 h, and then the cells were treated with a change of standard growth medium for 48 h before functional studies.

For stable transfection, 293A cells were transfected with pIRESpuro-derived expression vectors. After 16 h, the medium was changed with complete medium containing puromycin (2 μg/ml) to start the selection of stably transfected cells. The medium was changed every 3 days, and after about 12 days, the colonies surviving selection were lifted into 12-well plates, expanded with a maintenance concentration of 2 μg/ml puromycin, and screened for specific binding. All of the stock cultures were kept under constant selection pressure of 2 μg/ml puromycin, whereas cells seeded in dishes/wells were maintained without puromycin and used within 2–3 days.

**Total Inositol Phosphate Measurement**—Monolayers of transfected 293A cells grown in 12-well trays were labeled for 24 h with 2 μCi of [3H]inositol in 0.5 ml of inositol-free Dulbecco’s modified Eagles’ medium supplemented with 0.05% bovine albumin and penicillin-streptomycin. After equilibration in prewarmed Dulbecco’s modified Eagles’ medium containing 140 μM bicarbonate, 100 μM Captopril, and 25 mM LiCl for 15–30 min, the cells were stimulated with various ligands at the indicated concentrations for 20 min at 37 °C. The released total inositol phosphates (IP) were isolated using Bio-Rad AG1-X8 anion exchange columns (1 ml-volume) and quantified as described (15, 16).

**Radioligand Binding to Intact Cells**—Transfected 293A cell monolayers in 12-well plates were washed twice with Dulbecco’s modified Eagles’ medium and incubated at 4 °C with radioligand [125I]Sar-Tyr-εAhx-Lys-des-Arg^9-bradykinin (14) in the presence or absence of 5 μM of the unlabeled ligand in 0.5 ml of Dulbecco’s phosphate-buffered saline supplemented with 140 μM bicarbonate, 30 mM LiCl, 1 mM 1,10-phenanthroline, and 100 μM Captopril. The incubation lasted at least 3 h under gentle agitation. The cells were then rinsed twice with ice-cold phosphate-buffered saline with 0.3% bovine serum albumin followed by solubilization in 0.5 ml of 0.1 M NaOH. The radioactivity of the sample was quantified with a 1281 Mutigamma counter (Pharmacia Corp.). The cell number was determined in parallel wells.

**cGMP and cAMP Assays**—Stably transfected 293A cells grown in 6-well plates were preincubated for 15 min with Dulbecco’s modified Eagles’ medium containing 1 mM 3-isobutyl-1-methylxanthine and 100 μM Captopril and then stimulated with 1 μM DABK for 15 min. The stimulation was terminated by exchanging the incubation medium for 0.5 ml of ice-cold 1 M HCl. The cGMP and cAMP productions were determined by radioimmunoassays as described (17, 18).

**Construction of Wild-type and N130A Mutant Rat B1 Receptor Transgenes**—The bovine growth hormone poly(A) sequence in pcDNA3 was released with Asp1 and PvuII and inserted at Asp1/PvuII sites in prBluescript KS II (Stratagene). The Asp1/SmaI bovine growth hormone poly(A) sequence in pcDNA3 was released and inserted at Asp1/SmaI sites in the pcDNA3-derived wild-type and N130A receptor expression vectors described above. The human F2 enhancer was amplified by PCR from genomic DNA with the primers 5′-TAC TCG ATG GCA GCC CGC CCC CG-3′ and 5′-CTG GCC CCT TCA CCT TCA GAG AGC-3′ (19). After being cut with XbaI and Asp1, the 425 bp enhancer fragment was inserted at Asp1/SmaI sites resulting in final expression vectors. The B1 receptors were cut with NruI and Smal, and the linear transgenes were separated from the unneeded fragments with agarose gel and prepared for injection by QiaQuick gel extraction columns (Qiagen).

**Generation of B1 Receptor Transgenic Mice**—Transgenic mice were created by the Transgenic Facility at the Medical University of South Carolina and the Transgenic Facility of University of Ohio at Cincinnati. Linear transgene was injected into the pronuclei of one-cell mouse embryos, which were then surgically implanted into pseudopregnant female mice. Transgenic founder mice were identified by Southern blot analysis of genomic DNA isolated from tail biopsies. Positive founders identified from each line were bred with normal mice, and then F1 littermates were used as founders. Tail DNA was digested with restriction enzyme KpnI, run on 0.7% agarose gels containing ethidium bromide, and transferred to Immobilon-N membrane by capillary action with 10× SSC overnight, and the blots were hybridized to a rat kinin B1 receptor cDNA probe as described previously (14, 20).

**Expression of B1 Receptor Transgene**—Total RNA was extracted from mouse tissues using the RNeasy™ columns (Qiagen). Reverse transcription-PCR/Southern blot analysis was performed using the transgene-specific primers and internal probe as previously described (21). The upstream primer is 5′-ATG GCC TCC GAG TTC TT-3′; the downstream primer is 5′-GAC AAA CAC CAC ATG GC-3′; and the internal probe is 5′-TGG CAG CAA CGA CAG AG-3′.

**Membrane Preparation and Radioligand Binding Assays**—The mice were sacrificed by cervical dislocation. The kidney was removed, and the wet weight was determined. The tissue was homogenized using a Polytron in ~20 volumes of ice-cold 20 mM HEPES, pH 7.4. The membranes from the tissue were prepared, and binding assays were performed as described previously (7, 14).

**Blood Pressure Measurements by Tail Cuff**—Systolic blood pressure was measured using a computer system RTBP2000 (Kent Scientific) according to the manufacturer’s instruction. Briefly, the mice were placed into the prewarmed harness (38 °C). The tail was placed in the occlusion cuff/piezoelectric pulse sensor and distention caused by arterial blood pressure was detected by the sensor and read out onto the computer system. Pressure in the cuff was increased until the pulse was lost. Actual blood pressure was measured as the pressure at which a pulse was detected during cuff depressurization. Ten readings were taken for each animal.

**Blood Pressure Measurements by Artemial Cannulation**—The mice were anesthetized with 2,2,2-trimethoxyethanol in tert-amyl alcohol (Avertin, 20 mg/ml, 0.4 ml/25 g of body weight) and placed on a heated table to maintain body temperature. The right jugular vein and the left carotid artery were cannulated with PE-10 catheters (Clay Adams). After the animals were allowed to recover, blood pressure (in the carotid artery) and heart rate were recorded using a computer system MP100 (Biopac Systems Inc.). The mice were given a bolus injection of BK or DABK from right jugular vein. BK or DABK was serially diluted and administered at doses of 75, 150, and 300 ng in a volume of 50 μl of saline/mouse.

**Paw Edema**—Inflammation of one hind paw of mice was induced by intraplantar injection of 20 μl of 1% carrageenan (dissolved in saline), 3 μg of capsaicin in 10 μl of 5% ethanol, 10 μl of 5% tween 80 and 90% saline), or DABK (50 or 300 nmol in saline), whereas the contralateral paw received the same volume of vehicle. Thirty min post injection of capsaicin or DABK or 3 h after carrageenan administration, the mice were sacrificed, both hind paws were cut off at the ankle, and the difference between their weights, representing paw edema, was calculated.

**Response to Endotoxic Shock**—LPS was dissolved in sterile 0.9% NaCl. The mice were injected intraperitoneally with a single dose of LPS (24 mg/kg body weight), and the percentage of survivors was determined at 12-h intervals. Both of the control groups were injected with 0.9% NaCl.

**Statistical Analysis**—The data are expressed as the means ± S.E. The data were compared between experimental groups by one-way analysis of variance. Differences between groups were further evaluated by Fisher’s protected least squares differences. Differences were considered significant at a value of p < 0.05.

**RESULTS**

**Generation and Characterization of Rat B1 Receptor Mutants**—To generate the constitutively active mutants of B1 receptors, site-directed mutagenesis was directed at the Asn^154, Asn^130, and Asp^144 residues in the rat B1 receptor (Fig. 1). The amino acid replacements were N54A, N130A, D144Q, and D144T. The receptor expression vectors were transiently transfected into 293A cells for assessing constitutive activity by determining agonist-independent IP production. At optimal
transfection conditions, all of the mutants were expressed at a comparable level but significantly lower than the wild-type receptor (data not shown). As shown in Table I, the wild-type \( B_1 \) receptor displayed a marked ligand-independent, spontaneous activity (104% above control levels of the mock-transfected cells), and the N54A mutant showed an impaired basal activity compared with the wild-type receptor. Substitution of Ala for Asn130 resulted in significantly constitutive activation of the \( B_1 \) receptor (409% above control levels). In contrast, the wild-type receptor was abolished by the mutations. Interestingly, the maximal agonist-independent IP accumulations in 293A cells expressing N130A resulted in significantly constitutive activation of the \( B_1 \) receptor (409% above control levels).

To better characterize the N130A mutant receptor, stable transfection of 293A cells was established. Such stably transfected 293A cells (Table II) were used to evaluate the properties of some kinins to modulate IP production by the wild-type and N130A receptors. Using the stably transfected 293A cells, the dose-dependent DABK stimulation of IP production for the wild-type and N130A receptors was investigated. As shown in Fig. 2, the N130A mutant could be further activated by over 5-fold by saturation doses of DABK, whereas the wild-type receptor could be activated to a even higher degree (~16-fold).

**Table I**

| Receptor          | \( R_{max} \) | Basal IP |
|-------------------|---------------|----------|
| WT                | 1487 ± 112    | 104 ± 27 |
| N54A              | 433 ± 35      | 10 ± 4   |
| N130A             | 430 ± 18      | 409 ± 23 |
| D144Q             | 67 ± 7        | 0        |
| D144T             | 20 ± 9        | 0        |

* The \( R_{max} \) values are percentages above basal.

The N54A mutant showed an impaired basal activity compared with the wild-type receptor. Substitution of Ala for Asn130 resulted in significantly constitutive activation of the \( B_1 \) receptor (409% above control levels). In contrast, the wild-type receptor was abolished by the mutations. Interestingly, the maximal extent of DABK stimulation of IP production was significantly reduced for all mutants compared with the wild-type receptor. To better characterize the N130A mutant receptor, stable transfection of 293A cells was established. For comparison, stable expression of the wild-type \( B_1 \) receptor in 293A cells was also established. Such stably transfected 293A cells were analyzed for receptor density and their affinity for radioligand \([^{125}I]\)Sar-Tyr-eAhx-Lys-des-Arg\(^8\)-bradykinin and used in functional assays. The N130A receptor was expressed at a level of about 25% of the wild-type receptor in the stably transfected 293A cells (Table II). The maximum number of N130A receptor-binding sites \((B_{max})\) using \([^{125}I]\)Sar-Tyr-eAhx-Lys-des-Arg\(^8\)-bradykinin is 1.4 × 10\(^5\) sites/cell versus 5.3 × 10\(^5\) sites/cell for the wild-type receptor. In contrast, the mutation of Asn130 into Ala significantly increased the affinity for \([^{125}I]\)Sar-Tyr-eAhx-Lys-des-Arg\(^8\)-bradykinin. The dissociation constant \((K_d)\) for the binding of \([^{125}I]\)Sar-Tyr-eAhx-Lys-des-Arg\(^8\)-bradykinin by the wild-type and N130A receptors in intact stably transfected 293A cells were 2.54 ± 0.40 and 1.63 ± 0.18 nM, respectively.

To assess the mode of coupling between the \( B_1 \) receptor and adenylate cyclase and guanylate cyclase and the effect of the mutation of Asn130 into Ala on the DABK-induced cAMP and cGMP production, the intracellular cAMP and cGMP levels were measured in the stably transfected 293A cells. As shown in Table II, the wild-type receptor in 293A cells stably expressing the wild-type receptor were similar to those in the nontransfected 293A cells, whereas in the 293A cells stably expressing the N130A receptor, the basal cAMP and cGMP levels were elevated. DABK challenge increased intracellular cAMP and cGMP production in the 293A cells stably expressing the wild-type and N130A receptors: 116-fold increase in cAMP levels and 5.2-fold increase in cGMP levels for the wild-type receptor versus 14-fold increase in cAMP levels and 1.7-fold increase in cGMP levels for the N130A mutant.

Using the stably transfected 293A cells, the dose-dependent DABK stimulation of IP production for the wild-type and N130A receptors was investigated. As shown in Fig. 2, the N130A mutant could be further activated by over 5-fold by saturation doses of DABK, whereas the wild-type receptor could be activated to a even higher degree (~16-fold).

The properties of some kinins to modulate IP production by the wild-type and N130A receptors were evaluated. As shown in Fig. 3, the wild-type \( B_1 \) receptor agonist BK and antagonist Hoe-140 have no effects. Des-Arg\(^8\),[Leu\(^4\)]-bradykinin and Sar-Tyr-eAhx-Lys-Nal\(^7\),Ile\(^8\)-des-Arg\(^8\)-bradykinin are human \( B_1 \) receptor-specific antagonists. Sar-Tyr-eAhx-Lys-[N-\( \beta \)Nal\(^7\),Ile\(^8\)]-des-Arg\(^8\)-bradykinin is still an antagonist for the wild-type rat \( B_1 \) receptor, whereas des-Arg\(^8\),[Leu\(^4\)]-bradykinin becomes a partial agonist (70–80% of DABK), which provides support for the early observation that des-Arg\(^8\),[Leu\(^4\)]-bradykinin has partial agonist activity in a contraction assay of smooth muscle of rat duodenum and ileum (22, 23). In contrast, Sar-Tyr-eAhx-Lys-[N-\( \beta \)Nal\(^7\),Ile\(^8\)]-des-Arg\(^8\)-bradykinin becomes a partial agonist, and des-Arg\(^8\),[Leu\(^4\)]-BK becomes a potent agonist for the N130A receptor.

**Generation of Transgenic Mice**—Using the wild-type and N130A cDNAs, we have constructed two transgenes for development of transgenic mice. The transgene consists of the cytomegalovirus immediately early gene enhancer/promoter, the wild-type or N130A rat \( B_1 \) receptor cDNA, the human 4F2 megalovirus immediately early gene enhancer/promoter, the bovine growth hormone poly(A) sequence (Fig. 4). Three transmitting founder lines, including one wild-type line, WT2510, and two N130A lines, N130AS8 and N130AS2592, were identified by Southern blot analysis of genomic DNA. Heterozygous wild-type and N130A transgenic...
Characterization of the wild-type and N130A mutant receptors

293A cells stably expressing the wild-type (WT) and the N130A mutant (N130A) rat kinin B$_1$ receptor were established, and the receptor binding sites ($B_{\text{max}}$) and dissociation constant ($K_d$) were determined by using radioligand $[^{125}\text{I}]$Sar-Tyr-eAhx-Lys-des-Arg$^8$-bradykinin as described under "Experimental Procedures." The basal (Basal) and 1 $\mu$M DABK-stimulated (Maximal) intracellular cAMP and cGMP levels were also measured. The results are the means ± S.E. of three experiments.

| Receptor     | $K_d$ (nM) | $B_{\text{max}}$ (sites/cell × 10^{-5}) | cAMP (pmol/mg) | cGMP (pmol/mg) |
|--------------|------------|----------------------------------------|----------------|----------------|
| WT           | 2.54 ± 0.40| 530                                    | 16 ± 2         | 1858 ± 55      |
| N130A        | 1.63 ± 0.18| 140                                    | 43 ± 7         | 608 ± 53       |
| 293A cells   | 5.3 ± 1.3  | 105                                    | 12 ± 1         | 13 ± 5         |

**FIG. 4.** Rat kinin B$_1$ receptor transgene constructs. The open bar represents wild-type (WT) or mutant N130A rat kinin B$_1$ receptor cDNA. pCMV denotes the human cytomegalovirus immediately early gene enhancer/promoter. 4F2 represents the human 4F2 heavy-chain gene enhancer, and BGH polya represents the bovine growth hormone gene polyadenylation sequence.

**FIG. 5.** Expression of N130A mutant mRNA in transgenic mice. Reverse transcription-PCR Southern blot revealed the expression of transgene mRNA in the offspring. In the following studies heterozygous transgenic mice were used. Expression of B$_1$ Receptor Transgene—The distribution of transgene mRNA expression in F1 and F2 generation heterozygous mice was determined by reverse transcription-PCR/Southern blot analysis. As expected, both male and female transgenic mice showed significant overexpression of the transgene mRNA in the aorta, kidney, liver, heart, brain, and lung and in the prostate of males and the uterus of females. Fig. 5 shows the result from line N130A58. Using specific B$_1$ receptor radioligand $[^{125}\text{I}]$Sar-Tyr-eAhx-Lys-des-Arg$^8$-BK (14), strong B$_1$ receptor binding activity was detected in membranes prepared from the kidneys of all transgenic lines (data not shown). In contrast, neither transgene mRNA expression nor B$_1$ receptor (including endogenous) binding activity could be detected in the corresponding tissues of the nontransgenic control mice. Blood Pressure—All of the heterozygous transgenic mice were normotensive. The systolic blood pressures of transgenic mice 10 weeks old were 78.7 ± 8.5 mmHg (n = 11) for line WT2510, 75.0 ± 7.6 mmHg (n = 14) for line N130A58, and 76.2 ± 6.3 mmHg (n = 11) for line N130A2592 versus 80.9 ± 3.9 mmHg (n = 11) for age-matched nontransgenic control littermates. Intravenous injection of B$_1$ receptor agonist DABK via
the jugular vein produced a transient increase of mean arterial blood pressure (MABP) in anesthetized transgenic mice but not in nontransgenic control littermates (Fig. 6A). The duration of MABP increase lasted over 5 min. 75 ng of DABK led to an increase of blood pressure by up to 15 mmHg. In contrast, intravenous injection of B2 receptor agonist BK into transgenic mice caused a remarkable primary MABP reduction, followed by a blood pressure bounce-back going beyond basal level, whereas in nontransgenic mice the blood pressure just returned to basal level, after a similar primary MABP reduction (Fig. 6B). Single or subsequent injections of higher doses of DABK did not result in a further increase of blood pressure in transgenic mice (data not shown).

Inflammation—Intraplantar injection of carrageenan resulted in a marked inflammation seen by paw swelling in normal and transgenic mice (Fig. 7). But the paw edema induced in transgenic mice was more severe. The percentage of the weight increase of the carrageenan-injected paw over the contralateral vehicle-injected paw was 38.4 ± 6.7% for line WT2510, 42.1 ± 5.0% for line N130A58, and 37.4 ± 6.5% for line N130A2592 versus only 21.7 ± 5.8% for nontransgenic mice. However, there was no significant difference in paw weight increase between transgenic mice and nontransgenic mice after induction by either capsaicin or DABK (data not shown). We then evaluated the response of transgenic mice to the lethal effects of endotoxic shock. To this end, the mice were injected with a high dose of LPS (24 mg/kg of body weight). Fig. 8 shows the percentage of survivors after LPS injection. Within the first 36 h about 82% of transgenic mice from lines WT2510 and N130A58, but only 45% of nontransgenic mice died. After 3 days, the mortality rate of WT2510 and N130A58 mice reached 87 and 99%, respectively, whereas nontransgenic mice reached only 76%. In contrast, mock injection of vehicle did not cause death in either group.

DISCUSSION
In the absence of agonist, GPCRs spontaneously isomerize between the inactive and active conformations, with the equilibrium shifted toward the predominantly inactive conformation (24, 25). Some receptor mutations induce an agonist-independent shift in isomerization equilibrium toward the active conformation and evoke second messenger responses in the absence of agonist. Such constitutive activations of GPCRs are generally believed to result from an increase in receptor conformational flexibility caused by the loss of intramolecular constraints (26). Our present study shows that the wild-type rat kinin B1 receptor displayed a marked constitutive activity in the transiently or stably transfected 293A cells. During the preparation of this manuscript, Leeb-Lundberg et al. (27) reported that the human counterpart B1 receptor also exhibited a high level of constitutive activity in transiently transfected 293 cells and so did the rabbit wild-type B1 receptor, suggesting that a high constitutive activity might be a common characteristic of the B1 receptor.

Using single amino acid replacements based on findings from other GPCRs, we generated four mutants of rat B1 receptors. Asn54 in TMD I is highly conserved in the superfamily of
seven-TMD GPCRs (28). It was thought to be part of a highly conserved transmembrane “polar pocket,” involved in receptor activation (29). Substitution of the homologous Asn residue with Ala induced a moderate constitutive activation of the α1H-adrenergic receptor (29). However, in the case of the rat B1 receptor, similar mutation did not result in enhancement of constitutive activation. Asp144 is part of the highly conserved DRY motif (triplet of amino acids: Asp-Arg-Tyr) located at the boundary of TMD III and the second intracellular loop (Fig. 1). The DRY motif has played a pivotal role in the signal transduction pathway of GPCRs (30–32). Mutations of the aspartate residue are reported to lead to constitutive activity for some GPCRs (31–33). Similar mutation in the homologous position in the rat B1 receptor failed to induce detectable constitutive activity. However, this result is not unprecedented. For some adrenergic and muscarinic receptors, mutations in the aspartic residue of the DRY motif did not result in agonist-independent constitutive activity (34, 35). Asn130 in the rat B1 receptor is 14 residues N-terminal to the DRY motif. Mutations in this homologous position such as Cys128 in the α1H-adrenergic receptor (36), Cys116 in the β2-adrenergic receptor (37), and Asn111 in the angiotensin AT1 receptor (38) led to constitutive activity, suggesting that this particular amino acid position may function as a switch that regulates transition between distinct receptor conformations (37). Based on molecular modeling of the AT1 receptor and their finding that mutation of Asn111 to Ala led to constitutive activation of the AT1 receptor, Bonnafous and co-workers (38) proposed that Asp74, Asn111, and Trp255 were involved in the AT1 receptor activation. They found that these residues were conserved in the kinase B1 receptor and that mutation of Asn111 to Ala in the homologous position resulted in a high constitutive activation of the human kinin B2 receptor (39). Interestingly, these residues were also conserved in the B1 receptor (i.e., Asn90, Asn130, and Trp273 for the rat B1 receptor). Here we show that mutation of the homologous Asn130 to Ala in the rat B1 receptor induced a marked constitutive activation. Leeb-Lundberg et al. (27) reported that mutation of homologous Asn121 to Ala in the human B1 receptor also caused a further increase in constitutive activity, indicating that this Asn is indeed involved in constraining the B1 receptor in an inactive state. Our findings suggest that the molecular events associated to their activation processes are probably conserved between kinin B1 and B2 receptors.

The basal IP production by the N130A receptor in the stably transfected 293A cells was about 3-fold higher than that by the wild-type receptor in the stably transfected 293A cells. But if one considers that the expression level of the N130A receptor is about 25% of that of the wild-type B1 receptor and that increasing the expression of receptors could increase signal to phospholipase C as demonstrated by other researchers with other GPCRs (27, 31, 39), the N130A receptor would have a up to 12-fold increase in basal activity compared with the wild-type receptor.

Constitutively active mutants have been shown to be responsible for several hereditary and acquired diseases and have been used to produce transgenic mice serving as unique experimental models (40). Because the wild-type rat B1 receptor has a high basal activity and the N130A mutant is highly constitutively active, we expect that overexpressing these receptors in mice would allow the emergence of any pathophysiologically consequences associated with B1 receptors. B1-receptor-mediated hypotensive responses have been documented in rabbit, rat, pig, and dog (11). Unexpectedly, we found that all three B1 receptor transgenic lines were normotensive. One explanation for this finding is that some strong unknown compensatory in vivo mechanisms exist and that any such compensation might more likely dilute the manifestation of altered blood pressure phenotype. Alternatively, the B1 receptor may not be as important in the normal modulation of hemodynamics, which is compatible with the observation that B1-deficient mice are normotensive (12). It should be noted that the B1 receptor-mediated hypotensive effect is generally only observed following an inflammatory stimulus, such as endotoxin treatment (11).

Surprisingly, intravenous administration of DABK produced a MAP increase in transgenic mice, and a subsequent injection of DABK was almost as potent as the first injection, which can be explained by the lack of desensitization of B1 receptors (41). Intravenous injection of BK initially caused blood pressure reduction in transgenic mice, and then the blood pressure bounced back and went beyond the basal level, whereas in the control littermates BK only produced a transient blood pressure reduction. This discrepancy could be due to the conversion of some of the injected BK by kininase I into DABK in vivo (42), which then acted on the constitutively expressed B1 receptor, causing a blood pressure increase in transgenic mice. B1 receptor ligands have been reported to cause vasoconstriction of a range of blood vessels from several species (43). The DABK-mediated hypertensive effect in B1 receptor transgenic mice probably resulted from B1 receptor-induced peripheral resistance. However, the central role of B1 receptors in hypertension cannot be ruled out at this time. Alvarez et al. (44) reported that brain B1 receptor blockade lowers blood pressure in spontaneously hypertensive rats but not in normotensive rats. Similarly, we demonstrated that intracerebroventricular administration of B1 receptor agonists increases blood pressure in both Wistar Kyoto rats and spontaneously hypertensive rats, whereas B1 receptor blockade with antisense oligonucleotides reduced blood pressure in spontaneously hypertensive rats but not in Wistar Kyoto rats (45).

In B1 receptor transgenic mice, we found that carrageenan-induced paw edema was significantly enhanced compared with that in nontransgenic control littermates. It was reported that carrageenan, a water-extractable polysaccharide obtained from various seaweeds, could activate kinin release and induced the acute edema produced by kinins injection into rat paw showed mediation by a form of B1 receptor, without a significant involvement of B2 receptors (46, 47). This enhancement in B1 receptor transgenic mice is probably because released kinins could immediately activate the constitutively expressed B1 receptors without time delay for the B1 receptor induction as in nontransgenic control. Overexpression of B1 receptors appears to have no significant effects on DABK or capsaicin-induced acute edema. However, this finding is in line with the observation that the acute edema produced by kinins injection into rat paw showed mediation by a form of B1 receptor, without a significant involvement of B2 receptors (48), even if the animal was pre-treated with LPS (49). Compared with nontransgenic control mice, line WT2510 was prone to endotoxic shock, but N130A lines were more susceptible. The reason is not clear for the time being. But these findings clearly support the notion that B1 receptors play an important role in modulating inflammatory responses.

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Kinin B1 Receptor Transgenic Mice
Overexpression of Kinin B₁ Receptors Induces Hypertensive Response to Des-Arg⁹-bradykinin and Susceptibility to Inflammation
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