Tissue Kallikrein-binding Protein Reduces Blood Pressure in Transgenic Mice

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The kallikrein-kinin system participates in blood pressure regulation. One of the kallikrein-kinin system components, kallikrein-binding protein, binds to tissue kallikrein and inhibits its activity in vitro. To investigate potential roles of rat kallikrein-binding protein (RKBP) in vivo, we have developed transgenic mice that express an RKBP gene under the control of the mouse metallothionein metal-responsive promoter. Expression of the transgene, RKBP, was detected in the liver, kidney, lung, heart, pancreas, salivary glands, spleen, brain, testis, and adrenal gland at the mRNA and protein levels. Systolic blood pressures of homozygous transgenic mice were 90.5±0.8 mm Hg (mean ± S.E., n = 19, P < 0.001) for one line and 88.8±1.6 mm Hg (mean ± S.E., n = 19, P < 0.001) for another, as compared with 100.5±0.8 mm Hg (mean ± S.E., n = 18) for control mice. Direct blood pressure measurements of these transgenic mice through an arterial cannula showed similar reductions of blood pressure. Intravenous injection of purified RKBP into mice via a catheter produced a dose-dependent reduction of the mean arterial blood pressure. Our findings suggest that RKBP may function as a vasodilator in vivo, independent of regulating the activity of tissue kallikrein.

Tissue kallikreins (EC 3.4.21.35) are a group of closely related serine proteinases. The well characterized biological activities of tissue kallikrein are the abilities to cleave low molecular weight kininogen and to release kinin peptides that in turn mediate a broad spectrum of biological effects including vasodilation, inflammation, pain, and smooth muscle contraction and relaxation (1). Tissue kallikreins are regulated at the transcriptional level and by post-translational modifiers (2, 3). Potential endogenous kallikrein inhibitors or binding proteins have been identified. Human α1-antitrypsin (4) and protein C inhibitor (5) have been shown to bind to human tissue kallikrein in plasma. We have recently isolated and cloned new tissue kallikrein inhibitors from the human (kallistatin), rat (rat kallikrein-binding protein, RKBP), and mouse (mouse kallikrein-binding protein) (6–10). Kallikrein-binding protein forms a 1:1 stoichiometric complex with tissue kallikrein (6, 10) and inhibits both the amidolytic and kininogenase activities of tissue kallikrein in vitro (10, 11).

Kallikrein-binding proteins may potentially play a role in modulating the activity and function of tissue kallikrein in vivo. It has been shown that kallikrein-binding protein has the ability to bind to active kallikrein but not to inactive (latent) or active site-blocked kallikrein (12, 13). Co-expression and localization of tissue kallikrein and kallistatin in the kidney and other tissues, such as heart, lung, salivary glands, uterus, testis, and urine, suggest that kallistatin may regulate the activities of tissue kallikrein in vivo (14, 15). In addition, endogenous complexes of kallikrein and the binding protein have been identified in the serum, urine, and kidney of rats (12). The clearance rate of the radiolabeled kallikrein-kallistatin complex is longer than that of kallikrein alone in rat circulation, and the complex is mainly cleared from the circulation by the liver (16). Collectively, these results suggest that kallikrein-binding protein may play a role in regulating the bioavailability of tissue kallikrein in vivo.

Hypertension is a multifactorial and multigenic disorder. A potential physiological function of kallikrein-binding protein in blood pressure homeostasis has been implicated for two reasons. First, the expression levels of RKBP were shown to be significantly reduced in spontaneously hypertensive rats (SHR) when compared with normotensive Wistar-Kyoto rats (6, 13). Second, a restriction fragment-length polymorphism at or near the RKBP locus co-segregates with increased diastolic blood pressure of a stroke-prone SHR after salt loading (17). These findings suggest that RKBP may be one of the factors contributing to the hypertensive phenotype of this animal model. Since RKBP is deficient in SHR, we postulated that kallikrein-binding protein may potentially play a role in regulating vascular tone in vivo in addition to modulating the activity of tissue kallikrein. In order to elucidate its biological functions, we developed transgenic mice overexpressing RKBP and analyzed the potential function of RKBP in these animals.

EXPERIMENTAL PROCEDURES

Transgene DNA Preparation—The scheme for construction of the RKBP transgene was described previously (18). The transgene contains the mouse metallothionein metal-responsive element (MRE) fused to a modified RKBP gene in which the non-coding exon 1 and intron 1 sequences were deleted. The transgene MRE-RKBP DNA was released from the vector DNA by BglII digestion and purified using sucrose gradient centrifugation (19).

Generation and Identification of Transgenic Mice—Transgenic mice were generated as described previously (20). Three MRE-RKBP transgenic founder mice were identified by Southern blot analysis using an RKBP cDNA as the probe. Homozygotes of the transgenic mice were generated from each founder. Strains of C57BL/6 and DBA/2 mice having similar and normal blood pressures were used as control mice (20). Transgenic and control mice at a body weight of 25–30 g were used throughout the following studies.

Expression of RKBP Transgene—Total RNA was extracted from mouse tissues using the RNaseasy columns (Qiagen Inc., Chatsworth,
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CA). The first strand cDNA was synthesized using the murine leukemia virus reverse transcriptase (Life Technologies, Inc.) in a 20-μl reaction mixture containing 1× reverse transcription buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, and 3 mM MgCl2), 0.5 mM each dNTP, 0.5 μM downstream primer (CXXQ1101, 5′-GGG TGA AAT TCA GAA GA-3′), 0.01 mM dithiothreitol, and 1 μg of total RNA. The reaction mixture was incubated at 37°C for 60 min and then heated at 95°C for 5 min and chilled on ice. The subsequent PCR was performed in a 50-μl reaction mixture containing the total product of the reverse transcription, 1× PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl2, and 0.001% (w/v) gelatin, Sigma), 0.5 μM upstream primer (RK533, 5′-ACCC ATT TAA CCC CAA TG-3′). The PCR tubes were preheated at 94°C for 30 s followed by the addition of 2.5 units of Ampli-Taq DNA polymerase. The amplification was carried out following a program of 30 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C for the initial 5-min extension at 72°C. The PCR products were analyzed by Southern blot analysis using a 32P-labeled internal oligonucleotide probe (RK332, 5′-AAG GTC CTC CCA CTC GAG-3′).

Preparations of Tissue Extract and Urine Collection—Tissues collected from transgenic and non-transgenic control mice were homogenized with a Polytron (6). The total protein was determined by the Lowry protein assay using bovine serum albumin as the reference (21). The 24-h urine output of transgenic and control mice was collected using metabolic cages. Mice were fed overnight before the urine collection and were given water during the urine collection.

Enzyme-linked Immunosorbent Assay (ELISA)—An ELISA was performed as described previously (22). Briefly, a 96-well microtiter plate was coated with a non-iodinated RKBP (2 μg/ml in phosphate-buffered saline, 100 μl/well) at 4°C overnight. The plate was blocked with 200 μl of phosphate-buffered saline containing 1% bovine serum albumin before sample addition. RKBP standard (0.4 ng to 25 ng/ml), mouse serum, urine, and tissue extracts were added into each well. After 1.5 h of incubation at 37°C, a biotin-labeled RKBP IgG antibody (23) was added into each well at a concentration of 2 μg/ml followed by peroxidase-avidin, at a concentration of 1 μg/ml. After a 30-min color reaction, the absorbance was read with a Titertek ELISA reader at 414 nm.

Purification of Rat Kallikrein-binding Protein—Pooled rat sera were first dialyzed against 20 mM sodium phosphate buffer, pH 7.0, and then passed through an Affi-Gel Blue (Bio-Rad) column equilibrated with the same buffer at a flow rate of 12 ml/h. Flow-through fractions were collected and assayed for tissue kallikrein binding activity (6). The fractions containing the kallikrein-binding protein were combined and passed through an HPLC (PerSeptive Biosystems, Inc., Framingham, MA) anion-exchange column by perfusion chromatography (BioCAD™). Protein fractions were eluted using 20 mM sodium phosphate buffer, pH 7.0, over a linear NaCl gradient from 0 to 300 mM at a flow rate of 5 ml/min. The kallikrein-binding protein was eluted at 100–150 mM NaCl. Those fractions were combined and dialyzed against 20 mM sodium phosphate buffer, pH 7.0, and passed through a hydroxyapatite column (Bio-Gel HPHT™, Bio-Rad) by fast protein liquid chromatography at a flow rate of 0.5 ml/min. The kallikrein-binding protein was in the flow-through fractions. After concentration with a Centricon concentrator (Amicon, Inc., Beverly, MA), the kallikrein-binding protein was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) under reducing conditions.

Blood Pressure Measurements—Systolic blood pressure was measured with a programmed electromyogramanometer PE-300 (Narco Bio-Systems, TX) using the tail-cuff method (23). Male transgenic mice were placed into a small plastic holder mounted on a thermostatically controlled warm plate that was maintained at 33–35°C during the measurement. An average of 10 readings were taken for each mouse. Mean arterial blood pressure was measured via an intra-anastomosed cannula. Mice were anesthetized with 2,2,2-trichloroethanol in tert-amyl alcohol (20 mg/ml avertin, 0.4 ml/25 g of body weight intraperitoneally). PE-10 tubing (Clay Adams, Parsippany, NJ) filled with heparinized saline (50 units/ml) was used as a cannula. The cannula was inserted into the left femoral artery at one end, and at the other end, the cannula was connected to a physiological pressure transducer (Statham Laboratories Inc, Hato Rey, Puerto Rico) coupled to a model 7 polygraph (Grass Instrument Co., Quincy, MA).

Intravenous Injections of Purified RKBP—Mice were anesthetized with 2,2,2-trichloroethanol in tert-amyl alcohol. A cannula was inserted into the left femoral artery for direct blood pressure measurement. The right femoral vein was inserted with another cannula for injection of purified RKBP and Lys-bradykinin (kallidin) in a total volume of 50 μl of saline (0.9% sodium chloride). After each injection, the cannula was flushed with 50 μl of saline to remove residual injected substances.

RESULTS

Development and Identification of Transgenic Mice Expressing Rat Kallikrein-binding Protein—Fig. 1 shows the rat kallikrein-binding protein transgene placed under the control of the mouse metallothionein promoter (MRE). The RKBP gene is comprised of 5 exons interrupted by intronic sequences. The translation initiation site is located in exon 2. Exon 1 and intron 1 of the RKBP gene were removed from the full-length RKBP gene to eliminate the natural RKBP promoters (7). The remaining RKBP gene, approximately 5 kilobases in length, was placed immediately after the MRE promoter. Three independent founder mice, 5972, 5798, and 5975, harboring the RKBP transgene were identified by Southern blot analysis. Homozygotes were produced from lines 5972 and 5798 by mating heterozygotes within the same line. The homozygotes were confirmed by backcrossing with C57BL/6. Each homozygote produced at least 10 heterozygous offspring when mated with C57BL/6. The following studies were performed using the homozygotes from the 5972 and 5798 lines.

Expression of the RKBP Transgene in Transgenic Mice—Tissue distribution of the MRE-RKBP transgene was analyzed by an RT-PCR Southern blot. The RKBP mRNA was identified in the liver, kidney, lung, heart, pancreas, salivary glands, spleen, brain, testis, and adrenal gland of transgenic mice but not in the liver and kidney of the control littermates (Fig. 2). RKBP mRNA was not detected in other tissues of the control mice (data not shown). An ELISA, specific for RKBP, was used to quantitify the transgene product in mouse serum, urine, and many other tissues. High levels of immunoreactive RKBP were detected in transgenic mouse sera at levels up to 2 μg/ml and at much lower levels in various tissues (Table I). The linear displacement curves for the expressed RKBP in mouse serum and liver were parallel with the RKBP standard curve, indicating their immunological identity (Fig. 3). RKBP was barely detectable in the urine of transgenic mice and was not detected in sera, tissues, and urine of the control mice (data not shown).

Expression of RKBP Reduces Blood Pressure of Transgenic Mice—High expression levels of RKBP, under the control of the MRE promoter, caused a significant reduction of blood pressure in transgenic mice as measured by the tail-cuff method (Fig. 4). Transgenic mice from line 5972 had systolic blood pressure of 88.5 ± 0.8 mm Hg (mean ± S.E., male, n = 19, P < 0.001) and 90.9 ± 0.7 mm Hg (mean ± S.E., female, n = 12, P < 0.001), and mice from line 5798 had blood pressure of 88.8 ± 1.6 mm Hg (mean ± S.E., male, n = 19, P < 0.001) and 95.2 ± 1.2 mm Hg (mean ± S.E., female, n = 18, P < 0.01). Control mice had systolic blood pressure of 100.5 ± 0.8 mm Hg (mean ± S.E., male, n = 18) and 100.4 ± 0.8 mm Hg (mean ± S.E., female, n = 16). Male transgenic mice from both lines had an approximately 12-mm Hg reduction of blood pressure measured by the indirect tail-cuff method, which was further confirmed by the...
FIG. 2. Expression of RKBP in mouse tissues. RKBP mRNA was detected by RT-PCR followed by Southern blot analysis. 1 μg of total mouse RNA from various tissues was subjected to RT-PCR using two RKBP-specific oligonucleotide primers. RT-PCR products were then analyzed by Southern blot hybridization with a nested RKBP-specific oligonucleotide primer. Hybridization was carried out in 6 × SSC, 5 × Denhardt’s solution, 0.5% SDS, and 100 μg/ml herring sperm DNA at 45 °C overnight. The blot was washed to a final stringency of 1 × SSC at 45 °C and exposed to x-ray film. 10 × SSC = 3 M NaCl, 0.3 M sodium citrate, pH 7.0.

Rat kallikrein-binding protein in MRE-RKBP transgenic mice

Tissue extracts and sera from MRE-RKBP transgenic and control mice were subjected to an ELISA. RKBP levels were expressed as ng/mg total protein.

Purification and Characterization of Rat Kallikrein-binding Protein—In order to evaluate whether a bolus injection of RKBP into mouse circulation affects the blood pressure, we purified a large quantity of RKBP from rat plasma. Using a 3-step purification scheme, we obtained 2–3 mg of purified RKBP from 20 ml of rat plasma. The method was simple and efficient with a high yield as compared with our previously described purification procedures (6). Fig. 5, panel A, shows Coomassie Blue staining of protein fractions from each purification step on SDS-PAGE. Lanes indicate the molecular weight markers (lane 1), rat serum (lane 2), flow-through fractions from the Affi-Gel Blue column (lane 3), eluate from an HqM anion-exchange column (lane 4), and flow-through fractions from a hydroxyapatite HPHT™ column (lane 5). The purified RKBP migrates as a 60-kDa protein band on SDS-PAGE (panel A, lane 5). Its activity was assayed by the ability to bind to 125I-labeled rat tissue kallikrein. Fig. 5, panel B, shows the formation of a 92-kDa tissue kallikrein-RKBP complex on SDS-PAGE as visualized by autoradiography.

Intravenous Injections of Purified RKBP Reduces Blood Pressure of Mice—Intravenous injections of purified RKBP into anesthetized mice produced a transient reduction of mean arterial blood pressure in a dose-dependent manner (Fig. 6A). The duration of mean arterial blood pressure reduction lasted 0.5–1 min. RKBP (from 41 to 330 pmol/mouse) injected intravenously into mouse circulation through a cannula in the femoral vein produced a 5–13-mm Hg reduction of blood pressure (n = 10). The blood pressure lowering effect of RKBP was observed in both control and transgenic mice. A hypotensive

| Mouse | Rat kallikrein-binding protein | Control | Line 5972 | Line 5798 |
|-------|-------------------------------|---------|-----------|-----------|
| Serum | ND                            | 0.76 ± 0.01 | 0.82 ± 0.01 |
| Liver | ND                            | 0.97 ± 0.13 | 0.52 ± 0.02 |
| Kidney| ND                            | 0.79 ± 0.06 | 0.31 ± 0.12 |
| Lung  | 0.59 ± 0.09                   | 0.53 ± 0.02 |
| Heart | 0.29 ± 0.01                   | 0.29 ± 0.07 |
| Pancreas | 0.62 ± 0.11                  | 0.20 ± 0.04 |
| Salivary glands | 0.54 ± 0.06                | 0.90 ± 0.22 |
| Spleen| 0.28 ± 0.01                   | 0.34 ± 0.05 |
| Brain | 0.82 ± 0.09                   | 0.64 ± 0.09 |
| Testis| ND                            | 0.76 ± 0.04 | 0.40 ± 0.01 |

**TABLE I** Blood pressure of RKBP transgenic and control mice

Blood pressure of mice was measured by indirect (tail-cuff) and direct (cannulation) methods. Values are mean ± S.E. n indicates total number of mice in each group.

| Tail-cuff | Cannulation |
|-----------|-------------|
| Control   | 100.5 ± 0.8 (n = 18) | 92.1 ± 0.8 (n = 15) |
| Line 5972 | 88.5 ± 0.8 (n = 19)  | 81.4 ± 2.1 (n = 8)  |
| Line 5798 | 88.8 ± 1.6 (n = 19)  | 82.3 ± 3.6 (n = 8)  |

a P < 0.001 compared with the control (Student’s t test).
b P < 0.01 compared with the control (Student’s t test).

direct cannulation method (Table II). Mean arterial blood pressure measured by intra-arterial cannulation shows a similar reduction of blood pressure in transgenic male mice, line 5972 (81.4 ± 2.1 mm Hg, n = 8, P < 0.001) and line 5798 (82.3 ± 3.6 mm Hg, n = 8, P < 0.01), as compared with the control mice (92.1 ± 0.8 mm Hg, n = 15).
In the present study, we developed and characterized two homozygous transgenic mouse lines overexpressing RKBP. The blood pressures of both male and female transgenic mice from two homozygous lines were significantly lower than non-transgenic controls. Expression of the RKBP gene in these mice was identified at both the mRNA and protein levels. Immunoreactive RKBP was detected mainly in the circulation and in a small amount in various tissues of these mice. These results show that RKBP may play a role in regulating vascular tone in vivo. In order to substantiate this idea, we developed a simple and efficient purification scheme to purify a large quantity of RKBP from rat plasma. We show that bolus intravenous injections of purified RKBP cause transient and dose-dependent reduction of mean arterial blood pressure in mice. These findings suggest that kallikrein-binding protein may act as a vasodilator that contributes to blood pressure regulation.

We show that transgenic mice expressing RKBP had significant reductions in blood pressure compared with the control mice. Both male and female transgenic mice had lower blood pressure but to different extents. Blood pressure reductions in female transgenic mice were approximately 5–9 mm Hg (P < 0.01), whereas those of male transgenic mice were 12 mm Hg (P < 0.001). Since the blood pressure measurement was not performed at any particular time during the natural estrogen cycle for female mice, it is not clear whether sex hormones in the circulation performed at any particular time during the natural estrogen cycle for female mice, it is not clear whether sex hormones in the circulation and tissues are significantly reduced in SHR as compared with the control mice. In order to substantiate this idea, we developed a simple and efficient purification scheme to purify a large quantity of RKBP from rat plasma. We show that bolus intravenous injections of purified RKBP cause transient and dose-dependent reduction of mean arterial blood pressure in mice. These findings suggest that kallikrein-binding protein may act as a vasodilator that contributes to blood pressure regulation.

Direct bolus injections of purified RKBP via a catheter resulted in a dose-dependent reduction of blood pressure. Injection of 20 µg (330 pmol/mouse) of purified RKBP into mouse circulation produced an approximately 13-mm Hg reduction of blood pressure. This concentration (~8 µg/ml RKBP, calculated as if there were 7 ml of blood/100 g of body weight) is about several times higher than that in the circulation of the transgenic mice (0.8–2.0 µg/ml). However, in both cases, RKBP transgenic mice and mice injected with purified RKBP showed a similar degree of blood pressure reduction.

The expression of RKBP in transgenic mice was under the control of the MRE promoter. The RKBP gene spans approximately 10 kilobases on chromosome 6 (7, 24). Two promoters were identified in the RKBP gene (25). One promoter is located in the 5′-flanking region, and the other one is located in the first intron. Both promoters were found to have promoter activities in vitro, and a negative regulatory element was identified at the 5′-flanking region of the gene. In order to eliminate the regulating elements of the RKBP gene, we placed MRE immediately upstream of the translation initiation site (exon 2). As we expected, RKBP was widely expressed in many tissues as measured by RT-PCR Southern blot analysis. Northern blot analysis with an RKBP cDNA probe was also carried out to quantitatively determine the expression level of the transgene in mice. However, we found that the RKBP cDNA (8) cross-reacted with the mouse kallikrein-binding protein (data not shown), and the result was inconclusive. High levels of immunoreactive RKBP were quantified by an ELISA in transgenic mice. The result was inconclusive. High levels of immunoreactive RKBP were quantified by an ELISA in transgenic mice. The result was inconclusive.
pared with normotensive Wistar-Kyoto or Sprague-Dawley rats (6). Whether deficiency of RKBP contributes to the hypertensive phenotype of SHR is not clear at the present time. The present finding that transgenic mice expressing RKBP had lower blood pressure than non-transgenic mice suggests that RKBP may potentially play a physiological role in vasodilation. In a parallel study, we observed that RKBP caused vasoconstriction in isolated rat aortic rings. The vasorelaxing activity of RKBP in isolated aortic ring preparations was not inhibited by Hoe 140, a bradykinin B2 receptor antagonist.\(^2\) Collectively, these findings suggest that RKBP may participate in blood pressure regulation by a mechanism independent of its interaction with the tissue kallikrein-kinin system.

The tissue kallikrein-kinin system has been shown to play a role in blood pressure regulation (20, 26, 27). Kallikrein-binding protein may also modulate the bioavailability of kallikrein in vitro (10, 11). Kallikrein-binding protein may also regulate excess tissue kallikrein. These findings suggest that kallikrein-binding protein may also modulate the activity of kallikrein. On the other hand, RKBP could be consumed by binding to tissue kallikrein and thus regulates the activity of kallikrein. On the other hand, RKBP transgenic mice), RKBP could function as a vasodilator. This hypothesis is consistent with our present findings and the fact that RKBP is deficient in genetically hypertensive rats. The potential role of RKBP as a vasodepressor awaits further investigations with gene knockout mice or by direct gene delivery in hypertensive animal models.

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