Regulation of Cardiovascular Signaling by Kinins and Products of Similar Converting-Enzyme Systems

Role of bradykinin in angiotensin-converting enzyme knockout mice

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ANGIOTENSIN-CONVERTING ENZYME (ACE) plays a central role in the renin-angiotensin system (RAS). ACE is a carboxypeptidase that hydrolyzes the amino acid peptide angiotensin I into the potent vasoconstrictor angiotensin II (9). In addition to inducing vasoconstriction, angiotensin II increases blood pressure by a variety of physiological actions, including renal salt and water retention, the induction of drinking behavior, and the potentiation of sympathetic action. Another major substrate of ACE is the vasodilator bradykinin (47). ACE efficiently inactivates bradykinin by cleaving two amino acids from its COOH-terminus. Thus ACE may affect blood pressure through the production of the vasoconstrictor angiotensin II and the inactivation of the vasodilator bradykinin.

Two isozymes of ACE, somatic ACE and testis ACE, are encoded by the same genetic locus but are regulated by two different promoters (2). Somatic ACE is produced by a variety of tissues, but it is thought that the expression of this protein by vascular endothelium is critical for the regulation of blood pressure. In contrast, testis ACE is only produced by developing male germ cells and is thought to contribute to male reproductive ability. To explore the in vivo function of ACE, our laboratory used targeted homologous recombination in embryonic stem (ES) cells to produce three lines of mice with interrupted ACE expression (8, 16, 17). Mice called ACE.1 were completely null for all ACE expression. ACE.2 mice have ACE circulating within plasma but no tissue ACE in organs, such as the lung or the kidney. ACE.4 mice expressed no somatic ACE but retained normal testis ACE expression. All three lines of mice have a common phenotype: a marked decrease of blood pressure, impaired urine concentrating ability, renal morphological defects, and a decreased hematocrit.

The phenotype of ACE knockout mice is complicated and may result from a combination of the lack of angiotensin II production and the inability of these animals to eliminate bradykinin. Both angiotensinogen knockout mice and mice lacking the angiotensin II AT1 receptor have been studied (36, 44). These animals have a phenotype similar to ACE knockout mice and present the argument that the lack of angiotensin II is the major contributor to the pathology present in these animals. However, because of the multiple actions of ACE, it is not clear what role bradykinin plays in the overall phenotype of the ACE knockout mice. Physiologically, bradykinin may counterbalance the action of angiotensin II on blood pressure control and renal blood flow (40). The biological function of bradykinin is mediated mainly through the bradykinin B2 receptor (B2R), which is constitutively expressed by smooth muscle, neurons, and vascular endothelium (4, 37). In contrast, the bradykinin B1 receptor is not constitutive but is induced by tissue injury and inflammation. Evidence suggests that bradykinin mediates, at least partially, some effects of ACE inhibition through its action on the B2R (28, 43). B2R knockout mice had increased sensitivity to angiotensin II, as well as an increased prevalence for hypertension (6, 29).

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To investigate the role of bradykinin in the expression of the ACE knockout phenotype, we bred B2R knockout mice with ACE.4 knockout mice, thus generating a line of mice deficient in both the B2R and ACE. Surprisingly, these mice did not differ from ACE.4 knockout mice in blood pressure, urine concentrating ability, renal pathology, and hematocrit. Thus, rather than bradykinin accumulation, the defective production of angiotensin II was the major cause of the ACE knockout phenotype.

METHODS

Generation and genotyping of knockout mice deficient in both ACE and B2R expression. Male B2R knockout mice were obtained from the Jackson Laboratory (stock number 002641) and have been previously described (4). These mice originated on a mixed 129/C57BL/6J(B6) background and were then backcrossed onto a B6 background for two generations. ACE.4 knockout mice were created in our laboratory using targeted homologous recombination in ES cells (8). ACE.4 mice had a mixed 129/B6 background and were maintained by breeding of heterozygote mice. B2R knockout mice and ACE.4 knockout mice were first crossed onto a Swiss background (Taconic; Germantown, NY) for one generation. The resulting heterozygous mice were bred to obtain animals that were heterozygotes for both the B2R and ACE.4 alleles. Offspring of double heterozygote animals of various genotypes were used to breed double knockout animals and control animals matched for ACE and B2R genetic background. Of the 10 B2R/ACE.4 knockout mice used in this study, 2 resulted from the breeding of mice heterozygotes for both the B2R and ACE.4 alleles, 2 resulted from the breeding of mice heterozygotes for B2R and homozygous for the ACE.4 alleles, and 6 resulted from the breeding of mice heterozygotes for B2R and homozygous for the ACE.4 alleles with mice null for B2R and heterozygotes for the ACE.4 alleles. Animal procedure was performed according to the National Research Council’s Guide for the Care and Use of Laboratory Animals and approved by the Emory University Division of Animal Research.

Tail DNA was used for genotyping of mice by PCR and by genomic Southern blotting. PCR genotyping of the B2R alleles was performed using three primers: the sense primer for the wild-type allele (5′-GACTGAGCCTCTAATTCTC3′), the sense primer for the mutant allele derived from the neomycin cassette (5′-GATCTCTGTGATCCTAC3′), and the antisense primer (5′-GGACCCCTCAGATTA-CAAC3′). The primer set amplified an ~1-kb DNA fragment for the mutant allele and a 518-bp DNA fragment for the wild-type allele. Genotyping of B2R alleles was confirmed by genomic Southern blot. A DNA fragment 5′ to the B2R coding sequence was obtained by amplifying genomic DNA using the primer set 5′-AGTTCAGGAGAACAGCTGAAT-3′ and 5′TCATGGTGACATGGTTGAT-3′. This DNA fragment was digested with NsiI and BamHI. The resulting 600-bp fragment was used as the probe for Southern blot analysis as previously described (3). PCR genotyping of ACE.4 alleles was performed using the following three primers: the sense primer for the wild-type allele (5′-CAAGTGGATGCTCTGCTA3′), the sense primer for the mutant allele derived from the KAP promoter (5′-TTCTTCTCTGCAATCGGAACACTG-3′), and the antisense primer (5′-CTCTTCTCCTGGATGCTG-3′). This PCR primer set amplified a 793-bp DNA fragment for the wild-type allele and a 345-bp fragment for the mutant allele.

Blood pressure determination. Systolic blood pressure and heart rate were measured using a Visitech Systems BP2000 automated tail-cuff system as previously described with slight modification (17). Animals were trained for 5 consecutive days to be acclimated to the procedure. Blood pressure was then measured for 4 consecutive days immediately after the training period. On each day, animals were prewarmed on the Visitech blood pressure platform for 5 min, and two sets of 10 measurements were taken for each mouse. Blood pressure data were the average of the eight data sets for each mouse.

Urine collection and urine osmolality. Control samples were collected from mice with free access to water and standard rodent chow. Animals were then transferred to clean cages with no access to water but free access to food. Postdehydration urine samples were collected after 24 h of water deprivation. Body weight was measured immediately after urine sample collection. Urine samples were spun for 5 min at 5,000 rpm to remove particulate matter. Urine osmolality was determined using a Wescat 5,500 Vapor Pressure Osmometer (model 5500, Wescor; Logan, UT).

Tissue collection and kidney histology analysis. Animals were anesthetized with intraperitoneal injection of 125 mg/kg ketamine and 12.5 mg/kg xylazine. The heart and kidneys were removed, blotted, and weighed. Renal tissue was postfixed in 10% phosphate-buffered formalin and then embedded in paraffin. Tissue sections were stained with hematoxylin and eosin.

Blood collection and hematocrit analysis. Blood was collected from the tail vein in heparinized microcapillary tubes. Blood samples were centrifuged for 8 min at 12,000 g. Hematocrit was read manually using a microcapillary reader.

Blood pressure responses to infusion of bradykinin peptide. Mice were anesthetized by an intraperitoneal injection of a ketamine and xylazine (125 and 12.5 mg/kg body wt, respectively) mixture. Animals were then placed on a 37°C heating pad throughout the procedure. A polyethylene catheter was inserted into the carotid artery for direct blood pressure measurement using the Digi-Med BPA-400 system (MicroMed; Louisville, KY). Another catheter was inserted into the jugular vein for the injection of drugs. After blood pressure stabilized, 50 μl of heparinized saline (50 U heparin/ml 0.9% saline) were injected as a control. Bradykinin (30.0 μg/kg, Sigma; St. Louis, MO) was injected in 1 μl/g body wt. This was then flushed in using 50 μl of heparinized saline. Maximal blood pressure changes triggered by the bradykinin were recorded.

Statistical analysis. All data were expressed as means ± SE. The significance of the difference between two groups was obtained by an unpaired Student’s t-test. The significance of the difference among multiple groups was obtained by ANOVA analysis. A P value < 0.05 was considered statistically significant.

RESULTS

Generation of B2R/ACE.4 double knockout mice. The ACE.4 mice were previously generated in our laboratory using targeted homologous recombination in ES cells (8). In these mice, the somatic ACE promoter was replaced by a dysfunctional androgen-regulated protein (KAP) promoter. Mice homozygous for the ACE.4 mutation had essentially no somatic ACE expression and retained the phenotype of ACE null mice: low blood pressure, low urine concentrating ability, low hematocrit, and abnormal renal histology. Unlike mice
completely null for ACE, ACE.4 mice had testis ACE expression and thus were fully fertile. Breeding of mice heterozygote for both the B2R null allele and the ACE.4 allele yielded nine genotypes. Our laboratory has genotyped 232 offspring since 1999, of which 11 double knockout mice were viable. The ratio of double knockout offspring obtained, 4.7%, is not far from the expected Mendelian ratio of 6.3%. In this paper, we report B2R/ACE.4 double knockout mice generated through double heterozygote mating, as well as the mating of mice pairs homozygous for one of the genotypes and heterozygous for the other genotype. Both male and female mice were studied. Controls included littermates as well as nonlittermate mice matched for ACE and B2R genetic background.

Blood pressure and heart rate of the B2R/ACE.4 knockout mice. ACE knockout mice present with a marked decrease in blood pressure compared with wild-type mice. To evaluate the role of bradykinin in these animals, we measured the systolic blood pressure of the B2R/ACE.4 double knockout mice and compared it to that present in either the B2R or ACE.4 single knockout mice (Fig. 1A). The B2R knockout mice had a blood pressure of 121.1 ± 4.3 mmHg, which was not significantly different from the blood pressure of wild-type mice (112 ± 3.1 mmHg). As expected, the ACE.4 knockout mice had a blood pressure of 74.6 ± 2.7 mmHg, which was 37 mmHg lower than that of wild-type mice. Surprisingly, the B2R/ACE.4 double knockout animals had a blood pressure of 74.9 ± 1.2 mmHg, which was equivalent to that of the ACE.4 single knockout mice. Heart rate was not different among the four groups (Fig. 1B).

Blood pressure of B2R/ACE.4 knockout mice did not respond to bradykinin infusion. Two receptors for bradykinin have been described. Whereas the B2R is expressed constitutively, the bradykinin B1 receptor is typically expressed only during tissue injury or during inflammation (30). However, published reports (21, 31) indicate that ACE inhibitors may induce B1 receptor expression. B1 receptor function may also be upregulated in B2R knockout mice (12). To investigate whether the bradykinin B1 receptor may function in B2R/ACE.4 double knockout mice, we infused bradykinin intravenously into wild-type, B2R knockout, ACE.4 knockout, and B2R/ACE.4 double knockout mice and recorded blood pressure response. Bradykinin infusion resulted in a transient and significant decrease of blood pressure in wild-type mice of 34 ± 6.4 mmHg (Fig. 2). The pressure decrease was accompanied by a heart rate increase from 216 ± 18 to 337 ± 34 beats/min. In contrast, B2R knockout mice and B2R/ACE.4 double knockout mice showed a blood pressure response to bradykinin that was not different from that observed with the infusion of saline control. ACE.4 knockout mice showed a very small decrease in blood pressure that averaged 7.2 ± 1.0 mmHg. This was generally accompanied by an increase of heart rate. However, these changes were not sufficiently large as to be significant from those observed after saline injection. This may reflect the intrinsic low blood pressure of ACE.4 knockout mice, and the fact that with such a low basal blood pressure, significantly more mice may need to be examined to derive a statistically significant result. Nonetheless, these data suggest that the presence of the bradykinin B1 receptor does not influence the blood pressure of the B2R or B2R/ACE.4 knockout mice. If the B1 receptor was upregulated and physiologically significant, we would have anticipated a significant reduction of blood pressure following bradykinin infusion, a result not observed.

Renal concentrating ability in B2R/ACE.4 knockout mice. ACE knockout mice have a defect in renal concentrating ability; they produce large amounts of dilute urine. This phenotype seems intrinsic to a deficiency of the RAS (it is also observed in angiotensinogen and AT1 receptor knockout mice), and at least partly independent of hypotension, because it was not reported in other hypotensive animal models (27, 48). B2R transgenic mice were reported to have enhanced glomerular...
filtration rate and urine flow (46). Renal bradykinin was also shown to increase with ACE inhibition and might contribute to the renal hemodynamic changes induced by ACE inhibitors (32). We studied urine osmolality of B2R/ACE.4 double knockout mice to explore the possibility that bradykinin played a part in the renal function of ACE.4 knockout mice (Fig. 3A). In mice fed food and water ad libitum, wild-type mice and B2R knockout mice produced urine of equivalent osmolality (1,367 and 1,775 mosmol/kg). In contrast, ACE.4 knockout mice produced urine of decreased osmolality (650 and 676 mosmol/kg). This difference was more marked when mice were water deprived for 24 h. Both wild-type and B2R knockout mice responded to water deprivation by significantly increasing urine concentration (3,457 and 3,470 mosmol/kg). In contrast, the urine osmolality of ACE.4 knockout mice and B2R/ACE.4 double knockout mice did not differ significantly before or after water deprivation. Both ACE.4 knockout mice and B2R/ACE.4 double knockout mice lost significantly more body weight during the 24-h water deprivation compared with either wild-type or B2R knockout mice (Fig. 3B). There was no significant difference between ACE.4 knockout mice and B2R/ACE.4 double knockout mice with regard to weight loss or urine osmolality.

Fig. 2. Effect of bradykinin administration. Arterial blood pressure (BP) and heart rate were recorded through a carotid artery catheter while bradykinin was injected through the jugular vein. A: representative graph of BP and heart rate following bradykinin administration in WT, B2R, ACE.4, and the B2R/4 double knockout mice. Continuous line shows the BP; dashed line, heart rate. WT mice and ACE.4 knockout mice responded to bradykinin with a decrease in BP and an increase in heart rate. In contrast, B2R and B2R/4 mice did not respond to bradykinin injection. B: change of mean BP after injection of saline control or bradykinin. Number of mice in each group was 4 WT, 4 B2R, 3 ACE.4, and 4 B2R/4. All data are means ± SE. *P < 0.001 compared with saline control.
the B2R receptor itself appears important for renal effect of bradykinin in this pathology (45). Moreover, blockade of bradykinin signaling has suggested an angiotensinogen knockout animals, pharmacological angiotensin II, as indicated by a similar phenotype in (17, 20, 25). Although these changes are due to a lack of intrarenal arterioles and renal medullary hypoplasia changes characterized by abnormal wall thickening of ACE knockout mice have distinct renal morphological changes similar to those described in B2R knockout mice treated with high salt (Fig. 4B). Thus the lack of the B2R receptor does not seem to correct the phenotype present in animals lacking only ACE. If anything, the lack of the B2R contributes to a more complex renal phenotype.

B2R knockout did not change heart or kidney weight of ACE.4 knockout mice. Previously, we reported that ACE knockout mice have a mild decrease of kidney weight but no significant change of heart weight (17). In contrast, B2R knockout mice were reported to have cardiac hypertrophy (15, 29). We measured whole heart weight and kidney weight in mice 2–4 mo of age. Surprisingly, B2R knockout mice had similar heart weights to wild-type mice in our study. Both ACE.4 and B2R/ACE.4 knockout mice showed a very mild decrease of heart weight, compared with wild-type mice, but this difference did not reach statistical significance (P > 0.05) by ANOVA (Fig. 5). In contrast, the kidney weight (per gram body weight) of both ACE.4 knockout mice and B2R/ACE.4 double knockout mice was significantly lower than wild-type mice. However, there was no statistical significance between these two groups of knockout mice.

B2R/ACE.4 double knockout mice have a reduced hematocrit. ACE knockout mice have a hematocrit significantly less than littermate wild-type mice (7). Likewise, anemia had been reported clinically with ACE inhibitors (19). In our mice, wild-type animals had a hematocrit of 56% (Fig. 6). In contrast, ACE.4 knockout and B2R/ACE.4 double knockout mice had hematocrits of 41% and 45%, respectively. These two values were not statistically different, although they both were significantly different from the wild-type value (P < 0.001). Therefore, bradykinin does not seem to contribute to a decreased hematocrit in ACE knockout mice.

DISCUSSION

The kallikrein-kininogen-kinin system is thought to counterbalance the action of the RAS in cardiovascular function. Acute infusion of bradykinin significantly lowers blood pressure (38). Bradykinin is also known to play a role in renal function, including increasing renal sodium and water excretion (33). Overexpression of either the bradykinin B2 receptor or kallikrein, a precursor of bradykinin, reduced blood pressure (46). However, the physiological role of bradykinin in blood pressure control remains controversial. B2 knockout mice or kallikrein knockout mice have a blood pressure equal to, or only slightly higher than, the blood pressure of control animals (34, 35, 42). Nonetheless, bradykinin may contribute to blood pressure control in situations (such as ACE inhibitor administration) where the half-life of bradykinin is increased. In fact,
cough, a common side effect of ACE inhibition, is attributed to the effects of bradykinin (23).

In this study, we chose to investigate the effects of bradykinin in ACE knockout mice by breeding animals lacking both B2R and ACE. Genetically eliminating the B2 receptor did not modify any of the phenotypes found in ACE knockout mice, including blood pressure, renal morphology, urine concentrating ability, and hematocrit. The lack of a B2R knockout effect did not seem due to compensation by bradykinin B1 receptor upregulation, because a hypotensive effect of bradykinin infusion was completely absent in B2R or B2R/ACE.4 double knockout mice. Thus we conclude that bradykinin accumulation was not a significant factor in the ACE knockout phenotype.

In this study, we were not able to repeat some of the reported phenotypes of the B2 knockout mice, such as cardiac hypertrophy or hypertension (15, 29). There are discrepancies in the literature about the cardiovascular phenotype of B2R knockout mice. For instance, different groups have reported different effects of a B2R knockout on blood pressure. It has been suggested that these discrepancies resulted from different sensitivities of experimental measurements or different gen-

Fig. 4. Renal histology of the B2R/4 double knockout mice. Double knockout mice showed a renal histology similar to that seen in ACE knockout mice. This included medullary hypoplasia with widening of the renal pelvis (arrow, A), the thickening of intrarenal arterioles (C), and even perivascular inflammation with focal vasculitis (D). One B2R/4 double knockout mice also presented with rare renal cysts similar to the pathology occasionally noted in B2R knockout animals (*, B).
netic backgrounds found in the animals. Moreover, phenotypes such as cardiac hypertrophy and hypertension developed in older animals and/or animals with external stress, such as salt loading (1, 5, 15). The B2R knockout mice used in our study were relatively young (2–4 mo) and had a mixed genetic background. These factors may explain why we did not see a phenotypic difference between B2R knockout mice and wild-type mice. In contrast, the kidney weight (per gram body weight) of both ACE.4 knockout mice and B2R/4 double knockout mice was significantly lower than WT mice (B). However, there was no statistical significance between these two groups of knockout mice. *P < 0.001 compared with WT mice. Number of mice in each group was 9 WT, 14 B2R, 6 ACE.4, and 9 B2R/4. All data are means ± SE.

ACE is not the only enzyme that may degrade bradykinin. In humans, ACE and carboxypeptidase N are the two major enzymes for bradykinin degradation in human plasma (26). ACE accounts for >90% of bradykinin degradation at a physiological concentration of bradykinin, whereas carboxypeptidase N accounts for >90% of bradykinin degradation at a high dose of bradykinin. In rats, ~50% of bradykinin degradation in plasma is due to ACE activity, whereas carboxypeptidase N and aminopeptidase P represent the majority of the rest (11, 22). A membrane-bound peptidase, neutral endopeptidase, is the main enzyme for bradykinin metabolism in kidney and heart interstitium (10, 24). With ACE knockout or during long-term ACE inhibition, upregulation of these alternative enzymes may compensate for a lack of ACE and thus reduce bradykinin effects. Moreover, with chronic ACE inhibition and bradykinin accumulation, the B2 receptor may be desensitized and thus the response to bradykinin blunted. Therefore, the role of bradykinin in blood pressure control during chronic ACE inhibition may differ from the short-term effect of an ACE inhibitor (18, 41).

Fig. 5. Heart (A) and kidney weight (B) (expressed as mg/g body wt) of B2R/4 double knockout mice. WT, B2R, ACE.4 knockout, and B2R/4 double knockout mice were euthanized. Both ACE.4 and B2R/4 knockout mice showed a very mild decrease of heart weight, compared with WT, but this difference did not reach statistical significance (P > 0.05) by ANOVA (A). In contrast, the kidney weight (per gram body weight) of both ACE.4 knockout mice and B2R/4 double knockout mice was significantly lower than WT mice (B). However, there was no statistical significance between these two groups of knockout mice. *P < 0.001 compared with WT mice. Number of mice in each group was 9 WT, 14 B2R, 6 ACE.4, and 9 B2R/4. All data are means ± SE.

Fig. 6. Hematocrit of B2R/4 double knockout mice. Blood was collected from the tail vein and hematocrit was determined in WT, B2R, ACE.4 knockout, and B2R/4 double knockout mice. Both the ACE.4 and B2R/4 knockout mice had a significantly reduced hematocrit as compare with WT mice. However, there was no significant difference between the two groups. The B2R knockout mice had a hematocrit equivalent to that of WT mice. *P < 0.001 compared with WT mice. Number of mice in each group was 8 WT, 12 B2R, 7 ACE.4, and 8 B2R/4. All data are means ± SE.
In summary, the current study suggests that the ACE knockout phenotype, including low blood pressure, decreased renal concentrating ability, abnormal kidney morphology, and reduced hematocrit is not due to bradykinin action.

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