Dilated and Failing Cardiomyopathy in Bradykinin B₂ Receptor Knockout Mice

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Background—The activation of B₂ receptors by kinins could exert cardioprotective effects in myocardial ischemia and heart failure.

Methods and Results—To test whether the absence of bradykinin B₂ receptors may affect cardiac structure and function, we examined the developmental changes in blood pressure (BP), heart rate, and heart morphology of bradykinin B₂ receptor gene knockout (B₂⁻/⁻), heterozygous (B₂⁺/−), and wild-type (B₂⁺/+ ) mice. The BP of B₂⁻/⁻ mice, which was still normal at 50 days of age, gradually increased, reaching a plateau at 6 months (136 ± 3 versus 109 ± 1 mm Hg in B₂⁺/+ , P < 0.01). In B₂⁺/− mice, BP elevation was delayed. At 40 days, the heart rate was higher (P < 0.01) in B₂⁻/⁻ and B₂⁺/− than in B₂⁺/+ mice, whereas the left ventricular (LV) weight and chamber volume were similar among groups. Thereafter, the LV growth rate of B₂⁻/⁻ and B₂⁺/− mice was accelerated, leading at 360 days to a LV weight–to–body weight ratio that was 9% and 17% higher, respectively, than that of B₂⁺/+ mice. In B₂⁻/⁻ mice, hypertrophy was associated with a marked chamber dilatation (42% larger than that of B₂⁺/− mice), an elevation in LV end-diastolic pressure (25 ± 3 versus 5 ± 1 mm Hg in B₂⁺/+ mice, P < 0.01), and reparative fibrosis.

Conclusions—The disruption of the bradykinin B₂ receptor leads to hypertension, LV remodeling, and functional impairment, implying that kinins are essential for the functional and structural preservation of the heart. (Circulation. 1999;100:2359-2365.)

Key Words: bradykinin ■ angiotensin ■ myocardium ■ hypertrophy ■ heart failure ■ blood pressure ■ genes

Heart failure remains the leading cause of death in the industrialized countries and represents the emerging epidemics of the third millennium. 1 In ischemic and idiopathic dilated cardiomyopathies, in addition to extensive myocardial scarring and despite huge myocyte hypertrophy, a disproportion has been found between myocardial growth and left ventricular (LV) chamber enlargement, leading to a significant reduction in myocardial mass–to–chamber volume ratio. 2–6 Hypertension, myocardial hypertrophy, and ischemia accelerate the physiological drop-off of cardiomyocytes occurring with age, 3,4,7–9 and it can be assumed that cardiac dysfunction and failure may supervene when the number of remaining viable hypertrophied cells is too low to maintain cardiac performance. The mechanisms that provoke the ongoing myocyte loss remain unknown. Endocrine and paracrine factors, including catecholamines and angiotensin II (Ang II), may cause myocyte hypertrophy and myocyte death through apoptosis and necrosis. 10–15

Hence, the presence and functional relevance of a kallikrein-kinin system in the heart are firmly established 16–19 Kinins, cleaved by kallikrein from substrate kininogen, stimulate the release of NO and prostacyclin through the activation of bradykinin (BK) B₂ receptors. This action opposes Ang II–induced vasoconstriction and exerts anti-ischemic, antiproliferative, and antiatherosclerotic effects, preserving myocardial stores of energy-rich phosphates and glycogen. 16,20–24 Conversely, a dysfunctional kallikrein-kinin system may contribute to the pathogenesis of heart failure. In fact, reduced local kinin generation and blunted NO formation have been reported in failing human hearts. 20 Furthermore, icatibant, a selective antagonist of the BK B₂ receptor, reduces coronary blood flow and contractility and increases LV end-diastolic pressure in pacing-induced heart failure. 25

Thus, to test the hypothesis that the absence of the BK B₂ receptor may lead to cardiac dysfunction, we examined the func-
tional and structural changes occurring in the hearts of BK B₂ receptor knockout mice (B₂⁻⁻) during development. This genetic model has similarity with the natural history of patients with hypertension and ventricular hypertrophy, who are at high risk for cardiovascular complications and death.⁻⁻ The informative potential of heterozygous mice (B₂⁺⁻), resembling a condition of partial deficiency, has been added to test whether a gene-dose effect on the cardiovascular phenotype may be present.

Methods

All procedures complied with the standards for the care and use of animal subjects as stated in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Academy of Sciences, Bethesda, Md).

B₂⁻⁻ mice were kindly provided by Dr. Fred Hess (Merck Laboratories), and wild-type control animals (B₂⁺⁺) were obtained from Jackson Laboratories. B₂⁺⁻ mice were derived through the mating of B₂⁺⁻ and B₂⁻⁻. Two days after birth, the sex of the pups from B₂⁺⁻, B₂⁺⁺, and B₂⁻⁻ groups was determined, and each litter was culled to 5 male pups. The animals were identified and housed at a constant room temperature (24±1°C) and humidity (60±3%).

Hemodynamic Measurements

Body weight (BW), systolic blood pressure (SBP), and heart rate (HR) of unanesthetized B₂⁺⁺ (n=33), B₂⁻⁻ (n=12), and B₂⁺⁻ (n=38) mice were measured from 40 to 360 days of age with the use of tail-cuff plethysmography. At the end of the experiments, in unanesthetized mice, mean blood pressure was measured with the use of an intra-aortic catheter (PE-10; Clay Adams) with a Statham transducer (Gould). In six anesthetized mice (2,2,2-tribromoethanol, 88 mmol/100 g body wt IP; Sigma Chemical Co) of each group, LV pressure and dP/dt at 40 mm Hg were measured with a high-sensitivity pressure transducer (World Precision Instruments) in a closed-chest preparation.

Heart Morphology

In B₂⁺⁺, B₂⁺⁻, and B₂⁻⁻ mice at 40 (n=23, 28, and 20, respectively), 180 (n=10, 16, and 18, respectively), and 360 days of age (n=5, 5, and 7, respectively), hearts were arrested in diastole with cadmium chloride (100 nmol), excised from blood vessels, dissected free, rinsed in saline, blotted, weighed, and fixed in 10% buffered formalin. Lung wet weight was also determined (n=6 each group).

Ventricular Wall and Chamber Diameter Measurements

The free walls of the right ventricle (RV) and the LV, including the septum, were dissected free, and their weights were recorded. Myocardial volume was calculated by dividing its weight by the specific gravity of muscle tissue (ie, 1.06 g/mL). The major cavitary axis of the LV from the apex to the aortic valve was measured under a stereo microscope (Wild M 600) with a calibrated ocular accurate to 0.1 mm. Transverse chamber diameters and LV wall thickness were determined with an analyzer (Image Pro Plus 3.0; accuracy 0.01 mm) with images acquired with a videocamera (Sony) through the stereo microscope (magnification ×16). The cavity volume was computed with use of the Dodge equation.

Analysis of Ventricular Fibrosis

Transverse slices were embedded in paraffin, and 5-μm-thick sections were cut and stained with Masson’s trichrome. Sections were examined at a calibrated magnification of ×100 with an ocular reticle containing 42 sampling points (Wild Heerbrugg Instruments). This reticle defines a sectional area of 0.85 μm². The points overlaying the foci of perivascular or reparative fibrosis were counted separately to compute the volume fraction of myocardial fibrosis.

Myocyte Transverse Diameter and Sarcomere Length

The transverse diameter of longitudinally oriented myocytes of the LV was measured at the level of the nucleus at a magnification of ×1000. One hundred measurements were collected in each LV. At the same magnification, the lengths of ≥10 sarcomeres were evaluated, for a total of 200 measurements.
Reverse-Transcription Polymerase Chain Reaction Analysis

Total RNA was isolated from frozen hearts (n=3 each group) according to the RNAzol B method. cDNA was made from total RNA according to the manufacturer’s instructions (Stratagene). The primers for amplification of a 157-bp product of the α-skeletal actin transcript were α-actin L (5’-GCT CTC TCT CCT CAG GAC C-3’) and α-actin R (5’-GGA GCA AAA CAG AAT GGC TGG C-3’). These primers are specific to the 3’ untranslated region of α-actin RNA. Polymerase chain reaction amplification was performed under the following conditions: denaturing for 1 minute at 98°C, annealing for 1 minute at 61°C, and elongation for 1 minute at 72°C for 35 cycles. The primers used for amplification of a 287-bp product of angiotensin AT1a receptor isoform were AT1a L (GAT AAT TAT GGG CAT TGT GC) and AT1a R (TGC TCA TTT TCG TAG ACA GG). For amplification of a 303-bp product of angiotensin AT1b receptor isoform, the primers were AT1b L (ATT CAG TTT TCT GGA TGT GC) and AT1b R (TCC ACT TCA AAA CAA TAC GC). For both sets of primers, the conditions were denaturation at 98°C, annealing at 56°C, and elongation at 72°C for 30 cycles. RNA levels were normalized through amplification of glyceraldehyde-3-phosphate dehydrogenase.

Biochemical Assays

To determine the myocardial adenylate concentration, hearts (B2+/−, n=6; B2−/−, n=6; and B2+/−, n=9) were washed in saline, frozen, and then stored at −80°C until assay. Pulverized samples were suspended in 60% methanol (in 100 mmol/L Tris · HCl buffer), homogenized, and then centrifuged for 20 minutes at 10 000g. The supernatant was dried, resuspended in 25 μL of ultrapure water, and analyzed with capillary electrophoresis (P/ACE 2100 System; Beckman Instruments). Protein concentration was determined according to Lowry et al.30

Plasma concentration of atrial natriuretic factor and endothelin-1 (ET-1) was determined (n=6 each group) with the use of radioimmunoassays (Phoenix) after extraction of the peptides with Sep-Pack C-18 Cartridges (Waters).

Statistical Analysis

Data are expressed as mean±SEM. Multivariate repeated measures ANOVA was performed to test for interaction between time and grouping factor. In multiple comparisons among independent groups in which ANOVA and F test indicated significant differences, the statistical value was determined according to the Bonferroni method. Differences within and between groups were determined with paired or unpaired Student’s t tests, respectively. A P value of <0.05 was considered statistically significant.

Results

Hemodynamic Measurements

The SBP of B2−/− mice was normal at 50 days of age and gradually increased, reaching a plateau at 6 months (Figure 1A). In B2−/− mice, SBP elevation was delayed and matched the hypertensive levels of B2+/− mice after 6 months of age. Intra-arterial measurements confirmed the elevated BP of B2−/− and B2+/− mice (136±2 and 134±1 versus 115±1 mm Hg in B2+/− at 360 days, P<0.01 for both comparisons). Tachycardia was observed in B2−/− and B2+/− mice since the early phases of life through adulthood (Figure 1B). Initially, BW gain was slightly accelerated in B2−/− mice, but this trend was lost at 7 months (Figure 1C). LV end-diastolic pressure increased in B2−/− mice at 180 days (13±2 versus 6±1 mm Hg in B2+/−, P<0.05) and increased even more at 360 days (25±3 versus 5±1 mm Hg in B2+/−, P<0.01). At 180 days, the dp/dt at 40 mm Hg was similar in B2−/− and B2+/− mice (1725±73 versus 1704±112 mm Hg/s, respectively, P=NS), but at 360 days, it was reduced in B2−/− mice (1201±104 versus 1803±72 mm Hg/s in B2+/−, P<0.05).

Heart Morphology

Cross sections of the hearts of knockout and wild-type mice are shown in Figure 2. Due to a differential growth rate (Table), at 180 days, the LV weight (LVW) of B2−/− mice was 51% and 29% higher than that of B2+/− mice (P<0.01); at 360 days, however, no significant difference was detected among groups. A similar pattern was observed in RV weight (RVW). The early acceleration in LV growth of B2−/− mice was confirmed after normalization by body weight (BW): at 180 days, the LVW/BW ratio was 13% (P<0.01) and 28% (P<0.001) higher than that of B2+/− and B2−/− mice, respectively (Figure 3A). In the second interval, a 2.2-fold increase was seen only in B2+/− mice (P<0.05). Thus, at 360 days, the LV/BW ratio was 9% higher in B2−/− 17% higher in B2+/− than in B2+/− mice. No difference in RVW/BW ratio was observed among groups with maturation (Figure 3B). At 360 days, lung weight was greater in B2−/− mice, and the lung weight to BW ratio was 11% higher in B2−/− mice (P=0.01). The SBP of B2−/− mice after 6 months of age. For both comparisons. Tachycardia was observed in B2−/− and B2+/− mice since the early phases of life through adulthood (Figure 1B). Initially, BW gain was slightly accelerated in B2−/− mice, but this trend was lost at 7 months (Figure 1C). LV end-diastolic pressure increased in B2−/− mice at 180 days (13±2 versus 6±1 mm Hg in B2+/−, P<0.05) and increased even more at 360 days (25±3 versus 5±1 mm Hg in B2+/−, P<0.01). At 180 days, the dp/dt at 40 mm Hg was similar in B2−/− and B2+/− mice (1725±73 versus 1704±112 mm Hg/s, respectively, P=NS), but at 360 days, it was reduced in B2−/− mice (1201±104 versus 1803±72 mm Hg/s in B2+/−, P<0.05).

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A conspicuous increase in LV transverse chamber diameter occurred in B2−/− mice (1.8-fold), equally distributed in the 2 intervals (Figure 4A). This increase was greater (P<0.05) than that of B2+/+ or B2+/− mice (1.4- and 1.5-fold, respectively). A slight elongation in LV chamber length was observed in both B2+/+ and B2−/− mice, with no further change later (Figure 4B). As a result of the changes in these linear dimensions, a 4.0-fold enlargement of LV chamber was found in B2−/− mice compared with 2.6- and 2.3-fold increases in B2+/− and B2+/+ mice, respectively (P<0.001 for both comparisons).

At variance with the compensatory remodeling found in heterozygous mice, in B2−/− mice, the growth in wall thickness and myocardial mass was inadequate to compensate chamber enlargement (Table). In fact, at 360 days, the ratio of mass to chamber volume for knockout mice was 27% (P<0.01) and 24% (P<0.05) lower than that for B2+/+ and B2+/− mice, respectively (Figures 4C and 4D). Diastolic LV wall stress, calculated from hemodynamic and anatomic data, was 114% higher in B2−/− mice than in B2+/+ mice at 180 days (54±9 versus 25±6 dynes/mm²; P<0.001), and at 360 days, it reached 126±15 dynes/mm²; a value 655% higher (P<0.001) than that of B2+/+ mice (17±8 dynes/mm²).

As shown in the Table, at 360 days, the myocytes of B2+/− and B2−/− mice were 17% and 15% thinner than those of B2+/+ mice (P<0.05 for both comparisons). Sarcomere length was similar among groups at all ages examined.

At 40 days, the structure of ventricular myocardium did not reveal pathological alterations in any group. Later, distinct foci of fibrosis were present in the LV, mainly in the subendocardium but also scattered throughout the wall (Figure 5). Compared with B2+/+ mice (Figure 6), B2−/− mice showed an increase in perivascular and reparative fibrosis at 180 days (178% and 930%, respectively, P<0.05) and an even greater increase at 360 days (1013% and 1397%, respectively, P<0.01). No reparative fibrosis was seen in the RV.

Gene Expression in Heart

At 40 days, the mRNA levels of α-skeletal actin were 8.0- and 4.1-fold greater in the hearts of B2−/− and B2+/−, respectively, than B2+/+ mice (Figure 7). This pattern persisted in adult animals. By contrast, the expression of AT1 receptor isoforms was similar among groups at all ages examined (data not shown).

Biochemical Assays

At 360 days, the adenylate content was significantly (P<0.01) reduced in the hearts of B2−/− mice (22.62±1.04 mg/g body wt, P<0.002)
versus 30.22±2.15 and 32.66±1.77 nmol/mg protein in B₂⁺/⁻ and B₂⁺/+⁺, respectively). At the same age, plasma concentrations of ANF were increased in B₂⁻/⁻ mice (222±46 versus 18±11 pg/mL in B₂⁺/⁺, P<0.05), whereas no group difference was detected regarding circulating ET-1 levels (3.34±1.31 versus 4.15±0.87 pg/mL, P=NS).

Discussion

The relevance of kinins in the regulation of BP has been described repeatedly. In particular, the importance of the BK B₂ receptor has been demonstrated in genetically modified murine models. Transgenic mice in which the human B₂ receptor gene has been added to the murine gene are hypotensive, whereas B₂⁻/⁻ mice have mildly elevated BP levels. Furthermore, B₂⁺/⁻ mice, which mimic a condition of partial deficiency, are normotensive until 5 months of age, but with maturation, as demonstrated here, they show a delayed progressive elevation of BP. These data indicate that genetically determined changes in the level of expression of the BK B₂ receptor directly affect the BP of mice during development.

The tachycardia observed in B₂⁻/⁻, similar to that found in rats with prenatal blockade of the BK B₂ receptor, confirms that kinins are involved in the early programming of the cardiovascular phenotype. Because norepinephrine release is diminished in B₂⁻/⁻ mice, the elevated basal HR might be the consequence of sensitization of sinoatrial β-adrenoceptors to catecholamines. Other possible explanations include perturbation in the activity
of intrinsic cardiac cholinergic neurons,\(^{36}\) perturbation of transduction mechanisms linked to \(\beta\)-adrenoceptors, and alteration in the central baroreflex control of HR.\(^{27,38}\)

It appears likely that Ang II contributes to the development of cardiac remodeling in mice with partial or total deficiency of the BK \(B_2\) receptor. The results of our previous studies demonstrated that in \(B_2^{-/-}\) mice, the interrupted receptor signaling leads to blunted basal activation of NO pathway, leaving unbalanced Ang II–induced vasoconstriction.\(^{29}\) However, the elevation in BP was only moderate in \(B_2^{-/-}\) mice, and a load-independent mechanism may be operative.\(^{39}\) The observation that myocardial hypertrophy was present in heterozygous mice at 180 days, when BP was just starting to diverge from normal, is consistent with this hypothesis. In addition, reactivation of fetal \(\alpha\)-skeletal actin expression (an immediate-early gene response reportedly induced by Ang II independent of its vasoconstrictor action)\(^{40,41}\) in the heart of \(B_2^{-/-}\) mice precedes any increase in BP. The counterregulatory influence of kinins on Ang II–induced myocardial growth has been documented in animal models with renin-dependent hypertension\(^{42,43}\) and in vitro preparations of cardiomyocytes cocultured with endothelial cells.\(^{44}\) Consistently, we found that early, lifelong administration of an \(AT_1\) receptor antagonist prevents myocardial hypertrophy and fibrosis in \(B_2^{-/-}\) mice while only temporarily blunting the tendency to develop hypertension (Paolo Madeddu, unpublished results, 1999). Interestingly, plasma renin activity and cardiac expression of the renin gene are not altered in \(B_2^{-/-}\) mice at 6 months,\(^{27}\) and as demonstrated here, \(AT_1\) receptor expression is also unaffected by the mutation even at later stages of the life. ET-1 is well known to play a role in myocyte growth,\(^{45}\) and the formation of ET-1 can be inhibited with BK via the \(B_2\) receptor.\(^{46}\) However, we found that circulating levels of ET-1 are normal in \(B_2^{-/-}\) mice.

The development of ventricular dysfunction and failure in this knockout model is documented by anatomic, functional, and biochemical data. Combined, these abnormalities simulate the characteristic findings of decompensated cardiac hypertrophy seen in humans.\(^{47}\) In accordance with Laplace's law, the hearts of \(B_2^{-/-}\) mice are exposed to a substantial increase in the diastolic circumferential wall stress. It should be pointed out, however, that computed data may underestimate the stress on a per-cell basis due to the amount of myocyte replaced by reparative fibrosis. The distribution of myocardial fibrosis mainly in the subendocardium, the layer most exposed to the increased intraventricular diastolic pressure, again seems to confirm the importance of mechanical factors in the development of heart failure in this model. The decrease in myocardial adenylate content suggests an intrinsic alteration in myocardial energy reserves in the failing hearts of \(B_2^{-/-}\) mice. A shortage of myocardial energy reserves might have exaggerated the consequences of the pressure overload on the heart, leading to an abnormal growth response and accelerated myocyte death. A gene-dose effect influenced the severity of myocardial changes in mutant animals. Heterozygosity, in fact, was characterized by proportional increases in chamber volume and myocardial mass without cardiac dysfunction.

In conclusion, our results indicate that a partial deficiency of the BK \(B_2\) receptor causes LV hypertrophy and that total deficiency induces decompensated hypertrophy and failure similar to the hypertrophic hypertensive cardiomyopathy seen in humans, demonstrating the essential role of kinins in the preservation of myocardial structure and function.

**Note Added in Proof**

After the acceptance of this article, on the occasion of the 53rd Annual Fall Conference of the Council for High Blood Pressure Research, Pierra Meneton et al showed that disruption of tissue kallikrein gene triggers dilated cardiomyopathy in mice (Meneton P, Block-Favre M, Hagege A, Gasc JM, Huang W, Neubauer M, Duffy J, Menard J, Alhenc-Gelas F. Targeted disruption of the tissue kallikrein gene triggers cardiac abnormalities typical of a dilated cardiomyopathy. *Hypertension*. 1999;34:333. Abstract.) These data reinforce the view that the kallikrein kinin system is important for normal heart maturation.
Acknowledgments

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