Cardiac Hypertrophy and Microvascular Deficit in Kinin B2 Receptor Knockout Mice

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Abstract—Experimental and clinical evidence suggests kinin involvement in adaptive myocardial growth. Kinins are growth-inhibitory to cardiomyocytes. Knockout of kinin B2 receptor (B2R) signaling causes dilated and failing cardiomyopathy in 129/J mice, and a 9-bp deletion polymorphism of human B2R is associated with reduced receptor expression and exaggerated left ventricular growth response to physical stress. We reasoned that genetic background and aging may significantly influence the impact of B2R mutation on cardiac phenotype. The theory was challenged in C57BL/6 mice, a strain that naturally differs from the 129/J strain, carrying 1 instead of 2 renin genes. C57BL/6 B2R knockouts (B2R-KO) showed higher blood pressure and heart rate levels ($P<0.05$) compared with wild-type controls (WT) at all ages examined. At 12 months, left ventricular contractility and diastolic function were mildly altered ($P<0.05$) and histological and morphological analyses revealed ventricular hypertrophy and cardiomyocyte enlargement in B2R-KO ($P<0.01$). Reparative fibrosis was enhanced by 208% and capillary density reduced by 38% ($P<0.01$). Functional and structural alterations induced by B2R deletion in C57BL/6 mice were less severe than those reported previously in the 129/J strain. We conclude that interaction of B2R signaling with other genetic determinants influences aging-related changes in myocardial structure and function. These findings may help us understand the role of kinins in the development of cardiac failure. **(*Hypertension*. 2003;41:1151-1155.)**

Key Words: bradykinin ■ hypertension, essential ■ genes ■ heart failure ■ cardiac function

Left ventricular hypertrophy and failure constitute major risks of cardiovascular complications and sudden death among hypertensive subjects.[1] Dissection of the genetic diversities that favor pathological ventricular remodeling might help to prevent cardiac decompensation. Hence, targeted mutation of mouse genome is regarded as a powerful means for addressing the role of single genetic determinants in the progression toward cardiac insufficiency. Unfortunately, however, there have been reports of major physiological differences among wild-type strains that may disturb interpretation.[2] Furthermore, characterization of mutation in relatively young animals has obvious limitations when dealing with chronic diseases that progressively develop with aging.[3] These explanations may account for the discrepant phenotypes deriving from mutation of the kallikrein-kinin system (KKS). Kinins are generated from enzymatic cleavage of kininogen by kallikreins and exert their biological effects through activation of G protein–coupled B_1 (B1R) and B_2 (B2R) receptors.[4] Transgenic rats overexpressing tissue kallikrein (TK) develop less cardiac hypertrophy and fibrosis than do wild-type rats.[5] In addition, evidence provided by ourselves[3] and Meneton et al[6] indicates that genetically ablating B2R or TK in 129/J mice results in dilated decompensated cardiomyopathy. This is in keeping with the recent discovery that deletion polymorphism of human B2R gene is associated with an exaggerated cardiac growth response to physical training in healthy volunteers.[7,8] However, a recent report from Meneton's group[9] seems to negate the importance of KKS. In fact, at variance with previously published studies, including their own,[3,6] the authors documented that 3- to 5-month-old female TK knockouts (TK-KO) or B2R-KO, backcrossed on a C57BL/6 genetic background, do not display any obvious evidence of cardiovascular abnormality.[9]

It should be noted that wild-type 129/J and C57BL/6 mice differ from each other in that the former strain has a 2-renin gene, 10-fold higher plasma renin activity, and 100-fold higher plasma renin concentration.[10] Thus, the severe cardiomyopathy observed in 129/J with targeted deletion of B2R or TK gene may be reconciled with the unbalanced damaging action of an extraordinarily activated renin-angiotensin system (RAS).[3,6] This is in keeping with our observation that angiotensin II (Ang II) type 1 receptor blockade prevents cardiac remodeling in 129/J B2R-KO.[11] We reasoned that disruption of the B2R gene in the 1-renin gene C57BL/6 mice

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might lead to cardiomyopathy with aging, but at stages later than those addressed by Meneton et al[9] and in a milder form than in 2-renin gene mice.

To challenge these theories, the hemodynamic and structural characteristics of C57BL/6 B2R-KO and respective wild-type controls were followed until 1 year of age. Studies were conducted only in male mice, because in females confounding noise may derive from the influence of estrogen on cardiomyocyte growth and survival as well as on the expression of KKS components.[12,13] As a complementary aim, we evaluated whether the absence of B2R signaling may alter coronary microcirculation, eventually resulting in premature cardiomyocyte death and myocardial fibrosis.

Methods

All the experimental procedures were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health publication No. 93-23, revised 1985). B2R-KO, twice backcrossed to C57BL/6, and wild-type controls of the same strain (WT) were obtained from the Jackson Laboratory (Bar Harbor, Maine). Backcrossing to C57BL/6 was repeated another 4 times in our facilities and the levels of plasma renin concentration (under basal and high salt diet) were considered as a marker for inheritance of 1-copy renin gene. In fact, in preliminary experiments, we found that animals in which Ren1c homozygosity was documented by genotyping have significantly lower plasma renin concentration than do mice carrying the 2-renin gene. The animals were housed at a constant room temperature (24±1°C) and humidity (60±3%).

Hemodynamic Measurements

Body weight (BW) was recorded throughout the experimental period. Systolic blood pressure (BP) and heart rate (HR) of B2R-KO and WT mice (n=13 in each group) were monitored from 6 to 12 months of age with the use of tail-cuff plethysmography.[14] At time of death, mean BP (MBP) of the mice, instrumented 1 day in advance with an intra-arterial PE-10 catheter (Clay Adams), was measured by the use of a Statham transducer (Gould). Then, animals were anesthetized with 2,2,2-tribroethanol (88 mmol/100 g BW, IP) for closed-chest assessment of LV end-diastolic pressure (LVEDP) and dP/dt, by the use of a high-sensitivity pressure transducer (World Precision Instruments), as reported previously.[3]

Heart Morphology

After the collection of the hemodynamic measurements, the abdomen was opened and the aorta was cannulated with a PE-50 catheter connected to a perfusion apparatus.[15] In rapid succession, the heart was arrested in diastole by intravenous injection of 1 mL of cadmium chloride (100 nmol), the chest was opened, the right atrium was cut, and the coronary vasculature was shortly perfused with heparinized solution at a pressure equal to the mean arterial pressure measured in vivo. After a 15-minute perfusion at the same pressure with a solution containing 4% paraformaldehyde and 2.5% glutaraldehyde, the heart was removed by surgical excision of the major thoracic vessels.

Anatomical Measurements

The free walls of the right (RV) and left ventricle (LV), including the septum, were dissected free, and their weights were separately recorded. Myocardial volume was calculated by dividing its weight by the specific gravity of muscle tissue (ie, 1.06 g/mL).[15] The major cavity axis of the LV, from the apex to the aortic valve, was measured under a stereo microscope (Wild M600) with a calibration accurate to 0.1 mm. Transverse chamber diameter and LV wall thickness were determined with a stereo microscope (Zeiss, magnification X16) connected to a videocamera (Sony). The acquired images were processed by means of a software analyser (Image Pro Plus 4.0; accuracy 0.01 mm). The cavity volume was computed with the use of the Dodge equation.[16]

Determination of Myocardial Fibrosis

Transverse slices of the LV were embedded in paraffin and 5-μm-thick sections were stained with Masson's trichrome. Sections were microscopically examined at a calibrated magnification of ×200 with an ocular reticle containing 42 sampling points (Wild Heerbrugg Instruments). This reticle defines a sectional area of 0.2 mm^2. The points overlying the areas of collagen accumulation were counted separately to compute the volume fraction of myocardial fibrosis. The entire section of the LV including the septum of each heart was evaluated for this parameter.

Myocyte Transverse Diameter and Sarcomere Length

The transverse diameter of myocytes of the LV was measured at a magnification of ×1000, in longitudinally oriented cells at the level of the nucleus, when the bipolar distribution of mitochondria was apparent. For this measurement, 100 myocytes were collected for each LV. At the same magnification, the lengths of ≥10 sarcomeres of individual myocytes were evaluated, yielding a total of 200 measurements of this parameter for each LV.

Analysis of Capillary Density

The analysis of capillary density was performed in sections stained with silver methenamine, which defines basal membranes. Morphometric sampling at ×1000 magnification consisted of counting the number of capillary profiles in a measured area of tissue sections of both the epimyocardium and endomyocardium in which myocytes are transversally oriented. A square uncompressed tissue area of 9.8 μm^2 was delineated in the microscopic field by an ocular reticle containing 42 sampling points (Wild Heerbrugg Instruments). By counting the fraction of points lying over myocytes, the percentage of the area occupied by muscle cells was determined, and the number of capillaries per unit area of myocytes was computed. This approach was followed to eliminate the effects of variations caused by changes in the interstitial compartment. Sampling of capillary measurements involved a minimum of 20 and a maximum of 30 microscopic fields for each LV of each animal.

Statistical Analysis

Data are expressed as mean±SEM. Statistical evaluation was performed using multivariate analysis of variance (MANOVA), with the Bonferroni adjustment in case of repeated measurements. Correlation between hemodynamic and structural variables was tested by linear regression analysis. A value of $P<0.05$ was considered statistically significant. SPSS software (version 10.0 for Microsoft Windows) was used to run the above statistical tests.

Results

BW increased at the same rate in B2R-KO and WT (data not shown). Analysis of pressure profile from 6 to 12 months of age revealed slightly increased tail-cuff BP levels in B2R-KO (118±3 mm Hg) compared with WT (100±3 mm Hg, $P<0.02$). The difference was confirmed by intra-arterial measurements, with B2R-KO showing higher MBP (116±4 mm Hg) than WT (101±4 mm Hg, $P<0.05$). HR was elevated in B2R-KO at 8 (621±18 versus 570±26 beats/min in WT, $P<0.01$) and 12 months of age (615±19 versus 571±28 beats/min in WT, $P<0.05$).

Hemodynamic measurements obtained at sacrifice did not show evidence of cardiac decompensation. In details, LVEDP was not significantly altered in B2R-KO (data not shown), but a moderate impairment of systolic and diastolic performance was denoted by a decrease in $dP/dt_{40\ mm\ Hg}$ (1120±73

Cardiac Parameters in Wild-Type and B2R Knockout Mice

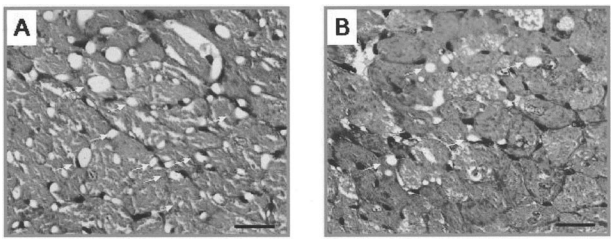
Parameter	Wild Type	B2R-KO	*P*
Heart weight, mg	127.94±4.13	158.31±6.26	0.001
LV weight, mg	105.91±3.79	127.83±4.82	0.002
RV weight, mg	22.02±0.43	30.48±2.21	0.001
Heart weight/body weight, mg/g	3.98±0.10	5.09±0.29	0.002
LV weight/body weight, mg/g	3.29±0.09	4.09±0.20	0.002
RV weight/body weight, mg/g	0.69±0.02	0.99±0.09	0.006
LV diameter, mm	3.87±0.21	3.14±0.14	0.009
LV thickness, mm	1.03±0.04	1.35±0.02	0.001
RV thickness, mm	0.44±0.02	0.53±0.03	0.017
Chamber volume, mm^3	52.83±6.42	39.16±1.52	0.51
LV thickness/chamber radius	0.02±0.01	0.03±0.01	0.0203
Myocyte transverse diameter, μm	12.26±0.37	15.47±0.29	0.0001
Myocytes in the myocardium, %	76.22±1.51	83.59±1.09	0.0032
Reparative fibrosis, %	0.07±0.02	0.19±0.04	0.028
Capillary density			
Number/mm^2 sectional area	4686±186	3235±68	0.001
Number/mm^2 myocytes	6251±317	3886±127	0.001

Values are mean±SEM.

versus 1495±96 mm Hg in WT, $P<0.02$) and dP/dt_{min} (−1480±377 versus −1740±221 mm Hg in WT, $P<0.05$), respectively.

At 1 year, B2R-KO showed increased LV and RV weight (Table, $P<0.01$ for both comparisons). The increase was confirmed after normalization by BW. The gravimetrical result was associated with morphometric evidence of LV ($P<0.005$) and RV wall thickening ($P<0.02$) and augmented LV wall thickness-to-chamber radius ratio ($P<0.05$). Thus, B2R-KO hearts showed distinct hemodynamic and structural features typical of compensated concentric hypertrophy. Microscopically, this result was confirmed by the observation of a 26% increase in transverse myocyte diameter ($P<0.0005$). Furthermore, the myocardium of B2R-KO contained small foci of reparative fibrosis corresponding to 0.19±0.04% of cross-sectional area. Collagen accumulation was 208% greater compared with the figure observed in WT hearts ($P<0.05$).

A representative picture of the microvascular deficit typical of B2R-KO hearts is provided in the Figure. Quantitative evaluation of the coronary microvasculature demonstrated a 31% decrease in the frequency of capillary profiles per unit area of whole tissue ($P<0.005$ versus WT). Capillary rarefaction was even more pronounced (38%) after normalization by myocyte density ($P<0.005$ versus WT).



Photomicrograph showing transversally oriented portion of LV myocardium of B2R-KO (B) and wild-type controls (A) at 12 months of age. Microvascular rarefaction in the myocardium of B2R-KO is evident. Capillaries are stained in black by silver methenamine. Magnification ×400; bar represents 30 μm.

Analysis of possible association between hemodynamic and structural parameters displayed a positive correlation of MBP with LV or RV weights in B2R-KO ($R=0.64$ and 0.69, respectively, $P<0.005$ for both correlations), but not with capillary density. On the other hand, the latter parameter was inversely correlated with LV ($R=-0.71$, $P<0.005$) and RV weight ($R=-0.55$, $P<0.05$). The result was confirmed after normalization of capillary density by myocyte density.

Discussion

Wild-type mice are polymorphic for the number of renin genes, with some inbred strains harboring 1 gene (Ren-1[c]) and other strains containing 2 genes (Ren-1[d] and Ren-2). Mice with the 2-renin gene, such as the 129/J strain, show extremely elevated plasma renin activity, higher basal BP levels that are Ang II–dependent, and increased BP sensitivity to salt and mineralocorticoids compared with 1-renin gene strains.[3,10] The present study indicates that the above differences are mirrored by significant cardiac effects. A comparative overview at 1 year of age shows increased heart weight, LV wall thickening, and enlarged myocyte diameter in wild-type 129/J[3] with respect to the 1-renin gene C57BL/6 studied here. Interestingly enough, the hearts of 129/J display a 3-fold increase in reparative fibrosis compared with C57BL/6, the exaggerated collagen deposition of the former strain being prevented by life-long treatment with an Ang II AT1 blocker.[11] Thus, the activity of endogenous RAS significantly influences myocardial growth and composition during normal aging.

RAS and KKS may exert counterbalancing actions on cardiac growth. Potentiation of KKS reportedly blocks cardiomyocyte hypertrophy promoted by exaggerated levels of Ang II.[17,18] Recent evidence indicates that increased cyclic GMP is essential for the antihypertrophic action of bradykinin in isolated rat hearts exposed to Ang II.[19] Conversely, deletion of B2R gene makes 2-renin gene 129/J uniquely prone to the development of decompensated cardiomyopathy with aging.[3] Even more precocious is the occurrence of ventricular dysfunction in 129/J lacking the TK gene.[6] Recent linkage analyses showed that a deletion polymorphism of the human B2R is associated with exaggerated LV growth response to physical training in young male volunteers.[7,8] The excessive adaptive response was additively enhanced in subjects carrying deletion variants of B2R and ACE genes, a combination that results in high levels of ACE responsible for accelerated Ang II formation and kinin degradation and low levels of kinin receptor.[7,8,20–23] However, in vitro studies on human umbilical veins have questioned the causative role of exon 1 B2R polymorphism, rather suggesting that the variant is a genetic marker in linkage disequilibrium with functional variants of neighboring genes, namely, the kinin B1R gene.[24] Interestingly, B2R-KO reportedly display up-regulated expression of the B1R at cardiac and renal level.[25] Thus, experimental and clinical studies suggest that cardiac remodeling associated with B2R mutation is augmented in the presence of a permissive genetic background and possibly modulated by counterregulatory activation of inducible B1R.

To gain additional insight into the role of genetic background, we have addressed the impact of B2R deletion on the cardiac phenotype of the 1-renin gene C57BL/6 strain. We found that 1-year-old B2R-KO display concentric hypertrophy associated with moderate LV dysfunction. Thus, functional and structural alterations induced by B2R deletion in 1-renin gene mice are less severe than those reported previously in the 2-renin gene strain.[3] Although genetic defect is likely to be directly responsible for the exaggerated cellular growth, we cannot exclude the possibility that reactive hypertrophy may represent an adaptive response to augmented hemodynamic load (as supported by the positive correlation with MBP levels) or to cell loss–mediated myocardial fibrosis.

Our data appear to complement, rather than contradict, a recent report from Meneton's group showing a normal cardiac phenotype in 3- to 5-month-old female C57BL/6 B2R-KO.[9] It seems plausible that the a longer period of time is necessary for the mutation to result in significant cardiac effects. In addition, the protective influence of female sex hormones might have prevented ventricular remodeling in animals with targeted mutation of KKS. Estrogen receptors are present and transcriptionally active in myocardium and in cardiac fibroblasts.[26] Furthermore, estrogen exerts direct antihypertrophic and antiapoptotic action on cardiomyocytes, modulates the antigrowth effects of converting enzyme inhibitors in genetic hypertension,[12,27] and differentially influences the expression of KKS.[28] Therefore, gender should be considered as an additional determinant capable of modulating the impact of KKS mutation on cardiac phenotype.

Recent studies have documented the importance of endogenous KKS in microvascular adaptive response to vascular occlusion.[29–32] A new finding of the present study consists of decreased capillary density in the hearts of B2R-KO. Interestingly, correlative analysis indicates that capillary density was increasingly reduced in parallel with increase of ventricular weight, but it did not correlate with MBP. Thus, the most hypertrophic hearts, independently of the load they were exposed to, were the ones more prone to the consequences of hypoperfusion. Microvascular rarefaction may compromise myocyte oxygenation, a deficit aggravated by the increased diffusion distance for O_2 characteristic of the hypertrophic heart, and thereby lead to progressive myocyte loss and reparative fibrosis. Collagen deposition was consistently augmented in B2R-KO, although less than previously observed in mice of the 129/J strain.[3] Such encasing foci of fibrosis may functionally segregate the viable cardiac myocytes from the coronary circulation, leading to regional perfusion imbalance typical of the progression phase toward myocardial insufficiency.[33] It should be noted that coronary reserve, as measured by double fluorescent microsphere methodology, is reportedly normal in the KKS mutants studied by Meneton's group.[9] Although assessment of maximal dilatation capacity provides functional estimation of coronary circulation, the method does not match histology in dissecting the structural profile of myocardial microvasculature. Eventually, differences in age and gender might explain the discrepancies between Meneton's studies and ours regarding the coronary microcirculation of B2R-KO.

In conclusion, our study indicates that genetic deletion of B2R favors pathological ventricular remodeling and myocardial capillary rarefaction with the permissive contribution of RAS, male gender, and senescence.

Perspectives

These discoveries may have significant implications for the diagnosis, follow-up, and treatment of patients with LV hypertrophy. In particular, evaluation of aggregated determinants, including the genes that encode for KKS, could be useful in screening patients at risk for cardiac failure or coronary events.

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