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Circ Res. 2008;103:1359-1362; originally published online November 6, 2008; doi: 10.1161/CIRCRESAHA.108.186577

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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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Bmx nonreceptor tyrosine kinase has an established role in endothelial and lymphocyte signaling; however, its role in the heart is unknown. To determine whether Bmx participates in cardiac growth, we subjected mice deficient in the molecule (Bmx knockout mice) to transverse aortic constriction (TAC). In comparison with wild-type mice, which progressively developed massive hypertrophy following TAC, Bmx knockout mice were resistant to TAC-induced cardiac growth at the organ and cell level. Loss of Bmx preserved cardiac ejection fraction and decreased mortality following TAC. These findings are the first to demonstrate a necessary role for the Tec family of tyrosine kinases in the heart and reveal a novel regulator (Bmx) of pressure overload--induced hypertrophic growth.

Bmx (also known as Etk) is a member of the Tec family of nonreceptor tyrosine kinases that has a critical role in B-cell development and proliferation.1,2 The Tec family was originally discovered by the observation that mutations in the Btk family member induce X chromosome--linked agammaglobulinemia (XLA),3,4 an immunologic disorder characterized by impaired antibody production and B-lymphocyte development. Although structurally similar to Btk, Bmx does not participate in XLA. Bmx has been shown to regulate wound healing in the epidermis5 and, more recently, the response of skeletal muscle to prolonged ischemia.6

Very little is known about the functional significance of Bmx or other members of the Tec family in the heart. An early study documented expression of Bmx mRNA in the endocardium and vasculature of the adult myocardium,7 but it was only recently that reports suggested that Bmx may be activated by nitric oxide8 or ischemic preconditioning9 in the heart. Whereas nonreceptor tyrosine kinases have been functionally implicated in cardiac phenotype, the role of the Tec family is unknown. In the present study, we demonstrate that absence of Bmx function prevents the hypertrophic response of the myocardium to pressure overload at the anatomic, functional, and cellular level. Our investigation uses genetic tools and physiological analyses to demonstrate a necessary role of this family of tyrosine kinase in cardiac function during stress.

Materials and Methods

The expanded Materials and Methods section in the online supplement, available at http://circres.ahajournals.org, provides a description of the mouse model of transverse aortic banding, the Bmx knockout (KO) mouse line, assessment of cardiac function by echocardiography, histology, gene expression analyses, and Western blotting.

Results

To determine whether Bmx nonreceptor tyrosine kinase is involved in cardiac signaling, we examined basal cardiac phenotype in homozygous Bmx KO mice.5 As monitored by echocardiography, these mice manifest no abnormalities in cardiac function at baseline resting conditions.

Given that Bmx has been implicated in growth and proliferative processes in noncardiac cells, we sought to examine the role of this protein in cardiac growth following pressure overload. Transverse aortic constriction (TAC) surgery was performed on adult male Bmx KO or strain-matched Balb/c wild-type (WT) controls after which mice were allowed to recover for up to 8 weeks with weekly monitoring of cardiac parameters by echocardiography (efficacy of TAC operation was determined by evaluation of carotid pressure gradient; data not shown). In WT mice, TAC caused robust hypertrophy at 8 weeks following the surgery, as evidenced by increased heart weight to body weight (Figure 1A) or heart weight to tibia length (Figure 1, A, in the online data supplement) ratios in TAC as compared to sham WT mice. Gross images also demonstrate changes in cardiac morphology, notably left atrial distention in TAC operated WT mice (Figure 1B). Remarkably, Bmx KO mice were resistant to pressure overload--induced hypertrophic growth even after 8 weeks of stress (Figure 1A and 1B and supplemental Figure 1, A), demonstrating a previously unrecognized role for Bmx signaling in the heart and implicating the molecule as a necessary component of cardiac hypertrophy following stress.

To evaluate the role of Bmx in functional adaptation of the myocardium to pressure overload, echocardiography was used to evaluate cardiac parameters every 5 to 7 days following TAC or sham surgery. Figure 1D shows M-mode images taken 8 weeks following surgery; note the preservation of wall motion in the TAC operated Bmx KO mice as compared with their WT counterparts (see supplemental Table I for additional cardiac parameters). This sustained function translated into preserved ejection fraction and frac-
NOS3-deficient mouse to pressure overload,13 in which the cardiac mass data, are reminiscent of the response of the aspects of the fetal gene program. These features, along with however, Bmx does not appear to be required for critical transcription in the heart10 and various cultured cell types11,12; other Tec family members have been implicated in regulating of the myosin heavy chain (MHC) switch following TAC and the role of Bmx. Ventricular hypertrophy induced by pressure overload or other stimuli can involve a conserved gene program that participates in reverting the myocyte to a more fetal-like state and precipitating pathological remodeling of the myocardium. We examined markers of this program in the Bmx KO myocardium or following TAC are unknown at this time. Because Akt is a known regulator of cardiac growth17 and has other processes to available active Bmx in the basal fraction,14,15 as well as caspase-dependent degradation of the total Bmx protein following TAC. Other investigators have implicated altered association of Bmx with membranous fractions,14,15 as well as caspase-dependent degradation of the protein,16 as means of regulation; the contribution of these and other processes to available active Bmx in the basal myocardium or following TAC are unknown at this time. Because Akt is a known regulator of cardiac growth17 and has been implicated in Bmx signaling in noncardiac cells,12,18 we identified regions of fibrotic deposition, in particular around macrovasculature, following TAC that appeared unaltered by the loss of Bmx (Figure 2D). We also used terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining to examine apoptosis in the hearts of WT and Bmx KO mice and observed no difference in the degree of TUNEL positivity between these groups (data not shown).

To investigate local signaling mechanisms through which Bmx is necessary for pressure overload hypertrophy, we examined known regulators of the protein also implicated in cardiac growth. In WT mice, we observed increased Bmx activation (as detected by phosphorylation on tyrosine 40; supplemental Figure I, C), as well as a trend toward increased total Akt protein and phosphorylation following TAC. Absence of Bmx function did not alter total Akt following TAC (supplemental Figure I, D). Likewise, proximal (protein kinase D) and distal (glycogen synthase kinase

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Figure 1. Loss of Bmx prevents pressure overload–induced hypertrophy: functional and anatomic indices. A, Mice were euthanized 8 weeks after TAC or sham surgery, and heart weight to body weight (HW/BW) ratio was determined. *P=0.004 vs WT sham for HW/BW, #P=NS vs Bmx KO sham. Bars are SEM. B, Images of hearts from mice euthanized 8 weeks after surgery. C, Kaplan–Meier survival curve (starting n values: WT TAC, 23; WT sham, 10; Bmx KO TAC, 9; Bmx KO sham, 7). D, M-mode echocardiographic images from WT and Bmx KO mice. E, Ejection fraction and left ventricular mass data obtained by echocardiography at 8 weeks after sham or TAC surgery. *P<0.01. Bars are SEM (n values for all groups: WT TAC, 6; WT sham, 9; Bmx KO TAC, 9; Bmx KO sham, 7).
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3B) signaling partners of Akt were ostensibly unaffected by the loss of Bmx. Furthermore, the behavior of Akt phosphorylation (at either T308 or S473), as detected by Western blotting, was not significantly changed between the normal and Bmx-deficient hearts (supplemental Figure I, D).

**Discussion**

This study provides the first definitive evidence for a role of the Tec family of tyrosine kinases in cardiac phenotype using genetic tools and physiological analyses. We demonstrate that Bmx is a necessary component of the morphological, functional, and cell level responses of the heart to pressure overload. Whether Bmx plays a role in other forms of cardiac hypertrophy, such as that induced by neural/hormonal stimuli, will be examined in future studies. A recent study showed that Bmx activity is necessary for acute and prolonged responses to ischemia in the rodent hindlimb. The short-term recovery required Bmx signaling not in the skeletal muscle but rather in bone marrow–derived cells. Given the increasingly recognized role of noncardiac cells in injury responses in the myocardium, we recognize that some of the affects Bmx exerts in the WT animal may arise from cells other than cardiomyocytes.

As mentioned previously, the Bmx KO phenotype following TAC shares several features with the NOS3 KO, including resistance to hypertrophic growth, preserved cardiac function, and undisturbed fetal gene activation. It is reasonable to hypothesize that Bmx modulates signaling via NOS3 during pressure overload; however, additional details regarding the relationship between these signaling systems will require further experimentation. The Bmx KO mice do not display altered cardiac development, indicating that although Bmx is indispensable in the response of the adult heart to pressure overload, it appears to not be required for normal embryonic cardiac development. These findings lend credence to task-specific roles for different signaling kinase family members and support the function of Bmx as a stress-activated isoform of the Tec family.

**Sources of Funding**

This work was supported by the NIH (to T.M.V. and Y.W.), American Heart Association (to T.M.V. and Y.W.), The Laubisch Endowment (University of California, Los Angeles) (to T.M.V. and Y.W.), and Helsinki University Hospital Funds (to K.A.).

**Disclosures**

None.

**References**


**Key Words:** tyrosine kinase ※ cardiac hypertrophy ※ signal transduction
Materials and Methods.

All experiments conform to the NIH Guide for the Care and Use of Laboratory Animals and were approved by the UCLA Animal Research Committee.

Antibodies, chemicals and reagents. Primary antibodies: GSK 3β #9315, phospho-GSK 3β (S9) #9336, AKT #9272, phospho-AKT (S473) #4058, phospho-AKT (T308) #9275, PDK1 #3062, phospho-PDK1 (S241) #3061, phospho-Bmx (Y-40) #3211 (from Cell Signaling Technologies); Bmx (BD Biosciences #610792), GAPDH (Abcam) ab9483. Secondary antibodies: Horse Radish Peroxidase (HRP) conjugate donkey anti-goat sc2304, goat anti-mouse sc2055 and chicken anti-rabbit sc 2936 (Santa Cruz Biotechnology). Staining and Immunohistochemistry: Texas red-X-conjugated wheat germ agglutinin was from Invitrogen and Masson’s Trichrome Stain Kit was from Sigma.

Mice. All experiments were performed on adult (8-12 week old) male homozygous (+/−) Bmx knockout mice, generated by replacement of the first coding exon of the Bmx gene with the lac-Z reporter cassette,1 or strain-matched balb/c wild type controls. Bmx knockout mice were back crossed to the balb/c background for at least eight generations prior to this study.
**Transverse aortic constriction surgery.** The murine model of transverse aortic banding-induced cardiac hypertrophy was performed as described previously.\(^2\) Animals were anesthetized with 3.0% isoflurane, intubated, and ventilated with 2.0% isoflurane in 98% O\(_2\) / 2% CO\(_2\). After shaving hair from the animal, the chest was entered from the left side via the third intercostal space, the aorta identified at the T8 region and a venous vascular clamp (Fine Science Tools), outfitted with a band of silastic tubing at the distal edge of one of the clamps, placed around the vessel. The internal diameter of the resulting modified clamp was that of a 27 gauge needle, a common diameter for aortic banding in the mouse; the clamp is of a size that does not impede pulmonary function. Once the aorta was clamped, distal blood flow was measured and quantified using a flow probe (Transonic Instruments). The chest was then closed using 6-0 proline suture, during which negative pressure in the thorax reestablished by removing air with a PE-50 chest tube attached to a syringe. SHAM operated mice underwent the same procedure without placement of aortic clamp.

**Echocardiography (ECHO).** ECHO was used to determine cardiac parameters in live mice as described,\(^2,3\) including the following indices: left ventricular size (end-diastolic and end-systolic dimension), wall thickness (intra-ventricular septum and posterior wall thickness), ventricular mass, ventricular function (ejection fraction), and blood flow. The mouse was sedated with isoflurane vaporized (2.5% for induction, 1.0% for maintenance) in oxygen, its chest shaved and positioned in the left lateral decubitus
position for ultrasonic imaging with Vevo 770 high-resolution ECHO system equipped with a 35 MHz transducer. The short axis view (M-mode) and the long axis view (Doppler) measurements were performed and data stored for off-line analysis. LV chamber dimensions, VST, PWT, and LV mass index (the ratio of LV mass to body weight) were obtained from M-mode images; LV systolic function is also assessed from these measurements by calculating EF. The peak velocity of early diastolic filling (E), the peak velocity of late filling associated with atrial contraction (A wave) and the E/A ratio were measured from the Doppler tracings to assess the LV diastolic function. All mice underwent ECHO analyses once before TAC or SHAM surgery, once a day after and then once every 5 days for the duration of the study. The ECHO data reported in the paper are only from mice that completed the study to eight weeks.

*Western immunoblotting.* Ventricular proteins were isolated in buffer containing: (in mmol/L) 150 NaCl, 20 Trs-HCl, 1 EDTA, 1 EGTA, 1 Na₃VO₄, 1 NaF, 1 PMSF phosphatase inhibitors, 1% Triton X-100 (Sigma) and protease inhibitor cocktail (Roche Diagnostics). After Bradford protein assay, proteins were separated by standard SDS-PAGE, transferred to nitrocellulose and blotted with corresponding antibodies. Protein loading was confirmed by Ponceau staining of membranes and GAPDH immunoblotting.⁴

*Histological Assessment.* Whole hearts were rapidly excised from animals, hung and perfused with 0.1M potassium (K⁺) solution, subsequently fixed using 4%
paraformaldehyde and then embedded in paraffin. Hearts were then sectioned at 4μm and placed on slides. *Wheat Germ Agglutinin*: Heart sections were incubated with Texas red-X-conjugated wheat germ agglutinin (1:100) for 90 minutes and assessed for cell size. ⁵,⁶ *Hematoxylin and Eosin Staining (H/E), Trichrome*: H/E staining and Masson’s Trichrome staining were performed according to the manufacturer’s protocols. All images were visualized using Nikon eclipse TE2000-U microscope and imaging and cell size analysis was performed with the SPOT software (Diagnostic Instruments Inc.).

**RNA Purification and Quantitative Real Time PCR Analysis.** Total RNA was isolated from the left ventricles of the hearts using TRIzol (Invitrogen) according to the manufacturer’s protocol. The total RNA was transcribed using SuperScript First-Strand Synthesis system for RT-PCR (Invitrogen) according to the manufacturer’s protocol to produce cDNA. For SYBR green qRT-PCR, 25μl iQ SYBR Green Supermix (BioRad), 0.5 μl of the specific primers and 2 μl of cDNA was mixed and incubated using the iCycler (BioRad) thermocycler for 40 cycles of denaturing at 95°C, annealing at 60°C and amplification at 72°C for 45 seconds each. Three samples were analyzed for each mouse type and were duplicated to account for pipetting errors. To calculate the fold change of expression the mean of the cycle number for each group was calculated and was normalized against GAPDH by the formula: mean sample – mean GAPDH. The normalized means were then applied to the formula \([2^{(ΔΔCt)}]\) to calculate the fold change.

**Statistical analyses.** All data are expressed ±SEM with p≤0.05 considered to be
statistically significant. Comparisons were made using student’s t-test for unequal variances.

References


Online Table I: Cardiac Parameters Determined by Echocardiography

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<th>Parameter</th>
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<th>4week TAC</th>
<th>8week TAC</th>
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<td>56.32±6.97b</td>
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<td>Bmx KO 57.21±3.63</td>
<td>46.65±4.36b</td>
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*a;p<0.01 compared to WT SHAM
b;p<0.05 compared to WT SHAM
c;p<0.01 compared to WT SHAM
d;p<0.05 compared to WT SHAM

<table>
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Online Figure I. A. Heart weight to tibia length (HW/TL) ratios were determined 8 weeks following TAC or SHAM surgery (* indicates p=0.007 vs. WT SHAM for HW/TL; 
# indicates p=NS vs. Bmx KO SHAM; bars are SEM). B. Fractional shortening indices as determined by ECHO 8 weeks following TAC or SHAM surgery (* indicates p<0.01, bars are SEM; n values for all groups: WT TAC, 6; WT SHAM, 9; Bmx KO TAC, 7; Bmx KO SHAM, 9). C. Expression and phosphorylation of Bmx in WT animals (* indicates p=0.002 and # indicates p=0.01 vs. SHAM). D. Local signaling responses involved in Bmx-dependent cell growth.
Online Figure I