Pathophysiological Defects and Transcriptional Profiling in the RBM20−/− Rat Model

Wei Guo1*, Jonathan M. Pleitner1, Kurt W. Saupe2†, Marion L. Greaser1*†

1 Muscle Biology Laboratory, University of Wisconsin-Madison, Madison, Wisconsin, United States of America, 2 Department of Medicine, University of Wisconsin-Madison, Madison, Wisconsin, United States of America

Abstract

Our recent study indicated that RNA binding motif 20 (Rbm20) alters splicing of titin and other genes. The current goals were to understand how the Rbm20−/− rat is related to physiological, structural, and molecular changes leading to heart failure. We quantitatively and qualitatively compared the expression of titin isoforms between Rbm20−/− and wild type rats by real time RT-PCR and SDS agarose electrophoresis. Isoform changes were linked to alterations in transcription as opposed to translation of titin messages. Reduced time to exhaustion with running in knockout rats also suggested a lower maximal cardiac output or decreased skeletal muscle performance. Electron microscopic observations of the left ventricle from knockout animals showed abnormal myofibril arrangement, Z line streaming, and lipofuscin deposits. Mutant skeletal muscle ultrastructure appeared normal. The results suggest that splicing alternations in Rbm20−/− rats resulted in pathogenic changes in physiology and cardiac ultrastructure. Secondary changes were observed in message levels for many genes whose splicing was not directly affected. Gene and protein expression data indicated the activation of pathophysiological and muscle stress-activated pathways. These data provide new insights on Rbm20 function and how its malfunction leads to cardiomyopathy.

Introduction

Recently our group cloned a novel splicing factor, RNA binding motif 20 (Rbm20), using a natural mutant rat and found that Rbm20 regulates titin isoform transition and the splicing of 30 other genes [1–3]. Rbm20 is one of a series of 48 putative RNA binding proteins that have been identified genomically by the presence of an RNA binding motif. Many of these proteins have been shown to be involved in RNA alternative splicing. The relationship of RBM20 mutations to human dilated cardiomyopathy was first reported by Brauch and coworkers [4]. They found five different mutations in eight families with cardiac enlargement (determined by echocardiography) and reduced ejection fraction. Li and coworkers [5] described an additional 4 mutations in the same gene. All are point mutations and 7 of the 9 are localized in the RS (arginine-serine) domain. Affected individuals often die suddenly in young adulthood (20s to 40s), and they display a varied degree of cardiac fibrosis and arrhythmia. Heart transplantation may be necessary in affected individuals [3–5]. RBM20 mutations may account for as many as 3% of dilated cardiomyopathy patients [6].

Titin is found in highest concentrations in heart and skeletal muscle. The single mammalian gene is expressed in multiple isoforms as a result of alternative splicing [7]. There are two major classes of cardiac titin isoforms: N2B and N2BA [8]. Before birth, mammalian heart mainly expresses the more compliant N2BA isoforms. During the perinatal period the larger N2BA (~3.7 MDa) is expressed almost exclusively throughout life. An intermediate sized N2BA isoform (~ 3.4 MDa) constitutes an increased proportion of the titin in the adult hearts of larger mammals, including humans [12,13]. Interestingly, titin splicing appears to be absent in the Rbm20−/− rat, and a larger titin isoform N2BA (~3.8MDa) is expressed almost exclusively throughout life.

A number of disease states (dilated cardiomyopathy, ischemia, hypertrophic cardiomyopathy) have been linked to
Materials and Methods

Animals and sample preparation

The study was performed with the Rbm20−/− rat [1–3]. Rats used in the current work were crosses of Sprague-Dawley (SD) and Fisher (F) 344 (SDF) [(SDxF344)F1-Rbm20−/−] or SD X F X Brown Norway (BN) (SDFBN) [(SDxF344)F1xBN-Rbm20−/−] (all strains were originally obtained from Harlan Sprague Dawley, Indianapolis, IN). Wild type animals [(SDxF344)F1-Rbm20+/+](SDxF344)F1xBN-Rbm20+/+ with the same genetic backgrounds were used for all comparisons. Animals were maintained on standard rodent chow. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Animal Use and Care Committee of the University of Wisconsin-Madison (protocol M01715). Hearts were removed immediately after euthanasia and the left ventricle, right ventricle, and atria were separated. Samples were obtained from animals ranging in age from 16 days fetal to 500 days after birth. Tissues were snap-frozen in liquid nitrogen and stored in a -80°C freezer prior to protein gel sample and RNA preparation.

Agarose gel electrophoresis

Titin isoforms were resolved using a vertical sodium dodecyl sulfate (SDS)-1% agarose gel electrophoresis (VAGE) system [16]. Protein samples of left ventricle were prepared with urea-thiourea buffer (8M urea, 2M thiourea, 75mM DTT, 3% SDS, 0.05% bromophenol blue, 0.05M Tris, pH 6.8) from wild type, and Rbm20−/− homozygous rats. Density analysis of titin bands was performed using NIH Image to determine isoform ratios. Myosin heavy chain proportions were determined by the method of Warren and Greaser [23]. Coomassie stained gels were analyzed by the method of Mitov and Campbell [24].

Exercise training and measurement of exercise capacity

Eight Rbm20−/− homozygous rats (4 males and 4 females) and 10 wild type rats (6 males and 4 females) with ages of 6 to 7 month were used. Rat exercise capacity determination was adapted from methods used previously [25]. Rats were acclimated to the treadmill by walking at a speed of 16 m/min, 5 min/d, for 2 weeks on a 4-lane Columbus Instruments treadmill. After this acclimatization period, rats in each age group were then assigned to training at a speed of 20 m/min, 10 min/d, for another two weeks. At the end of the 4-week period, maximal exercise capacity was measured twice for each rat in tests separated by 2 days. The protocol for the maximal exercise capacity test consisted of walking at 14m/min for 5 minutes followed by 2 m/min increases in speed every 2 minutes until the rat reached exhaustion. Rats were considered exhausted when they failed to stay off of a shock bar. All the tests were conducted by the same individual.

Light and electron microscopy

Wild type (n=3) and homozygote mutant (n=3) adult rats (1-1.5 years of age) were anesthetized and maintained on isoflurane. Heparin (2000 IU units per kg body weight) was injected intraperitoneally. After 10 minutes, the chest cavity was opened and 3M KCl was injected into the left ventricle to arrest beating. The aorta was cut, and a cannula attached for connection to a Langendorf perfusion apparatus. Blood was washed out by perfusing with 20-30 ml of high potassium Ringers solution (60 mM NaCl – 60 mM KCl – 1.2 mM NaH₂PO₄ - 25 mM HEPES – 11 mM glucose – 1.2 mM MgCl₂, pH 7.2). The heart was subsequently perfused with Karnovsky fixative (2% paraformaldehyde – 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2) (80-90 ml at a flow rate of about 10 ml/min). The left ventricle free wall was cut into 2 X 1 X 1 mm pieces and transferred to fresh fixative for 3 hr at room temperature. After several rinses with buffer, the tissue pieces were fixed for 1 hr in 1% osmium tetroxide in 0.1 M phosphate buffer, rinsed in water, and dehydrated in an ethanol series to
70% ethanol. Samples were stained in block with 5% uranyl acetate in 70% ethanol for 1 hr. They were subsequently transferred to 100% ethanol, then a 50:50 ethanol:propylene oxide mixture, and then into 100% propylene oxide. Samples were transferred into 25% Epon-Araldite resin - 75% propylene oxide for 1 hour, then in 50% resin for 3 hours, in 75% resin for 4 hours, and in 100% resin in a vacuum oven overnight at room temperature. Samples were cured in a vacuum oven for 48 hours at 60°C. Thin sections of 60-70 nm were obtained and stained with lead citrate and uranyl acetate. Digital images were obtained using a Philips CM 120 transmission electron microscope. Sections were obtained from 5 to 10 different blocks for each animal.

Skeletal muscle electron microscopy was conducted using the same animals described above. Small fiber bundles from the tibialis anterior, longissimus dorsi, soleus, and psoas major were tied to wooden dowels and immersed in the same composition paraformaldehyde-glutaraldehyde fixative used for cardiac muscle samples. The remaining preparation methods were identical to those described above. Sections from approximately 10 different regions of each fiber bundle preparation were observed and digitally recorded.

Gene expression analysis

Total RNA was extracted from left ventricle (~50mg) of three wild types and three Rbm20-/- homozygous rats at day 49 with 1ml TRIzol reagent (Invitrogen) separately and further purified with RNeasy columns (Qiagen) according to the manufacturer’s protocols. RNA concentration was measured with a NanoDrop ND 1000 spectrometer (NanoDrop Technologies, Wilmington, DE), and RNA integrity was assessed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Double stranded cDNA was synthesized from total RNA (SuperScript II system; Invitrogen). An in vitro transcription reaction was then performed to obtain biotin-labeled cRNA from the double-stranded cDNA (Enzo BioArray High Yield RNA Transcript Labeling kit; Enzo Diagnostics, Farmingdale, NY). The cRNA was fragmented before hybridization, and then mixed in a hybridization mixture containing probe array controls, BSA, and herring sperm DNA. A cleanup procedure was performed on the hybridization cocktail using an RNeasy spin column (Qiagen), after which it was applied to the Affymetrix Rat 230 2.0 probe array. Three wild type (Wt) and three Rbm20-/- homozygote (Hm) rats (age 49 days) were analyzed with 6 Affymetrix GeneChip Rat Genome 230 2.0 arrays. Hybridization was allowed to continue for 16 h at 45°C in a Hybridization oven, after which the arrays were washed and stained with phycoerythrin-conjugated streptavidin (Molecular Probes, Eugene, OR). Images were scanned using a GeneChip scanner (Agilent Technologies, Palo Alto, CA).

Chip quality and hybridization experiments were assessed by methods previously described [26]. After passing the quality control, GeneChip raw data were subsequently processed by the log scale robust multi-array analysis (RMA) method [27]. After RMA normalization, one way ANOVA was performed to detect expression differences in allele status and a Student t-test with a p value cut-off of 0.01 and a minimum 1.5-fold change between two specified genotype groups was used to identify genes that were significantly regulated between the conditions being compared. The complete data set is publicly available in the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo; accession numbers GSE11137).

Quantitative real time RT-PCR

Total RNA was extracted using TRIzol, following manufacturer instructions. For reverse transcription, 60 ng of RNA were mixed with 5 uM random hexamers, 1 mM each dNTP, 7.5 mM MgCl2, 40 U RNasin (Promega, Madison, WI), 1X PCR buffer II (Applied Biosystems, Foster City, CA) and 250 U of SuperScript II reverse transcriptase (Invitrogen). The reaction mixture was incubated at 25°C for 10 min, 48°C for 45 min, and 95°C for 5 min, then cooled to 4°C. For reverse transcription, two volumes of ethanol were mixed with the cDNA product before transfer to a -20°C freezer for 30 minutes. The tubes were then centrifuged for 15 minutes at 16,000 X g, the pellets washed with 75% ethanol, and the product finally dried. The cDNA was re-suspended in distilled water and used as template for SYBR Green quantitative real-time PCR using primers designed for selected genes (Table S1). All primer pairs produced a single PCR product as determined by the dissociation curve and gel analysis. Real-time PCR was performed in a 20 ul reaction, 96-well format and 1 X SYBR PCR Master Mix (Applied Biosystems). Reaction plates were incubated in an Opticon 2 real-time PCR machine (MJ Research) for 40 cycles consisting of denaturation at 95°C for 15 s and annealing/extension at 58–60°C for 1 min. Three biological repeats at each development stage were analyzed in quadruplicate, with a minimum of two independent experiments. The relative amount of target mRNA normalized to GAPDH was calculated [28].

Western blot analysis

Western blots were performed with rabbit anti-MARPs (kindly supplied by Siegfried Labelt), RBM20 (Home-made), Fhl1 (Santa Cruz), and anti-GAPDH (Santa Cruz). Total protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membrane. Membrane was first blocked in phosphate-buffered saline-0.05% Tween 20 (PBST) with 5% nonfat dry milk overnight and then incubated with primary antibody diluted in blocking buffer for 3 hours at room temperature. After 3 washes with PBST, the membrane was incubated with horseradish peroxidase conjugated secondary antibody in PBST with 5% nonfat dry milk for 2 hours. After another 3 washes, the blot were developed with ECL western blotting substrate (Pierce) and exposed to CL-Xposure film (Thermo Scientific). The same membrane was then stripped and re-probed with anti-GAPDH which serves as a control to show that the total protein loads in different lanes are similar.

Statistical analysis

GraphPad prism software was used for statistical analysis. Results are expressed as means ± SEM or SD. Statistical significance between groups was determined using one way ANOVA with a Tukey post test, or a 2-tailed t-test for
Results

RBM20⁺⁻ rats express more protein and mRNA for the titin N2BA isoform

Titin normally undergoes a series of isoform expression changes with development as a result of alternative splicing (Figure 1A). The titin isoform protein proportions in wild type animals were ~25% N2B at day 1 after birth and ~85% N2B at day 20 and older rats (relative to total titin) respectively (Figure 1C). This developmental and adult expression pattern is disrupted when Rbm20 is absent [1,2]. Ventricle tissue from wild type, heterozygotes, and homozygote knockout rats had different agarose gel phenotypes at each time period after birth [2]. Homozygous knockouts expressed primarily a giant titin (~3.8 MDa) of the N2BA type at all ages examined (Figure 1B) [2,22] and the giant N2BA isoform constitutes almost 100% of total titin (Figure 1D). Quantitative real-time RT-PCR was performed to measure the level of titin isoform mRNAs in normal and knockout ventricular tissue at ages 1, 20, and 49 days (Figure 1E and F). Primer pair Ex49-50 (spanning exons 49 and 50) was designed to quantify total (N2B+N2BA) cardiac titin since these exons are constitutively expressed in both isoform classes [8,9]. Wild type N2B mRNA level (primer pair Ex50-219 spanning exons 50 and 219) comprised approximately 24% of the total titin message at day 1 and 81-84% at days 20 and 49. However, titin N2B-mRNA was nearly undetectable in homozygote knockout rats at all stages examined. Wild type N2BA mRNA proportion (estimated using primers from exon 108) was approximately 81% at day 1 but dropped to 15-17% at the two later time points (Figure 1E). Total N2BA-mRNA is almost equal to total titin-mRNA in homozygous knockout rats at all three ages. These results are consistent with protein proportion of titin isoforms in the different genotypes and ages (Figure 1D).

Quantitative estimates of two splice variants (Ex50-71 and Ex50-91) from the middle Ig region of the N2BA class (N2BA-A1 and N2BA-A2) were also measured (Figure 1F). Both Ex50-71 and Ex50-91 are almost undetectable in wild type hearts at day 1, but the Ex50-71 constituted about 8.5% of total titin message at day 20 and 49. Ex50-91 accounted for about 7.6% at day 20 and 49 in wild type rats. Homozygous knockout rats expressed only traces of the Ex50-71 and the Ex50-91 at all three ages, mirroring the lack of the corresponding intermediate sized titin protein isoforms (Figure 1C and D).

RBM20⁺⁻ rats have reduced maximal exercise capacity

Maximal exercise capacity in wild type and homozygote knockout rats was used to assess the effect of the mutation on in vivo whole animal physiological function. Because the determination of exhaustion in a rat can be somewhat subjective and variable, we verified the reproducibility by conducting the maximal capacity test on each rat in duplicate. The correlation of the two measurements is demonstrated by a large R² value, near unity of slope, and a small intercept. Figure 2A demonstrates not only very high reproducibility, but also clear stratification between the two groups. Maximal exercise capacity defined as time to exhaustion on a standardized treadmill running protocol was highly significantly lower in knockout rats (Figure 2B). The average of the maximal exercise capacity of the wild type is 9.3 minutes (p<0.001) longer than that of the homozygous knockout rats, and this was not due to differences in age, gender or body weight (Table S2 and Figure S1).

RBM20⁺⁻ rat hearts have altered ultrastructure

Titin functions as a template in sarcomere assembly and for maintenance of sarcomere integrity [29,30]. Thus changes in titin size might affect the sarcomeric structure and integrity. Samples from left ventricle of one year old wild type and homozygote knockout rats were examined by electron microscopy. A typical example from a wild type heart is shown in Figure 3A. Myofibrils with well formed sarcomeres are visible, and these are interspersed regularly with mitochondria. Most areas of the homozygote knockout samples had a fairly normal appearance. However, a number of irregularities were observed that were never seen in age matched wild type hearts. These modifications included Z line streaming (ZS) (Figure 3B), lipofuscin granules (LF) and myofibril disarrayfilaments both longitudinal (L) and perpendicular (P) (Figure 3D), and regions of myofibril degeneration (DG) (Figure 3F). In addition a couple novel structural patterns were observed. Figure 3C shows an area with an extremely wide myofibril. Widths (perpendicular to the sarcomere longitudinal axis) exceeded 5 microns in several instances while widths greater than 2 microns were not seen with wild type hearts. Myocytes with these wide myofibril structures were often accompanied by a marked, irregular clustering of mitochondria (M) (Figure 3E). The appearance of the mutant mitochondria, however, was normal with membrane and cristae structure indistinguishable from wild type controls.

Electron microscopic observations of skeletal muscles from homozygote mutants were compared with those from wild type animals. Representative micrographs from tibialis anterior and longissimus dorsi are shown in Figure S2. The myofibril sarcomeres and filament lattice of the mutants were indistinguishable from wild type. Similarly there was no evidence of the mitochondrial clustering, wide sarcomeres, or degenerating regions seen in the mutant skeletal muscle samples. The only structural abnormalities observed in the mutants were a couple occurrences of Z line streaming, but the vast majority of the muscle regions appeared normal.

RBM20⁺⁻ rats have changes in heart failure related genes

We compared gene expression of homozygous knockout rat left ventricles versus wild type at 49 days of age using Affymetrix arrays. The 49 day age was selected as a time point where changes in titin splicing should be completed in wild type and potential secondary changes in the mutants would be limited. A total of 136 gene-identified transcripts were differentially up or down-regulated in knockout rat hearts (http://www.ncbi.nlm.nih.gov/geo/; accession numbers GSE11137) (Table S3) Among them, several muscle stress response and pathophysiological related genes (Table 1) were selected and
Figure 1. Developmental changes in expression of titin protein isoforms and mRNA in wild type (Wt) and homozygote (Hm) mutants. Proteins were separated by SDS agarose electrophoresis [16]. D: days after birth; Std: mixture of human soleus (3.7 MDa) & rat N2B (3.0 MDa); T2, titin fragment; Ex, exon. A. Larger N2BA isoforms (3.6 to 3.7 MDa) were present at one day after birth and disappeared with age in normal rats during development. B. A different larger N2BA isoform (3.8 MDa) appeared in mutant homozygote rats; the smaller N2B and other shorter N2BA isoforms were essentially absent at all ages. C. The smaller N2B isoform increased in proportion with age and reached approximately 85% (P<0.01) from 20 days of age and older in wild type. D. The larger N2BA isoform in homozygous mutants constituted nearly 100% (P<0.05) of the total titin. E. Relative titin mRNA expression in wild type and mutant rats determined by Q-PCR. All data are from an average of triplicates (P<0.05). Primers for exons 49 to 50 would prime both N2B and N2BA titin isoforms; primers for exons 50 and 219 would amplify the N2B only, and primers for exon 108 would amplify the N2BA isoforms only. F. Relative mRNA expression of other pre-identified titin isoforms to total titin in wild type and mutant rats. Total titin of 1 day Wt was defined as 1.0 for all the comparisons.

doi: 10.1371/journal.pone.0084281.g001
verified with quantitative PCR measurements (Figure 4). CARP and DARP were significantly up-regulated at both the RNA and protein levels in knockout rats (Figure 4A and 4C). Arpp levels were not different between genotype groups. Rbm20 was undetectable at both the RNA and protein levels in knockout rats (Figure 4A and 4C), a finding consistent with our recent
Transcriptional level of beta myosin heavy chain increases in 49 day knockout rats detected by microarray (Table 1) and quantitative PCR (Figure 4A). However, there were no significant differences in the myosin heavy chain protein proportions of the different phenotypes at age 49 days or any other age up to > 2.7 years (Figure 4 B and D).

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Gene Symbol</th>
<th>Gene Description</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>64032</td>
<td>Ctgf</td>
<td>connective tissue growth factor</td>
<td>+2.48</td>
</tr>
<tr>
<td>81809</td>
<td>Tgb2</td>
<td>Transforming growth factor, beta 2</td>
<td>+2.33</td>
</tr>
<tr>
<td>445442</td>
<td>Tsp1</td>
<td>thrombospondin 1</td>
<td>+2.05</td>
</tr>
<tr>
<td>27064</td>
<td>Ankrd1</td>
<td>Ankyrin repeat domain 1 (cardiac muscle)(CARP)</td>
<td>+1.60</td>
</tr>
<tr>
<td>316330</td>
<td>Ankrd23</td>
<td>ankyrin repeat domain 23 (DARP)</td>
<td>+3.68</td>
</tr>
<tr>
<td>25177</td>
<td>Fhl1</td>
<td>four and a half LIM domains 1</td>
<td>+2.58</td>
</tr>
<tr>
<td>28557</td>
<td>Myh7</td>
<td>myosin, heavy chain 7, cardiac muscle, beta</td>
<td>+3.92</td>
</tr>
<tr>
<td>24692</td>
<td>Nppa</td>
<td>natriuretic peptide precursor A</td>
<td>+3.10</td>
</tr>
<tr>
<td>309544</td>
<td>Rbm20</td>
<td>RNA binding motif protein 20</td>
<td>-20.36</td>
</tr>
</tbody>
</table>

Wild type values were set at 1.00.

doi: 10.1371/journal.pone.0084281.t001

Figure 4. Expression levels of selected genes. A. Quantitative real time RT-PCR verification of microarray results for selected genes; wild type value set at 1.00 for each gene; all data are from an average of triplicates (P<0.05). C. Western blot analysis of MARPs and Rbm20. B and D. Left ventricle beta myosin heavy chain proportion and electrophoresis with various ages in the different phenotypes. D: day; Wt: wild type; Hm: Homozygote mutant.

doi: 10.1371/journal.pone.0084281.g004

Discussion

Titin has been implicated as being responsible for passive tension, and other work has shown that the proportions of different sized titins affect passive tension [12,31,32]. The proportions are also altered with heart disease [14,15,21,33]. Previous studies with knockout cardiomyocytes expressing only the larger titin N2BA isoform showed they had markedly reduced passive tension [2]. Ca++- activated force development

results [3]. Transcriptonal level of beta myosin heavy chain increases in 49 day knockout rats detected by microarray (Table 1) and quantitative PCR (Figure 4A). However, there were no significant differences in the myosin heavy chain protein proportions of the different phenotypes at age 49 days or any other age up to > 2.7 years (Figure 4 B and D).
by skinned trabeculae was also reduced in the homozygote mutants [34]. Such changes might result in inadequate cardiac filling and/or in a smaller ejection fraction. These phenotypes have been observed with echocardiography in one and a half year old Rbm20-/- animals [3]. Therefore, it is reasonable to speculate the Rbm20-/- rats might have a lower maximal cardiac output (i.e. impaired cardiac reserve), and the observation of reduced maximal exercise capacity in the Rbm20-/- rats further supported this idea. Alternatively, maximal exercise capacity is determined by a complex, interacting set of factors including motivation, the ability of the lungs to oxygenate blood, and the ability of the skeletal muscles to extract and utilize oxygen for generation of ATP. Structurally, there appeared to be fewer electron microscopic abnormalities observed in mutant skeletal muscles than in cardiac. Other work from our laboratory has indicated that the protein profiles were essentially identical between wild type and mutant tibialis anterior [35]. Finally, the myosin heavy chain ratios of tibialis anterior and soleus were indistinguishable between wild type and mutants [36]. Thus we believe that the reduced exercise capacity of the mutants is due primarily to changes in the heart rather than in skeletal muscle. In the context of highly motivated otherwise healthy subjects, exercise capacity is often taken as a surrogate for maximal cardiac output. This can reflect inadequate cardiac filling, ejection or chronotropy, but titin’s putative role in the Frank-Starling response [29] and thus the exercise capacity remains to be determined.

Heterozygous and homozygous Rbm20-/- rat hearts had left ventricular dilatation, an increased percentage of sudden death, and increased fibrosis [3]. The electron microscopy results indicated that additional structural changes (Z line streaming, accumulation of lipofuscin granules, and myofibril disarray), and these phenotypes have been associated previously with human cardiac structural pathology [37-42]. Two additional unusual patterns were also observed – wide myofibrils (Figure 3C) and extensive mitochondrial clumping (Figure 3E). The former might be explained by the reduction in transverse forces due to the larger titin since such forces have been proposed as a mechanism for myofibril formation [43,44]. The mitochondrial grouping may be due to increased myofibril lysis within the Rbm20-/- cardiomyocytes by an unknown mechanism. No ultrastructural evidence of changes in mitochondrial arrangement, density, or myofibril damage was observed in skeletal muscles from the homozygote mutants (Figure S2).

The mechanisms that explain these alterations in muscle structure remain elusive. Since so many changes in gene isoform expression occur in the RBM20 mutant rats (the splicing of at least 29 genes are affected in addition to titin [3]), it would be pure speculation to ascribe them only to this latter protein. It is remarkable that the structural changes observed in these mutants have so many similarities to those observed in other species, including humans.

Gene analysis indicated that disproportionality of titin isoform in cardiac muscle is consistent with a transcriptional change (measured by Q-PCR) to alter the isoform expression in the Rbm20-/- rats. Message levels of N2B titin normally increase in the early neonatal period andthose for N2BA decline as a proportion of the total titin message. The levels of message for the middle Ig splice variants (Ex50-71; Ex50-91) were consistent with the low levels of minor N2BA protein isoforms in wild types and virtual absence in the homozygous Rbm20-/-.

The latter also had an essential absence of N2B message at all three ages examined. These results indicated that the Rbm20-/- rats express almost exclusively the largest titin isoform (N2BA-G) [2], suggesting consistency between genotype and phenotype.

Because titin isoform transition of 49 day old rats has been completed either with development in wild type or in the Rbm20-/- rat [2,3], 49 day cardiac tissues were used for gene expression analysis. Transcriptional changes of global genomic expression demonstrated that CTGF, TGF-β, TSP1, Marps, Fhl1, Myh7, and Nppa are over-expressed in the Rbm20-/- heart (Table 1). The first three genes interact in extracellular matrix (ECM) synthesis [45,46]. CTGF is an important mediator of TGF-β signaling in the heart and abnormal expression of this gene has been used as a diagnostic marker for cardiac fibrosis [47]. TSP1 can activate latent TGF-β by stimulation of growth factors such as Ang II and endothelin-1 (ET-1) [48,49]. Both CTGF and TSP1 are up regulated by TGF-β [50]. Furthermore, CTGF and TSP1 can promote the disassembly of focal adhesions and thus seem to be actively involved in tissue remodeling [51,52]. It should be noted that the up-regulation of these fibrosis-related genes is already present at 49 days of age even though the increase in trichrome positive staining material is not evident till rats are at least 3 months of age [3].

MARP5 participate in muscle stress-activated pathways and are up-regulated in both cardiac and skeletal muscles after mechanical or metabolic challenge. Cyclic stretching of cultured cardiomyocytes induced expression of CARP and DARP both in the nucleus and in the sarcomeric I-bands [53], and end-stage failing human DCM hearts showed increased expression levels of MARPs [21,54]. Fhl1 interacts directly with the N2B region of titin to form a novel complex, and it has been proposed to act as a biomechanical sensor to myofibrillar passive tension generated upon stretch [55,56]. A dose-dependent increase in Fhl1 can reduce the titin N2B phosphorylation by interfering with the binding of Erk2 [57]. Therefore, the increase of Fhl1 in Rbm20-/- rats is consistent with markedly reduced passive tension [2]. An increase of natriuretic peptide precursor A (Nppa), a blood pressure-dependent marker [58,59], suggests a supplementary mechanism to respond to (1) inadequate cardiac filling and ejection or (2) the increased cardiomyocyte size [2] in the Rbm20-/- rats. Previous description on the up-regulation of these genes is consistent with the phenotype of the Rbm20-/- rats that causes heart failure and signs of cardiomyopathy. These results suggest that the pathophysiological phenotype of the Rbm20-/- rat results from changes in multiple genes triggered by modulating gene splicing [3]. Interestingly, almost all stress and heart failure related genes in the Rbm20-/-rat are up-regulated (Table 1). This could be due to adaptive response to change in stress signaling [54].

Previous studies showed that both 3,5,3'-triiodo-L-thyronine (T3) and insulin can restore or increase N2B proportion in cultured wild type neonatal cardiomyocytes [54,60]. A
hypothyroidism rat model shows increased expression of a larger titin isoform [20]. Whether the Rbm20 is related to PI3K/AKT/mTOR signaling pathway or insulin signaling pathway remains to be determined. Finally, our previous results showed that there appeared to be no significant effect on the troponin T developmental time course in the Rbm20-/- animals [2]. Although the myosin heavy chain β (Myh7) is up-regulated in mRNA level in knockout rat heart, there were no phenotype differences in protein levels between genotypes (Figure 4B, D). The left ventricle beta myosin heavy chain proportion increases with age in both knockout and wild type.

In conclusion, altered splicing resulting from Rbm20 deficiency in our natural mutant rat model is related to the pathogenesis of cardiac muscle, but the mechanism can involve the regulation and interaction of multiple genes. The model should be invaluable for developing therapeutic treatments for the Rbm20 human phenotype.

Supporting Information

Table S1. Primers for quantitative real-time RT-PCR.

Table S2. Animal phenotype data. Sex and age paired animals were used for the running protocol. F: Female; M: Male; Wt: Wild type; Hm: Homozygote mutant.

Table S3. Left ventricle microarray comparisons of transcripts from wild type and homozygous Rbm20 knockout rats at 49 days of age.

References


Measurement of cardiac function using pressure–volume conductance catheter technique in mice and rats

Pál Pacher1, Takehiro Nagayama2, Partha Mukhopadhyay1, Sándor Bátkai1 & David A Kass2

1Section on Oxidative Stress Tissue Injury, Laboratories of Physiological Studies, National Institutes of Health/NIAAA, 5625 Fishers Lane, MSC-9413, Bethesda, Maryland 20892-9413, USA. 2Division of Cardiology, Johns Hopkins Medical Institutions, Ross Research Building 835, Johns Hopkins University Hospital, 720 Rutland Avenue, Baltimore, Maryland 21205, USA. Correspondence should be addressed to EP (pacher@mail.nih.gov) or D.A.K. (dkass@jhmi.edu).

Published online 14 August 2008; doi:10.1038/nprot.2008.138

Ventricular pressure–volume relationships have become well established as the most rigorous and comprehensive ways to assess intact heart function. Thanks to advances in miniature sensor technology, this approach has been successfully translated to small rodents, allowing for detailed characterization of cardiovascular function in genetically engineered mice, testing effects of pharmacotherapies and studying disease conditions. This method is unique for providing measures of left ventricular (LV) performance that are more specific to the heart and less affected by vascular loading conditions. Here we present descriptions and movies for procedures employing this method (anesthesia, intubation and surgical techniques, calibrations). We also provide examples of hemodynamics measurements obtained from normal mice/rats, and from animals with cardiac hypertrophy/heart failure, and describe values for various useful load-dependent and load-independent indexes of LV function obtained using different types of anesthesia. The completion of the protocol takes 1–4 h (depending on the experimental design/endpoint).

INTRODUCTION

Around 30 years ago, Sagawa and colleagues1 embarked on a systematic and detailed analysis of canine ventricular function using pressure–volume (PV) relationships. Their work led to the appreciation that such relations provided a uniquely powerful approach to quantifying heart function, particularly in vivo1–3. Subsequent studies in large animals4 and humans5 generated PV loops in real time, both under steady-state conditions and during transient reduction of inflow to the heart. This work established the methodology as the most comprehensive yet available for assessing ventricular performance independent from loading conditions, yet simultaneously quantifying load and the interaction of heart and vasculature. The most convenient way to obtain these data was the use of an impedance (or conductance) and pressure-measuring catheter, inserted to lie along the long axis of the ventricle, to provide a real-time volume signal as well as micromanometer pressure signal. This catheter was first used in large animals and humans starting in the mid-1980s. About 15 years later, technical development in miniature sensors made it feasible to apply this approach to very small mammals (Fig. 1a).6 This method provided simultaneous measurement of both pressure and volume signals from the intact beating mouse6–10 and rat11–20 hearts. Despite its invasiveness, this sophisticated methodology has great potential for characterizing cardiac function in various genetically manipulated mouse models of cardiovascular disease, and testing the effects of various drugs under physiological and pathological conditions. Noninvasive methodologies for measuring cardiac function (echo and MRI) are limited by their application to steady-state conditions and reliance on motion parameters that can be influenced by loading conditions and thus lack specificity to the ventricle itself. Their advantage is that they can be repeated in the same animal and provide direct quantification of absolute volumes, whereas the conductance catheter signal is proportional to volume but must be appropriately calibrated to provide accurate absolute volume measurements.

Ventricular pressure measurements have been commonly used for decades, but real-time volume measurements have historically been problematic. A technique by Baan et al.21 made it possible to correlate the change in ventricular volume to a change in electrical resistance of the blood pool within the LV chamber. The conductance catheter has multiple ring electrodes placed along its length (Fig. 1a), and a high-frequency low-amplitude constant current is passed through the outer pair of electrodes to generate a local electric field between these electrodes (E4, E1). The field passes through the blood, muscle wall and surrounding structures, with field strength declining by the square of the distance from the electrodes. Electric theory indicates that if voltage potentials are measured within this field, they will be similar along planes that are perpendicular to the current field lines. The potential difference between two intervening electrodes will be inversely proportional to the amount of conductive material at that site. For the small rodent catheter, this measurement is made between two inner sensing electrodes (E2, E3), providing a time-varying signal. The resistivity of blood is about 1/3 that of the heart muscle, so the signal combines both the blood pool and chamber muscle wall. However, the latter is essentially constant,22 whereas the former varies with the cardiac cycle, so the time-varying component of the conductance signal is due to blood volume changes in the cavity.

As noted earlier, this conductance signal is itself noncalibrated. There is a fairly linear relation between absolute volume and this signal, and the slope (gain) and offset are related to the geometry of the heart and surrounding structures and their conductivity. Some have used external reservoirs filled with conductive material to mimic the heart to obtain a calibration curve (cuvette calibration; see also manufacturer’s instructions). However, the accuracy of this approach may be questionable under all conditions or disease models. More accurate volume calibration can be accomplished using an independent measure of cardiac output (e.g., ultrasound
flow probe) from which stroke volume is derived to calculate the gain of the signal, defined as gain = flow probe stroke volume/conductance stroke volume. The offset is due to the fact that the ventricular cavity is not a perfect insulator, and a portion of the current leaks into the muscle. This offset must be subtracted to obtain absolute volume. It is usually estimated by the hypertonic saline dilution method.8,12,13

The unique advantage of the PV methodology over all other available approaches to measure cardiac function is that it enables more specific measurement of the LV performance independently from loading conditions and of heart rate (for commonly used load-independent indices of systolic (Fes or Emax), dP/dtmax—and diastolic volume; preload-recruitable stroke work (PRSW) and diastolic function (EDPVR)), see Table 1. Emax or Emax defines chamber end-systolic stiffness and can be a useful measure of contractile function, particularly to assess acute changes.22–23,28. Chronic changes in Emax from heart disease can also reflect cardiac morphometry—that is, hypertrophy, fibrosis—and thus is not simply a reflection of ‘contractility’. The dP/dtmax—end-diastolic volume relation also provides a load-independent contractility index, as preload dependence of dP/dtmax is effectively reduced by using this regression. PRSW23 is a similar type of index, plotting stroke work versus end-diastolic volume for the set of load-altered loops.

The following protocol describes the procedures (summarized in Fig. 1b) for this method (anesthesia/analgesia, intubation techniques (Fig. 2), surgical techniques for LV catheterization (open and closed chest approaches; Fig. 3), vena cava inferior occlusion methods (Fig. 4a) and calibrations (Figs. 4b–c and 5) to convert the raw conductance signals to true volumes), provides movies of the key processes/steps performed in our laboratories (Supplementary Movies 1–5 online), presents representative examples of PV loops and various calculated useful hemodynamics indices (Table 1 and Figs. 5–7) and gives troubleshooting advice (see TROUBLESHOOTING).

**Figure 1** | Pressure–volume (PV) catheters and main steps of the protocol. (a) Mouse and rat PV catheters (magnified image) and working principle. (b) Flow chart indicates main procedures and important considerations of the PV protocol.

---

**Experimental design**

**Anesthesia, body temperature control and intubation.** The following injectable or gas anesthetics/analgesics can be used (see also ref. 25): Injectable

- Ketamine (50 mg kg⁻¹)
- Ketamine (50 mg kg⁻¹) + fentanyl 250 μg kg⁻¹
- Ketamine/diazepam (40–80/5–10 mg kg⁻¹)
- Ketamine/xylocaine (80–100/10 mg kg⁻¹)

Inhalants (induction 3–4%, maintenance 1.5% mixed with 100% oxygen)

- Halothane
- Isoflurane
- Methoxyflurane

Analgesia: morphine (1 mg kg⁻¹) or fentanyl (50–250 μg kg⁻¹)

Muscle relaxant: pancuronium (2 mg kg⁻¹)

Euthanasia: pentobarbital
TABLE 1 | Hemodynamic parameters and indices of systolic and diastolic function derived from PV relations in mice and rats.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mouse&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Mouse&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Rat&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Rat&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW (g)</td>
<td>20–30</td>
<td>20–34</td>
<td>20–34</td>
<td>250–500</td>
</tr>
<tr>
<td>HR (min&lt;sup&gt;−1&lt;/sup&gt;)</td>
<td>490–655</td>
<td>470–620</td>
<td>340–510</td>
<td>370–420</td>
</tr>
<tr>
<td>MAP (mm Hg)</td>
<td>81–105</td>
<td>92–118</td>
<td>104–125</td>
<td>72–90</td>
</tr>
<tr>
<td>ESP (mm Hg)</td>
<td>2–8</td>
<td>1–6</td>
<td>1–9</td>
<td>2–8</td>
</tr>
<tr>
<td>ESV (µl)</td>
<td>2–12</td>
<td>7–21</td>
<td>9–20</td>
<td>10–29</td>
</tr>
<tr>
<td>EDV (µl)</td>
<td>20–33</td>
<td>25–53</td>
<td>25–39</td>
<td>28–54</td>
</tr>
<tr>
<td>SV (µl)</td>
<td>14–26</td>
<td>17–30</td>
<td>14–21</td>
<td>17–25</td>
</tr>
<tr>
<td>CO (ml min&lt;sup&gt;−1&lt;/sup&gt;)</td>
<td>7–16</td>
<td>8–16</td>
<td>6–10</td>
<td>6–13</td>
</tr>
<tr>
<td>CI (ml min&lt;sup&gt;−1&lt;/sup&gt; × kg)</td>
<td>280–557</td>
<td>350–580</td>
<td>225–400</td>
<td>114–227</td>
</tr>
<tr>
<td>Ea (mm Hg µl&lt;sup&gt;−1&lt;/sup&gt;)</td>
<td>4–6</td>
<td>3–7</td>
<td>5–9</td>
<td>4–6</td>
</tr>
<tr>
<td>TPR (mm Hg ml&lt;sup&gt;−1&lt;/sup&gt; × min)</td>
<td>—</td>
<td>6–12</td>
<td>10–19</td>
<td>7–14</td>
</tr>
</tbody>
</table>

**Systolic indices**

| EF (%) | 50–88 | 55–72 | 49–63 | 44–62 | 49–89 | 42–87 |
| dP/dt<sub>max</sub> (mm Hg s<sup>−1</sup>) | 9,500–16,000 | 8,200–14,200 | 7,700–14,480 | 6,900–11,000 | 8,900–13,100 | 7,600–11,500 |
| SW (mm Hg × µl) | 1,200–2,700 | 1,500–2,600 | 1,600–2,200 | 1,100–2,100 | 13,500–22,900 | 12,800–21,000 |
| E<sub>a</sub>/E<sub>max</sub> (mm Hg µl<sup>−1</sup>) | 6–14 | 7–14 | — | 6–9 | 2–7 | 2–4 |
| dP/dt<sub>max</sub>–EDV (mm Hg s<sup>−1</sup> ml<sup>−1</sup>) | 360–600 | 180–470 | — | 160–390 | 40–120 | 28–90 |
| PRSW (mm Hg) | 70–130 | 58–93 | — | 51–86 | 50–170 | 50–140 |
| Efficiency (%) | 70–85 | 60–75 | — | 55–68 | 60–90 | 50–80 |

**Diastolic indices**

| −dP/dt<sub>min</sub> (mm Hg s<sup>−1</sup>) | 6,000–12,000 | 6,700–10,500 | 6,900–10,400 | 5,400–9,500 | 5,900–11,900 | 5,970–9,970 |
| Tau (W) (ms) | — | 4.4–7.5 | 4.8–8.5 | 4.9–10 | 7–9.6 | 7–9.9 |
| Tau (G) (ms) | 7–9 | 7–12 | 8–13 | 7–15 | 10–13 | 10–13 |
| EDPVR | 0.04–0.12 | — | 0.06–0.2 | 0.01–0.04 | 0.01–0.04 | 0.01–0.04 |

In obese animals, considerable differences may occur in the distribution of the injectable anesthetics, similar to that in aging animals or in animals with impaired liver function. Animals with various models of heart failure and shock may be more sensitive to the cardiodepressive effects of these agents. Even a slight overdosage, especially with ketamine/etomidate or pentobarbital sodium, may profoundly decrease the heart rate and cardiac function. For most injectable anesthetics, the intubation of the animals will significantly improve the hemodynamic variables (this is especially critical with pentobarbital, which markedly increases mucus secretion in the respiratory tract). With the proper use and careful optimization, it is possible to achieve reasonable results with almost all anesthetic agents, and we will show examples with ketamine/etomidate, pentobarbital sodium, urethane+etomidate+morphine and isoflurane in our protocol (see Table 1). However, because of the ease of overdosing and decreasing heart rate with some of the above-mentioned agents, we recommend using urethane + etomidate + morphine/fentanyl or isoflurane for anesthesia. There are reports describing extreme cardiodepressive effects of various anesthetics (e.g., ketamine/etomidate or pentobarbital), but most likely a significant part of these effects may actually be attributed to the lack of proper temperature control, intubation and overdosing.

Most of the above-mentioned agents require special handling because of the drug regulation laws and possible toxic effects. Urethane is carcinogenic (avoid contact with the skin) and prolonged use in animals may lead to hemolysis, making urine red. Similarly, avoid contact with and inhalation of gas anesthetics (especially methoxyflurane). Use downdraft table to avoid exposure to waste gases.

**Surgical procedures for LV catheterization.** For drug testing and more prolonged experiments, the closed-chest approach (see Step 8A, Fig. 3a and Supplementary Movie 2 online) is more suitable because it is less invasive and animals are more stable for a longer period. An additional advantage of this approach in various animal models (e.g., in heart failure) is that arterial pressure records can easily be obtained from the carotid artery at the start or at the end of the experiment, allowing the calculation of total peripheral resistance (TPR) later (TPR = (mean arterial pressure—mean venous pressure)/cardiac output). The carotid approach should
also be used in a chronic heart failure model induced by ligation of the left anterior coronary artery because of the scar formation in the apex area. Retrograde insertion via the LV apex does have some methodological advantages even for drug testing or other protocols as proper placement of the catheter in the LV is easier to confirm, and the procedure is done very quickly. In addition, if the carotid artery is severely atherosclerotic (e.g., in ApoE mice fed with a high-fat diet), or when the aortic valve is calcified (e.g., in advanced aging models) or in transverse aortic constriction (TAC)-induced hypertrophy and heart failure models, the open chest approach (Step 8B, Fig. 3b and Supplementary Movie 3 online) is appropriate.

Conductance catheter calibration. The conductance signal is itself noncalibrated and must be carefully converted to absolute volumes if such information is required. The primary equation relating conductance to volume is \( V = 1/x \left( \rho L^2 (G - G_0) \right) \), where \( \rho \) is the blood resistivity, \( L \) is the distance between sensing electrodes, \( G \) is the conductance (measured as a voltage and utilizing a constant current circuit, this is directly proportional to \( G \)), \( G_0 \) is the parallel conductance due to conductivity of the muscle wall and surrounding tissues and \( x \) is a gain coefficient (volume correction/calibration factor). The simplistic model of this approach is that the electric field is as if applied from parallel plates, so the current lines are straight and parallel to the catheter shaft. The fact that we use point source electrodes means that the field lines are curved, and this introduces a value of \( x \) that is not unity, and some nonlinearity to the volume signal calibration. This nonlinear behavior is more problematic the larger the heart, and actually, the mouse heart may be the best designed for this technology, given the small distances from the catheter that are involved. In this mammal, the relationship between catheter and Doppler-measured stroke volume, for example, is highly linear over a broad loading range. The small heart and local electric field distribution also have implications for the parallel conductance, which can be much large in larger mammals, as right ventricular (RV) volumes are clearly registered in the signal. In the mouse, \( G_0 \) appears mostly due to the muscle wall, and there is little far field (i.e., RV or other chamber volume) contribution. This was tested and previously demonstrated. With regards to the actual calibration procedure, there are two approaches generally taken. One is to use mock-up cylinders with known volumes, and the catheter is placed in each using fluid matching the conductivity of blood (or blood), and a calibration curve generated. In our experience, this can yield inaccurate values as the cylinders do not exactly replicate the field distribution in the mouse heart, and conductances can vary between different types of hearts (e.g., heart failure models, hypertrophy and infarction). Rather, we prefer the direct approach where both the gain and offset of the calibration are determined. The gain is assessed by measuring the cardiac output by Doppler flow probe. This can be done with a flow-velocity probe that can be placed on the ascending (or descending) aorta (Craig Hartley, Houston company) multiplied by a measure of cross-sectional diameter or using a volume flow probe (Transonic) that provides flow without requiring area assessment. For practical reasons, we prefer the latter and use the descending thoracic aorta as the placement position. Although this clearly excludes some relevant blood flow, the anatomy of the mouse (small upper body) implies that fairly little flow is missed, and this proportion is fairly constant among animals.

Data acquisition, analysis, noise filtering. In our experience, sampling data at 1–2 kHz is optimal; higher rates simply increase the file size. If concomitant electrical analysis is being performed, higher digitization rates would be useful. The default setting of the PVAN in PowerLab (PV analysis program) uses 1 kHz. The data analysis using PVAN module integrated into Chart is straightforward and quick (see the manufacturer’s instructions). More experienced users may also create algorithms in chart to calculate various selected parameters, but as PVAN is included with the Millar PV
system, this is not required. Some users have developed their own custom software for PV analysis. Noise filtering may be a critical issue under some circumstances. In general, we do not recommend using noise filters. In 99% of cases, noise (mostly appearing at volume channel) can be prevented by very simple things (see TROUBLESHOOTING for details). In most of these cases, noise at volume channel actually originates from electric network and replugging the system into stabilized circuit outlet may solve the problem. If the noise problem cannot be solved and the noise pattern is regular, most likely one of the filters built into PowerLab/Chart may improve the signal. In these cases, filtering of volume signal may be acceptable (this will only minimally affect derived parameters). However, do not try to filter the pressure signal because it may profoundly affect derived parameters (e.g., +dP/dt) (see Fig. 7 for representative examples). However, if any filter is applied, it should be used for all conditions.

**MATERIALS**

**REAGENTS**
- Anesthesia/analgesia
  - Urethane (Sigma-Aldrich, cat. no. U2500)
  - ! CAUTION Use gloves during handling.
- Etomidate injection (Bedford Laboratories, cat. no. Div. ETIMP02)
- Morphine sulfate injection (Baxter Healthcare Corp.)
- Nembutal 50 mg ml⁻¹ pentobarbital sodium, USP (Ovation Pharmaceuticals, cat. no. NDC no. 67386-501-55)
- Ketavet 100 mg ml⁻¹ ketamine, USP (Vedco, cat. no. NDC no. 50989-248-06)
- AnaSed 20 mg kg⁻¹ xylazine solution (Lloyd Laboratories, cat. no. NADA no. 139-236)
- Forane isoflurane, USP (Baxter, cat. no. NDC no. 10019-360-40)
- ! CAUTION Do not inhale; use downdraft table during handling.
- Other
  - 0.9% (wt/vol) sodium chloride injection, USP (Hospira, cat. no. NDC no. 0409-4888-50)
  - Surgical tape
  - Alconox (Alconox Inc.) for catheter cleaning

**EQUIPMENT**
- Tabletop anesthesia machine, single (Harvard Apparatus, cat. no. 72-3012)
- Anesthetizing box, large (Harvard Apparatus, cat. no. 50-0116)
- MiniVent ventilator for mice (Harvard Apparatus, cat. no. 73-0043)
- Harvard ventilator model 683 for rats (Harvard Apparatus, cat. no. 35-0000)
- Tracheotomy cannula, 1.5 mm outer diameter (od.) for mice (Harvard Apparatus, cat. no. 73-2730)
- Tracheotomy cannula, 3.0 mm od. for rats (Harvard Apparatus, cat. no. 73-2733)
- Tubing kit (Harvard Apparatus, cat. no. 72-1049)
- Gaymar TPump circulating water blanket (Gaymar Industries, cat. no. TP-400) or Homothermic Blanket (Harvard Apparatus, cat. no. 50714-16)

**Stereo microscope** (Carl Zeiss Optical Inc., cat. no. Stemi 2000)
- Boom stand for microscope (Diagnostic Instruments Inc., cat. no. SMS6B)
- Cole-Parmer Illuminator 50 W (Cole-Parmer, cat. no. 41720)
- Battery-operated Thermal Cautery Unit (Fine Science Tools Inc., cat. no. 18015-00)
- Dumont no. 55 Dumostar Forceps (Fine Science Tools Inc., cat. no. 11295-51)
- Graef forceps, curved (Fine Science Tools Inc., cat. no. 11052-10)
- Moria MC31 forceps (Fine Science Tools Inc., cat. no. 11370-31)
- Mayo scissors (Fine Science Tools Inc., cat. no. 14512-15)
- Iris scissors (Fine Science Tools Inc., cat. no. 14041-10)

**Figure 3** Surgical procedures for LV catheterization. (a) Closed-chest approach: insertion of the catheter into the LV through right carotid artery (see also Supplementary Movie 2 online). Sequentially numbered panels indicate stages of procedure. Image 11 shows mouse PV catheter in the LV on an echo image. (b) Open-chest approach: insertion of the catheter into the LV following stab of the apex with a 25–30 gauge needle through the stab wound (see also Supplementary Movie 3 online). Sequentially numbered panels indicate stages of procedure.
• Halsey needle holder (Fine Science Tools Inc., cat. no. 12501-13)
• Olsen–Hegar needle holder (Fine Science Tools Inc., cat. no. 12002-12)
• Vannas–Tubingen spring scissors, titanium (Fine Science Tools Inc., cat. no. 13610-98)
• Durarobe disposable underpads (Kendall/Tyco Healthcare, cat. no. 1038)
• Gauze sponges, sterile (Dukal, cat. no. 62280)
• Cotton-tipped applicators, sterile (Solon, cat. no. 368)
• Surgical suture, black braided silk 4.0 (Surgical Specialties Corp., cat. no. SP116)
• Monoject 1 cc tuberculin syringes (Sherwood Medical, cat. no. 501400)
• Lo-dose insulin syringe 1/2 cc (Becton Dickinson, cat. no. 329461)
• Millar PV system MPV-300/400 or MPV-3 Ultra (Millar Instruments Inc.). The system includes calibration cuvette for mice and rats; MPV-3 Ultra includes resistivity calibration cuvette.
• For mice: PVR-1045, sensor tip PV catheter, 1F, or SPR-839, 1.4F (Millar Instruments Inc.); FT111B, 1.2F (SciSense)
• For rats: SPR-869, Sensor Tip PV Catheter, 2F small rat; or SPR-838, 2F normal/large rat (Millar Instruments Inc.); FT211B, 1.6F (SciSense)
• PowerLab 4/30 with Chart Pro (AD Instruments Inc., cat. no. ML866/P)
• MiniARCO trimmer (animal clipper; Wahl, cat. no. 8787-450A)
• Intradermic tubing PE-10 (Becton Dickinson, cat. no. 427401)
• Intradermic tubing PE-50 (Becton Dickinson, cat. no. 427411)
• Needle assortment (18, 25 and 30 gauge; Thomas Scientific)
• Various-sized syringes
• Perivascular flow module TS420 (Transonic Systems Inc.)
• 1-mm perivascular probes (Transonic Systems Inc., cat. no. 1PRB)
• Visual Sonic Echo system (optional; Vevo 770 High-Resolution In Vivo Imaging Systems; RMV 707B High Frame Rate Scanhead, Vevo Integrated Rail System I. I, Imaging Kit, Aquasonic ultrasound gel)

Figure 4 | Occlusion techniques, aortic flow measurements and jugular vein injection. (a) Vena cava inferior occlusion techniques. (b) Aortic flow measurements. (c) Jugular vein injection (see also Supplementary Movies 4 and 5 online). Sequentially numbered panels indicate stages of procedure.

PROCEDURE

▲ CRITICAL All animal procedures described or shown in videos were approved by the Institutional Animal Care and Use Committees of NIAAA or Johns Hopkins Medical Institutions and were performed in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Anesthesia, body temperature control and intubation ▼ TIMING 10–30 min
1] Deliver intraperitoneal injection of chosen anesthetics in 0.5 ml of physiological saline solution (we recommend urethane (800 mg kg⁻¹) + etomidate (5–10 mg kg⁻¹) + morphine (2 mg kg⁻¹)) or put animal into the anesthesia chamber containing gas anesthetic (we recommend isoflurane 3–4% for induction). See Experimental design for further information on anesthetics/analgesics.
▲ CRITICAL STEP It is critical to optimize the dose of any anesthetic used for the given mouse/rat strain and the pathological model evaluated (see Experimental design for further details).

2] When the animal is anesthetized (not responding to tail or ear pinch), shave the neck and chest area with a clipper and carefully move the animal to the heating pad with the water temperature set to 40 °C; in the case of a servo-controlled homeothermic blanket, insert the temperature feedback probe into the rectum and set the desired body temperature to 37–37.5 °C.
▲ CRITICAL STEP The temperature control in anesthetized mice and rats is critical (especially in mice). Without proper heating, the heart rate in anesthetized mice can reduce almost to half of the normal rate.

3] Using surgical tape, tape down the front paws and one distal paw of the mouse to the heating blanket (use the remaining distal paw to monitor the depth of anesthesia: movement of the paw can indicate decrease of the depth of the anesthesia). Also, place a thin piece of tape across the tip of the snout, and tape this down to pull the head back slightly to create traction on the trachea. Instead of the tape for the latter procedure, a needle holder can also be used.
▲ CRITICAL STEP Do not obstruct the nostrils because mice are obligate nose breathers.

4] Following midline neck incision, pull the skin away from the underlying muscles and cut it off. Pull the pretracheal muscles apart gently with forceps and dissect the underneath of the trachea to free it from surrounding tissues.
▲ CRITICAL STEP Be careful not to damage carotid arteries and vagus nerves, which run alongside the trachea.

5] Pass a #4 surgical silk suture underneath the trachea, make a small cut onto the surface, insert the tracheotomy cannula (1.3 mm o.d. for mice and 3.0 mm for rats) and tie down with the suture (Fig. 2b; Supplementary Movie 1 online).
Figure 5 | Representative examples of rat and mouse baseline PV loops and occlusions. Examples show representative (a) rat and (b) mouse PV loops before the calibration (in RVUs) and following cuvette and saline calibrations (in microliters) obtained by closed-chest approach with Millar PV system and ana-lyzed by PVAN3.5. Upper rows (a and b), left-hand panels show examples of baseline uncalibrated PV loops (rectangular shape, left side of the panel), volume signal (red trace), pressure signal below volume (blue trace) and ΔP/Δt and ΔP/dt derived from the pressure signal (green trace). Upper rows (a and b) in middle and right show examples of hypertonic saline injections (rightward ship of PV loops without decrease in the amplitude) and derived V̇e values. Lower rows (a and b) show uncalibrated (in RVUs) and calibrated (in microliters) normal rat and mouse baseline PV loops (left two panels) and loops following inferior vena cava occlusions (right two panels). See also Supplementary Movies 2 and 4 online, and Figure 6a.

Alternatively, intubation can also be performed without a tracheal incision as shown in Fig. 2a; Supplementary Movie 1 online, but this procedure requires more experience.

6) Connect the tracheotomy cannula to the respirator in case of gas anesthesia providing mixture of 100% oxygen and 2% isoflurane (later, isoflurane can be decreased to 1–1.5%).

7) Calculate and set the recommended ventilation settings for mice or rats, depending on the animal weight, according the following formulas: tidal volume (Vt, ml) = 6.2 × M−0.62 (M = animal mass, kg); respiration rate (RR, min−1) = 53.5 × M−0.26. For example, in a 30-g mouse, V̇e = 179.6 ul and RR is 133 min−1. In a 400-g rat, V̇e = 2,457 ul and RR is 68. Regularly monitor the depth of the anesthesia by checking the response to a tail pinch and make necessary adjustments if required. In the case of an injected anesthetic, after 20–40 min if required, inject approximately 15–20% of the initial amount i.p. or i.v. to maintain the level of anesthesia.

Surgical procedures for LV catheterization • TIMING 10–30 min

8) The PV catheter can be inserted to the LV through right carotid artery without opening the chest cavity (closed-chest approach, option A; see Supplementary Movie 2 online, and Fig. 3a) or after the chest opening and stabbing the apex of the left ventricle with a 25–30 gauge needle through the stab wound (open-chest approach, option B; see Supplementary Movie 3 online, and Fig. 3b). Perform these procedures (see outlined below) under a stereomicroscope (× 10 magnification).

(A) Closed-chest approach (right carotid artery catheter insertion)

(i) In an immobilized anesthetized animal, make an inverted T-shaped middle-neck incision from mandible to the sternum. Move aside parotid glands and with forceps, bluntly dissect the thin muscle layer around the throat to expose and isolate the right carotid artery (Fig. 3a, panels 1–3; Supplementary Movie 2 online).

▲ CRITICAL STEP It is very important to separate carotid artery from vagus before ligation.

(ii) Secure suture around the proximal end of the artery, gently pull it and tape it to the table (or use a needle holder instead of the tape) and insert two additional sutures beneath carotid artery. Place a very loose knot to the middle suture and gently pull the distal suture (without securing it) with a needle holder and clamp it to the skin of the animal to fix it in the desired position (Fig. 3a, panels 4–6).

(iii) Put a couple of drops of physiological saline in the vessel area, make a tiny incision near the proximal end of the artery with a microincision scissor and extend the incision longitudinally for a short distance (Fig. 3a, panel 7).

(iv) Pull back slightly on the arterial flap while inserting the catheter tip into the vessel followed by gently securing the middle suture (Fig. 3a, panels 7–9).
Figure 6 | Representative normal and pathological mouse baseline PV loops and occlusions, and example of hemodynamic effects of drug in mice and rats. (a) Normal calibrated baseline PV loops and occlusions (calibration was performed on the basis of flow measurements and hypertonic saline injections). Normal baseline PV loops from mice (left two panels) and loops during vena cava inferior occlusions (right two panels) obtained from LV catheterization through right carotid artery (carotid approach) or through ventricular apex (see also Fig. 5 and Supplementary Movies 2–3 online).
(b) Characteristic changes in PV relationships obtained by vena cava inferior occlusions in control (sham) mice, 1 and 9 weeks following TAC, and in dilated cardiomyopathy (DCM).
(c) Characteristic changes in baseline (blue continuous trace) PV relationships during isoproterenol (ISO, red) infusion.
(d) Characteristic changes in rat LV volume (red trace) and pressure (blue trace), –dp/dt and –dP/dt derived from LV pressure signal (green trace), and arterial pressure (purple trace) before (at baseline) or after an administration of a drug with known cardiodepressive properties (a cannabinoid type 1 (CB1) receptor agonist HU210), followed by the recovery after the administration of the CB, antagonist SR144716 (drug administrations are indicated by arrows). Lower panels show characteristic PV loops at baseline and following the drug administrations. Note that even without any calibrations, the volume traces and PV relationships are very informative.

(v) Simultaneously, release the distal suture and quickly advance the presoaked catheter (for 30 min into physiological saline solution) into the left ventricle until the PV signal is displayed in the monitor (Fig. 3a, panel 10, Figs 5a,b; also see Supplementary Movie 2 online).

**A CRITICAL STEP** Never force the catheter and never grab the sensors by forceps (this can damage it and perforate vessel or ventricle). If some resistance is encountered while advancing the catheter tip, gently pull back and try advancing again until the catheter is inside the ventricle; be patient. If required, gently rotate the catheter shaft to achieve optimal placement of the tip along the axis of the left ventricle (Fig. 3a, panel 10). After stabilization of the signal for 10–15 min, record the baseline PV loops at a steady state or at varying preloads during the vena cava inferior occlusions (see below and also Figs. 4a and 5).

(vi) It is advisable to cannulate left jugular vein at this point by inserting a plastic tube (P10 in mice) or cannulation needle connected to tubing for later possible fluid/drug or saline injections/infusions as shown in Figure 4c and Supplementary Movie 5 online.

(vii) To test the effect of a new drug that may also affect peripheral resistance, it is advisable to cannulate one of the femoral arteries with a plastic tube and connect it to a pressure transducer recording on separate channel at PowerLab. This way, you can also calculate the time course of the TPR changes during the experiment.

(B) Open-chest approach (catheter insertion after the chest opening and stabbing the apex of the left ventricle with a 25–30 gauge needle through the stab wound)
(i) In anesthetized, fixed and intubated/respirated animals, make an incision over the xyphoid process and separate the skin from the chest wall by blunt lateral dissections with scissors and/or heat cautereation. Starting around the xyphoid process,
cut through the chest wall moving laterally on both sides with the cautery until the diaphragm is clearly visible from beneath. Cut through the diaphragm to expose the apex of the heart. Alternatively, begin the chest incision (anterior thoracotomy) above the xiphoid process, cut across the sternum to fully expose the chest cavity and retract the chest walls either by pulling sutures through the rib cage or by using a chest retractor (Fig. 3b, panels 1–9 and Supplementary Movie 3 online).

(ii) Gently remove the pericardium from the heart with forceps (Fig. 3b, panel 10).

(iii) Using a 25–30 gauge needle, make a stab wound near the apex of the heart into the left ventricle and remove the needle. Do not push the needle in more than 2–4 mm (Fig. 3b, panels 11 and 12).

(iv) Insert the catheter tip retrograde into the left ventricle until the proximal electrode on the catheter (E4) is just inside the ventricular wall. Adjust the position of the catheter to obtain rectangular-shaped pressure volume (in diseased animals, the shape may not be rectangular) loops (Figs. 5 and 6).

(v) After stabilization of the signal for 10–15 min, record baseline PV loops at steady state or at varying preloads during the inferior vena cava occlusions. This latter procedure is used to derive various load-independent indices of systolic function.

(vi) Vena cava inferior occlusions can be performed in open-chest respirated animals by pulling of a suture placed around the vessel, by lifting the vessel with a small stick or compressing it with a forceps (Fig. 4a, and Supplementary Movie 4 online). In case of the closed-chest approach, it is possible to obtain reasonable vena cava occlusions by compression of the inferior vena cava through diaphragm with a cotton tip applicator from the opened abdominal cavity (without chest opening). However, this requires considerable experience and may not be as reproducible as the other above-mentioned methods. During the data collection, shut off the small animal respirator for a few seconds to acquire data without lung motion artifact.

(vii) At the conclusion of the experiment, remove the catheter by gently pulling it back through the stab wound and euthanize the animal by an overdose of pentobarbital. Upon removal, immediately place the tip of the catheter into a syringe filled with saline to prevent clotting.

(viii) Clean the catheter with the detergent provided (Alconox) according to Millar’s instructions (see also Millar training CD). Proper care of the catheter will considerably prolong its useful life.

**Catheter calibrations, data analysis **• TIMING 15–60 min

Catheter calibrations and data analysis can be carried out by relative volume unit (RVU) calibration (option A), cuvette calibration with fresh heparinized warm blood (option B), saline calibration (option C) or aortic flow calibration (option D) (see Supplementary Movie 5 online, and Figs. 4b and 5a,b).
(A) RVU calibration
(i) This is the default built-in calibration in the Millar PV systems (converts conductance to RVUs). Perform the calibration according to the manufacturer’s instructions and always record raw data in raw RVUs (do not attempt to create charts with predefined formulas that immediately convert the RVUs to true volume signal based on cuvette and saline calibrations during the online streaming because if there is a mistake in calibrations, original data may not be retrievable). Analysis of RVU signals even without conversion to absolute volumes can be very informative in most pathological models.

(B) Cuvette calibration with fresh heparinized, warm blood
(i) With the calibration cuvette, an actual blood sample from the animal is used to make a more accurate assessment of LV blood volume and convert the volume data from RVUs to units of true volume (µl): use a mouse or rat insulator-type calibration cuvette with known diameter provided by the manufacturer, place it on a heating pad or into a 37 °C prewarmed water bath and fill quickly the first 4–5 holes with freshly taken warm blood from heparinized animals (Supplementary Movie 5 online).

(ii) Hold the catheter tip centered above the well of the cuvette (guides provided by manufacturer may be used), quickly submerge it in the blood (all 4 electrodes should be submerged) and hold it as steady as possible for 10–20 s in each well (normally using the first 1–5 wells is sufficient).

(iii) Record the conductance changes in the volume channel in RVUs. Calculate the volume of the wells (or check in the manufacturer’s instructions) using the equation for the volume of a cylinder, where the radius is that of the cuvette well and the length is based on the length between the inner two sensing electrodes (E2 and E3) on the catheter tip. The conductance output from dipping the catheter tip in the wells can be correlated with the known volumes to develop a calibration equation that converts the data from RVUs into units of true volume (µl). According to our experience, the calculated slope of the equation in mice based on 2–5 point cuvette calibrations varies from 4 to 7 and the intercept varies from ~10 to ~25; in rats, these values are from 10 to 20 and from ~14 to ~80, respectively.

(iv) Enter the well volume and corresponding blood conductance values into PVAN (or the slope and intercept values of the equation) and make conversion of RVUs to more realistic first estimate of true volumes (µl). However, these estimated volume signals are still larger than expected because of the parallel conductance, which refers to the conductivity of the heart muscle that surrounds the LV blood pool. To account and correct for this change, perform intravenous hypertonic saline calibration as described below.

(C) Saline calibration
(i) Ideally, the current applied to the excitation electrodes on the catheter should go through the blood only, but in reality, some of the applied current flows into the surrounding muscle, which is also a conductor, often causing an overestimation of the blood volume within the ventricle. As the heart muscle acts as a shunt to the applied current, this effect is referred to as parallel conductance or parallel resistance, or in volume calculations as parallel volume (Vp). To obtain a value for Vp, perform a saline bolus calibration with hypertonic saline (30%) bolus injection into the animal at the conclusion of the experiment as follows: firstly, cannulate jugular vein as shown in Supplementary Movie 5 online, and Figure 4c.

(ii) After turning of the respirator for a few seconds (and during the injection), inject 5–10 µl hypertonic (30% saline solution) i.v. into mice and 20–40 µl into rats to obtain visible shift to the right in PV loops (change in conductance) without significant decrease in the pressure signal amplitude (e.g., see Supplementary Movie 5 online, and Fig. 5a,b). The parallel volume is calculated by solving a system of linear equations to locate the intersection of two lines, one represented by the saline calibration data (plotting VEd versus VEs for each beat during the phase where the volume signal appears to rise following the hypertonic saline bolus) and the other by VEd = VEs. The VEd = VEs line represents equal end-systolic and end-diastolic volumes or the equivalent of a heart chamber devoid of blood. The value at the intersection of the VEd = VEs line and the saline calibration line represents the parallel volume of muscle tissue only (Vp) and is calculated by PVAN program. According to our experience, Vp values in mice (depending on size and strain) vary from 17 to 42 µl and in rats from 130 to 280 µl. Details of the method and its theory have been reported previously[6,21].

(iii) Because of the possible variability, perform at least 2–3 saline calibrations in each animal. Every time, wait a few minutes for the volume signal to recover. Use only the data during the rising phase of this intervention.

(iv) Enter the calculated Vp value from saline injection to PVAN together with the parameters derived from cuvette calibrations and convert volume to true volumes in microliters (see Fig. 5 and Table 1 for examples).

(D) Aortic flow calibration
(i) Following the collection of the data, the conductance signal can also be converted to true volumes by performing the saline calibration as described above in conjunction with aortic flow determinations[8]. Perform open chest surgery as described previously (Step 88).

(ii) Slowly turn the animal onto its left side, paying attention to only minimally disturb the volume signal. Make a lateral thoracotomy between T3 and T5 to create a small window and gently dissect a small part of the thoracic aorta running parallel to the spinal column with forceps (see Supplementary Movie 5 online, Fig. 3b, panels 6–9, and Fig. 4b).
(iii) Use either a perivascular flow probe (Transonic) placed around the aorta to assess cardiac output or a Doppler flow probe to provide flow velocity. The latter is then multiplied by the aortic cross-sectional area (echo image) to determine volume flow. The phasic flow data are then integrated to determine stroke volume, and this is assessed while simultaneous measurements of the PV loops are made. The latter provides stroke volume (SV) from the catheter, determined by the mean width of the PV loop. The ratio provides the calibration gain.

**TROUBLESHOOTING**

**TIMING**
The total amount of time necessary for anesthesia, surgery and calibrations for one animal may largely depend on the experience and surgical skills of the investigator and experimental protocol used. This time can vary from 1 to several hours. Plan around 1 week for a study with several groups with 8–14 animals each. It is advisable to start with a few control animals on the day of the experiment to make sure that everything is optimized and working well, followed by measurements in pathological states or groups. This will also minimize the potential errors in case the catheter needs to be replaced in the middle of the study by another one with slightly different properties.

**TROUBLESHOOTING**
Troubleshooting advice can be found in Table 2.

### TABLE 2 | Troubleshooting table.

<table>
<thead>
<tr>
<th>Potential problem(s)</th>
<th>Possible source(s) and solution(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>From the beginning of the experiment, the uncalibrated volume signal is in the normal range (in mice 4–7; in rats 5–10 RVUs), pressure signal and dP/dt is in the lower range, the heart rate is very low (see Table 1 for normal values)</td>
<td>The temperature of the animal may have dropped or the anesthetic is overdosed. Check the temperature of the animal and the temperature control unit; also check the depth of the anesthesia and make sure that the anesthetic was not overdosed. If so, in case of inhalation anesthesia, decrease the concentration by 1–1.5%, or for a short period, use just 100% oxygen. Make adjustments and allow 10–15 min for stabilization. In case of injectable anesthetic overdose, i.e., fluid injection and 100% oxygen can help recovery. Make sure that animals are always intubated if injectable anesthesia is performed and trachea tubes are not blocked by mucus. If so, insert a small plastic tube (P10 in mice) to the tube connected to a 10–20 ml syringe and suck out obstructing mucus.</td>
</tr>
<tr>
<td>From the beginning of the experiment, pressure signal and dP/dt is low</td>
<td>Animal may have lost too much blood during the surgery and/or fluid via evaporation through the surgical surface. Anesthesia/analgiesia may also be overdosed or not sufficient. Use battery-operated electrocautery during the surgery to minimize blood loss and always moisten the surgical surface with physiological saline to decrease drying out and evaporation. If the blood/liquid loss is significant, slowly inject/infuse physiological saline solution into the jugular or femoral veins; in more severe cases, inject albumin-containing solution. If the abdominal cavity is intact, saline solution can also be injected i.p. Check the anesthesia/analgiesia depth. Paradoxically, if animals feel pain and distress, it can also lead to hemodynamic instability; if so, adjust the drug concentrations</td>
</tr>
<tr>
<td>From the beginning of the experiment, pressure signal and dP/dt is normal, but volume signal is very low</td>
<td>Most likely, the catheter is not in the right position. Try to improve the volume signal by gently readjusting the position of the catheter</td>
</tr>
<tr>
<td>The pressure and/or volume signal is noisy, regular 50 Hz (Europe) to 60 Hz (USA) noise pattern</td>
<td>The most likely cause is electric interference, which can originate from pumps, electrocautery devices, ungrounded operating tables, ventilators, fans and electric warming blankets. Try to isolate any sources of electrical interference by moving them away from the catheter and by sequentially unplugging the possible interfering appliances. If the noise is coming from the power supply, sometimes just unplugging the MPVS system and plugging into different outlet, preferably with stabilized power supply for the laboratory equipment, can eliminate the problem. If the noise problem from the electrical network cannot be resolved for any reasons, the built-in 50- and 60-Hz filter in PowerLab can sometimes improve results</td>
</tr>
<tr>
<td>The pressure and/or volume signal is noisy and shows irregular pattern</td>
<td>If all of the above fail and the noise is irregular, check if it disappears with another catheter. If so, the first catheter is most likely damaged. Check catheter for damage under the microscope, and if the coating is disrupted, clean and send it immediately to the manufacturer for further evaluation and repair. Always have at least one or two backup catheters before starting a study</td>
</tr>
</tbody>
</table>

(Continued)
TABLE 2 | Troubleshooting table (continued).

<table>
<thead>
<tr>
<th>Potential problem(s)</th>
<th>Possible source(s) and solution(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>The pressure sensor shows an excessive amount of drift, and/or it is not possible to ‘zero balance’ the pressure transducer output</td>
<td>Fluid may have gotten inside the catheter, or the pressure sensor diaphragm may be cracked or broken. It is possible to check the functionality of the pressure sensor by connecting an ohmmeter (resistance gauge) across the pins of the pressure sensor connector. The bridge on the Millar sensor should have symmetrical input and output impedances of approximately 1,000 ohms. If either reading is dramatically different from 1,000 ohms, there are chances that the pressure sensor is broken because of the above-mentioned reasons. Please contact Millar Instruments’ customer support.</td>
</tr>
<tr>
<td>The pressure sensor appears to be functional, but the readings are off by approximately 25 or 100 mm Hg</td>
<td>The pressure calibration in the data acquisition hardware is incorrect. Recalibrate the pressure signal in the data acquisition software according to the manufacturer’s instructions. Briefly, the pressure control side of the MPVS-300/400 has three calibration settings. The settings are 0, 25 and 100 mm Hg. The standardized pressure output from the MPVS-300/400 is 1 V per 100 mm Hg. Therefore, the 0 mm Hg calibration setting corresponds to 0 V output, 25 mm Hg corresponds to 250 mV (0.25 V) output, and 100 mm Hg corresponds to 1 V output. In the case where the pressure readings are off by exactly 25 or 100 mm Hg, the wrong units label has been assigned to the voltage output coming out of the MPVS-300/400 for a particular calibration setting (e.g., the 25 mm Hg units label was assigned to 0 V output rather than 0 mm Hg units label).</td>
</tr>
<tr>
<td>The zero balanced pressure sensor in saline or distilled water drifts a few mm Hg after insertion into the biological environment</td>
<td>The most likely reason is that the catheter was not properly prepared before inserting it to the animal. Presoak the catheter tip in saline at body temperature for at least 30 min before use (e.g., by inserting the tip (but not the connectors) through the tip of a 1-ml syringe containing physiological saline solution).</td>
</tr>
<tr>
<td>The volume/conductance signal output from the MPVS-300/400 appears to drift</td>
<td>Electronic hardware has some drift present in the signal as a result of the electronic components heating up. To minimize conductance signal drift from the MPVS-300/400, turn it on and let it warm up at least 30 min before calibrating the output and using the catheter to collect PV data.</td>
</tr>
<tr>
<td>The calibrated volume signal is larger than expected</td>
<td>Following the cuvette calibration, the volume signal (converted from RVUs to uls) is larger than expected. The reason for this is that the parallel volume contribution of the myocardium (parallel conductance, which refers to the conductivity of the heart muscle that surrounds the left ventricular blood pool) has not been taken into account. Perform hypertonic saline calibration to correct for $V_p$.</td>
</tr>
<tr>
<td>The conductance/volume signal is out of range</td>
<td>In larger animals with dilated cardiomyopathy, the conductance signal may go out of the range. Measure the ventricular length of the animal in question and make sure that the catheter being used has electrode spacing that matches this length (e.g., a 6-mm signal electrode spacing is appropriate for small-sized rats and a 9-mm signal electrode spacing is appropriate for larger rats). In some cases, custom catheter design may be required; contact the manufacturer with such request.</td>
</tr>
<tr>
<td>The volume/conductance outputs from the cuvette calibration are not consistent</td>
<td>Make sure that the cuvette with fresh heparinized blood (to avoid clotting) is at body temperature. Always properly clean the cuvette between uses. Center the catheter tip within the cuvette and submerge it (all four electrodes should be submerged) in the blood, and hold it as steady as possible for 10–20 s in each well (usually using the first 1–5 wells is sufficient).</td>
</tr>
</tbody>
</table>

ANTICIPATED RESULTS

Using miniature PV catheters advanced in the left ventricle via right carotid artery or ventricular apex following stabbing with a needle, this sophisticated methodology allows simultaneous measurements of both pressure and volume signals from the mouse with intact heart beat and rat hearts both at steady state and during manipulation or decreasing of the preload through VCI occlusions. The estimation of the absolute volume from the raw conductance measurements is possible by various calibrations (RVU (built into the Millar PV systems), external cuvette with blood and hypertonic saline calibrations in live animals) or by combination of the use of independent measure of cardiac output and hypertonic saline calibration as described in this protocol (for representative examples of baseline PV loops and occlusions before and after saline calibrations, see Figs. 5 and 6; Supplementary Movies 2, 4 and 5 online). With both surgical techniques, very similar results can be obtained, given the
careful optimization of the temperature regulation, fluid balance and anesthesia/analgesia during the surgery and ultimately the surgical procedures, which are the major keys to the success of this whole approach (see Supplementary Movies 1–5 online, and Figs. 2–4).

A large number of hemodynamic parameters and indices of systolic and diastolic function can be derived from PV relations both from steady state data and from data measured at decreasing preloads (for normal ranges of selected, commonly used both load-dependent and load-independent hemodynamics variables obtained in our laboratories with various anesthesia protocols and approaches, see Table 1). For representative mouse PV loops and occlusions at baseline and during the development of myocardial hypertrophy and failure 1 and 9 weeks following TAC, in dilated cardiomyopathy and following drug additions, see Figure 6.

Note: Supplementary information is available via the HTML version of this article.

ACKNOWLEDGMENTS This research was supported by the Intramural Research Program of NH/NIAAA (to P.P.), NH/NHLBI HL-077180; HL-95408. We are indebted to Huntly Millar and Tim Daugheerty for reading the protocol and valuable suggestions and acknowledge Millar Instruments for the permission to use animations in the movies and for providing background information on the use of Millar PV systems. P.P. dedicates this protocol to his beloved mother Irene Bolfert and grandmother Ilona Kerenyi.

Published online at http://www.natureprotocols.com/
Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions/

Too much or not enough of a good thing — The Janus faces of autophagy in cardiac fuel and protein homeostasis

Jun Ren a,b,⁎,1, Heinrich Taegtmeyer c,⁎⁎,1

a Shanghai Institute of Cardiovascular Diseases, Zhongshan Hospital, Fudan University, Shanghai 200032, China
b Center for Cardiovascular Research and Alternative Medicine, University of Wyoming College of Health Sciences, Laramie, WY, USA
c Department of Internal Medicine, Division of Cardiology, The University of Texas Health Science Center, Houston, TX, USA

⁎ Correspondence to: J. Ren, Center for Cardiovascular Research and Alternative Medicine, University of Wyoming College of Health Sciences, Laramie, WY 82071, USA. Tel.: +1 307 766 1613; fax: +1 307 766 2953.
⁎⁎ Corresponding author. Tel.: +1 713 500 6569; fax: +1 713 500 0637.
E-mail addresses: jren@uwyo.edu (J. Ren), Heinrich.Taegtmeyer@uth.tmc.edu (H. Taegtmeyer).
1 Both authors contributed equally to this work.

Abstract

Cells respond to changes in their environment and in their intracellular milieu by altering specific pathways of protein synthesis and degradation. Autophagy is a highly conserved catabolic process involved in the degradation of long-lived proteins, damaged organelles, and subcellular structures. The process is orchestrated by the autophagy related protein (Atg) to form the double-membrane structure autophagosomes, which then fuse with lysosomes to generate autophagolysosomes where subcellular contents are degraded for a variety of cellular processes. Alterations in autophagy play an important role in diseases including cancer, neurodegenerative diseases, aging, metabolic diseases, inflammation and cardiovascular diseases. In the latter, dysregulated autophagy is speculated to contribute to the onset and development of atherosclerosis, ischemia/reperfusion injury, cardiomyopathy, diabetes mellitus, and hypertension. Autophagy may be both adaptive and beneficial for cell survival, or maladaptive and detrimental for the cell. Basal autophagy plays an essential role in the maintenance of cellular homeostasis whereas excessive autophagy may lead to autophagic cell death. The point and counterpoint discussion highlights adaptive vs. maladaptive autophagy. In this review, we discuss the molecular control of autophagy, focusing particularly on the regulation of physiologic vs. defective autophagy.

Article Info

Article history:
Received 4 September 2014
Received in revised form 23 February 2015
Accepted 2 March 2015
Available online 11 March 2015

Keywords:
Autophagy
Heart diseases
Survival
Cell death

1. Point

Autophagy is an evolutionarily conserved catabolic process to maintain cellular and organ homeostasis in response to a wide spectrum of stress, such as nutrient starvation, protein aggregates, damaged organelles and invasion of cells by infective organisms. Autophagy is essential to the controlled destruction of organelles under physiological conditions, mainly in a “housekeeping” fashion to degrade defective or unnecessary proteins and/or organelles [1–3]. As a result, autophagy may alleviate abnormal protein aggregation, at the same time providing amino acids for the maintenance of ATP levels and anabolic pathways such as protein synthesis [1–3]. Although some of these “quality control” features are shared with the ubiquitin-proteasome system (UPS), autophagy is rather unique in its ability to degrade large molecules or organelles such as mitochondria (aka, mitophagy), endoplasmic reticulum (ER; reticulophagy), peroxisomes (pexophagy), or triglyceride droplets (lipophagy) [4–6]. Autophagy may be activated in response to different stresses such as ischemia, pressure overload and endotoxemia [2,7,8]. Autophagy functions to protect cells against potential damage upon exposure to various stresses, and thereby helps to defend cells and, subsequently, organs against dysfunction. Multiple lines of evidence are available that autophagy possesses disease-preventing effects and, if disease still occurs, autophagy limits disease severity [8]. For example, autophagy appears to play a key role in delaying the aging process [2,3]. Reduced autophagy is commonly present in aging, resulting in the accumulation of damaged proteins and organelles. Measures such as caloric restriction and exercise may facilitate autophagy to delay aging and extend lifespan [9]. Stimulation of autophagy using rapamycin may serve as a novel strategy to prolong lifespan and ameliorate aging-associated diseases [7,9], possibly associated with mitochondrial biogenesis [2,10].

Initially described as a cellular survival mechanism in starvation, autophagy has recently gained much attention in cellular self-renewal and in cell death in a number of diseases including heart failure [11]. In the case of ischemic injury, hypoxia promotes formation of autophagosomes adjacent to swollen and fragmented mitochondria [8,12], indicating facilitated autophagy of mitochondria (as depicted in Fig. 1). This is in line with the observation that autophagy inhibitors prompt cardiomyocyte death following glucose starvation, suggesting an essential role for autophagy in the replenishment of energy stores and removal of...
damaged organelles. Induction of autophagy is considered to be cardioprotective under ischemia [13]. During ischemia, cellular ATP is consumed to trigger autophagy to recycle amino acids and fatty acids for ATP generation to adapt to energy deprivation [9]. Likewise, chloramphenicol succinate, an agent that purportedly upregulates autophagy, ameliorates ischemia/reperfusion injury through induction of autophagy [3]. Further scrutiny has revealed an essential role for AMPK in ischemia-induced autophagy where autophagy induction promotes survival by eliminating damaged mitochondria which may otherwise serve as a source of ROS and pro-apoptotic mediators [8,14,15]. With reperfusion following ischemia, a new surge of autophagy is triggered, and the number of autophagosomes are increased compared to hypoxia alone [8]. The Bcl-2 adenovirus E1B 19-kDa interacting protein 3 (BNIP3) was recently implicated in ischemia/reperfusion injury downstream of hypoxia-inducible factor 1α (HIF1α). BNIP3 permeabilizes mitochondria, leading to mitochondrial injury and cardiomyocyte death during ischemia/reperfusion injury. Forced expression of BNIP3 stimulates autophagy in cardiomyocytes whereas inhibition of autophagy promotes BNIP3-induced cardiomyocyte death by preventing removal of damaged mitochondria [16]. Recent work also suggested that the histone deacetylase (HDAC) inhibitor suberoylanilide hydroxamic acid reduced ischemia/reperfusion injury by upregulating autophagy, therefore attenuating cell death in the border zone [17].

While basal autophagy plays a housekeeping role under physiologic conditions, defective autophagy is responsible for Danon cardiomyopathy [6]. Along the same line, defective autophagy is seen in some genetic causes of cardiomyopathy and skeletal myopathy such as Lamin A/C (also known as LMNA) cardiomyopathy and muscular dystrophy [13,18,19]. Moreover, autophagy is a key to the protection of cardiomyocytes against proteotoxic stress as shown in experimental models of cardiac proteinopathy [13]. Likewise, increased autophagy may be an adaptive response to limit cardiac hypertrophy. A heart-specific deficiency of Atg5 in adult mice leads to cardiac hypertrophy, left ventricular dilatation and contractile dysfunction associated with accumulation of ubiquitinated proteins and ultra-structural changes. Collectively these and other findings support the notion of a cytoprotective role for autophagy [20]. Recent evidence also suggests a pivotal role for autophagy in terminal cardiomyocyte differentiation [8,12]. Given the promises of therapeutic application of stem cells in ischemic and degenerative heart diseases [21], these findings are of great importance. It should be noted that autophagy plays a critical role in preventing heart disease beyond cardiomyocyte differentiation [12].

2. Counter-point

The phenomenon of autophagy first was described by the late Christian de Duve nearly half a century ago [6]. Thus, in principle, the phenomenon is not new and needs to be considered in the context of fundamental research on acid proteases, lysosomal biology, and intracellular protein turnover [22]. What is new today is a detailed understanding of the proteins involved in autophagy and the capability to measure autophagic flux. What is also new is a raging debate whether autophagy is beneficial or deleterious for the survival of the cell, the organ, or the organism as a whole. Furthermore, accumulating evidence has demonstrated that autophagic flux may be affected by various stresses including starvation, ischemia/reperfusion, pressure overload, type 2 diabetes, alcoholism and obesity [14,23,24].

Autophagy is induced under ischemia and with reperfusion, although its function may be paradoxical in ischemia and reperfusion [19]. It is perceived that autophagy may be adaptive in the context of ischemia/reperfusion injury [14]. Autophagosomes sequester damaged organelles such as mitochondria, ER and ribosomes for degradation, thereby preventing release of ROS and cell death [25]. However, during reperfusion after ischemia, autophagy is over-activated, and when autophagy exceeds a critical threshold, excessive degradation of proteins and organelles may injure the myocardium [14]. Excessive autophagy has been documented in severely damaged cardiomyocytes, contributing to the onset and progression of pathological conditions such as reperfusion injury, pressure overload-induced cardiac remodeling and heart failure [26,27] (as shown in Fig. 1). Angiotensin II (AngII) and its type 1 receptor (AT1) have been reported to play a pivotal role in the etiology of heart failure possibly in association with autophagy induction [28]. Sadoshima et al. reported an increase in both autophagosomes and autolysosomes following myocardial ischemia/
reperfusion, denoting increased autophagy and autophagic flux in association with oxidative stress in ischemia/reperfusion injury. Treatment with an antioxidant attenuated ischemia/reperfusion-induced myocardial infarct size and rises in oxidative stress, autophagic flux, and Beclin-1 expression, suggesting that oxidative stress plays an important role in mediating autophagy, autophagic flux and myocardial injury during ischemia/reperfusion injury. Such beneficial effects of antioxidant were duplicated in Beclin-1 haploinsufficient (Beclin-1<sup>+/−</sup>) mice, with no additive effect between the two [25]. These findings were supported by the observation that inhibition of hypoxia and reoxygenation (H/R)-induced autophagy protected cardiomyocytes in vitro [29]. Induction and inhibition of autophagy, using rapamycin and 3-MA respectively, promoted cell survival against hypoxia and H/R, respectively [14]. Taken together, these results suggest a key role for oxidative stress in mediating autophagy and autophagic flux during ischemia/reperfusion. Increased accumulation of autophagosomes has been observed in cardiac tissues of patients suffering from ischemia or pressure overload [30]. However, work from Ma et al. challenged the concept that Beclin-1 haploinsufficiency merely prevents autophagy initiation, suggesting a role in promoting autophagic processing [16]. Typically, increased autophagosome build-up may represent either increased autophagy initiation or defective lysosomal clearance (autophagy flux) — which is often considered a mis-interpretation of “excessive autophagy”. Ma et al. reported elevated ROS production coupled with upregulated Beclin-1 and decreased lysosome-associated membrane protein-2 (LAMP-2), a critical determinant of autophagosome-lysosome fusion, after reperfusion, denoting decreased autophagic processing (as opposed to increased autophagic flux observed by Sadoshima et al. [25]). Diwan et al. also noted that restoration of LAMP-2 synergizes with partial Beclin-1 knockdown to promote autophagosome processing capacity, facilitating cell survival after hypoxia-reoxygenation [16]. These findings suggest that autophagic flux is compromised in ischemia/reperfusion resulting in poor autophagosome clearance along with ROS production, mitochondrial permeabilization, and, ultimately, cell death. These findings confirm the notion that autophagy should be essential to cardiomyocyte survival although excessive autophagic activation may be detrimental during reperfusion [14]. It is somewhat puzzling with regards to the disparate findings on autophagic flux from these labs using the Beclin-1 haploinsufficiency (Beclin-1<sup>+/−</sup>) model. Possible differences in the ischemia/reperfusion protocol (30 min/90 min versus 45 min/120 min, for the Diwan and Sadoshima studies, respectively) or autophagic flux assessment technique (fluorescent overlay versus ratio in the absence and presence of chloroquine) may contribute to the disparate results between the two groups. Our study in failing hearts of patients with dilated cardiomyopathy noted increased markers of autophagy which may be downregulated with mechanical unloading by a left ventricular assist device [5]. Although autophagic flux was not measured, we speculated that reduced demand for energy providing substrates reduced the need for cellular self-cannibalism.

Lastly, there is excessive myocardial autophagy in response to hypertrophic stimuli such as aortic banding and angiotensin-II administration [24,31]. Along the same line, accumulation of autophagic vacuoles containing mitochondria, glycogen granules and degraded remnants were observed in human myocardium in transition from compensated hypertrophic cardiomyopathy to heart failure [5]. This was supported by upregulated autophagy in experimental heart failure [18]. These findings suggest a possible role for dysregulated autophagy in the poor protein quality control during the transition of cardiac hypertrophy to heart failure.

However, it must be kept in mind that the structural changes and the appearance of prenylation product LC3-II are mere footprints rather than a reflection of true autophagic flux. To assess flux, we must measure LC3-I to LC3-II conversion by a turnover assay involving inhibitors of autophagy (Bafilomycin A1 or Hydroxycholoroquine). An increase in LC3-II protein levels would then be indicative of an increase in autophagic flux, while unchanged levels of LC3-II would suggest no flux [31]. Alternatively, one can also look at changing p62/SQSTM1 protein levels as an assessment of autophagic flux. Autophagy degrades the p62 protein during the formation of the autophagolysosome. Thus, degradation of p62 is also a marker of autophagic flux [32].

3. Conclusion – Ren and Taegtmeyer

Autophagy is a key cellular catabolic process that is induced under not only physiological but also pathophysiological conditions. Maintenance of a fine balance between protein synthesis and degradation is essential for eukaryotic cell survival. Autophagy can be regulated by a wide variety of signaling mediators, including the Atg family and mTOR kinase. The proteins encoded by 30+ Atg genes regulate the nucleation of autophagic vacuoles, formation of the isolated double membrane (i.e., phagophore), maturation of the autophagosome, and fusion with lysosomes forming autolysosomes [33]. The other major regulator of autophagy is mTOR, the mammalian target of rapamycin [34], an inhibitor of autophagy. In this point-counter-point, we provide a brief overview of pros and cons for autophagy and autophagic flux in the homeostasis of cardiomyocyte survival. While autophagy is usually deemed cytoprotective with the removal of damaged or dysfunction organelles and proteins, the process may be detrimental under certain conditions, such as alcoholic cardiomyopathy, reperfusion and heart failure [16,18,24,25,35]. However, despite the current literature, several questions remain unanswered. For example, how does the cell decide to switch from “adequate or basal” to “uncontrolled” autophagy? Is this strictly a temporal event linking autophagy initiating proteins (such as Beclin-1) to downstream autophagy regulatory proteins? At what point does the controversial autophagy initiating Beclin-1 switch the cells from adaptive autophagy to maladaptive autophagy resulting in death? What are the brakes on the maladaptive autophagy in the heart? For example, disruption of Beclin-1 has generated some conflicting conclusions on its effects on autophagic flux and its role in ischemia reperfusion [16,25]. Given that autophagy is a highly dynamic process involving multiple-step processes in a specific sequent manner, changes or interruptions at any step between initiation and degradation may generate distinct outcomes for the fate of the cell. Resolving these controversies surrounding the roles of autophagy in cardiac health and disease will undoubtedly lead to new clinical approaches based on manipulating autophagy.

Conflict of interests

None declared.

Acknowledgment

Work in the authors’ labs is supported by the U.S. Public Health Service (SR01HL061483 to HT). The authors wish to express their sincere apology to those authors whose important work could not be included in this review due to space limitation. We thank Mrs. Roxy A. Tate for the expert editorial assistance and the reviewers for their constructive comments.

References

II type 2 receptor antagonizes angiotensin II type 1 receptor-mediated cardiomyo-
autophagy. Annual Review of Physiology; 2010 45
Gottlieb RA, Mentzer Jr RM. Autophagy during cardiac stress: joys and frustrations of
1363
Rothermel BA, Hill JA. Autophagy in load-induced heart disease. Circ Res 2008;103:
ischemia/reperfusion. Antioxid Redox Signal 2011;14:2179–90.
Hariharan N, Zhai P, Sadoshima J. Oxidative stress stimulates autophagic
potential for myocardial ischemia. Stem Cells Cloning 2010;3:57
Zhang Y, Ren J. Autophagy in ALDH2-elicited cardioprotection against ischemic heart
Zhang Y, Ren J. Autophagy in ALDH2-elicited cardioprotection against ischemic heart

Jun Ren, MD, PhD, FAHA, is a Professor of Pharmacology at the University of Wyoming College of Health Sciences and an Adjunct Professor of cardiology at the Zhongshan Hospital, Fudan University. He earned his Ph.D. in 1994 from the University of Alberta, Canada, in cellular physiology, following his medical training in China (Beijing University and Peking Union Medical College). He then conducted his post-doctoral training at the Wayne State University School of Medicine and served as a faculty at the University of North Dakota School of Medicine and Health Sciences before joining the University of Wyoming in 2002. His major area of research focuses on cardiac pathophysiology in alcoholism, diabetes mellitus, obesity and aging. His main scientific contributions include elucidation of cardiac excitation–contraction coupling defects in these comorbidities at the cardiomyocyte level and how overeating and diabetes modulate those defects. Recently his primary research interest is switched to the autophagy regulation of mitochondrial function in the heart and liver. His studies have enhanced our understanding of the molecular mechanisms for autophagy in the pathogenesis and therapeutics of cardiometabolic diseases.

Heinrich Taegtmeyer, MD, DPhil is a Professor of Medicine at the University of Texas Medical School at Houston. He was born and educated in Germany. He received his medical degree “summa cum laude” from the University of Freiburg and a PhD (DPhil) in metabolic research from the University of Oxford. He is teaching and practicing cardiovascular medicine in an academic setting since completion of his residency and fellowship at the Harvard Medical School (Boston City Hospital, and the Peter Bent Brigham Hospital). The focus of his research is the metabolism of energy-providing substrates in the normal and diseased heart. This has resulted in the discovery of transcriptional and post-translational mechanisms of metabolic adaptation and de-adaptation, including the return to the fetal gene program in the heart. More recent work in the laboratory has discovered intermediary metabolites as signals for intracellular protein turnover, resulting in the concept of the self-renewing cardiomyocyte. His laboratory has been supported by grants from the NHLBI of the U.S. Public Health Service for nearly 40 years.