Increased Sarcolipin Expression and Adrenergic Drive in Humans With Preserved Left Ventricular Ejection Fraction and Chronic Isolated Mitral Regurgitation

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Background—There is currently no therapy proven to attenuate left ventricular (LV) dilatation and dysfunction in volume overload induced by isolated mitral regurgitation (MR). To better understand molecular signatures underlying isolated MR, we performed LV gene expression analyses and overlaid regulated genes into ingenuity pathway analysis in patients with isolated MR.

Methods and Results—Gene arrays from LV tissue of 35 patients, taken at the time of surgical repair for isolated MR, were compared with 13 normal controls. Cine-MRI was performed in 31 patients before surgery to measure LV function and volume from serial short-axis summation. LV end-diastolic volume was 2-fold (P=0.005) higher in MR patients than in normal controls, and LV ejection fraction was 64±7% (50%–79%) in MR patients. Ingenuity pathway analysis identified significant activation of pathways involved in β-adrenergic, cAMP, and G-protein-coupled signaling, whereas there was downregulation of pathways associated with complement activation and acute phase response. SERCA2a and phospholamban protein were unchanged in MR versus control left ventricles. However, mRNA and protein levels of the sarcoplasmic reticulum Ca2+ ATPase (SERCA) regulatory protein sarcolipin, which is predominantly expressed in normal atria, were increased 12-and 6-fold, respectively. Immunofluorescence analysis confirmed the absence of sarcolipin in normal left ventricles and its marked upregulation in MR left ventricles.

Conclusions—These results demonstrate alterations in multiple pathways associated with β-adrenergic signaling and sarcolipin in the left ventricles of patients with isolated MR and LV ejection fraction >50%, suggesting a beneficial role for β-adrenergic blockade in isolated MR. (Circ Heart Fail. 2014;7:194-202.)

Key Words: heart ventricles • mitral valve insufficiency

Isolated mitral regurgitation (MR) is characterized by left ventricular (LV) dilatation and augmented stroke volume mediated by the Starling mechanism and facilitated by LV ejection into the low-pressure left atrium.1 These favorable conditions for LV shortening dynamics can falsely elevate LV ejection fraction despite underlying cardiomyocyte contractile impairment. Therefore, to preserve LV systolic function and improve survival, corrective mitral valve surgery is recommended if LV ejection fraction (EF) falls <60%.2,3 Nevertheless, a decrease in LV systolic function remains a risk after mitral valve repair, and mechanisms involved in the transition to irreversible cardiomyocyte damage in chronic isolated MR remain elusive. There is currently no effective medical therapy that attenuates progressive LV remodeling in isolated MR, and mechanisms of LV myocardial remodeling specific to chronic MR are poorly understood.2,3

The measurement of plasma or LV interstitial fluid catecholamines identified increased sympathetic drive early and throughout the course of isolated MR in both animal models4–6 and humans, respectively.7 There is evidence of myocardial dysfunction from LV muscle strips and derangement of calcium-handling proteins in patients with isolated MR despite LVEF >55%.6,8–11 Although a comprehensive analysis of LV tissue in human-isolated MR is lacking, there is evidence that β1-adrenergic receptor blockade, initiated either immediately or 3 months after MR induction in the dog, improves LV...
contractility and cardiomyocyte calcium transients and cardiomyocyte fractional shortening. In a retrospective analysis of patients with severe MR and normal LVEF, β1-adrenergic receptor blockade imparts a significant independent survival benefit with or without coronary artery disease. Furthermore, results of a recent phase IIb clinical trial demonstrate a beneficial effect of β1-adrenergic receptor blockade versus placebo on LVEF in patients with isolated MR over a 2-year follow-up period.15–16

To understand whether β-adrenergic signaling, or other signaling pathways, is important in patients with isolated MR, we determined the cardiac transcriptome from LV biopsies taken from patients with isolated MR and mean LVEF >60% at the time of mitral valve corrective surgery. This analysis in patients with largely class I to II New York Heart Association (NYHA) symptoms demonstrates increased activity of adrenergic signaling pathways, lending further support for early β1-adrenergic receptor blockade treatment in isolated MR.

Methods

Study Subjects

The protocol was approved by the institutional review board of the University of Alabama at Birmingham, and informed consent was obtained from all participants. The study group consisted of 35 patients (mean age, 44±14 years; median, 54 years; range, 34–71 years) with severe isolated MR secondary to degenerative mitral valve disease, who were referred for corrective mitral valve surgery. Patients with obstructive coronary artery disease (>50% stenosis), aortic valve disease, or concomitant mitral stenosis were excluded from the study. Severe isolated MR was documented by echocardiographic/Doppler studies, and 31 patients underwent cine-MRI. Each patient with severe isolated MR had cardiac catheterization before surgery. Results of cine-MRI of the MR group were compared with those of a normal, healthy cohort from previous studies in our laboratory15,17,18 (age, 40±3 years; median, 38 years; range, 21–62 years), who have no history of cardiovascular disease.

At the time of surgery, LV tissue was taken from the lateral endocardial wall of the left ventricle at the level of the tips of the papillary muscles in all patients. Portions of the biopsy sample were then placed in RNA later or frozen and stored at −80°C for future analysis.

Cardiac MRI

A 1.5-T MRI scanner (Sigma GE Healthcare, Milwaukee, WI) optimized for cardiac application was used to perform all MRIs (Figure 1).19–21 LV volumes were computed by summation of volumes defined by contours in each short-axis slice, as previously described in our laboratory.19–21

RNA Isolation and Microarray

Total RNA was extracted from LV biopsies using Qiagen RNeasy Fibrous Tissue Mini Kit (Qiagen Sciences, MD) and cleaned with Ambion TURBO DNase. The integrity of RNA was evaluated by BioRad Experion (Bio-Rad Laboratories, CA). Samples containing an OD ratio of 260/280 >1.8 and 28S/18S >1.7 were selected for microarray processing. Thirteen normal human RNA samples (12 men; ages, 41, 44, 21, 25, 23, 27, 29, 36, 27, 24, 21, 66, 50 years) were extracted from the LV tissue of motor vehicle accident subjects (mean age, 44±14 years; median, 54 years; range, 34–71 years) with no history of cardiovascular disease.19–21 LV tissue from 3 normal and 4 MR hearts was electrophoretically separated on 8% (for SERCA2a and calsequestrin [CSQ]) or 14% (for phospholamban [PLN] and triadin) SDS-PAGE gels. Images were adjusted appropriately for background fluorescence.

Verification of Gene Expression Using Real-Time RT-PCR

Quantitative real-time PCR was performed using the Bio-RAD iCycler IQ system (Bio-Rad Laboratories) on 500 ng total RNA from microarray samples to verify array data. Table I in the Data Supplement demonstrates the selected genes and primer sequences

Genespring, Ingenuity Pathway Analysis, and Statistical Analysis

Microarray data were analyzed by Genespring GX 11.5. Raw data were log2-transformed and then normalized to the 75th percentile of
all values on a chip. MR and normal samples were compared using t test to examine for differentially expressed genes. A list of genes with ≥2-fold change was generated first and tested by Benjamini–Hochberg multiple testing correction. Significant genes were selected with a cut-off of P<0.05 and fold change ≥2.0. The list of altered genes was then imported into ingenuity pathway analysis (IPA) as previously described in our laboratory.21 Fisher exact test was applied by IPA to predict the likelihood that the association between the set of altered genes and a related pathway is not due to random association. RT-PCR and Western blot were analyzed by t test. Significance was set at P<0.05.

Results

Clinical Characteristics

Clinical characteristics of 51 normal controls and 35 MR patients are outlined in Table 1. The MR group is older than the control group (55±12 versus 44±14 years; P<0.0001). There are no significant differences in body surface area and sex between the 2 groups. Heart rate and diastolic blood pressure are similar in the 2 groups. Table 1 also summarizes individual patient medications, history of hypertension, and NYHA functional class.

Magnetic Resonance Imaging

Thirty-one MR patients had MRI performed <1 month before surgery (Table 2). MR patients have greater LV end-diastolic volume, LV end-systolic volume, and LV stroke volume normalized to body surface area compared with normal controls. MR patients have higher LV end-diastolic and LV end-systolic dimensions, similar LVEF, but a higher LV mass compared with normal controls. Figure 1 demonstrates the spherical remodeling and thinning of the LV wall in a representative MR patient.

Microarray Analysis

The microarray analysis identified 724 differentially expressed genes (2-fold change) in MR patients versus normal controls (P<0.05), including 353 upregulated and 371 downregulated genes. The heatmap in Figure 2A demonstrates a consistent pattern of change in these genes in 35 MR left ventricles and 13 normal left ventricles. A principal components analysis plot (Figure 2B) verifies the quality of the array. In this plot, samples representing the same experimental conditions are more similar to each other than samples representing different experimental conditions. Table II in the Data Supplement lists genes well established in the pathophysiology of cardiovascular disease identified by IPA. Among the 724 genes, the gene with the highest fold increase (22-fold) is natriuretic peptide A (NPPA); NPPB is also increased by 5.13-fold. The upregulation of these marker genes for hypertrophy underscores the quality of gene expression profiles from patients with severe MR and higher LV mass compared with control left ventricles.

Validation of Microarray With Quantitative PCR

Table III in the Data Supplement demonstrates microarray results validated by PCR for PLN, SLN, NPPA, 5′-AMP–activated protein kinase subunit β-2, natriuretic peptide receptor C, peroxidoredoxin 3, desmocollin 1, Kv channel interacting protein 2, and FOS. There is excellent agreement between microarray and quantitative PCR (Table III in the Data Supplement).

IPA Canonical Pathway Analysis

Activation of Cardiac β-Adrenergic Signaling in MR Hearts

The 724 altered genes are analyzed by IPA. The top network with a score of 38 is associated with cardiovascular disease. Canonical pathway analysis identifies the significant activation of cardiac β-adrenergic signaling pathway in MR hearts (Figure 3A). Figure 3B demonstrates the altered genes and their relation with calcium channel regulation. PLN is a 52-amino acid SR membrane protein expressed abundantly in cardiac muscle. In its dephosphorylated form, PLN interacts with SERCA2a to inhibit Ca2+ transport by lowering SERCA2a’s affinity to Ca2+. When PLN is phosphorylated, its inhibitory effect on SERCA2a is relieved. The 31-amino acid SR membrane protein, SLN, has a similar ability to inhibit either SERCA1a or SERCA2a. In human MR heart, the mRNA of PLN and SLN is increased by 2.5- and 12.4-fold, respectively.
Protein kinase A, cAMP dependent regulatory type 1α, and PRKA anchor protein 7, which direct or indirectly bind to PLN and regulate its phosphorylation, are increased 2-fold. There is a significant decrease in phosphodiesterase 4D (PDE4D) and PDE3B that degrade cAMP and cGMP, which inactivate PKA.

**Activation of Intercellular and Second Messenger Signaling in MR Hearts**

Adrenergic receptors belong to G-protein–coupled receptor family, and a major activity of β-adrenergic receptors is to modulate the level of the second messenger cAMP. Thus, we analyzed the activation of canonical intercellular and second messenger signaling in MR hearts. Figure 4 demonstrates the significant activation of G-protein–coupled receptor and cAMP signaling. The corresponding heatmaps represent the normalized signal intensity values for genes in these 2 signaling pathways in normal and MR left ventricles. The significant activation of cAMP-mediated and G-protein–coupled receptor signaling further supports the highly activated β-adrenergic signaling in the MR hearts.

**Downregulation of the Humoral Immune Response and Cytokine Signaling in MR Hearts**

We further analyze the regulation of inflammatory signaling pathway in the MR heart. Figure 5 demonstrates an extensive downregulation of humoral immune response and cytokine signaling in these patients with isolated MR and predominantly class I to II symptoms and well-preserved LVEF.

**Quantification of SLN and PLN Proteins in Normal and MR Hearts by Western Blot**

The increase in SLN transcription is further verified by a 6-fold increase in SLN protein in the MR left ventricle (Figure 6A and 6B), whereas PLN protein levels are not altered in the MR heart. The protein levels of SERCA2a, CSQ, and triadin are also unaltered in the MR left ventricle compared with the control group.

To determine the influence of age on the microarray results, we performed a linear regression analysis between SLN or PLN mRNA expression and age of the 35 patients. Figure IA and IB in the Data Supplement demonstrates no significant correlation between age and expression of SLN or PLN mRNA in MR hearts. To rule out the influence of sex on gene array results, SLN and PLN mRNA expression was compared between the male control (n=12) and male MR (n=24) hearts. Figures IIA and IIIA in the Data Supplement demonstrate that SLN and PLN mRNA expression is significantly higher in male MR versus male normal hearts. SLN and PLN mRNA expression was then compared in male and female patients. Figures IIB and IIIB in the Data Supplement demonstrate that SLN and PLN mRNA expression does not differ between male and female MR patients. Taken together, these results demonstrate that sex does not influence the expression of SLN or PLN.

**Immunofluorescence Staining of SLN**

Figure 6C demonstrates immunofluorescence staining of SLN in normal and MR heart. Protein expression of SLN (red) is
low in normal left ventricle, but dramatically increases in MR left ventricle. Furthermore, immunohistochemical costaining (Figure 6D) with SERCA2 (green) demonstrates that SERCA2 and SLN (red) are colocalized in MR heart.

**Discussion**

The results of the current investigation support the significant role played by the adrenergic nervous system and the expression and a potential functional role of SR Ca\(^{2+}\) regulatory protein SLN in the nonfailing left ventricle of patients with isolated MR. The new finding of SLN upregulation, which is normally an atrial-specific SERCA regulator, has important implications for its role in mediating human cardiac muscle SR Ca\(^{2+}\) transport and LV function in patients with isolated MR.

To gain a comprehensive insight into cellular processes in these patients with isolated MR and well-preserved LVEF, we imported the list of altered genes into IPA to predict the association between the set of altered genes and a related function. Statistical analysis for this process is presented in Figure 3, which demonstrates a significant activation of cardiac β-adrenergic signaling pathway (P=0.017). The height of the bars indicates the percentage of genes that changed in the particular pathway. Red bar indicates upregulated; and green bar, downregulated. Pathways (orange square and dotted line) to the right of the threshold (blue dashed line) are significantly activated. Heatmap represents the normalized signal intensity values for genes in the selected signaling pathway in normal controls (left) and MR patients (right). Red indicates higher expression; and blue, lower expression. **B**, Diagram of cardiac β-adrenergic signaling demonstrates that altered genes are centered in the control of Ca\(^{2+}\) flux. Green indicates decreased; and red, increased.
G-protein–coupled receptor signaling further supports the highly activated β-adrenergic signaling in MR hearts. As opposed to the end-stage dilated cardiomyopathy, where there is global decrease in β-adrenergic signaling, the current study includes MR patients with a well-preserved LVEF and a majority of patients presenting in class I or II NYHA heart failure (class I, n=18; class II, n=13; class III, n=4). It is of interest that previous studies have reported that in patients with isolated MR who largely present in class III to IV heart failure, there is a downregulation of SERCA2a LV protein levels and a negative correlation between SERCA2a protein levels and LV end-diastolic diameter. Thus, as with the failing

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**Figure 5.** Ingenuity pathway analysis (IPA) demonstrates the downregulation of canonical humoral immune response (A) and cytokine signaling (B) in mitral regurgitation patients. As in Figures 3 and 4, stacked bar charts demonstrate IPA-generated humoral immune response and cytokine signaling.
heart, the decompensated MR heart has decreased SERCA2a expression, whereas in our patients who largely presented in class I and II symptoms, β-adrenergic signaling is increased and SERCA2a mRNA and protein expression is unchanged at this earlier stage in isolated MR.

Among the 724 altered genes, SLN is the secondmost upregulated gene, increasing from a low intensity of 144 in normal left ventricles to 3085 in MR left ventricles. SLN is normally expressed at high levels in atria, and its expression is at very low/undetectable levels in the ventricles.24–29 However, the role of SLN in ventricular function in normal and diseased myocardium in human or in animal models is not understood. In patients with Takó-Tsubo cardiomyopathy, SLN is upregulated acutely in the dysfunctional LV segment and returns to normal at the 12-day recovery phase when LVEF returned to normal.30 Our study is the first to demonstrate SLN protein upregulation in the left ventricle of patients with isolated MR. Studies using transgenic and knockout mouse models suggest that SLN functions as a regulator of SERCA2a by lowering its Ca2+ affinity.25–30 These studies also show that the inhibitory function of SLN is independent of PLN. Thus, increased expression of SLN in MR hearts may be an earlier compensatory alteration in the setting of increased adrenergic drive. Nevertheless, the question remains whether ventricular expression of SLN is a detrimental effect because its expression, although to a lower level, is present in the basal state.

We previously reported xanthine oxidase, lipofuscin accumulation, and myofibrilar loss in patients with isolated MR,16 whereas in the current study, IPA pathway analysis demonstrates a significant downregulation of genes associated with acute phase response, in particular cytokines and the complement system (Figure 5). Oral et al31 report a relationship between tumor necrosis factor-α expression and severity of LV remodeling, suggesting that TNF-α, and an increase in cytokines in general, may occur in the decompensated LV in patients with isolated MR. In support of this contention, we previously reported gene array results from the volume overload of aortocaval fistula in the rat where 24-hour,32 2-week, and 15-week aortocaval fistula time points are marked by global inflammatory gene expression, whereas the 5-week interval was relatively quiescent for inflammatory gene expression.33 In the MR heart, there is extensive downregulation of humoral immune response and cytokine signaling in these patients with isolated MR and predominantly class I to II symptoms and well-preserved LVEF (Figure 6). Thus, the expression of inflammatory cytokines in the MR heart may be dependent on the clinical stage of LV remodeling or heart failure symptoms, whereas cardiomyocyte oxidative stress may be a persistent finding throughout the course of volume overload.

There are several potential limitations in the current study. In addition to a small sample size, the RNA and protein control samples are not obtained from the same hearts. To avoid potential differences of LV region on gene array results in MR hearts, biopsies were taken from the same region of the left ventricle in all patients in the lateral LV endocardial wall at the tip of the papillary. However, the mean age of the control group (33 years) was lesser than that of the MR group (55 years), which may have affected the results. In addition, heart samples from deceased donors may have unpredictable warm ischemic time before collection. To ensure reliability of microarray data and comparability of the expression profiles between normal and MR hearts, (1) RNA samples were screened by checking their integrity and genomic contamination. Only samples that met the requirement of an OD ratio of 260/280 >1.8 and 28S/18S >1.7 were selected for microarray processing. (2) The excellent homogeneity within control and MR cohort demonstrated by cluster heatmap (Figure 2A) and principal components analysis plot (Figure 2B) indicates the high quality of RNA samples used in this study. (3) The marked increase of cardiac hypertrophic markers, NPPA
and NPPB, in the MR heart compared with control samples 
ascertains the trustworthiness of the microarray data. Finally, 
mRNA and protein analysis was not performed on the same 
samples. However, for the purpose of defining the status of 
SLN, we think that similar RNA, protein, and immunohisto-
chemistry findings truly reflect the fact that the left ventricle 
have little SLN expression in control hearts and is markedly 
upregulated in MR heart.

The patient population of the current investigation was 
homogeneous in the marked degree of LV remodeling defined 
by MRI-derived volumes, preserved LVEF, all but 4 patients 
with class I to II symptoms, and no significant epicardial coro-
nary artery disease. However, the majority of patients were 
taking either β-blockers or renin–angiotensin system block-
ers. Unfortunately, the small number of patients did not allow 
for an evaluation of gene response in the face of drug therapy. 
Despite this variability in drug therapy, the gene array results 
clearly identify enhanced β-adrenergic receptor signaling and 
related cAMP and G-protein–coupled signaling. The new 
finding is the marked upregulation of SLN, a key regulator of 
atrial SERCA pump. Further work will fill the gap in knowl-
dge about the processes leading to cardiac dysfunction in 
response to volume overload, in particular the question of how 
SLN expression is related to Ca2+ homeostasis in the setting of 
increased adrenergic drive in isolated MR.

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**Disclosures**

None.

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## Supplementary Tables

### Supplementary Table 1. Primer sequences for validating microarray by real-time PCR

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The p-values are Benjamini-Hochberg FDR p-values.
Supplementary Figures

Supplementary Figure 1.

A

![Supplementary Figure 1A](image1)

B

![Supplementary Figure 1B](image2)

Supplementary Figure 2.

A

![Supplementary Figure 2A](image3)

B

![Supplementary Figure 2B](image4)

Supplementary Figure 3.

A

![Supplementary Figure 3A](image5)

B

![Supplementary Figure 3B](image6)
Supplementary Figure Legends

**Supplementary Figure 1. Regression analysis demonstrates SLN and PLN expression in the MR hearts are not influenced by age.** A, Correlation analysis of SLN signal intensity with patients’ ages (n=35). B, Correlation analysis of PLN signal intensity with patients’ ages (n=35).

**Supplementary Figure 2. Gender does not influence the expression of SLN in MR heart.** A, Comparison between male normal hearts (n=12) with male MR hearts (n=24) demonstrates SLN is significantly increased in MR patients. B, Comparison between male (n=24) and female (n=11) MR patients demonstrates no significant difference on SLN expression between the male and female patients.

**Supplementary Figure 3. Gender does not influence the expression of PLN.** A, Comparison between male normal hearts (n=12) and male MR hearts (n=24) demonstrates PLN is significantly increased in MR patients. B, Comparison between male (n=24) and female (n=11) MR patients demonstrates no significant difference in PLN expression between the male and female patients.