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# Increased Expression of Adenosine Triphosphate-Sensitive K<sup>+</sup> Channels in Mitral Dysfunction

## Mechanically Stimulated Transcription and Hypoxia-Induced Protein Stability?

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### Objectives

The aim of this study was to test whether adenosine triphosphate-sensitive K<sup>+</sup> (KATP) channel expression relates to mechanical and hypoxic stress within the left human heart.

### Background

The KATP channels play a vital role in preserving the metabolic integrity of the stressed heart. However, the mechanisms that govern the expression of their subunits (e.g., potassium inward rectifier [Kir] 6.2) in adult pathologies are mostly unknown.

### Methods

We collected biopsies from the 4 cardiac chambers and 50 clinical parameters from 30 surgical patients with severe mitral dysfunction. Proteins and messenger ribonucleic acids (mRNAs) of KATP pore subunits and mRNAs of their known transcriptional regulators (forkhead box [FOX] F2, FOXO1, FOXO3, and hypoxia inducible factor [HIF]-1 $\alpha$ ) were measured respectively by Western blotting, immunohistochemistry, and quantitative real-time polymerase chain reaction, and submitted to statistical analysis.

### Results

In all heart chambers, Kir6.2 mRNA correlated with HIF-1 $\alpha$  mRNA. Neither Kir6.1 nor Kir6.2 proteins positively correlated with their respective mRNAs. The HIF-1 $\alpha$  mRNA related in the left ventricle to aortic pressure, in the left atrium to left atrial pressure, and in all heart chambers to a decreased Kir6.2 protein/mRNA ratio. Interestingly, in the left heart, Kir6.2 protein and its immunohistochemical detection in myocytes were maximal at low venous PO<sub>2</sub>. In the left ventricle, the Kir6.2 protein/mRNA ratio was also significantly higher at low venous PO<sub>2</sub>, suggesting that tissue hypoxia might stabilize the Kir6.2 protein.

### Conclusions

Results suggest that post-transcriptional events determine Kir6.2 protein expression in the left ventricle of patients with severe mitral dysfunction and low venous PO<sub>2</sub>. Mechanical stress mainly affects transcription of HIF-1 $\alpha$  and Kir6.2. This study implies that new therapies could aim at the proteasome for stabilizing the left ventricular Kir6.2 protein. (J Am Coll Cardiol 2012;59:390–6) © 2012 by the American College of Cardiology Foundation

Mitral regurgitation (MR) is a common disease and increases in frequency with age: 19% by a mean age of 54 years in the Framingham cohort (1), and exceeding 80% by 80 years of age (2). Nearly 20% to 30% of patients experience MR after myocardial infarction (3). Structural changes

involving left ventricular geometry, the mitral annulus, and the valvular/subvalvular apparatus often appear with MR. They result in clinically significant valve incompetence (3), and malfunction of the valve itself due to tethering. When the insufficiency becomes more severe, surgical correction is often required, but not all patients can tolerate the surgical procedure (4). Consequently, a better understanding of cardiac adaptation to MR could lead to therapies for delaying the surgical intervention.

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The adenosine triphosphate-sensitive potassium (KATP) channels are beneficial in the adaptation to cardiac stress. They partly mediate ischemic pre-conditioning in human

myocardium (5,6). In humans, missense and frameshift mutations of the regulatory sulfonyl urea receptor (SUR) 2A subunit confer susceptibility to dilated cardiomyopathy (7) and adrenergic atrial fibrillation (8). The KATP channels play a vital role during vigorous exercise in mice and men as demonstrated, for example, in a study on transgenic mice with knockout of the pore-forming high conductance KATP-channel subunit potassium inward rectifier (Kir) 6.2, or over-expression of dominant negative Kir6.1 (9,10). These mice only tolerate one-half of the workload compared with wild-type mice, and one-half of them die. In children who have congenital heart disease (11), venous hypoxemia was an underlying factor for over-expression of forkhead box (FOX) O1, Kir6.1, and SUR2. In the infarct border zone of the rat left ventricle (LV), FOXO1 is linked to increased Kir6.1 expression (12), whereas in rat atrial myocytes, hypoxia-inducible factor (HIF)-1 $\alpha$  is causally involved in hypoxia-induced activation of FOXO1 (11).

Several important questions remain unanswered. Mechanical stress accompanies different cardiac pathologies, but its role in Kir6.1 and Kir6.2 expression is still unknown. The signaling pathway for Kir6.2 expression is not established, and intriguingly, during venous hypoxemia, no correlation exists between Kir6.2 mRNA and Kir6.2 protein (11). Moreover, the signaling pathways may differ between right atrial Kir6.1 in children with congenital heart disease, and left ventricular Kir6.2 in adults with MR. In the present study involving MR patients, we focused on identifying the most important, potential determinants of KATP-channel pore expression. We related biochemical measurements on cardiac biopsies to blood pressures and sizes of the cardiac chambers, and to blood gases and blood chemistry. The results suggest that mechanical stress activates transcription of Kir6.2 mRNA, whereas tissue hypoxia likely operates through post-transcriptional mechanisms by inhibiting Kir6.2 protein degradation.

## Methods

**Experimental approach.** We conducted this study on a surgical patient population with MR because specific protein and mRNA contents of cardiac biopsies can be related to a large array of clinical data. This approach also has the advantage of finding a broad range of values of the clinical parameters within a relatively homogenous patient population. Highly significant correlations emerging from the statistical analysis thus point to novel potential determinants of KATP subunit expression. Although Kir6 knockout mice with various degrees of MR would be an interesting model, mice models of MR do not yet exist. A rat model of MR has been established (13), but as in mice, fewer parameters are measurable when compared to human studies.

**Patient population.** This study reports results from 30 adults (22 women, 8 men), ages 60 to 79 years (and 1 at age 45 years). The patients had severe mitral dysfunction accompanied by mild to moderate tricuspid valve disease, with indications for mitral and tricuspid valve repair. They

were prospectively enrolled between January 2007 and June 2009. All patients gave their informed written consent to have myocardial biopsies performed and specimens used for experimentation. This study was approved by the local institutional ethics committee (MU 301390-07).

**Tissue samples.** All myocardial biopsies were performed during cardiopulmonary bypass, immediately after cardioplegic cardiac arrest. A Scholten Bioprote (Scholten Surgical Instruments, Lodi, California) was used for the left and right ventricle specimens (3-mm-thick samples weighing between 10 mg and 25 mg) obtained close to the base of the papillary muscles. A strip 4 mm in length of left and right atrial free walls was excised at the atriotomy level.

**Protocol.** At the beginning of anesthesia, artificial respiration was switched during 5 min from 50% to 21% oxygen. After this equilibration—designed to reach the blood gas levels presumably prevailing before anesthesia—venous and arterial blood samples (0.5 ml) were withdrawn for immediate analysis of pH, blood gases, and plasma concentrations of hemoglobin, glucose, lactate, Ca<sup>++</sup>, Na<sup>+</sup>, K<sup>+</sup>, Cl<sup>-</sup>, and calculated bicarbonate (Online Table 1). Blood pressures were measured in all cardiac chambers and aorta. Part of the biopsies (see the preceding text) was collected in liquid nitrogen for protein and RNA extractions, and part was washed in phosphate-buffered saline and fixed in 3% paraformaldehyde for histology.

**Data.** We collected data on a total of 50 parameters such as age, sex, echocardiographically determined size and thickness of all cardiac chambers (obtained during pre-operative consultation), and parameters cited previously (Online Table 1). Comparison of the effect of MR on hemodynamic parameters with normal values (14) indicates an increase of right and left atrial pressures (Online Table 1), in accordance with reported values (15–17).

**Western blot tests.** Pieces of right atrium (RA), left atrium (LA), right ventricle (RV), and LV tissues were placed in homogenization/lysis buffer (25 mM Tris, 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid [1% vol/vol], TritonX-100, pH 7.4, supplemented with protease inhibitor cocktail [Roche, Basel, Switzerland]), and was further homogenized, centrifuged, and processed as described (12). Supernatants were loaded on a polyacrylamide-sodium dodecyl sulfate gel (10%), and blotted (12). Loading controls were performed by red Ponceau staining. Reference samples were co-applied in all gels to allow for intergel comparisons of Kir6 signals. Polyclonal goat anti-Kir6.1 antibody (R14 SC-11224) and anti-Kir6.2 (G16 SC-11228,) were diluted 1/400 and

## Abbreviations and Acronyms

<b>FOX</b>	= forkhead box
<b>HIF</b>	= hypoxia-inducible factor
<b>KATP</b>	= adenosine triphosphate-sensitive potassium
<b>Kir</b>	= potassium inward rectifier
<b>LA</b>	= left atrium
<b>LV</b>	= left ventricle
<b>MR</b>	= mitral regurgitation
<b>mRNA</b>	= messenger ribonucleic acid
<b>RA</b>	= right atrium
<b>RV</b>	= right ventricle
<b>SUR</b>	= sulfonyl urea receptor

1/300, respectively. Specificity of antibodies was verified as described (11). Quantification of the detected proteins was performed by scanning and with MetaMorph software (Universal Imaging, Downingtown, Pennsylvania).

**RNA extraction, reverse transcription, and real-time PCR.** After RNA extraction and reverse transcription, real-time PCR was performed with specific primers for human KATP channel subunits Kir6.1, Kir6.2, SUR2A, SUR2B, and transcription factors FOXO1, FOXO3, FOXF2, and HIF-1 $\alpha$ . The primers were designed by Primer Express software, version 2.0 (Applied Biosystems, Foster City, California), and synthesized by Mycosynth (Balgach, Germany) (Online Table 2), as described (12). Standard curves were established with a mix of all samples, and values normalized by hypoxanthine phosphoribosyl transferase mRNA, which was chosen as the representative normalization gene, as in previous human studies (11).

**Histology and immunohistochemistry.** After fixation, tissues were embedded in paraffin, and 5- $\mu$ m-thick sections were cut and fixed on glass slides. Representative sections from each sample were stained with hematoxylin and eosin for light microscopy. Immunohistochemical studies were carried out using a rabbit polyclonal antibody recognizing Kir6.2 (Ab79171, Abcam, Cambridge, England) that was biotinylated using EZ-Link Biotinylation Kits (Thermo Fisher Scientific, Rockford, Illinois) as described (18). Immunoreactivity was intensified by pressure cooker treatments for 5 min in citrate buffer 10 mM, pH 6.0. To quench endogenous peroxidase, slides were incubated with methanol containing 0.5% hydrogen peroxide for 10 min. Sections were then incubated with anti-Kir6.2 (1/100) for 1 h, followed by monitoring with the EnVision Kit (Dako, Glostrup, Denmark) with a high-sensitivity alkaline-phosphatase-based second-generation visualization. This treatment contains 3 steps interspersed with rinsing in wash buffer: 30 min incubation with the Linker solution, 30 min with the Enhancer solution, and 8 min with the substrate added permanent red chromogen. Slides were counterstained with Hemalun and mounted in Eukitt (Kindler, Freiburg, Germany).

**Image analysis.** For quantitative immunohistochemistry, sets of images were acquired with a high-sensitivity camera of a Mirax system (Carl Zeiss, Jena, Germany) using a Plan-Apochromat 20x/0.8 objective. In the scanning process, the preview camera captured an overview of the whole sample, and the software determined the scanning areas containing the tissue areas. The unstained portion of the specimen automatically discriminated the immunostaining. Sixty sections and their negative controls were scanned and studied with MetaMorph software (Universal Imaging). Pixels were selected according to hue (dominant color tone), lightness (color intensity), and saturation (color purity) components (19). The negative control, in which only the second antibody was applied, was used to determine the threshold of positive staining above the background. Cardiomyocytes, connective tissue, adipose tissue, and layers of vessels were manually circumscribed using MetaMorph

software. Two calculations were performed: the ratio of the sum of the Kir6.2-positive areas of all types relative to the total area; and the percentage of the average integrated intensity of each region (cell type) relative to the integrated intensity of all regions.

**Statistical analysis.** Data are presented as mean  $\pm$  SEM, with “n” representing the number of patients. Mean values obtained were compared by the unpaired analysis of variance test with a Tukey method for post-hoc comparisons. Parameters and levels of proteins and mRNAs were classed by rank. Linear and second-degree polynomial correlations were performed on ranks with SPSS software (Chicago, Illinois) and/or Origin 6.0 software (OriginLab Corp., Northampton, Massachusetts). To minimize type-1 errors (false positives) due to multiple tests, we mainly considered pre-planned correlations in data interpretation. Such correlations involve the oxygen levels, parameters related to mechanical stress, and transcription factors FOX and HIF-1 $\alpha$ . Figures 1, 2, 3, 4, and 5 display color-coded ranges of p levels; Online Figures 1 through 6 indicate exact p, N, and R. We do not show non-pre-planned correlations, such as those involving serum levels of glucose and electrolyte. There were no sex-specific differences in outcome.

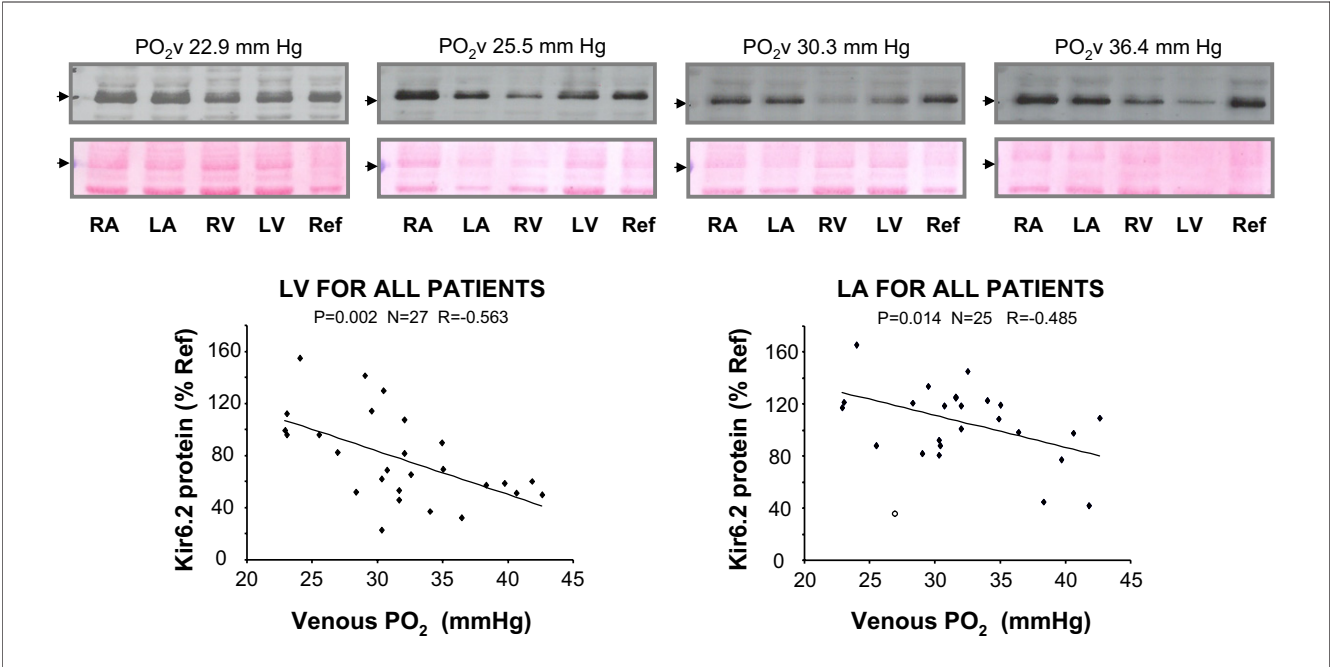
## Results

**Expression of left heart Kir6.2 protein depends on venous PO<sub>2</sub> but not Kir6.2 mRNA.** Quantification by Western blot of LV and LA Kir6.2 protein indicates that the high expression correlates with low venous PO<sub>2</sub> (Fig. 1). Because tissue extracts contain not only cardiomyocytes but also blood vessels, connective tissue, and adipocytes, tissue sections were immunostained for Kir6.2 by a sensitive technique (Fig. 2, Online Fig. 1). The morphometric analysis indicates that most immunoreactive Kir6.2 is contained in cardiomyocytes. Morphometry of tissue areas with Kir6.2 containing myocytes from LV and LA from 15 patients shows a strong negative correlation of cardiomyocyte Kir6.2 staining with increasing venous PO<sub>2</sub> (p < 0.01, R = -0.544), indicating that the Western blots mirror the Kir6.2 content of cardiomyocytes. The adipocyte content of LA and LV is negligible, and the minor amount of Kir6.2 staining in connective tissue or blood vessel walls does not correlate with decreasing venous PO<sub>2</sub> (p > 0.6).

A statistical analysis of left heart mRNAs and proteins (Fig. 3, Online Fig. 2) shows that Kir6.2 mRNA bears no significant correlation with venous PO<sub>2</sub>. The Kir6.2 protein does not depend on arterial PO<sub>2</sub>, and the Kir6.1 protein correlates with neither arterial nor venous PO<sub>2</sub>. In the right heart, neither Kir6.1 nor Kir6.2 protein correlates with arterial or venous PO<sub>2</sub> (Online Fig. 3).

**Kir6.2 mRNA relates to HIF-1 $\alpha$  mRNA expression.** In all heart chambers, Kir6.2 mRNA strongly associates with HIF-1 $\alpha$  mRNA (Fig. 3, Online Fig. 3). The SUR2B mRNA expression also strongly and positively associates



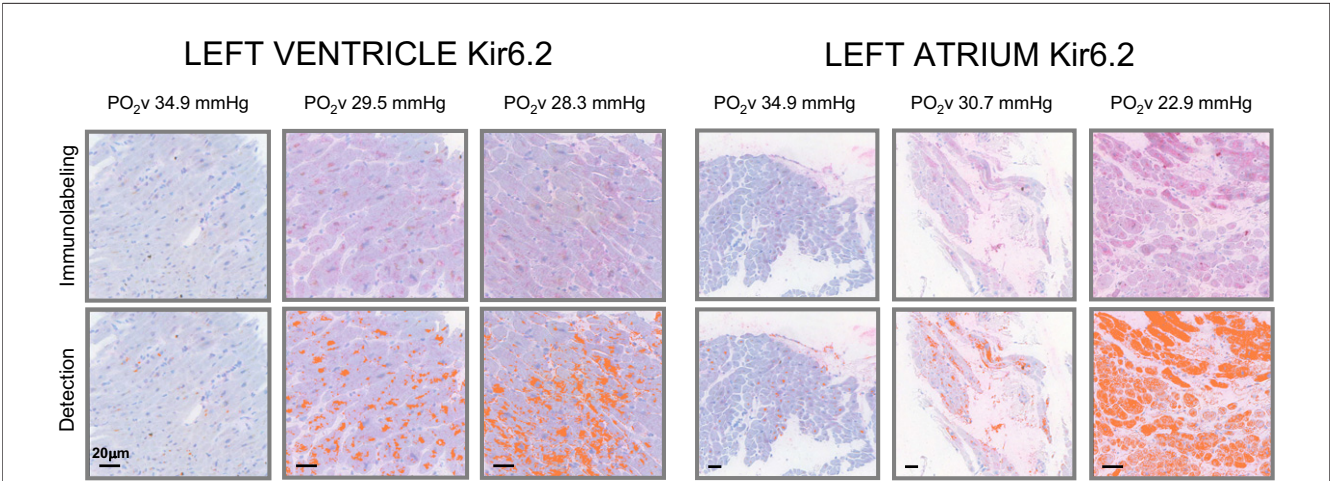


**Figure 1** Expression of Kir6.2 Protein in Left Heart at Different Venous PO<sub>2</sub>

(Top) Examples of Western blots of potassium inward rectifier (Kir) 6.2 protein of right atrium (RA), left atrium (LA), right ventricle (RV), and left ventricle (LV) for 4 patients, with various venous PO<sub>2</sub>. Red Ponceau staining mostly shows equal loading of wells. (Bottom) Significant inverse correlations in left heart of all patients between Kir6.2 and venous PO<sub>2</sub> (lowest LA not considered). Correlations for right heart were not significant (not shown).

with HIF-1 $\alpha$  mRNA ( $p < 0.01$ ) (Online Fig. 4). In addition, both left heart Kir6.1 and Kir6.2 mRNA correlate with one of the FOXOs. Strikingly, HIF-1 $\alpha$  mRNA does not correlate at all with venous or arterial PO<sub>2</sub>, raising the question of whether other clinical parameters might be underlying factors for HIF-1 $\alpha$  mRNA expression.

**Expression of LV HIF-1 $\alpha$  and Kir6.2 mRNA relates to mechanical and echocardiographic parameters.** Left ventricular HIF-1 $\alpha$  mRNA expression significantly associates with systolic aortic pressure and size of the LV, while LV Kir6.2 mRNA correlates with both systolic and diastolic aortic pressure (Fig. 4, Online Fig. 5). The LA HIF-1 $\alpha$  expression



**Figure 2** Immunohistochemistry and Detection of Kir6.2 in Tissue Sections

(Top) Representative regions show tissues immunolabeled with anti-potassium inward rectifier (Kir) 6.2 antibody, (Bottom) The same tissues where all cells with Kir6.2 labeling above threshold were evenly stained using MetaMorph software. The pairs of panels of different patients are displayed in descending order of venous PO<sub>2</sub>. Note the increased staining at low PO<sub>2</sub>.

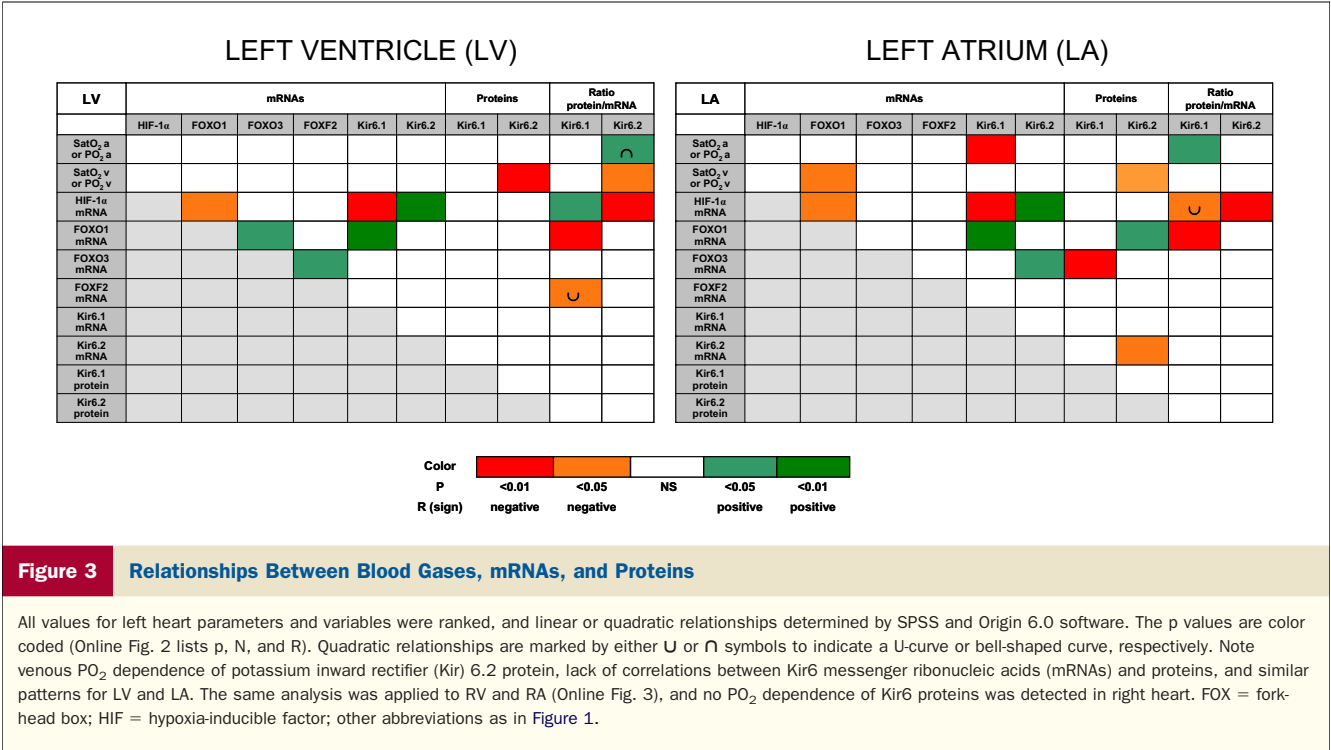


Figure 3

Relationships Between Blood Gases, mRNAs, and Proteins

correlates with LA wedge pressure. In LA, however, Kir6.2 mRNA inversely correlates with LA cross-section and systolic ventricular pressure. In striking contrast, the right heart HIF-1α and Kir6 mRNA expressions are barely affected by mechanical or echocardiographic parameters, but there are prominent associations between these parameters and RA FOXO mRNA and Kir6 proteins (Online Fig. 6).

**Kir6.2 protein/mRNA ratios relate to venous PO<sub>2</sub> and HIF-1α.** The ratio of Kir6.2 protein/Kir6.2 mRNA is of interest to quantify the dissociation between mRNA and protein. Indeed, the LV ratio for Kir6.2 significantly increases at low venous PO<sub>2</sub> (p = 0.024, R = −0.441, n = 26). As Kir6.2 mRNA is unchanged at low venous PO<sub>2</sub> (Fig. 3), this increased ratio indicates an increased protein

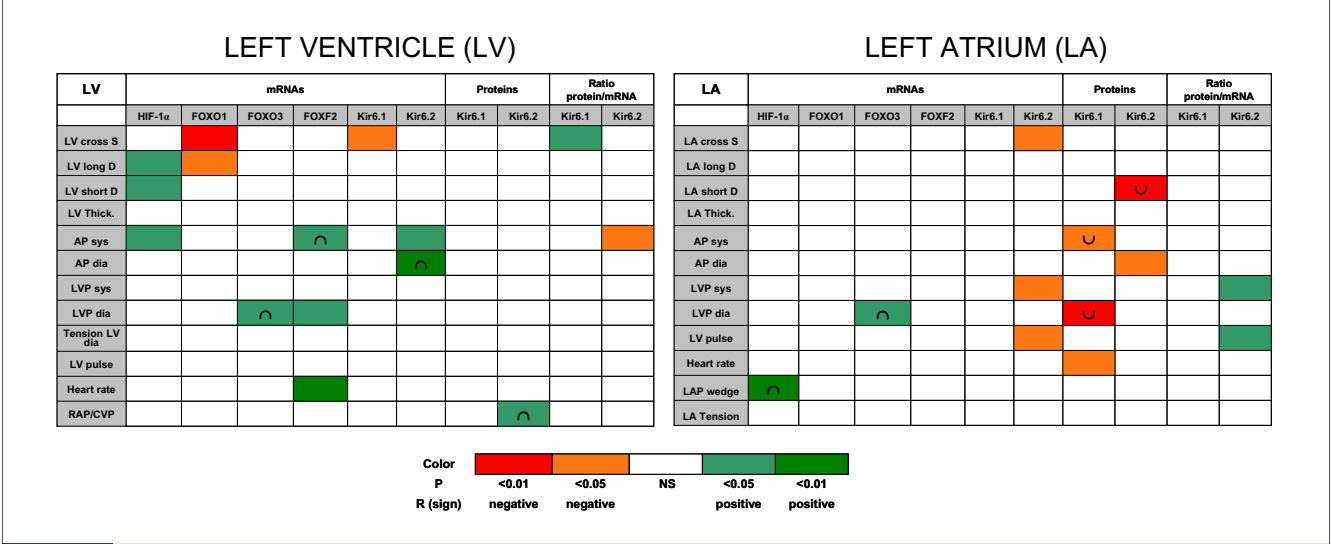
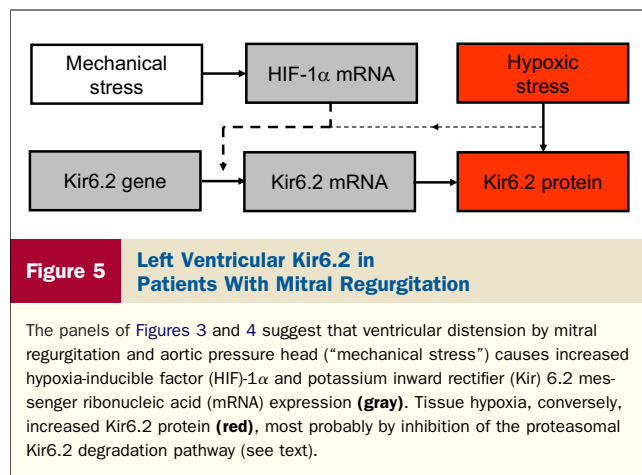


Figure 4

Relationships Between Mechanical and Echocardiographic Parameters, mRNAs, and Proteins



accumulation. Conversely, the decreased ratio observed with increased HIF-1 $\alpha$  and Kir6.2 mRNA expression (Fig. 3) indicates that LV Kir6.2 protein does not change significantly with changes in HIF-1 $\alpha$  mRNA.

## Discussion

Increased KATP channel expression is of vital importance during cardiac stress (5,6,9,10), but the mechanisms for this expression are mostly unknown. This is the first study to focus on the expression of KATP channel pore subunits and relevant transcription factors within the 4 chambers of the human heart, exposed, due to MR, to considerable mechanical and hypoxic solicitations. The latter were sufficiently severe to lead to surgical valve repair in the 30 patients of this study.

**Salient features of LV Kir6.2 expression.** The main results on LV suggest an interesting dual, transcriptional and post-translational regulation of Kir6.2 expression (Fig. 5). The Kir6.2 protein expression appears to be dictated by tissue hypoxia, independently of Kir6.2 mRNA expression. The hypoxia results from low cardiac output (20) and is revealed by a low venous PO<sub>2</sub>. Mechanical factors, in contrast, determine the Kir6.2 mRNA expression but do not directly affect Kir6.2 protein. The HIF-1 $\alpha$  is a candidate mediator of increased Kir6.2 gene transcription in response to mechanical stress. Bioinformatics indeed indicates a binding site for HIF-1 $\alpha$  on the Kir6.2 gene promoter (but not on the Kir6.1 gene; results not shown).

The mechanism for increased LV Kir6.2 protein expression, due to hypoxic stress, most likely results from hypoxic inhibition of the proteasomal degradation pathway of Kir6.2 (21–23). Alternatively, hypoxia, although usually an inhibitor of protein translation, may increase Kir6.2 translation, as shown for vascular endothelial growth factor (24). However, no studies have yet been performed for Kir6.2 translation efficiency. In addition to increased protein stability of Kir6.2 in hypoxia, repression of transcription by excess protein (25) might explain the striking dissociation of Kir6.2 protein and mRNA. Precedents of such dissociation

have previously been noted for other potassium channels (26) and regulatory proteins (27).

**Common and distinct features for the regulation of cardiac KATP- channel expression.** This study has resulted in the analysis of nearly 1,700 relationships among expression of KATP channels, relevant transcription factors, pressures, partial blood gas pressures, blood chemistry, and echocardiographic parameters. For the whole heart, 138 of the 812 relationships shown in this article are significant at the <0.05 or <0.01 level. Of 34 significant relationships in the LV, 12 are also found in the LA. Of 22 significant relationships in the RV, 10 are also found in the RA. The HIF-1 $\alpha$ -Kir6.2/SUR2B mRNA pathway is unique in being common to all heart chambers. The high percentage of significant associations common to different heart chambers supports the notion that the number of type-1 errors were minimal in pre-planned correlations.

The LA Kir6.2 expression follows a similar pattern as shown for the LV (see preceding discussion), such as hypoxic control of Kir6.2 protein expression, the potential influence of mechanical stress on HIF-1 $\alpha$  mRNA, and the correlation of HIF-1 $\alpha$  with Kir6.2 and SUR2B mRNA. Another common feature is the FOXO1-Kir6.1 mRNA axis and potential effects on the Kir6.1 protein/mRNA ratio. The differences relative to the LV are that the LA Kir6.2 mRNA is negatively linked to the LV systolic and pulse pressure, and mechanical and transcription factors in the LA negatively affect Kir6.1 protein expression.

The regulation of left heart Kir6.1 expression appears to be more complex than for Kir6.2. Transcription factor HIF-1 $\alpha$  is negatively linked to FOXO1 and Kir6.1 mRNA expression, possibly resulting in opposite effects on Kir6.1 relative to Kir6.2. As inferred from previous studies (28,29), the potential implication is a reduced dominant negative influence of Kir6.1, and thus, an enhanced Kir6.2 expression and channel pore conductance.

In the right heart, 8 of 15 significant relationships found between mRNAs in the RV are also true in the RA. These concern the HIF-1 $\alpha$ -FOXO3-Kir6.2 mRNA axis leading to a decreased Kir6.2 protein/mRNA ratio, and the associations among FOXO1-FOXO3, FOXO3-SUR2A/B, and HIF-1 $\alpha$ -SUR2B mRNAs. Although few correlations emerge with FOXF2 in left heart, RA KIR6.1 protein is highly and negatively associated with FOXF2 mRNA. However, no mechanical parameters appear to drive RA FOXF2 transcription; thus, the role of FOXF2 in stress-induced KATP channel expression remains unknown.

## Conclusions

This study tests the hypothesis that hypoxic and mechanical stress determine expression of KATP channel subunits, by taking advantage of the wide range and large array of clinical parameters in surgical patients. We encountered unexpected findings. Most importantly, LV Kir6.2 protein but not mRNA expression depends on tissue hypoxia. Conversely, LV Kir6.2

mRNA but not protein links to mechanical stress, most probably through the transcription factor HIF-1 $\alpha$ . Other studies could explain the lack of correlation between Kir6.2 mRNA and protein, for example, by a hypoxia-induced increased stability of Kir6.2 protein, or repression of Kir6.2 gene transcription by excess Kir6.2. Although an HIF-1 $\alpha$ -Kir6.2 mRNA axis is also detectable in the right heart, the right heart HIF-1 $\alpha$  mRNA does not respond to mechanical stress, or alternatively, the mechanical stress is not sufficient to activate HIF-1 $\alpha$  mRNA expression.

The clinical implications of these findings are 3-fold. First, as gauged by the expression of LV Kir6.2 protein, venous hypoxemia clearly is a sensitive marker of hypoxic cardiac stress in MR. The measurement of venous PO<sub>2</sub> is available to many clinicians confronted with cardiac pathologies. Second, the proteasome could represent a pharmacological target for delaying the surgical therapy of MR. And third, clinicians will be increasingly aware of possibly distinct regulations of mRNA and protein expression in heart disease.

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**Key Words:** ischemia ■ Kir6.2 expression ■ mechanical stress ■ mitral regurgitation ■ transcription.

#### APPENDIX

For supplemental tables and figures, please see the online version of this article.