Gender Modulates the Expression of Calcium-Regulating Proteins in Pediatric Atrial Myocardium

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A differential expression of sarcoplasmic reticulum calcium-ATPase (SERCA2a) and phospholamban (PLB) characterizes the remodeling process in heart failure and atrial arrhythmias in adult patients. Gender is known to modulate the course and prognosis of different forms of heart disease. We hypothesized that gender plays a role in molecular changes of myocardial calcium regulating components already in childhood. Moreover, we studied the influence of volume overloaded (VO) on SERCA2a and PLB in pediatric patients. Quantitative reverse transcription-polymerase chain reaction was used to measure mRNA expression of SERCA2a and PLB in atrial myocardium from 30 pediatric patients (12 girls, 18 boys). Eighteen patients had VO right atria, and 12 patients had not-overloaded atria (NO). Protein expression was studied by Western blot. In the entire population, SERCA2a and PLB expression was not different between girls and boys. If hemodynamic overload was taken into account, SERCA2a mRNA expression was significantly reduced in the VO group compared with the NO group ($P = 0.021$). The VO versus NO difference was restricted to boys, which corresponds to a highly significant interaction of gender versus VO status ($P = 0.002$). The PLB to SERCA2a protein ratio was significantly lower in girls ($P = 0.028$). The decrease in SERCA2a mRNA expression in VO atrial myocardium and the PLB to SERCA2a ratio of protein expression was modulated by gender in this pediatric population. To our knowledge, this study is the first to show the impact of gender on the differential expression of calcium-regulating components in pediatric cardiac patients. Exp Biol Med 230:853–859, 2005

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Introduction

Congenital abnormalities of the heart are the most common types of birth defects, occurring in about 8 per 1000 live births (1). Ca^{2+} is a key component for the regulation of cardiac contractility, relaxation, and intracellular signaling. One of the main regulators of the cytosolic Ca^{2+} level is the sarcoplasmic reticulum calcium-ATPase (SERCA2a). Its remodeling in different forms of myocardial overload and disease (2) has been shown to be the main factor for the elevated cytosolic Ca^{2+} (3, 4). Phospholamban (PLB) is closely linked to cardiac function, as it is determining the SERCA2a activity (5). It has been shown that PLB ablation leads to enhanced contractility (6), whereas an overexpression leads to reduced contraction (7). Moreover, the regional differences in Ca^{2+} handling and the resulting longer duration of contraction in ventricle compared with atrium might be the functional consequence of the regionally different PLB and SERCA2a expression in the human heart (8). To date, SERCA2a and PLB have been studied in different animal models and in adult humans, mainly with end-stage heart failure. Having in mind their developmental regulation in different species (9–11) and their species specificity (12), we investigated if SERCA2a and PLB expression might be changed already in childhood. It has been shown that molecular events (13), morbidity, and mortality in various forms of heart disease are influenced by gender (14). For example, premenopausal women have a better prognosis after myocardial infarction than male patients (15). We studied the influence of gender on the susceptibility of the atrial myocardium to volume overload (VO). Information on the molecular changes of SERCA2a and PLB could be of relevance for patients as it could serve as an early marker for disease and future complications. A better understanding of the molecular basis of myocardial

Table 1. Nucleotide Sequence of the Primers Used in the Study

Gene			Product length (bp)[a]
PLB	fwd	TCACAGCTGCCAAGGCTACC	204
	rev	TAGATTCTGTAGCTTTTGACGTGC	
SERCA2a	fwd	AGCGGTTACTCCAGTATTGCAG	188
	rev	CTGTCCATGTCACTCCACTTCC	

[a] bp, base pairs; fwd, forward primer; rev, reverse primer.

disease in children with congenital heart defects may improve decision making in these patients.

Materials and Methods

Patients. A total of 30 patients who were undergoing open heart surgery were studied. In all patients the surgical procedure required a right atriotomy, and tissue samples of the right atrial free wall were obtained during the surgery. An echocardiography (Acuson Sequoia 512; Siemens Medical Solutions, Mountain View, CA) was preoperatively performed, and the areas of the right atrium (RA) and left atrium (LA) were calculated in all patients. According to the atrial dimensions and the ratio between the RA area and the LA areas, the patients were assigned to a group having an RA VO and a group with not-overloaded (NO) RA. All parents of the patients gave written informed consent. The study protocol has been approved by the local ethics committee (request 168/2000).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Quantitative RT-PCR was used to measure mRNA expression of SERCA2a and PLB and defined as arbitrary units (1 unit corresponding to 1 mRNA molecule per 10,000 28S rRNA molecules). Atrial myocardium, which was removed during corrective surgery, was immediately snap frozen in liquid nitrogen and stored at –80°C until further processing. RA myocardium (10 mg) was used for the extraction of total cellular RNA. Frozen samples were homogenized with a mortar and pestle, and the lysate was passed 10 times through a 20-gauge needle fitted to a syringe. DNase I treatment was performed, and RNA extraction was performed with the RNeasy Mini-Kit (Qiagen, Hilden, Germany) using the protocol for heart tissue, including proteinase K digestion. The amount of isolated RNA was determined, specifically measuring RNA concentration according to the manufacturer's instructions, using RiboGreen dye (Molecular Probes, Eugene, OR) in a fluorescent assay. For quantitative 1-step RT-PCR, a OneStep RT-PCR Kit (Qiagen) was used. Two µl (~20 ng) of total RNA were reverse transcribed and directly amplified in the glass capillary of a LightCycler system (Roche, Rotkreuz, Switzerland). Online fluorescence monitoring was performed by adding SYBR-Green I (Sigma-Aldrich Corp., St. Louis, MO). The final reaction volume was 20 µl, and final reaction concentrations were as follows: 1× OneStep RT-PCR Buffer, 400 µ*M* of each dNTP, 0.6 µ*M* of each primer, 7.5 units RNase inhibitor (Roche), freshly prepared SYBR-Green I, 1:2105 diluted in H_2O (v/v), and 1 µl of OneStep RT-PCR Enzyme Mix. LightCycler (Roche) conditions were as follows: reverse transcription, 30 mins at 50°C; initial RT inactivation and polymerase activation step, 15 mins at 95°C; PCR rapid cycling for 40 cycles (denaturation at 95°C for 30 secs, annealing at 58°C for 30 secs, elongation at 72°C for 15 secs, and final melting curve analysis from 58°C to 95°C with a slope of 0.1°C/sec). Fluorescence was measured at the end of the elongation phase. LightCycler (Roche) fluorescence settings were F1 Gain 5 and Channel setting F1.

Primer Design. The primer pairs were designed to discriminate between the different isoforms. Each primer pair contained at least one exon-exon–bound spanning primer to prevent amplification of contaminating genomic DNA. The calculated annealing temperatures were identical for both primer pairs. Building of primer dimers was excluded using software OLIGO 6.1 (MedProbe, Oslo, Norway). The size of the amplicons was 204 bp for PLB and 188 bp for SERCA2a, respectively (Table 1).

PCR Product Characterization. In addition to the quantitative analysis of PCR product amplification and melting curve analysis using the LightCycler (Roche) system, RT-PCR products were analyzed by polyacrylamide gel electrophoresis (PAGE) and silver staining to confirm amplification specificity (Fig. 1). Reagent samples without template were included to identify contamination. Additional confirmation of product identity was performed by

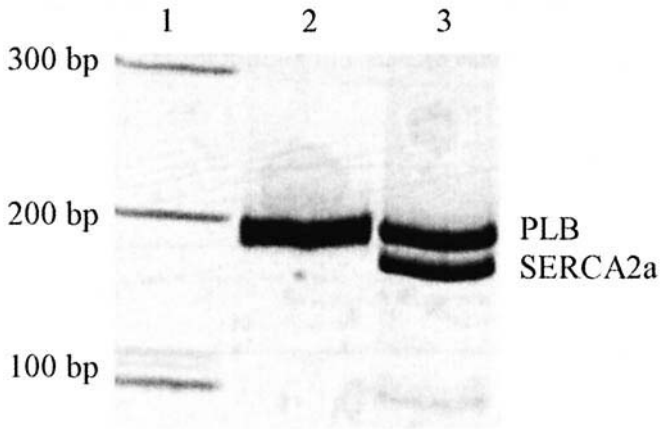

Figure 1. The PAGE of the RT-PCR products. (Lane 1) Molecular weight marker. (Lane 2) 28S rRNA transcript (control). (Lane 3) PLB and SERCA2a transcripts.

sequencing of forward and reverse strands with the PRISM Ready Reaction Dye Deoxy Terminator Sequencing Kit (Perkin-Elmer/ABI, Huenenberg, Switzerland) on an ABI 373 DNA Sequencer.

Protein Analysis. For preparation of myocardium for protein analysis, we used a modification of a previously described method (16). The frozen atrial tissue (10 mg) was pulverized in a precooled steel mortar and suspended in 50 µl homogenization buffer containing 30 m*M* Tris-HCl (pH 7.6), 2 m*M* EDTA, 0.6 *M* NaCl, protease-inhibitor mix (10 µg/ml each of leupeptin, aprotinin, and antipain and 2.5 µg/ml of benzamidine), and 1 m*M* phenylmethylsulfonyl-fluorid (PMSF). The resulting homogenates were extracted with 0.5% Triton X-100 on ice for 30 mins. To precipitate myofibrillar proteins, the sample was diluted with dilution buffer (30 m*M* Tris-HCl [pH 7.6], 2 m*M* EDTA, protease-inhibitor mix, and 1 m*M* PMSF) to a final concentration of 0.1 *M* NaCl. After centrifugation at 10,000 *g* for 20 mins at 4°C, the supernatant was collected. The protein concentration was determined by the Lowry method. For immunoblot of SERCA2a and PLB, 5 µg protein was resolved on a 12% SDS-polyacrylamide gel in the Mini-Protean II Cell-system (Bio-Rad, Hercules, CA). Subsequently, proteins were electroblotted onto a PVDF membrane (No. 162-0182; Bio-Rad), and nonspecific binding was blocked by 5% nonfat milk in Tris-buffered saline with 0.1% Tween 20. The blots were incubated with two monoclonal antibodies: antiSERCA2a (1:1000 dilution; Affinity Bioreagents, Golden, CO) and anti-PLB (1:500; Affinity Bioreagents). For detection of immune-complex formation, incubation of the blots with a secondary antibody (anti-mouse IgG, 1:2000) labeled with horseradish peroxidase was performed. After the chemiluminescence reaction (ECL-Kit; Amersham Biosciences Europe, Dübendorf, Switzerland), the blots were exposed on x-ray film. For evaluation of the method, the same amount of protein from each patient was loaded and separated on the polyacrylamide gel. The densitometric intensity of the bands after immunoblotting was expressed as arbitrary units. Linear regression of the amount of loaded protein and densitometric intensity showed a good linearity in the range of 0–20 µg protein loaded ($r^2 = 0.94$; Fig. 2).

Statistical Analysis. The levels of mRNA and protein are expressed as median (range). Because of the small sample size and skewed data distribution, nonparametric exact methods were used throughout. The three clinical variables of gender, VO versus NO, and infants up to 12 months of age versus older children were used to form groups of patients. For differences of mRNA and protein levels among the groups, their Hodges-Lehmann estimates and exact 95% confidence intervals (CI) were calculated and exact Wilcoxon-Mann-Whitney tests applied. For analysis of gender-overload interactions and multivariate analysis of the three clinical variables, exact polytomous regression applying the adjacent category logit model was used. This is

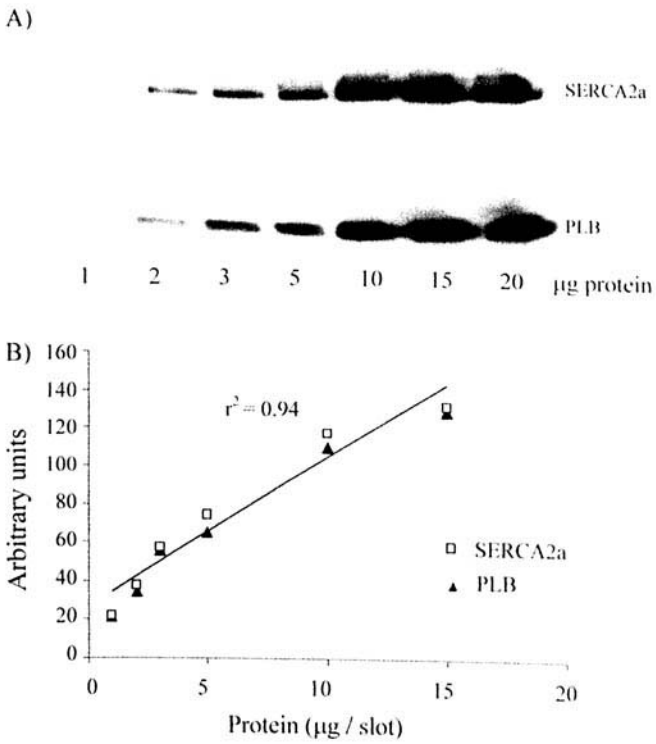

Figure 2. An immunoblot of SERCA2a and PLB. (A) The densitometric intensity of the bands after immunoblotting was expressed as arbitrary units. (B) Linear regression of the amount of loaded protein and densitometric intensity showed a good linearity in the range from 0–20 µg protein loaded ($r^2 = 0.94$).

a nonparametric exact method suited to the analysis of this small dataset, corresponding functionally to the well-known multivariate linear regression that is applicable in larger datasets. Continuously measured mRNA and protein levels were transformed into ordinal variables with eight levels each to allow for regression analysis. Results of the regression analysis are expressed as beta-regression coefficients with their exact 95% CI. Two-sided tests were used throughout, and P levels <0.050 were considered statistically significant. Because of the exploratory nature of the study, no correction for multiple comparisons was performed.

Results

Patients. Thirty patients (12 girls and 18 boys) were included in the study. Eighteen patients had a congenital heart defect with left-to-right shunt at atrial level, causing RA VO. Twelve patients had NO heart defects of the RA (Table 2). Gender distribution was not significantly different between the VO and NO groups (girls, 7 VO and 5 NO; boys, 11 VO and 7 NO; Fisher's exact test; $P = 0.71$). Clinical and echocardiographic data are summarized in Table 3. The RA area per m^2 body surface area (RA [cm^2]/m^2) was significantly higher ($P = 0.002$) in the VO group (median, 20.4; range, 6.2–38.8) compared with the NO group (median, 12.4; range, 5.3–26.4). The difference between the VO and NO groups remained significant if the population was divided in boys and in girls. The ratio of

Table 2. Heart Defects

	VO	NO
n	18	12
ASD I[a]	2	0
ASD II[b]	11	0
AVSD[c]	3	0
SV ASD	1	0
VSD	1	7
PS	0	3
DORV	1	2
TOF	0	2
TI	2	0
TAPVR	1	0

[a] ASD I, primum type.
[b] ASD II, secundum type.
[c] AVSD, atrioventricular septum defect; SV ASD, sinus venosus atrial septal defect; VSD, ventricular septum defect; PS, pulmonary stenosis; DORV, double outlet right ventricle; TOF, tetralogy of Fallot; TI, tricuspid insufficiency; TAPVR, total anomalous pulmonary venous return.

the RA area and the LA area (RA:LA) was significantly higher in the VO group compared with the NO group in girls ($P < 0.0001$) and in boys ($P < 0.0001$). The types of diagnoses, age, weight, degree of VO, and length of hospital stay was not different between female and male patients. In two male patients from the VO group, a severe dilatation of the RA was not the result of a left-to-right shunt due to an atrial septal defect (ASD) or an atrioventricular septal defect, but was caused by a severe insufficiency of the tricuspid valve. Only these two, and one newborn boy with ASD and ventricular septal defect, had congestive heart failure and were on medical therapy (i.e., spironolactone and hydrochlorothiazide). All patients were in sinus rhythm. One girl and one boy from the VO group had additional complex cardiac and noncardiac malformations and died in the postoperative course.

SERCA2a and PLB mRNA Expression. Median SERCA2a mRNA expression was 22.5 (1 unit corresponding to 1 mRNA molecule per 10,000 28S rRNA molecules). Although there were no significant differences as to gender and age group (data not shown), SERCA2a mRNA expression was significantly reduced in the VO group compared with the NO group (estimated difference, 8; 95% CI, 1–27; $P = 0.021$; Fig. 3). This difference remained significant in multivariate analysis; that is, it was independent from the other factors (beta, 0.60; 95% CI, 0.05–1.41; $P = 0.028$). The VO versus NO difference was restricted to boys (estimated difference, 11; 95% CI, 2–48; $P = 0.010$), although there was no significant difference in girls (estimated difference, 3; 95% CI, −13–40; $P = 0.77$). This fact corresponded to a highly significant interaction of gender versus VO status (beta, 1.58; 95% CI, 0.32–3.79; $P = 0.002$). Median PLB mRNA expression was 58.5 (range, 25–445). There were no significant differences in the gender, age group, or VO status or the interaction of gender and VO status (data not shown). The PLB to SERCA2a mRNA ratio was not different between the VO group (median, 3.05; range, 1.48–5.84) and the NO group (median, 2.78; range, 1.27–6.44; $P = 0.4$; data not shown).

SERCA2a and PLB Protein Expression. Protein expression was investigated in 16 patients (8 girls and 8 boys: 7 NO/9 VO, 6 infants/10 older children). The densitometric intensity of the bands after immunoblotting was expressed as arbitrary units. Median SERCA2a protein level was 36.6 kU (range, 17.2–93.7). There were no significant differences as to gender, age group, or VO status or the interaction of gender and VO status (data not shown). Median PLB protein level was 143.1 kU (range, 89.8–255.1). The median level was significantly lower in infants (estimated difference, 41.0 kU; 95% CI, 7.4–86.9; $P = 0.031$; data not shown). This difference remained significant in multivariate analysis; that is, it was independent of the other factors (beta, 0.87; 95% CI, 0.04–1.64; $P = 0.033$). There were no significant differences as to gender, VO status, or the interaction of gender and VO status (data not shown). The median PLB to SERCA2a protein ratio was 2.86 (range, 1.66–6.71). The median ratio was significantly higher in boys (estimated difference, 1.36; 95% CI, 0.28–

Table 3. Patients' Characteristics[a]

	VO	NO	*P*
Girls (*n*)	7	5	
Age (months)	35 (0.2–132)	27 (6–46)	0.38
Weight (kg)	15 (2.2–24.6)	11.2 (6.5–18)	0.57
Hospital stay (days)	12 (10–36)	10 (9–17)	0.43
RA $(cm^2)/m^2$	22.4 (6.2–28)	12.1 (6.3–13.8)	0.029
RA to LA ratio	2.08 (1.56–2.89)	0.6 (0.45–1.23)	<0.0001
Boys (*n*)	11	7	
Age (months)	41 (0.5–153)	11 (5–165)	0.60
Weight (kg)	12.5 (3.7–37)	7.1 (4.6–46.2)	0.95
Hospital stay (days)	11 (6–35)	10 (6–18)	0.70
RA $(cm^2)/m^2$	25 (15.6–38.8)	16.7 (5.3–26.4)	0.017
RA to LA ratio	2.11 (1.5–2.78)	0.94 (0.74–1.38)	<0.0001

[a] Values expressed as median and range.

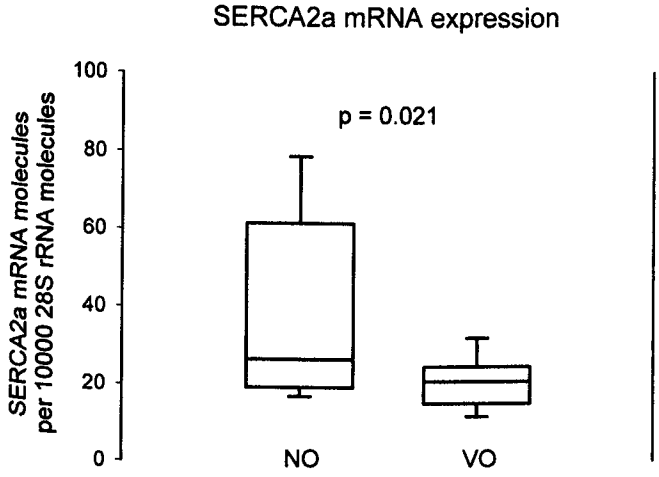

Figure 3. The influence of hemodynamic overload on SERCA2a mRNA expression. SERCA2a mRNA expression was significantly reduced in the VO group compared with the NO group (estimated difference, 8; 95% CI, 1–27; $P = 0.021$).

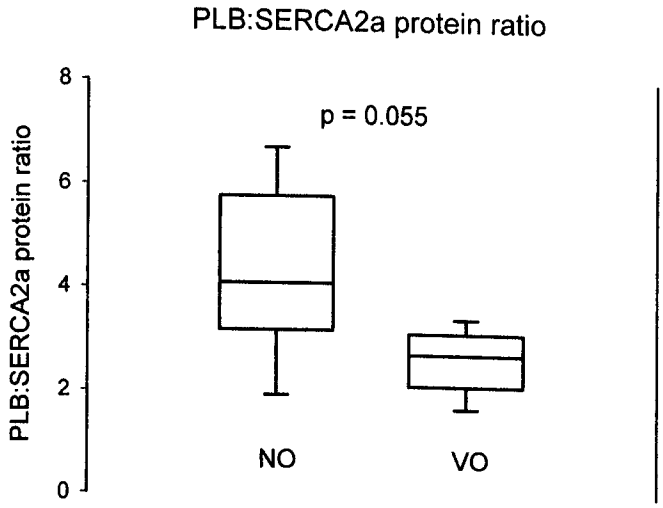

Figure 5. The influence of hemodynamic overload on the PLB to SERCA2a protein ratio. Tendency toward a lower ratio in VO (estimated difference, 1.77; 95% CI, –0.17–3.61; $P = 0.055$).

3.61; $P = 0.028$; Fig. 4). This difference remained significant in multivariate analysis; that is, it was independent of the other factors (beta, 0.75; 95% CI, 0.07–1.683; $P = 0.025$). There was a tendency toward a lower ratio in VO (estimated difference, 1.77; 95% CI, –0.17–3.61; $P = 0.055$; Fig. 5). There were no significant differences as to age group or the interaction of gender and VO status (data not shown).

Discussion

Various studies conducted in animal models (17, 18) and myocardium from adult humans with ventricular remodeling (19) and atrial remodeling (20) confirmed the central role of calcium and calcium-handling proteins in healthy and diseased myocardium. Data cannot be adopted from other studies as the expression of these proteins is age dependent and species specific (13, 15, 21). We aimed to study if the expression of SERCA2a and its regulator PLB is changed in VO of atrial myocardium. We hypothesized that differential expression is evident in pediatric patients and that gender plays a role already in childhood.

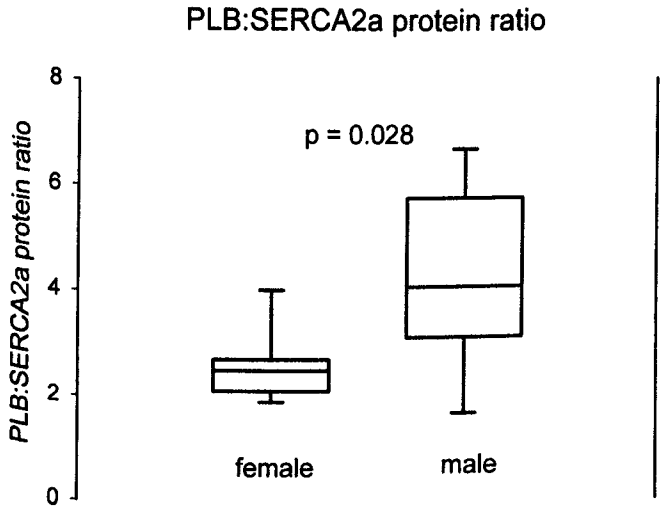

Figure 4. The influence of gender on the PLB to SERCA2a protein ratio. The median PLB to SERCA2a protein ratio was 2.86 (range, 1.66–6.71). The median ratio was significantly higher in males (estimated difference, 1.36; 95% CI, 0.28–3.61; $P = 0.028$).

Differential Expression of SERCA2a mRNA. The major finding of the present study is that the mRNA expression of the calcium-pump SERCA2a was significantly reduced in pediatric myocardium from VO RA compared with NO atria. Diminished SERCA2a mRNA expression has been found in other studies in adult patients with various atrial pathologies (22–24). No changes were found in the PLB mRNA expression. Moreover, the PLB to SERCA2a mRNA ratio was not different between the VO and the NO groups. This is in line with data from adults with atrial dilatation due to fibrillation (21).

Protein Expression of SERCA2a and PLB. Regarding the SERCA2a and PLB protein expression, VO did not influence the single values of these components. This is in line with some studies (25, 26), although it is generally accepted that decreased SERCA2a (27) and PLB (28) protein expression are hallmarks of heart failure and compensated cardiac hypertrophy. However, there was a tendency toward a lower PLB to SERCA2a protein ratio in the VO atrial samples. Keeping in mind that this was dilated, but probably not failing, atrial myocardium, one might hypothesize that this could be a compensatory functional mechanism in the dilated atrium, which should lead to less inhibition of SERCA2a and, therefore, enhance relaxation and contraction. In contrast, failing ventricular myocardium displays an increased PLB to SERCA2a ratio, leading to more inhibition of SERCA2a, rising cytosolic calcium (29, 30), and functional deterioration. As SERCA2a and PLB interact, apart from the single values of both components, the ratio is known to be an important determinant for functional consequences like duration of contraction, relaxation (31), and the force-frequency relationship (32). Changes of the mRNA expression are not necessarily accompanied by

differences of protein expression. This has also been described for SERCA2a and PLB (33), and there is evidence that transcriptional and post-transcriptional mechanisms (34, 35) are responsible for this phenomenon.

Influence of Gender. To date, there are no reports on gender-related changes of SERCA2a or PLB expression in human heart disease. Although the extent of RA dilatation was not different between female and male patients from the VO group, the significantly reduced SERCA2a mRNA expression in the VO group compared with the NO group was restricted to boys, showing a highly significant interaction of gender versus hemodynamic overload status. If the hemodynamic situation was not taken into account, this gender-related difference was found also at the protein level, showing a higher PLB to SERCA2a protein ratio in boys. Several reports have shown gender-related differences such as a better prognosis in aortic stenosis (36), hypertension (37), and congestive heart failure (38) in female patients. In contrast, after coronary artery bypass operations, women have a higher morbidity (39) and mortality (40) and less functional gain compared with men (41). In pediatric patients, the risk of death due to malignant arrhythmias (42), the incidence of supraventricular tachycardia at birth (43), and the outcome after pediatric cardiac surgery (44) was different according to the gender of the patients. In our patients, the clinical course and postoperative recovery was not different between male and female patients. Molecular studies revealed that myocyte necrosis and apoptosis is more severe in male patients with heart failure (45) and after myocardial infarction (46). Several recent studies have shown that not only sex-steroid hormones are involved in gender-related cardiovascular pathophysiology (47); their effects are propagated by a highly complex interplay of specific receptors (48), coactivator and corepressor factors (49), and post-translational modifications (50). To date, the exact mechanisms of gender differences, particularly in infancy and childhood, are still under investigation. However, sex-hormone–mediated changes of calcium-regulating proteins are likely involved in gender-specific outcomes of heart failure (48).

In conclusion, the molecular mechanisms for gender-related differences in cardiovascular disease still remain to be elucidated. Due to species and developmental differences in the expression of calcium-regulating proteins and the expression of gender-specific, sex-steroid–hormone pathways, data cannot be deduced from adult cardiac disease or animal models. Although, due to the small number of patients our findings are preliminary and need to be confirmed, we believe that our study in children contributes new data in this field. Knowledge of molecular changes could help in decision making and therapy in pediatric heart disease in the future.

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