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Molecular changes in children with heart failure undergoing LVAD therapy

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Abstract

Objective—To determine whether left ventricular assist device (LVAD) treatment in children with heart failure would result in modification of molecular pathways involved in HF pathophysiology.

Study design—Forty-seven explanted hearts from children were studied (16 non-failing control, 20 failing, and 11 failing post LVAD implantation (F-LVAD)). Protein expression and phosphorylation states were determined by receptor binding assays and western blots. mRNA expression was measured with Real-time quantitative polymerase chain reaction. To evaluate for interactions and identify correlations, 2-way Analysis of Variance (ANOVA) and regression analysis were performed.

Results—Treatment with LVAD resulted in recovery of total β -AR expression (Bmax) and β_1 -AR in failing hearts to normal levels (Bmax: 67.2 ± 11.5 fmole/mg failing vs 99.5 ± 27.7 fmole/mg non-failing, 104 ± 38.7 fmole/mg F-LVAD, $P = 0.01$; β_1 -AR: 52.2 ± 10.3 fmole/mg failing vs 83.0 ± 23 fmole/mg non-failing, 76.5 ± 32.1 fmole/mg F-LVAD $P = 0.03$). The high levels of G protein-coupled receptor kinase-2 were returned to non-failing levels after LVAD treatment (5.6 ± 9.0 failing vs 1.0 ± 0.493 non-failing, 1.0 ± 1.3 F-LVAD). Interestingly, β_2 -AR expression was significantly higher in F-LVAD (27.5 ± 12 ; $P < 0.005$) hearts compared with non-failing (16.4 ± 6.1) and failing hearts (15.1 ± 4.2). Phospholamban phosphorylation at serine 16 was significantly higher in F-LVAD (7.7 ± 11.7) hearts compared with nonfailing (1.0 ± 1.2 , $P = 0.02$) and failing

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(0.8 ± 1.0 , $P=0.01$). Also, atrial natriuretic factor (0.6 ± 0.8) and brain natriuretic peptide (0.1 ± 0.1) expression in F-LVAD was significantly lower compared with failing (2.8 ± 3.6 , $P=0.01$ and 0.6 ± 0.7 , $P=0.02$).

Conclusion—LVAD treatment in children with heart failure results in reversal of several pathologic myocellular processes and GRK2 may regulate β_1 -AR but not β_2 -AR expression in children with HF.

Keywords

β -adrenergic receptor; phospholamban; pathological gene program

The β -adrenergic pathway plays an essential role in the regulation of cardiac function. The two main receptors in the heart are the β_1 -adrenergic receptor (β_1 -AR) and the β_2 -adrenergic receptor (β_2 -AR)¹. In adults with heart failure, elevations in circulating catecholamines lead to chronic stimulation of β -ARs resulting in β_1 -AR down-regulation and desensitization due to an increase in receptor phosphorylation by the upregulated G protein-coupled receptor kinase-2 (GRK2)¹.

During end-stage heart failure, mechanical circulatory devices such as left ventricular assist devices (LVADs) are used as a form of therapy when drug treatments are no longer effective. Unloading the heart can lead to reverse remodeling such as improved calcium homeostasis and cycling in myocytes as well as alterations in gene expression in various components of the β -adrenergic system².

Heart failure in children is not as common as in adults; therefore, there is a lack of focused research specific to this patient group. Previous work has demonstrated significant differences in the regulation of the β -adrenergic system and a number of downstream molecules suggesting that the molecular effects of heart failure and interventions in adults cannot be extrapolated to children^{3–5}. There is also no evidence that drugs such as β -blockers are as efficacious when utilized for pediatric heart failure patients^{6–8}. However, as seen in adults, there is an improvement in structure and function in pediatric hearts implanted with LVADs^{9, 10}. A study on acute LVAD treatment of pediatric patients reveals that some genes are differentially regulated after 10 days of LVAD treatment; however, the study did not address changes in the GRK regulation of the β -adrenergic system, the pathologic gene program, or phosphorylation of its downstream targets¹¹. These data suggest that there are functional and molecular changes occurring in pediatric hearts implanted with LVADs, and we therefore hypothesized that LVAD therapy will modify the β -AR system in children with heart failure. This study analyzes the molecular changes in the left ventricles of pediatric patients implanted with an LVAD and determines whether changes in GRK2 may underlie changes in β -AR expression.

Methods

Human subjects were males and females under the age of 17 years who donated their hearts to the pediatric transplant tissue bank at the University of Colorado. The study was approved by the institutional review committee and informed consent was obtained. Non-failing

control hearts were donor hearts not transplanted for technical reasons. Explanted diseased hearts were from children suffering from advanced non-ischemic idiopathic dilated cardiomyopathy (IDC) without or with an LVAD. A detailed description of the pediatric patients can be found in the Table (available at www.jpeds.com). At the time of cardiac transplantation, the explanted hearts were immediately cooled in ice cold oxygenated Tyrodes in the operating room. The left ventricle was rapidly dissected, flash frozen and stored at -80°C until further use. The failing and non-failing groups were age-matched as best as possible to failing patients treated with an LVAD (F-LVAD) a priori to mitigate any potential confounding between groups.

The Engel method was used with crude membrane preparations and [^{125}I]-iodocyanopindolol (CYP) to determine total and β -AR subtype protein expression as previously published by our group^{3, 12}.

Real-time quantitative polymerase chain reaction (RT-qPCR) of the pathologic gene program was performed as previously described¹³. Primers have been previously published by our group³. Data were normalized to the expression of ribosomal RNA 18s.

Western blots were performed as previously described^{3, 14}. GRK2 antibody (SC-562, 1:1000) & GAPDH (SC-20357, 1:10,000) were purchased from Santa Cruz Bio Tech. Phospholamban serine 16 (Ser16) (A010-12, 1:15,000) and threonine 17 (Thr17) (A010-13, 1:15,000) antibodies were purchased from Badrilla. Phospholamban total (05-205, 1:1000) antibody was purchased from Millipore. The HRP (115-035-146) anti-mouse and anti-rabbit secondaries were purchased from Jackson Laboratories. Antibodies were diluted in 1X TBS (20mM Tris 500mM NaCl pH 7.5) containing 5% BSA and 0.1% Tween. Blots were incubated overnight at 4°C .

Statistical analyses

The Shapiro-Wilk method was used to determine normality in all continuous data¹⁵. Data that was not normally distributed was log transformed and retested prior to analysis. Statistical analysis was performed using Statview software (SAS Institute, Cary, NC, USA). To evaluate for interactions, 2-way Analysis of Variance (ANOVA) was performed on all outcomes. Regression analysis was performed when indicated to identify correlations. Statistical significance was set to $P < 0.05$ and all data are presented as mean $\pm$ SD (mean $\pm$ SEM in Figures 1–4). For log-transformed data, the mean and error measurements are given for the raw data for visual clarity.

Results

The clinical characteristics of each cohort are described in the Table. Importantly, there was no significant difference in age between failing and F-LVAD or non-failing and F-LVAD patients; however, the non-failing cohort average age was higher than the failing patients ($P = 0.01$). Although four different LVADs were used, the overwhelming majority of the devices were pulsatile flow Berlin Hearts and only two were continuous flow devices.

β-adrenergic receptor density

Total AR density (Bmax) in the failing hearts (67.2 ± 11.5 fmole/mg) was lower when compared with nonfailing (99.5 ± 27.7 fmole/mg, $P=0.01$); however, Bmax was similar to non-failing in F-LVAD patients (104.0 ± 38.7 fmole/mg) (Figure 1, A). Similar results were seen for the β_1 receptor (non-failing 83.0 ± 23.0 fmole/mg; failing 52.2 ± 10.3 fmole/mg; F-LVAD 76.5 ± 32.1 fmole/mg; non-failing to failing $P=0.005$ and F-LVAD to failing $P=0.03$) (Figure 1, B). β_2 -AR was significantly higher in F-LVAD patients (27.5 ± 12.0 fmole/mg, $P<0.005$) when compared with both nonfailing (16.4 ± 6.1 fmole/mg) and failing (15.0 ± 4.2 fmole/mg) (Figure 1, C).

GRK2 protein levels

GRK2 protein expression was significantly higher in the failing pediatric heart (5.6 ± 9.0 , Figure 2). Expression in the F-LVAD group (1.0 ± 1.3) was significantly lower than the failing group and no different from GRK2 expression in the non-failing hearts (1.0 ± 0.493).

Phospholamban phosphorylation

There was no significant difference in total phospholamban protein levels between the three groups (data not shown); however, there was significantly higher Ser16 phosphorylation (protein kinase A site) in F-LVAD treated patients (7.7 ± 11.7 , $P<0.05$) compared with non-failing (1.0 ± 1.2) and failing (0.8 ± 1.0) (Figure 3, A). Although phosphorylation at Thr17 (the calcium calmodulin kinase II site) appeared lower in F-LVAD patients (0.6 ± 0.5), the change was not significant compared with nonfailing (1.0 ± 1.2) and failing (1.3 ± 1.7) patients (Figure 3, B). A regression analysis was performed to determine whether age, time on LVAD, medical therapy, β -AR density or GRK2 levels were responsible for the variability in phospholamban phosphorylation within this population, but no correlation was found (data not shown).

Changes in pathologic gene program messengerRNA

Atrial natriuretic factor (ANF) levels were significantly higher ($P=0.03$) in failing hearts (2.8 ± 3.6) compared with nonfailing (1.0 ± 1.8), but were similar between non-failing and F-LVAD patients (0.6 ± 0.8). Although there was no significant difference in brain natriuretic peptide (BNP) levels between non-failing (1.0 ± 1.6) and failing hearts (0.6 ± 0.7), there was significantly less in F-LVAD hearts (0.1 ± 0.1) when compared with nonfailing ($P=0.05$) and failing ($P=0.02$) samples. Sarcoendoplasmic reticulum calcium transport ATPase (SERCA) and α -myosin heavy chain (α -MyHC), were both significantly lower in the failing hearts (SERCA: 0.4 ± 0.3 ; α -MyHC: 0.1 ± 0.1) compared with nonfailing (SERCA: 1.0 ± 0.7 , $P=0.03$; α -MyHC: 1.0 ± 0.6 , $P<0.0001$), and β -MyHC was significantly higher in the failing heart (1.5 ± 0.3) compared with nonfailing (1.0 ± 0.6 , $P=0.03$). However, there was no effect of LVAD therapy, and expression of these genes remained significantly different from non-failing levels (SERCA: 0.3 ± 0.2 , $P=0.003$; α -MyHC: 0.2 ± 0.2 , $P<0.0001$; β -MyHC 1.6 ± 0.7 , $P=0.01$).

Discussion

Mechanical circulatory support has become an important tool for heart failure treatment. Adjunctive therapies may be especially important for children because drug treatments lack the same efficacy as seen in adults and there are far fewer donor hearts available to children. Therefore, understanding the molecular remodeling produced with LVAD therapy in the failing pediatric heart is necessary. The current study demonstrates for the first time the effect of LVAD treatment on the β -adrenergic receptor expression, phospholamban phosphorylation, and the pathologic gene program of pediatric patients suffering from heart failure. Furthermore, our findings suggest that GRK2 may be responsible for regulation of the β_1 -AR, but not β_2 -AR in pediatric heart failure.

Both pulsatile and continuous flow LVADs in adult populations have been demonstrated to normalize total cardiac β -AR and β_1 -AR densities^{16, 17}. Although limitations in our population do not allow us to definitively separate by LVAD type, total β -AR and β_1 -AR densities also approximate normal levels in children with heart failure after treatment with an LVAD. The broad range of treatment times in our population allowed us to perform a regression analysis (data not shown) that demonstrated a positive correlation between the number of days the patient was treated with changes in total β -AR and β_1 -AR densities. We have previously demonstrated unique age-dependent regulation of the β_2 -AR with heart failure in children³ and the data extends this finding into the LVAD treated population by demonstrating that β_2 -AR levels were significantly and substantially higher in LVAD hearts compared with both failing and nonfailing hearts. Because the β_2 -AR is thought to play a cardioprotective role in the heart¹⁸, a change in this receptor population may underlie age-differences in mechanisms of disease.

In adult heart failure there is an upregulation of GRK2 that correlates with the clinical severity of the disease¹⁹ and downregulation of the β_1 -AR. In adults, interventions that mitigate the disease process, such as β -blockers or LVADs lower GRK2 and increase β_1 -AR expression^{16, 20}. Our results show that GRK2 levels are significantly higher in pediatric failing hearts, but return to non-failing levels in response to LVAD therapy and correlate with β_1 -AR expression providing novel evidence for a similar regulatory process in children. In contrast, the changes in GRK2 expression aren't associated with the changes in β_2 -AR expression in pediatric patients, suggesting that a different mechanism is responsible for adaptations in this receptor.

Similar to milrinone therapy⁷ Ser16 phosphorylation is significantly higher in the F-LVAD group. The preservation of Ser16 phosphorylation in the absence of milrinone in the children with LVADs strongly suggests a positive remodeling process. In addition, and consistent with a positive remodeling process, ANF and BNP levels were significantly lower in the F-LVAD group than in the failing heart on medical therapy alone. These data are similar to the prior gene expression data in children¹¹ and the response of the failing adult heart to LVAD therapy²¹. Improvement in the natriuretic peptides may be due to unloading of the left ventricle or positive remodeling.

There were several limitations to this study. First, there are limitations inherent to the cross-sectional study design. We have attempted to control for possible confounding factors through age-matching and limiting our failing population to IDCs. Second, because it is impossible to use “normal” hearts from children, brain dead donor hearts were utilized as non-failing controls for this study. Brain death can affect the heart²² however, it is important to note that any changes in AR activation would be more likely to bias our results against finding a difference. Third, there were four different types of LVADs used to treat patients in this study; however, the overwhelming majority (9/11) was the pulsatile Berlin Heart which makes the result most applicable to this device. A final concern is the potential heterogeneity within the IDC population, as demonstrated in Figure 3. Despite performing multiple correlations of various clinical variables such as patient medication profile, no correlations were identified to account for these results.

In conclusion, these studies provide the first insight into the molecular changes that occur in the β -adrenergic pathway, phospholamban phosphorylation, and pathologic gene program in LVAD treated pediatric failing hearts. These changes are most likely due to unloading of the heart. Importantly, these results suggest that GRK2 may be a regulatory mechanism for the β_1 -AR but not the β_2 -AR in the failing pediatric heart. These changes are indicative of reverse remodeling and suggest that LVADs may have the potential to be used as pathway to recovery. Additional studies will be important to determine whether any of these findings can be utilized as biomarkers of myocardial recovery or as a means of identifying novel medical adjunctive therapies to promote ventricular recovery and device explant in this population.

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Abbreviations

LVAD	Left ventricular assist device
F-LVAD	Failing post LVAD implantation
β-AR	β -adrenergic receptor
Bmax	Total β -adrenergic receptor expression
GRK2	G protein-coupled receptor kinase-2
SERCA	Sarcoendoplasmic reticulum calcium transport ATPase
α-MyHC	α -myosin heavy chain

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Figure 1.

β -AR density of non-failing, failing, failing hearts treated with an LVAD (F-LVAD). A) B_{max} , total receptor density, B) β_1 -AR density, and C) β_2 -AR density. Total and β_1 -AR density in LVAD treated hearts is similar to non-failing levels. β_2 -AR density in LVAD treated hearts is significantly higher compared with nonfailing and failing levels.

Figure 2.

GRK2 expression in pediatric non-failing (NF), failing (F), and failing hearts treated with an LVAD (F-LVAD, L) with representative Western blots. Blots were normalized to GAPDH. GRK2 expression levels in LVAD treated hearts are similar to non-failing.

Figure 3. Phospholamban Ser16 (Panel A) and Thr17 (Panel B) phosphorylation in pediatric non-failing, failing, and failing hearts treated with an LVAD (F-LVAD, L) with representative Western blots normalized to GAPDH. Phospholamban Ser16 phosphorylation is significantly higher in LVAD treated hearts compared with nonfailing and failing. (*) $P < 0.05$. PLN, phospholamban.

Figure 4.

Pathologic gene program mRNA levels of non-failing, failing, and LVAD treated patients (F-LVAD). A) ANF and B) BNP levels are significantly decreased by LVAD treatment, and no change occurs in C) SERCA, D) α -MHC, and E) β -MHC. Normalized to 18s.

Table 1

Pediatric subjects characteristics

Characteristics	NF	F	F-LVAD
Number in cohort	16	20	11
Male, n (%)	9 (56)	10 (50) (P=0.7)	6 (54) (P=0.9)
Mean age at tissue collection (years)	11 ± 5	6 ± 6 (P=0.01)	7 ± 6 (P=0.08)
Mean days on LVAD (range)			71(12–210)
Type of LVAD (n)			Berlin Heart (8) Thoratec LVAD (1) Pedicor (1) Heartware HVAD (1)
Mean EF ±SD (%)	49 ±15	18 ± 8 (P=0.0004)	
Milrinone, n (%)		10 (50)	3 (19)
PDE5 inhibitor, n (%) *			4 (36)
Beta-blocker, n (%)	2 (13)	4 (20)	4 (36)
ACE inhibitor, n (%)		18 (90)	3 (27)
Non-PDEi Inotrope, n (%) [†]	9 (56)	3 (15)	1 (9)

NF, non-failing; F, Failing; F-LVAD, failing patients treated with an LVAD; M, male; EF, ejection fraction; SD, standard deviation; PDE, phosphodiesterase; PDEi, phosphodiesterase inhibitor and ACE, angiotensin-converting enzyme.

* PDE5 inhibitor, sildenafil or tadalafil.

[†] Non-PDEi Inotropes include: dopamine, dobutamine, epinephrine, & norepinephrine. P-values are compared to non-failing.