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Reversal of Impaired Myocardial β -Adrenergic Receptor Signaling By Continuous-Flow Left Ventricular Assist Device Support

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Abstract

Background—Myocardial β -adrenergic receptor (β -AR) signaling is severely impaired in chronic heart failure (HF). The objective of this study is to determine if LV β -AR signaling can be restored following continuous-flow LVAD support.

Methods—Paired LV biopsies were obtained at the time of HeartMate II LVAD implant (HF group) and transplant (LVAD group). The mean duration of LVAD support was 152 ± 34 days. Myocardial β -AR signaling was assessed by measuring adenylyl cyclase (AC) activity, total β -AR density ($B_{\max}$), and GRK2 expression and activity. All patients underwent LVAD implant as a bridge to transplant ($n=12$). LV specimens from non-failing hearts (NF) were used as controls ($n=8$).

Results—Basal and isoproterenol (ISO)-stimulated AC activity was significantly lower in HF vs. NF indicative of β -AR uncoupling. Continuous-flow LVAD support restored basal and ISO-stimulated cyclase activity to levels similar to NF. $B_{\max}$ was decreased in HF versus NF and increased to near normal in the LVAD group. GRK2 expression was increased 2.6-fold in HF versus NF and was similar to NF following LVAD support. Similarly, GRK2 activity was 3.2-fold greater in HF versus NF and decreased to NF levels in the LVAD group.

Conclusions—Myocardial β -AR signaling can be restored to near normal following continuous-flow LVAD support. This is similar to previous data for volume displacement, pulsatile LVADs. Decreased GRK2 activity appears to be an important mechanism and indicates that normalization of the neurohormonal milieu associated with HF is similar between continuous-flow and pulsatile LVADs. This may have important implications for myocardial recovery.

Introduction

Left ventricular assist devices (LVADs) are becoming an increasingly used treatment for patients with end-stage heart failure (HF). The most common indication is as a bridge to transplant, however, there is significant interest in implanting these devices as a bridge to recovery in patients with chronic HF. Although this has been reported for a very small

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percentage of patients, novel multi-modality therapies are being investigated. Chronic HF is characterized by severely impaired myocardial β -adrenergic receptor (β -AR) signaling [1]. These signaling defects include a decrease in total β -AR density, known as receptor downregulation, and impaired signaling through remaining receptors, a process known as homologous desensitization [2]. These abnormalities are thought to be due, in large part, to phosphorylation of agonist-occupied β -ARs by G protein-coupled receptor kinase-2 (GRK2), a member of the family of serine-threonine kinases known as G protein-coupled receptor kinases (GRKs) [3]. Following phosphorylation by GRK2, receptors are targeted for binding by β -arrestins which sterically inderdict further receptor coupling leading to receptor internalization [4]. In the setting of chronic HF in humans and animal models, myocardial expression and activity of GRK2 is upregulated approximately 3-fold [5,6] and this is thought to be a major mechanism of impaired β -AR signaling as circulating levels of catecholamines are significantly increased in an attempt to increase cardiac output. Inhibition of GRK2 has been shown to reverse the impaired β -AR signaling in animal models of HF and restore ventricular function [7,8,9].

Our laboratory and others have shown that myocardial β -AR signaling can be restored to near normal following support with the pulsatile HeartMate XVE LVAD (Thoratec Corp., Pleasanton, CA) [10,11,12]. There was normalization of circulating catecholamine levels and, importantly, a decrease in myocardial GRK2 expression and activity. These studies are particularly important with regard to myocardial recovery as the β -AR signaling system is the most critical pathway in the regulation of cardiac systolic and diastolic function. The primary goal of this study is to investigate whether the abnormal β -AR signaling characteristic of HF can be reversed by a continuous-flow (CF) LVAD, specifically, the HeartMate II LVAD. These data may be important in determining if CF devices can be used as a platform for achieving myocardial recovery in patients with long-standing HF in combination with novel pharmacologic, cell-based, or genetic therapies.

Methods

Study Population

We collected myocardial tissue and blood samples from 12 consecutive patients who underwent LVAD implant and subsequent orthotopic heart transplantation. The indication for LVAD implantation in all patients was end-stage (NYHA Class IV) HF with deterioration of cardiac function despite maximal medical therapy. The clinical characteristics of this study population are presented in Table 1. Right heart catheterization had been performed within eight weeks prior to transplant. All procedures for tissue procurement were performed in compliance with institutional guidelines for human research and an approved institutional review board protocol at the University of Chicago Medical Center.

Myocardial Tissue Collection

The left ventricular apical core excised during implantation of the HeartMate II LVAD for each patient was snap frozen in liquid nitrogen and stored at -80°C . A section of the apex of the left ventricle was then excised and stored in identical fashion following LVAD explant and cardiectomy at the time of heart transplantation. All samples were paired from LVAD implant to transplant (n=12). Non-failing control left ventricular apical tissue was obtained from organ donors whose hearts were unsuitable for transplantation but had normal ventricular function and no structural heart disease (n=8).

Lymphocyte Samples

For both pre-LVAD (HF) and post-LVAD (LVAD) samples, blood was collected intraoperatively and anti-coagulated with EDTA. Lymphocytes were isolated by Ficoll

gradient using Histopague-1077 (Sigma), frozen, and stored at -80°C . All blood samples were paired from LVAD implant to explant ($n=10$). Blood samples were unable to be obtained from non-failing organ donors.

Protein Immunoblotting

Tissue was homogenized in lysis buffer (25 mmol/L Tris-HCl [pH 7.5], 5 mmol/L EDTA, 5mmol/L EGTA). Nuclei and tissue were separated by centrifugation at 800g for 20 minutes. The crude supernatant was then centrifuged at 20,000g for 20 minutes. Sedimented proteins (membrane fraction) were resuspended in 50 mmol/L HEPES (pH 7.3) and 5 mmol/L MgCl_2 . The immunodetection of myocardial levels of GRK2 (Santa Cruz Biotechnology, Inc, CA) was performed on cytosolic and membrane extracts (80 μg) electrophoresed through 12% Tris/glycine gels and transferred to nitrocellulose. Membranes were blocked in 5% nonfat dried milk for 1 hour at room temperature. The protein was visualized using a horseradish peroxidase-linked secondary antibody and ECL detection (Amersham).

Measurement of GRK Activity

The membrane fractions of the myocardial extracts were used to determine GRK activity. Extracts (100 μg of protein) were incubated with rhodopsin-enriched rod outer-segment membranes as previously described [13]. After incubating in white light for 15 minutes at room temperature, reactions were quenched with ice-cold lysis buffer and centrifuged for 15 minutes at 13,000g. Sedimented proteins were resuspended in protein-gel-loading dye and treated with 12% SDS-PAGE. Phosphorylated rhodopsin was visualized by autoradiography and quantified using a Molecular Dynamics PhosphorImager.

Radioligand Binding Assays

Total β -AR density (B_{max}) was determined by incubating 25 μg of cardiac sarcolemmal membranes with a saturating concentration of [^{125}I] cyanopindolol and 20 $\mu\text{mol/L}$ alprenolol to define nonspecific binding. Sarcolemmal membrane samples were studied in triplicate with 80 pmol/L [^{125}I] cyanopindolol and 10^{-4} mol/L in 250 μL of binding buffer (50 mmol/L HEPES [pH 7.3], 5 mmol/L MgCl_2 , and 0.1 mmol/L ascorbic acid). Assays were done at 37°C for 1 hour and then filtered over GF/C glass fiber filters (Whatman) that were washed twice and counted in a gamma counter. Data were analyzed by nonlinear least-square curve fit (GraphPad Prism).

Sarcolemmal Membrane Adenylyl Cyclase Activity

Cardiac sarcolemmal membranes (20 μg of protein) were incubated for 15 minutes at 37°C with [α - ^{32}P]ATP under basal conditions, with 10^{-4} mol/L isoproterenol, or 10 mmol/L NaF. Sodium fluoride directly stimulates the G protein subunit, $G_{\alpha s}$, which activates adenylyl cyclase. This determines whether the G protein and cyclase moiety are intact and establishes whether uncoupling is occurring at the level of the receptor or downstream. cAMP production was quantified by standard methods described previously [14].

Semi-Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction

RNA was extracted from tissue samples, converted to cDNA, and used as the template in a polymerase chain reaction (PCR) that included the fluorophore SYBR Green. Increased fluorescence was detected by use of a real-time PCR machine (Applied Biosystems). PCR primers for GRK2 and GRK5 were designed to span introns that characterize their genomic DNA and prevent genomic DNA contamination of the RNA and subsequent reverse transcription PCR. GRK2 mRNA was determined (forward, 5'-GAACACATGCACAATCG-3'; reverse, 5'-CCAGGGAGAACCAGTC-3'). GRK5 mRNA

was determined (forward, 5'-GAAGGTTAAGCGGGAAGAGG-3'; reverse, 5'-TCCAGGCGCTTAAAGTTCAT-3').

Statistical Analysis

Repeated-measures analysis of variance was used to analyze serial data over time within treatment groups. Analyses were conducted using Statview 4.01 software (Abacus Concepts Inc, Berkeley, CA). Experimental groups were compared using Student's *t* test or 1-way ANOVA, as appropriate. The Bonferroni test was applied to all significant ANOVA results using SigmaStat software. *P* values of less than 0.05 were considered statistically significant. All results are expressed as mean $\pm$ SEM.

Results

Patient Population

Patient demographics are summarized in Table 1. All patients underwent HeartMate II LVAD implant for bridge to transplant. They were all NYHA Class IV at the time of implant and Class I or II by the time of LVAD explant and transplant as a result of mechanical circulatory support. The study population included 8 males and 4 females. Four patients had ischemic cardiomyopathy and 8 had non-ischemic dilated cardiomyopathy. Ten patients were receiving dobutamine prior to LVAD implant. All patients received dobutamine and/or epinephrine following LVAD implant for a range of 3–10 days. None were receiving inotropic therapy at the time of transplant and β -blockade was not resumed after LVAD implantation. The mean duration of LVAD support was 152 ± 34 days, after which all patients underwent LVAD explant and heart transplantation. The mean LVAD flow was 5.0 ± 0.7 liters per minute at a speed of $9,450 \pm 55$ rpm. Cardiac hemodynamics were measured by right heart catheterization prior to LVAD implant and within 2 months of transplant (Table 2). Following LVAD implant, there was a significant decrease in right atrial pressure, pulmonary artery wedge pressure, mean pulmonary artery pressure, and a significant increase in mean and diastolic blood pressure and cardiac index. For all tissue studies, non-failing (NF) left ventricular myocardium was obtained from organ donors with normal ventricular function when the heart was not procured for non-cardiac issues.

Myocardial β -Adrenergic Signaling

β -AR signaling was assessed in left ventricular tissue samples from NF, failing (HF), and LVAD-supported (LVAD) hearts by measurement of total β -AR density ($B_{\max}$) and β -AR-coupled adenylyl cyclase activity. These assays were performed after preparation of sarcolemmal membranes from the LV tissue specimens. Consistent with previously reported data, total β -AR density was significantly decreased in the HF group compared to the NF group (36.2 ± 3.7 versus 76.1 ± 6.2 fmol/mg protein, $P < 0.05$). LVAD support led to a significant increase in β -AR density in these failing hearts (71.4 ± 9.5 versus 36.2 ± 3.7 fmol/mg protein, $P < 0.05$). $B_{\max}$ in the LVAD group was similar to the NF control group (Figure 1).

β -AR-effector coupling was studied by measuring sarcolemmal membrane adenylyl cyclase activity under basal conditions and following stimulation with the β -agonist isoproterenol (Figure 2). Basal cyclase activity was lower in the HF group compared to NF although this did not reach statistical significance (data not shown). Isoproterenol-stimulated activity was severely attenuated in the HF group (40.3 ± 4.6 versus 89.2 ± 7.2 % increase of ISO-stimulated cyclase activity over basal, $P < 0.05$) consistent with uncoupling of β -AR signaling. There was a significant increase in ISO-stimulated adenylyl cyclase activity following LVAD support (81.5 ± 9.9 versus 40.3 ± 4.6 % increase of ISO-stimulated cyclase activity over basal, $P < 0.05$). There was no difference in NaF-stimulated cyclase activity between all three groups (data not shown), indicating that the uncoupling is at the level of the β -AR and not downstream. This

normalization of β -AR signaling after CF LVAD support is similar to that seen following long-term pulsatile LVAD support [10].

Myocardial GRK Expression and Activity

To investigate potential mechanisms by which β -AR signaling is restored in failing hearts by CF LVAD support, we measured the expression and activity of the two GRKs known to be present in the human myocardium, GRK2 and GRK5 (Figure 3). In the HF group, LV protein levels of GRK2 were increased greater than 2-fold compared to NF controls (22.3 ± 4.1 versus 7.8 ± 2.8 arbitrary densitometry units, $P < 0.05$). In contrast, following mechanical unloading, GRK2 protein expression was decreased to levels similar to the NF group (9.9 ± 2.5 versus 7.8 ± 2.8 arbitrary densitometry units, $P > 0.05$). This decrease in GRK2 protein expression correlated with a decrease in GRK2 mRNA in the LVAD group relative to HF (0.45 ± 0.03 versus 0.78 ± 0.10 pg of RNA, $P < 0.05$) (Figure 4). There was no significant difference in LV GRK2 mRNA expression between the NF and LVAD groups (Figure 4). In contrast to GRK2, there was no increase in GRK5 protein or mRNA expression in the HF group compared to NF and this was unchanged after LVAD support (data not shown). This is not unexpected considering that GRK5 is expressed in the heart at very low levels and its role in cardiovascular pathology is unclear.

In order to assess the functional significance of increased GRK2 protein expression, GRK2 activity was measured in sarcolemmal membrane preparations using an *in vitro* rhodopsin phosphorylation assay (Figure 5). GRK2 activity was increased in the HF group nearly 3-fold compared to the NF group (30.8 ± 4.0 versus 12.1 ± 2.3 densitometry units, $P < 0.05$). After CF LVAD support, GRK2 activity was decreased to levels similar to the NF group and significantly lower than the HF group (15.4 ± 2.5 versus 30.8 ± 4.0 densitometry units, $P < 0.05$). These data support the concept that GRK2 plays an important role in the regulation of β -AR signaling in human myocardium.

Lymphocyte GRK Expression

Lymphocyte GRK2 protein expression was measured by immunoprecipitation and immunoblotting analysis of paired blood samples from the time of LVAD implant and at transplant. Similar to the myocardium, GRK2 protein levels were significantly decreased in lymphocytes after mechanical unloading with a CF LVAD (LVAD 58.6 ± 6.1 versus HF 101.7 ± 12.3 densitometry units, $P < 0.05$). These data are consistent with a recent study showing a strong correlation between GRK2 expression in the myocardium and the peripheral lymphocytes in patients with HF [15].

Discussion

The myocardial β -AR signaling pathway plays a critical role in the regulation of cardiac contractility. β -ARs (β_1 and β_2 subtypes) are the primary myocardial targets of the sympathetic neurotransmitter norepinephrine and the adrenal hormone epinephrine. Activation of β -ARs in the heart by these two catecholamines leads to positive chronotropic and inotropic action via stimulation of adenylyl cyclase and subsequent increases in cAMP and intracellular Ca^{2+} release [16]. Continued exposure of β -ARs to agonists results in a rapid decrease in responsiveness known as desensitization. Agonist-dependent desensitization can be initiated by the phosphorylation of activated receptors by members of the family of G protein-coupled receptor kinases (GRKs) [17]. GRK2 specifically phosphorylates activated β_1 - and β_2 -ARs, leading to desensitization *in vitro* and *in vivo* [18,19]. In addition, cardiac-specific overexpression of GRK2 (3-fold) in genetically engineered animal models leads to a decrease in baseline and β -agonist-stimulated contractility [20,21]. In contrast, inhibition of myocardial GRK2 in transgenic mice or via adenoviral-mediated gene transfer significantly enhances

cardiac function and can rescue several models of heart failure [22,23,24]. These studies demonstrated that GRK2 is a critical mediator of ventricular function and remodeling.

Heart failure (HF) in humans is characterized by specific alterations in the β -AR signaling system. These include selective down-regulation of β_1 ARs by approximately 50% and desensitization of remaining β -ARs, which leads to the blunting of agonist-mediated stimulation [25]. The enhanced desensitization of myocardial β -ARs is most likely due to the elevated expression and activity of GRK2 ($\approx$ 3-fold) present in human HF [26]. It is generally thought that these changes in the β -AR system in HF are triggered by increased sympathetic stimulation of the heart in this disease state [27]. The dysfunctional β -AR signaling, including increased GRK2 expression and activity, is a contributing factor to the impaired myocardial contractility present in chronic HF.

This study demonstrates that impaired myocardial β -AR signaling, which is a hallmark of chronic HF, can be reversed by mechanical unloading with a continuous-flow rotary pump. This finding was similar to the effect of a pulsatile, volume displacement pump, on restoration of this critical signaling pathway [10]. Importantly, the improvement in myocardial β -AR signaling is likely a result of decreased GRK2 expression and activity, and appears to be due to relative normalization of the neurohormonal milieu associated with end-stage HF. In addition to the left ventricle, right ventricular response to catecholamine stimulation should also be improved. Our data show that peripheral lymphocyte GRK expression mirrors what is seen in the myocardium and may serve as a novel biomarker for the status of cardiac β -AR signaling. Although limited by the number of patients in this study, the restoration of β -AR signaling in the heart and lymphocytes correlates with the hemodynamic improvement. Thus, measurement of lymphocyte GRK2 expression may represent a novel non-invasive method to assess the status of cardiac β -AR signaling.

Haft et al. recently compared hemodynamic and exercise performance between pulsatile and continuous-flow LVADs and found no difference in pressure unloading and cardiopulmonary exercise testing at 3 months following implant, despite a greater degree of LV volume unloading with the pulsatile pumps [28]. It is unknown to what extent the differences observed in LV volume unloading have on the potential for LV recovery. Interestingly, Thohan and colleagues observed similar changes in the degree of regression of myocyte hypertrophy between these two pump designs [29]. In conjunction with our data, this may indicate that differences in pump design (pulsatile versus continuous-flow) may have little influence on the degree of LV reverse remodeling and β -AR signaling.

In conclusion, this study is the first to demonstrate that the dysfunctional left ventricular β -AR signaling characteristic of chronic HF can be restored to normal levels with a continuous-flow LVAD. In addition, this study provides further evidence that myocardial GRK2 expression can be monitored in peripheral lymphocytes which could provide a mechanism to follow changes in myocardial β -AR signaling following an intervention. Restoration of β -AR signaling alone clearly does not lead to recovery of normal ventricular function allowing LVAD explant as this is a very infrequent clinical outcome and the basic science of chronic HF is extremely complex. However, this may provide a platform for adjunctive therapies (novel pharmacologic, cell-based, or potentially gene therapy) in restoring and maintaining long-term cardiac function since this signaling pathway is fundamental to the regulation of myocardial contractility.

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Figure 1. Total myocardial β -adrenergic receptor density ($B_{\max}$)

NF, non-failing controls (n=8); HF, heart failure group, pre-LVAD (n=12); LVAD, post-LVAD implant (n=12). * $P < 0.05$ versus NF; # $P < 0.01$ versus HF and $P > 0.05$ versus NF.

Figure 2. Myocardial sarcolemmal membrane adenylyl cyclase activity

NF, non-failing controls (n=8); HF, heart failure group, pre-LVAD (n=12); LVAD, post-LVAD implant (n=12). * $P < 0.01$ versus NF; # $P < 0.05$ versus HF and $P > 0.05$ versus NF. All experiments performed in triplicate.

Figure 3. Left ventricular protein expression of G protein-coupled receptor kinase-2 (GRK2) with a representative Western blot

NF, non-failing controls (n=8); HF, heart failure group, pre-LVAD (n=12); LVAD, post-LVAD implant (n=12). * $P < 0.05$ versus NF; # $P < 0.01$ versus HF and $P > 0.05$ versus NF.

Figure 4. GRK2 mRNA expression measured by semi-quantitative real-time reverse transcription polymerase chain reaction

NF, non-failing controls (n=8); HF, heart failure group, pre-LVAD (n=12); LVAD, post-LVAD implant (n=12). * $P < 0.05$ versus NF; # $P < 0.02$ versus HF and $P > 0.05$ versus NF. All experiments performed in triplicate.

Figure 5. GRK2 activity in left ventricular tissue preparations measured by rhodopsin phosphorylation. A representative autoradiogram of phospho-incorporation into Rho following gel electrophoresis

NF, non-failing controls (n=8); HF, heart failure group, pre-LVAD (n=12); LVAD, post-LVAD implant (n=12). * $P < 0.05$ versus NF; # $P < 0.01$ versus HF and $P > 0.05$ versus NF. Rho, rhodopsin.

Figure 6. Lymphocyte GRK2 protein expression in paired blood samples

HF, heart failure group, pre-LVAD (n=12); LVAD, post-LVAD implant (n=12).

* $P < 0.04$ versus HF.

Table 1

Clinical Data

Group	M/F	Age (y)	DCM/ICM	Medications
NF (n=8)	5/3	41 ± 8	-	Dop, PE
HF (n=12)	8/4	47 ± 11	8/4	Dob, AI, BB
LVAD (n=12)	8/4	48 ± 11	8/4	AI, Amio

M, male; F, female; DCM, dilated cardiomyopathy;

NF, normal function/non-failing; HF, heart failure;

LVAD, post-LVAD implant; Dop, dopamine; PE, phenylephrine;

Dob, dobutamine; AI, ACE-inhibitor; BB, beta-blocker;

Amio, amiodarone

Table 2**Hemodynamic Data**

	HF	LVAD
Heart rate, bpm	88±19	79±18
Mean arterial pressure, mm Hg	67±8	85±11 *
Cardiac index, L/M ² /min	1.9±0.3	3.0±0.7 *
Pulmonary wedge pressure, mm Hg	24±7	11±5 *
Mean pulmonary artery pressure, mm Hg	37±8	20±5 *
Right atrial pressure, mm Hg	14±4	8±3 *

* $P < 0.05$ versus HF. N=12 in each group.