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Dynamic Changes in Lymphocyte GRK2 Levels in Cardiac Transplant Patients: A Biomarker for Left Ventricular Function

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Abstract

G protein-coupled receptor kinase 2 (GRK2), which is upregulated in the failing human myocardium, appears to have a role in heart failure (HF) pathogenesis. In peripheral lymphocytes, GRK2 expression has been shown to reflect myocardial levels. This study represents an attempt to define the role for GRK2 as a potential biomarker of left ventricular function in HF patients. We obtained blood from 24 HF patients before and after heart transplantation and followed them for up to 1 year, also recording hemodynamic data and histological results from endomyocardial biopsies. We determined blood GRK2 protein by Western blotting and enzyme-linked immunosorbent assay. GRK2 levels were obtained before transplant and at first posttransplant biopsy. GRK2 levels significantly declined after transplant and remained low over the course of the study period. After transplantation, we found that blood GRK2 significantly dropped and remained low consistent with improved cardiac function in the transplanted heart. Blood GRK2 has potential as a biomarker for myocardial function in end-stage HF.

Background

Enhancing our prognostic capabilities in terms of response to evidence-based therapies in heart failure (HF) may lead to improved patient outcomes and a more cost-effective approach to this complex syndrome. Biomarkers appropriately satisfy this role by providing the potential to enhance our diagnostic, therapeutic, and prognostic approach to the complex treatment of HF patients. There have been many research endeavors examining various natriuretic peptides, extracellular and intracellular molecules, neurohormones, and inflammatory mediators with the hope of providing insight into the pathophysiologic mechanism(s) of left ventricular (LV) systolic dysfunction, the volume-overload state, decompensation, and prognosis.[1] Despite these discoveries, we still lack a complete understanding of the complex pathophysiology of HF and response to various therapies.

Evidence-based medical therapies, including β-adrenergic receptor (βAR) antagonists (β-blockers), angiotensin-converting enzyme (ACE) inhibitors, and aldosterone receptor antagonists, are the cornerstones of pharmacologic therapy for LV systolic dysfunction and congestive HF. It is unclear as to why LV systolic function improves and sometimes even normalizes in some patients, while others show no improvement in ejection fraction or LV remodeling reflected in ventricular dimensions.

We, and others, have previously studied how these G protein-coupled receptors (GPCRs) are dysregulated in the heart.[2,3] Much of this work has focused on GPCR kinases (GRKs), which are responsible for the desensitization of βARs and other receptors in the heart dampening signal transduction throughout the myocyte. G protein-coupled receptor kinase 2 (GRK2) is the most abundant GRK expressed in cardiac myocytes, and it has been known for over a decade that this kinase is elevated in failing myocardium.[4] Importantly, we have shown that human myocardial levels of GRK2 are mirrored by levels found in white blood cells.[5] Moreover, levels of blood GRK2 expression were inversely correlated with LV systolic function, and higher levels of expression were seen with more advanced New York Heart Association (NYHA) functional classes of HF.[5]

Of note, we have previously found that modalities that improve cardiac function and reverse LV remodeling can decrease GRK2 levels in both cardiac tissue and white blood cells.[6] This was shown in severe HF patients undergoing implantation of mechanical LV assist devices (LVADs), which are an important therapeutic option for many patients needing additional hemodynamic support either as a bridge to ventricular recovery or cardiac transplantation or as destination therapy.[7] LVAD patients were followed with cardiac and blood samples taken at the time of LVAD implantation and also 2–3 months later during cardiac transplantation, and LV unloading caused a significant downregulation of cardiac GRK2 and improved βAR signaling, which was mirrored in decreased lymphocyte levels of GRK2.[6] This finding supports our hypothesis that blood GRK2 levels may be a biomarker for LV functional status. In the current study to further support GRK2 as a biomarker of cardiac function, we explored the hypothesis that cardiac transplantation would result in downregulation of GRK2 expression consistent with normalized cardiac function gained from the transplanted heart.

Methods

Study population

We collected blood samples from 24 patients who had severe HF and were listed for cardiac transplantation at Thomas Jefferson University Hospital from October 2005 to October 2008. The study was approved by our Institutional Review Board, and patients voluntarily gave informed consent at the time of enrollment. Patients unable to provide informed consent and who were unavailable for follow-up were excluded from this study. Enrolled patients had GRK2 levels drawn before transplant. GRK2 levels were measured from isolated white blood cell

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fraction from whole blood (see below). After heart transplant, patients had blood GRK2 drawn at the time of routine surveillance endomyocardial biopsies for 1 year. In addition, hemodynamic data from routine posttransplant right heart catheterization were obtained. At the time of the right heart catheterization, cardiac index was calculated using the Fick method.

Peripheral blood lymphocyte and protein isolation

Blood was collected and peripheral lymphocytes isolated by Ficoll gradient as previously described.[5,6] Total cytosolic protein was then prepared from the lymphocyte extracts using a mixture of leupeptin/aproptinin, phenylmethylsulfonyl fluoride (PMSF), and radioimmunoprecipitation (RIPA) buffer solutions. The cell mixture was then centrifuged for a total of 1 hour. The purified cytosolic protein fraction was stored at −80°C until the day of the assay.

GRK2 protein quantification

Protein immunoblotting

The purified cytosolic protein from lymphocytes was subsequently measured by protein spectrophotometry utilizing the Bradford assay. A total of 20 µL of cytosolic protein and RIPA buffer in addition to 5 µL of 5× loading buffer are added to each well of a 4–20% Tris ethylenediaminetetraacetic acid (EDTA) gel. The gel is then run at 145 V for 1.5 hours, after which it is transferred to a membrane with 45 V for 70 minutes. The membrane is first incubated with blocking buffer for 1 hour. The membrane is then incubated with an anti-GRK2 human polyclonal rabbit primary antibody (Santa Cruz Biotechnology Company, Santa Cruz, CA, USA) GRK2 (C-15: sc-562) for 1 hour followed by incubation with an antirabbit goat secondary antibody (Alexa Fluor 680; Invitrogen, Carlsbad, CA, USA). The 80-kDa GRK2 protein was visualized using standard enhanced chemiluminescence. Quantification of immunoreactive GRK2 was done by scanning the autoradiography film with an Odyssey scanning system (LI-COR Biosciences, Lincoln, NE, USA), which provides a calculated measure of protein amount based on band size and intensity.

GRK2 enzyme-linked immunosorbent assay

Frozen, dry white blood cell pellets were suspended in 100 µL RIPA (89901; Pierce Biotechnology, Rockford, IL, USA) with leupeptin/aprotinin and PMSF (Sigma, St. Louis, MO, USA). Lysates were passed through a 27-G needle and placed on ice. Spun first at 4°C at 13,000 RPM, the resultant supernatant was then spun at >30,000 RPM at 4°C. Bradford assay (Pierce Biotechnology) determined accurate protein concentrations of all lymphocyte samples. Sandwich enzyme-linked immunosorbent assay (ELISA) plates were prepared by Ortho-Clinical Diagnostics (Johnson & Johnson, Raritan, NJ, USA) using C5/1.1 antibody (Upstate/Millipore, Billerica, MA, USA) bound to well solid phase as capturing antibody and C15 GRK2 antibody (Santa Cruz) as capture antibody. An eight-point calibration curve was performed for accurate GRK2 determination using recombinant GRK2 protein (ADRBK1; PV3361; Invitrogen) in RIPA buffer (89901; Pierce Biotechnology). To each well, assay buffer [0.01M PBS (p3813-10; Sigma-Aldrich), pH 7.4 with 1% Bovine Albumin (82-046-3; Celliance-Millipore) and 0.1% Tween-20 (Bio-Rad EIA Grade, Bio-Rad, Hercules, CA, USA)] was added with cell extract or calibrator. Assay plate was sealed and shook (Ortho Shaker Incubator; Johnson & Johnson) at 37°C. Each well was washed four times with wash buffer (0.01M PBS, pH 7.4 with 0.05% Tween-20). The detecting antibody was C15 GRK2 antibody (Santa Cruz) and the secondary antibody used was Horseradish Peroxidase (HRP)-conjugated, goat-antirabbit antibody (Pierce Biotechnology), diluted 1:1000. Chemiluminescence solution (OCD ECI Signal Reagents A&B, Rochester, NY, USA), was used and luminescence plate reading was performed in 10 minutes. Good well-to-well consistency (SD = 427.6 and %cv = 4.702) and a concordance study of calculated GRK2 concentrations in three repeated patient lysate assays ($r^2 = 0.9697$, $r^2 = 0.9406$, $r^2 = 0.9487$) confirmed a high-quality assay.

	(*n* = 24)
Age (years)	51.8 ± 13.9
Male (%)	16 (67)
Caucasian (%)	11 (46)
African American (%)	12 (50)
Asian (%)	1 (4)
Etiology of HF	
Ischemic (%)	9 (38)
Sarcoidosis (%)	2 (8)
Valvular (%)	1 (4)
Peripartum (%)	2 (8)
Myocarditis (%)	2 (8)
Idiopathic (%)	8 (33)
VAD (%)	6 (25)
ICD (%)	18 (75)
CRT-D (%)	9 (38)
SBP (mm Hg)	114 ± 252.3
Laboratory values	
BNP (pg/mL)	1136 ± 1175
Serum sodium (mmol/L)	137 ± 2.99
Serum creatinine (mg/dL)	1.2 ± 0.376
Hemoglobin (g/dL)	12.1 ± 1.99
LVEF	13 ± 4.4
Medications	
ACE inhibitor (%)	12 (50)
ARB (%)	3 (12)
Aldosterone antagonist (%)	8 (33)
Beta-blocker (%)	11 (46)
Loop diuretic (%)	11 (46)
Digoxin (%)	7 (29)
Nitrate (%)	3 (13)
Milrinone (%)	16 (67)
Dobutamine (%)	3 (13)
Nesiritide (%)	1 (4)

Data presented as n (%) or mean ± standard deviation.

ACE = angiotensin-converting enzyme; ARB = angiotensin receptor blocker; BNP = B-type natriuretic peptide; CRT-D = cardiac resynchronization-defibrillator therapy; LVEF = left ventricular ejection fraction; HF = heart failure; ICD = implantable cardio-verter defibrillator; SBP = systolic blood pressure; VAD = ventricular assist device.

Table 1. Clinical characteristics of the study population.

Statistical analyses

The data were summarized using means and variances. Comparison of GRK2 levels pre- and posttransplant for the 15 individuals for whom both levels were available was done using the Wilcoxon signed-rank test. Comparisons of posttransplant GRK2 levels between subgroups of patients were done using the Wilcoxon rank-sum test. All hypothesis tests were two-sided, and p values <0.05 were considered statistically significant. All graphics and computations were carried out using statistical software from the R Foundation for Statistical Computing (Vienna, Austria).

Results

Demographics of HF patients listed for cardiac transplant

Among the 24 patients enrolled in the study, the mean age was 52 years. The majority of the patients were male (67%). The etiology of HF in this study population was primarily ischemic (38%) and idiopathic dilated (33%). Six patients required an LVAD as a bridge to transplant. The mean LV ejection fraction was 13%. Most of the patients (84%) were either on intravenous inotropes and/or vasodilators prior to cardiac transplant. See *Table 1* for a complete list of patient demographics.

Hemodynamic profiles of HF patients before and after transplant

Hemodynamic parameters measured during right heart catheterization prior to cardiac transplant demonstrated elevated left- and right-sided filling pressures and low cardiac index consistent with severe HF. After heart transplant, the pulmonary capillary wedge pressure and pulmonary artery pressure significantly declined. The cardiac index also significantly increased (3.49 ± 0.90) compared to pretransplant values (2.23 ± 0.49, $p < 0.001$). Full hemodynamic profiles for all patients are shown in *Table 2*.

Lymphocyte levels of GRK2 significantly decline after cardiac transplantation

Lymphocyte levels of GRK2 protein were measured by immunoblotting and ELISA from paired samples of blood. Blood GRK2 levels, measured at the first posttransplant endomyocardial biopsy approximately 1 week after transplant, significantly declined (mean GRK2 pretransplant, 9.22 ± 2.30 nmol/g of protein vs. posttransplant 2.78 ± 1.20 nmol/g of protein, $p = .01$, *Figure 1A*) corresponding to a 70% drop in protein. To illustrate the change in GRK2 expression before and after heart transplant, paired patient data are graphically represented in *Figure 1B* demonstrating the significant drop in GRK2 after transplant. Levels of GRK2 also declined after transplant in those patients with LVADs implanted as a bridge to transplantation. In addition, although a trend toward increased GRK2 levels was evident after transplant over time, mean GRK2 expression remained low over the duration of the study period (*Figure 2*).

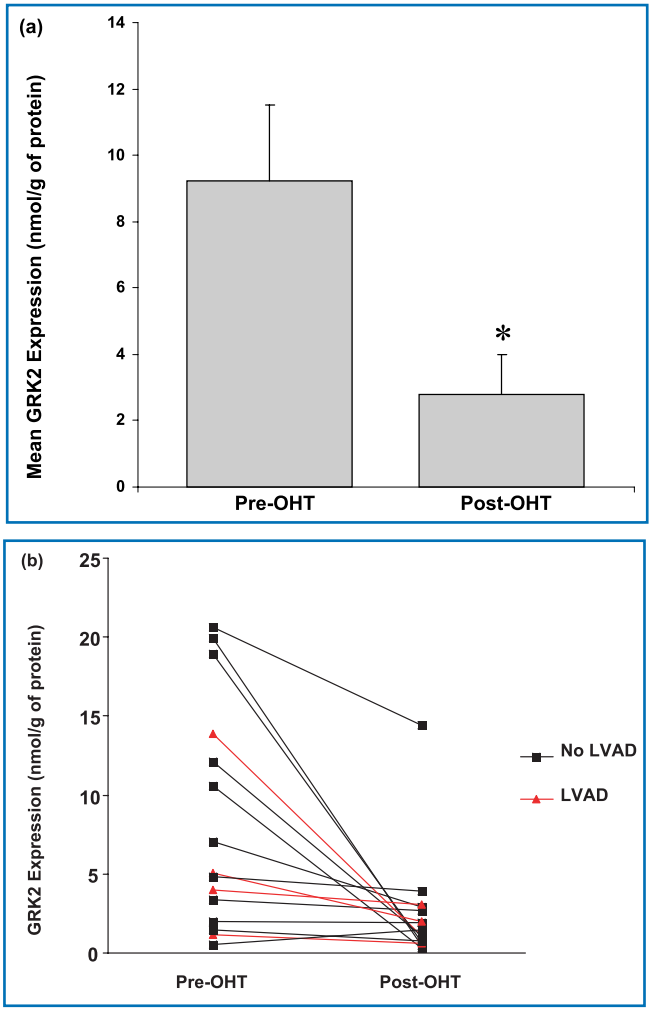

Figure 1. (**A**) GRK2 expression in paired patient samples without LVAD ($n = 11$) pre- versus post-OHT. *$p = 0.01$ pre- versus post-OHT. Results are shown as mean ± SEM. GRK2 = G protein-coupled receptor kinase 2; LVAD = left ventricular assist device; OHT = orthotopic heart transplant; SEM = standard error of the mean. (**B**) Graphical representation of the change in blood GRK2 expression in paired samples pre-OHT versus post-OHT in the study group, with or without LVAD ($n = 15$). GRK2 = G protein-coupled receptor kinase 2; LVAD = left ventricular assist device; OHT = orthotopic heart transplant; SEM = standard error of the mean.

	Pretransplant		Posttransplant		Difference		
	Mean	SD	Mean	SD	Mean	SEM	p value
RA	7.6	5.77	6.29	3.93	−1.33	5.88	0.352
RV sys	40.4	12.58	36.09	7.24	−3.91	13.86	0.266
RV dia	9.9	6.87	8.31	3.81	−1.59	7.11	0.322
PA sys	40.4	13.28	33.00	7.76	−7.41	13.25	0.013
PA dia	18.5	8.72	11.5	4.51	−7.00	9.61	0.004
PCWP	18.3	8.38	13.39	4.88	−4.91	9.36	0.016
CI	2.23	0.49	3.49	0.906	1.26	1.04	< 0.001

CI = cardiac index; PA sys = pulmonary artery systolic pressure; PA dia = pulmonary artery diastolic pressure; PCWP = pulmonary capillary wedge pressure; RA = right atrial pressure; RV sys = right ventricular systolic pressure; RV dia = right ventricular diastolic pressure; SD = standard deviation; SEM = standard error of the mean.

Table 2. Hemodynamic variables pre- versus posttransplant.

Discussion

The present study examines lymphocyte GRK2 expression in HF patients before and after cardiac transplantation. This investigation includes long-term follow-up during visits for endomyocardial biopsying. Our major finding is that GRK2 levels decrease dramatically after transplantation. Therefore, it appears that, with the introduction

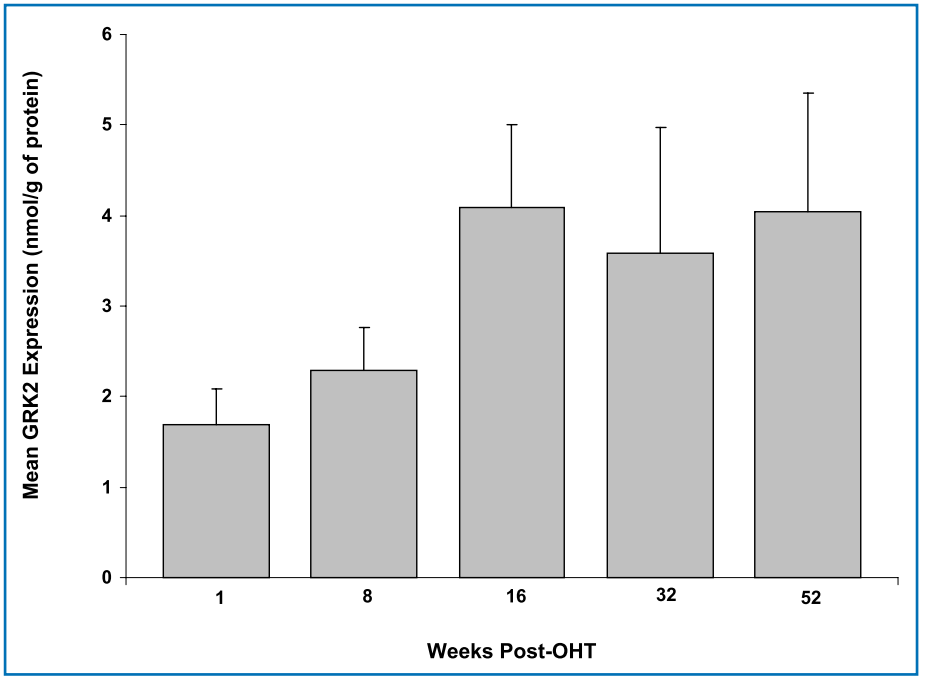

Figure 2. GRK2 expression after transplant over time (n = 24). Bars show representative mean GRK2 expression (±SEM) over the study period.

of a healthy heart, having normal function leads to a feedback mechanism that significantly lowers levels of GRK2 measured in white blood cells. These data support our hypothesis that GRK2, measured in peripheral lymphocytes, may represent a biomarker reflecting positively on cardiac function.

These data are also consistent with previous findings in which blood GRK2 protein levels inversely correlated with lower LV ejection fraction and increased in a correlative manner with NYHA functional HF class.[5] This may be somewhat surprising; however, previous studies had revealed that characteristics of the βAR system of peripheral lymphocytes were reflective of the myocardial situation in humans.[8–10] In addition to the above study in ambulatory HF patients,[5] we have also previously found that both myocardial and blood GRK2 mRNA and protein were significantly decreased in failing human hearts after a period of mechanical unloading with LVADs.[6] The current study provides further evidence supporting a role for blood GRK2 being a surrogate marker for response to treatment because when cardiac function was improved (after LVAD-mediated reverse remodeling or transplantation), GRK2 levels were significantly lowered. Interestingly, in the patients enrolled in our study who had a LVAD placed prior to transplant, GRK2 levels were on average lower than in the other patients; however, they had a further decline in GRFK2 after transplant. This finding is important because several studies, including a recent study in a cardiac-targeted GRK2 knockout mouse, showed that the decreasing cardiac GRK2 activity improves cardiac function and can even reverse HF.[3,11,12]

The inability to perform multicenter clinical trials due to cost and difficulties in enrollment underscores the need to develop surrogate markers of therapeutic response for HF treatments. Currently, there are no proven outcomes both in terms of biomarkers or anatomical markers that can serve as surrogates. Our hypothesis is that GRK2 will be an improved biomarker over what is available currently in HF.

We believe the significant decline in GRK2 protein expression after transplantation reflects the normalization of LV systolic function from the transplanted heart. We also report herein that after cardiac transplantation blood GRK2 remained low and steady over time, a finding that is consistent with improved cardiac function from the transplanted heart.

Limitations

The strength of our data is limited by the small sample size of our patient population. In addition, our study has other limitations. Some patients did not have GRK2 measured prior to heart transplant (9 out of 24). In these patients, we do not know if GRK2 was elevated compared to values after heart transplant. Nevertheless, we believe that the significant decline in GRK2 expression posttransplant is a clinically meaningful finding. Another limitation of our study is that we lack insight about the potential impact immunosuppressive regimens may have on GRK2 expression in these patients (posttransplant, all patients were on calcineurin inhibitors, mycophenolate mofetil, and steroids). This may be a significant issue because we are measuring the expression of GRK2 in peripheral lymphocytes. The immune system in heart transplant recipients is greatly suppressed, and this immunosuppression may affect GRK2 expression levels. Along these lines, in an experimental animal model of lung airway inflammation, dexamethasone was shown to significantly downregulate GRK2 expression.[13] Further study is warranted to better define the influence immunosuppression has on GRK2 expression. Finally, our small study population did not allow us to correlate GRK2 expression with the degree of LV systolic dysfunction prior to transplant.

Conclusions

Our study provides supporting evidence that GRK2 is a potential biomarker of LV systolic function in HF patients on the basis that GRK2 significantly declined after heart transplant consistent with normalization of LV function from the donor graft. Further study is required to better define the role of this novel biomarker in the management and treatment of patients with HF.

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