Myocardial Expression of the Arginine:Glycine Amidinotransferase Gene Is Elevated in Heart Failure and Normalized After Recovery
Potential Implications for Local Creatine Synthesis

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Background—Combination therapy consisting of mechanical unloading using a left ventricular assist device (LVAD) and pharmacological intervention can promote recovery from end-stage heart failure, but the mechanism is unknown. Preliminary microarray analysis revealed a significant and unexpected decrease in myocardial arginine:glycine amidinotransferase (AGAT) gene expression during recovery in these patients. The aim of this study was to evaluate the expression and role of AGAT expression in heart failure and recovery.

Methods and Results—We used quantitative real time (TaqMan) polymerase chain reaction to examine myocardial AGAT mRNA expression in implant and explant samples from recovering patients after combination therapy (n=12), end-stage heart failure (ESHF) samples from stable patients undergoing transplantation without LVAD support (n=10), and donor hearts with normal hemodynamic function (n=8). AGAT mRNA expression was significantly elevated in all heart failure patients relative to donors (4.3-fold \( P < 0.001 \) and 2.7-fold \( P < 0.005 \) in LVAD and ESHF relative to donors, respectively) and returned to normal levels after recovery. AGAT enzyme activity was detectable in both human and rat myocardia and was elevated in heart failure.

Conclusions—Our data highlight local and potentially regulated expression of AGAT activity in the myocardium and suggest a specific response to heart failure involving elevated local creatine synthesis. These findings have implications both for the management of recovery patients undergoing combination therapy and for heart failure in general.

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Key Words: genes \( \bullet \) heart-assist device \( \bullet \) metabolism \( \bullet \) myocardium \( \bullet \) remodeling

The high prevalence and poor prognosis of heart disease has lead to increasing interest in the use of left ventricular assist devices (LVADs) to support the failing heart, principally as a bridge to transplantation. However, several studies have revealed that LVAD support can lead to recovery from heart failure,\(^1\) although at a low and unpredictable frequency, currently 5% to 10% worldwide.\(^2\) In an attempt to maximize the rate and durability of recovery, we have developed a combination therapy consisting of mechanical unloading using an LVAD and pharmacological therapy to maximize reverse remodeling, followed by the use of the \( \beta_1 \)-adrenergic agonist clenbuterol to stimulate physiological hypertrophy and improve cardiac function, resulting in recovery in patients with nonischemic dilated cardiomyopathy.\(^3,4\) Combination therapy aims to maximize recovery, with currently two thirds of these patients recovering without the need for transplantation. However, the mechanisms and molecular markers underlying the recovery process remain obscure.

To screen for genes and pathways that may provide insight into mechanisms of recovery, we performed a preliminary microarray analysis (Dr Hall, unpublished data, 2005) of paired myocardial samples taken at LVAD implantation and explantation from 6 patients who underwent combination therapy and recovered. Among the genes showing differential expression, myocardial arginine:glycine amidinotransferase (AGAT) gene expression had a highly significant (2.8-fold) decrease during recovery. This was unexpected, as AGAT is the rate-limiting enzyme that catalyzes formation of guanidinoacetate (GAA), the immediate precursor of creatine (Cr), and is more commonly associated with kidney and liver. It has not previously been shown to be expressed in human myocardium or to change in response to cardiac disease.
The potential for local myocardial creatine synthesis is of interest because creatine/phosphocreatine (PCr) is central to energy metabolism in tissues with high energy demand, such as the myocardium.\(^5\) Dysregulation of this process in heart failure of various etiologies has led to the emerging concept that the failing heart is energy starved.\(^6\) Studies in both humans and animal models indicate that PCr and free creatine levels decrease by up to 70% in heart failure. Moreover, alteration of high-energy phosphate availability in creatine kinase knockout mice,\(^7\) guanidinoacetate methyltransferase knockout mice,\(^8\) or mice overexpressing the creatine transporter CRTR\(^9\) has adverse consequences for myocardial function.

Given the importance of creatine in cardiac energy metabolism and its dysregulation in heart failure, we sought to characterize AGAT expression and activity in normal and failing myocardium, and during recovery in LVAD patients after combination therapy.

**Methods**

**Patient Groups**
The study was approved by the Royal Brompton and Harefield ethical review committee and informed consent was obtained from patients. Twelve nonischemic cardiomyopathy patients who required LVAD implantation because of deteriorating clinical status with evidence of secondary organ dysfunction in the context of low cardiac output despite inotropic support, and who subsequently recovered sufficient ventricular function to have the device removed, were studied as previously described\(^10\) (LVAD group). Ten were male and 2 were female. Mean age was 36.5 years (range 15 to 56 years). Mean duration of heart failure symptoms was 47 months (range 1 to 56 months). Mean ejection fraction at time of LVAD implantation was 11±5%. During LVAD support, patients received a combination therapy consisting of mechanical support and \(\beta\)-blockers, angiotensin-converting enzyme inhibitors, angiotensin II receptor antagonists, and spironolactone, followed by administration of clenbuterol to stimulate hypertrophy and improve cardiac function.\(^11\) Immediately before explantation, ejection fraction measured after the pump had been turned off for 15 minutes was 65±6%. Mean duration of LVAD support was 321±206 days.

Ten patients with stable end-stage failure (ESHF group) undergoing transplantation were also studied as previously described.\(^10\) Six were male and 4 were female. Mean age was 43 years (range 9 to 64 years). Mean duration of heart failure symptoms was 42 months (range 1.5 to 192 months). Seven had dilated cardiomyopathy and 3 had ischemic heart disease.

**Myocardial Sample Analysis**
Paired samples taken from patients showing recovery consisted of left ventricle (LV) myocardial “core” samples taken at the time of device implantation (n=12) and LV biopsies taken at explantation after functional recovery (n=10). These were compared with LV biopsies from patients in stable end-stage heart failure that were taken at the time of transplantation (n=10). Right ventricular biopsies from donor organs with normal hemodynamic function were used as normal controls (n=8).

**RNA Analysis**
RNA was extracted using methods adapted for maximal recovery from endomyocardial biopsies\(^11\) and accurately quantified using RiboGreen (Invitrogen, Ltd) to ensure equal loading of polymerase chain reaction (PCR) reactions. Reverse transcription quantitative PCR was performed essentially as described\(^12\) using a PRISM 7700 (Applied Biosystems, Warrington, UK) and TaqMan chemistry. Expression levels were normalized using 18S rRNA with output data analyzed as described in the figure legends. mRNA abundance is shown normalized to 18S rRNA and relative to average donor value (\(^2\)-\(^{\Delta\DeltaCT}\) method) ± SEM. The AGAT TaqMan assays were purchased as pre-optimized kits from Applied Biosystems (human- Hs00155208_m1; rat- Rn00578954_m1).

**AGAT Enzyme Activity**
AGAT enzyme activity was measured in cardiac and kidney samples using a newly developed, sensitive and specific liquid chromatography/mass spectrometry assay developed to detect the low activity expected in the heart specimens. Tissue homogenates were prepared in buffer as previously described.\(^13\) Assays were run with arginine-glycine substrate versus arginine alone blanks. After incubation at 37°C for 3 hours, the reaction was stopped with 60% acetonitrile, followed by centrifugation, freeze-drying of the supernatant, and analysis of the reconstituted sample using high-performance liquid chromatography with mass detection. Chromatographic separation was achieved with a 15×0.2 cm column packed with 3 μm BDS-Hypersil (Phenomenex). Elution was carried out at 0.15 mL/min in gradient mode from 100% buffer A (10 mmol/L nonafluoropentanoic acid) to 50% buffer B (100% acetonitrile). A post-column stream of acetonitrile at 0.15 mL/min was added to reduce spray current and improve ionization efficiency. Column effluent mixed with acetonitrile was introduced into an LCQ Deca XP (Thermo Finnigan) ion-trap mass detector and detection was carried out in positive single ion monitoring mode for substrates and products. This procedure was verified positively for reproducibility and linearity with regard to time and the amount of the homogenate (data not shown).

**Determination of Creatine Metabolites**
Metabolite concentration was analyzed using chromatography as previously described.\(^14\) Samples of ventricular core collected during LVAD implantation (~50 mg) were freeze dried and extracted with 0.4 mol/L perchloric acid. As controls, extracts obtained from left ventricular biopsies of hearts with normal function taken from patients undergoing coronary artery bypass graft (CABG) were reanalyzed for creatine metabolite content.\(^15\)

The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

**Results**
Real-time quantitative PCR was used to measure AGAT mRNA in the group of 6 recovery patients used in the microarray analysis, together with samples from an additional 6 recovery patients. In agreement with the microarray data, AGAT mRNA levels were consistently and significantly (4.3-fold, \(P=0.001\); Figure 1A) higher at implantation compared with explantation, with all paired samples showing a decrease in AGAT expression over the recovery period (Figure 1B).

Consistent with the change in AGAT expression during recovery, we found an inverse correlation of AGAT mRNA levels with ejection fraction and a positive correlation with pulmonary capillary wedge pressure (\(R=-0.57, P=0.01\), and \(R=0.68, P<0.0008\), respectively; both measured before explantation with the device switched off for 15 minutes) in individual recovery patients.

AGAT mRNA levels seen in the LVAD patients were compared with those seen in normal functioning donor myocardium and in myocardium from ESHF patients undergoing cardiac transplantation without LVAD support (Figure 2). Levels in both the LVAD implantation group and the ESHF transplantation group were higher than in donors (4.3-fold, \(P=0.0007\), and 2.7-fold, \(P=0.0014\), respectively). AGAT mRNA levels were higher in the LVAD implantation...
group than the ESHF transplantation group, although this failed to reach statistical significance (P = 0.0603). After recovery, AGAT mRNA levels in explant samples returned to levels indistinguishable from donors.

As expression in myocardium was unexpected, we compared AGAT mRNA levels in a panel of human tissue RNAs. As shown in Figure 3, AGAT mRNA is expressed at different levels in several tissues, with low levels in the lung, high levels in the kidney, and intermediate levels in the brain, skeletal muscle, and heart, suggesting that AGAT is expressed both in tissues synthesizing creatine intermediates (kidney and liver) and in tissues with high energy requirements, such as striated muscle.

AGAT enzyme activity was assayed in both rat and human myocardial tissue extracts by measurement of GAA production. Rat myocardial activity was 15% of the levels seen in kidney, a major site of activity of this enzyme (2.6 ± 1.2 and 17.1 ± 4.6 nmol GAA/g wet wt per min, respectively). Analysis of AGAT enzyme activity in deteriorating human myocardium taken at the time of LVAD implantation from 2 different patients revealed elevated levels (2.2 and 2.5 nmol GAA/g wet wt per min) when compared with donor myocardium (0.9 nmol GAA/g wet wt per min), in agreement with mRNA measurements.

As expected, the total creatine pool (Cr + PCr) was decreased by 30% to 40% in the myocardia of failing hearts at the time of LVAD implantation in comparison to myocardium from hearts with normal hemodynamic function (Table), both when expressed as absolute concentration and in relation to either total adenine nucleotide (ATP, ADP and...
Creatine Levels

<table>
<thead>
<tr>
<th></th>
<th>Total Creatine (PCr + Cr)</th>
<th>Total Creatine/TAN</th>
<th>Total Creatine/TNAD</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVAD implant (n=3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>35.70</td>
<td>1.62</td>
<td>11.44</td>
</tr>
<tr>
<td>SEM</td>
<td>5.83</td>
<td>0.36</td>
<td>2.16</td>
</tr>
<tr>
<td>Control (n=17)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>58.54</td>
<td>2.38</td>
<td>19.57</td>
</tr>
<tr>
<td>SEM</td>
<td>2.56</td>
<td>0.10</td>
<td>1.21</td>
</tr>
<tr>
<td>P value (t test)</td>
<td>0.003</td>
<td>0.012</td>
<td>0.016</td>
</tr>
</tbody>
</table>

Creatine levels are decreased in failing hearts at the time of LVAD implantation. The concentration of creatine, adenine, and nicotinamide metabolites was measured by an liquid chromatography/mass spectrometry assay. Source tissues consisted of myocardial core samples taken during LVAD implantation and normal myocardial biopsies taken at the time of CABG.

Values are expressed in μmol/g dry wt. TAN indicates totals adenine nucleotides; TNAD, total nicotinamide metabolites.

AMP; TAN) or nicotinamide metabolite (NAD and NADH; TNAD) pools.

Discussion

The data presented herein show the previously undescribed mRNA expression and enzyme activity of AGAT in human myocardium and its response to heart failure. Our data show that myocardial AGAT mRNA levels are elevated relative to normal levels in failing hearts, both in deteriorating heart failure at the time of LVAD implantation and in stable ESHF. After a period of combination therapy leading to functional normalization and recovery, AGAT mRNA levels in the LVAD patients return to levels seen in donor right ventricle. Two important questions arise from these data. First, to what extent can AGAT mRNA levels in donor right ventricular samples be taken to reflect those of normal LV? Second, is decreased AGAT expression a feature of recovery or general to all patients receiving LVAD support? To address the first question, we examined AGAT mRNA expression levels in rat myocardial samples taken from the left ventricular apex, free wall, and septum and compared these to the right ventricular free wall. No significant differences in expression were observed (mean relative values 1.00, 0.99, 1.04, and 0.90 respectively; n=4 each group, P=NS). With regard to the second question, we reviewed a previous microarray analysis of patients undergoing LVAD support who did not recover myocardial function. No change of AGAT expression was detected (mean fold change, explant versus implant =0.89; n=19, P=0.37). Taken together, these data indicated that the levels at explantation correspond to a return to normal and that the decrease in AGAT expression at explantation reported here is not a general feature of LVAD support but is specific to recovery.

We have shown that AGAT transcripts in human myocardium are translated into functional protein with measurable enzymic activity, as measured by the appearance of GAA, one of the products of the AGAT reaction, and that this activity is increased in heart failure. Although low in comparison to its major site of action in kidney, the presence of myocardial AGAT mRNA and enzyme activity, as well as myocardial expression of guanidinoacetate methyltransferase, the gene product of which catalyzes creatine formation from GAA, suggest that the components of the creatine synthesis pathway are available and functional in mammalian heart. Taken together, our data show local and potentially regulated expression of AGAT in the myocardium and, in addition, a specific response to heart failure involving elevated local expression of the creatine synthesis pathway in the context of decreased intracellular creatine.

It is well known that changes in circulating levels of creatine have a reciprocal effect on the levels of AGAT enzyme activity. Rats fed a diet with increasing levels of creatine supplementation had decreasing levels of both AGAT mRNA and enzyme activity in kidney. This likely represents end-product feedback inhibition by creatine, although the molecular mechanisms involved remain unknown. Increased extracellular creatine also depletes the levels of creatine transporter in skeletal muscle cells, leading to the correct titration of intracellular creatine. Given the known depletion of intracellular Cr and PCr levels in myocardial tissue of patients with heart failure and seen in our patients (Table), it is possible that the increased level of AGAT expression newly described in this study is a direct response to the depletion of the myocardial creatine pool.

This raises the possibility that there is a mechanistic connection between intracellular creatine levels and AGAT expression in myocardium and that myocardial tissue itself is able to initiate localized synthesis of creatine as an adaptive response to lower intracellular levels. Although there is ample evidence of Cr/PCr dysregulation in heart failure, there is currently no direct evidence that creatine synthesis occurs in the myocardium as a consequence. Whether upregulation of AGAT represents an adaptive response to creatine depletion in heart failure therefore awaits further experiments.

Our data also indicate that recovery entails the reestablishment of correct levels of intracellular Cr/PCr and the concomitant normalization of AGAT mRNA levels and presumably AGAT activity. The effect of heart failure on the expression of myocardial creatine expression of certain metabolic genes (GLUT1, GLUT4, mCPT-1 and UCP3, CK) has been noted previously, with subsequent LVAD support as a bridge to transplantation only partially reversing these changes, despite some clinical improvement. In contrast, the return of AGAT expression from its elevated level to normal levels in recovery may be a consequence of the combination therapy that the patients in this study have undergone. Consistent with this idea, it has been shown that various β2-agonists including clenbuterol can stimulate creatine uptake by increasing transmembrane [Na+]-gradient, which indirectly stimulates the [Na+]-creatinine cotransporter. As part of the combination therapy at Heartfield, clenbuterol is administered to elicit LV hypertrophy after maximal pharmacologically induced reverse remodeling but whether it may also have the beneficial effect of helping to restore intracellular creatine levels to the energy-starved heart remains to be determined.

Conclusion

The data presented here indicate that the myocardium expresses AGAT and may therefore be able to synthesize...
creatinine locally. Moreover, AGAT mRNA expression and enzyme activity are increased in heart failure, suggesting that local creatine synthesis is responsive to intracellular creatine availability. These findings have implications both for the management of recovery patients undergoing combination therapy and for heart failure in general.

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Disclosures
None.

References
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