Alterations of gene expression in failing myocardium following left ventricular assist device support

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RECENT STUDIES HAVE DEMONSTRATED that chronic unloading of the failing heart with a left ventricular assist device (LVAD) can result in profound structural reorganization in failing myocardium following left ventricular assist device support.

LVAD support of the failing heart with a left ventricular assist device (LVAD) can decrease cardiac mass and myocyte size and has the potential to improve contractile function. To study the effect of chronic ventricular unloading on myocardial gene expression, a microarray (U133A, Affymetrix) profiling gene expression was compared before and after LVAD support in seven patients with idiopathic dilated cardiomyopathy and end-stage heart failure. On average, 1,374 ± 155 genes were reported as “increased” and 1,629 ± 45 as “decreased” after LVAD support. A total of 130 gene transcripts achieved the strict criteria for upregulation and 49 gene transcripts for downregulation after LVAD support. Upregulated genes included a large proportion of transcription factors, genes related to cell growth/apoptosis/DNA repair, cell structure proteins, metabolism, and cell signaling/communication. LVAD support resulted in downregulation of genes for a group of cytokines. To validate the array data, 10 altered genes were confirmed by real-time RT-PCR. Further study showed that the phosphoinositide-3-kinase-forkhead protein pathway and proteins related to nitric oxide synthase, including eNOS and dimethylarginine dimethylaminohydrolase 1 (DDAH1), an enzyme regulating endogenous nitric oxide synthase activity, were significantly increased during the cardiac remodeling process. Increased eNOS and DDAH1 expression after LVAD support may contribute to improved endothelial function of the failing hearts.

Increased eNOS and DDAH1 expression after LVAD support reacts with NO to form peroxynitrite and thereby to increase superoxide produced in the failing heart avidly attaches with NO bioavailability (3). The analysis was confined with the intent that a more uniform patient group would reduce the variability in response to ventricular unloading.

During the analysis we noted upregulation of the dimethylarginine dimethylaminohydrolase 1 (DDAH1) gene. DDAH is the enzyme that hydrolyzes asymmetric dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide synthase (NOS); increased DDAH activity would be expected to enhance NO production (17, 18).

Because endothelial NO bioavailability is decreased in heart failure (3), we paid special attention to changes in expression of other genes that might enhance NO bioavailability following LVAD support, including endothelial NOS (eNOS) and inducible NOS (iNOS), as well as genes related to oxidative stress, since increased superoxide produced in the failing heart avidly reacts with NO to form peroxynitrite and thereby to decrease NO bioavailability (3). The analysis was confined to patients with idiopathic dilated cardiomyopathy; patients with ischemic cardiomyopathy were excluded with the intent that a more uniform patient group would reduce the variability in response to ventricular unloading.

MATERIALS AND METHODS

Tissue sources. Patients with end stage congestive heart failure (CHF) secondary to idiopathic dilated cardiomyopathy received a Heartmate VE vented electronic left ventricular assist device (Cardiosystems, Woburn, MA) as a bridge to cardiac transplantation. Paired specimens of LV myocardium were obtained from seven patients with end-stage CHF at the time of LVAD placement and again at the time of cardiac transplantation (Table 1). An initial myocardial specimen (1–2 g) was obtained from the core of LV apex removed for placement of the inflow cannula of the LVAD. All patients...
subsequently underwent cardiac transplantation 194 ± 58 days after LVAD implantation (Table 1). At the time of transplantation, 3–4 g of myocardium was removed from the LV free wall near the apex. Myocardium was quickly frozen in liquid nitrogen and maintained at −80°C until study.

Total RNA isolation and Affymetrix oligonucleotide microarray. Total RNA was extracted from ~200-mg myocardial tissue samples with TaqMan RNA (Ambion, Austin, TX). RNA was quantified by spectrophotometry (absorbance 260 nm), and the quality of RNA was assessed by electrophoresis through formamide/formaldehyde agarose gel. Total RNA was first purified using an affinity resin column (RNeasy; Qiagen, Chatsworth, CA). Purified total RNA (8 μg) was converted to cDNA using the Superscript Choice cDNA synthesis kit (Gibco-BRL Life Technologies, Gaithersburg, MD), incorporating a T7-(dT)24 primer (Genset, Paris, France). Double-stranded cDNA was then purified by phase lock gel (Eppendorf, Westbury, NY) with phenol/chloroform extraction. Purified cDNA was used as a template for in vitro transcription reaction for the synthesis of biotinylated cRNA using an Enzo BioArray HighYield RNA transcription labeling kit (Affymetrix, Santa Clara, CA), and further purified using an affinity resin column. After purification, in vitro cRNA was fragmented in buffer containing magnesium at 94°C. Fragmented cRNA was hybridized onto the Affymetrix U133A GeneChip microarray, which contains 22,283 probe sets. Briefly, 20 μg fragmented cRNA was added along with control cRNA (BioB, BioC, and BioD), herring sperm DNA, and BSA to the hybridization buffer. The hybridization mixture was heated at 99°C for 5 min, incubated at 45°C for 5 min, and injected into the microarray. After hybridization at 45°C for 16 h, the array was washed and stained with the Affymetrix Fluidics Protocols-antibody amplification for Eukaryotic Targets, and scanned using an Affymetrix microarray scanner system at 570 nm. To minimize experimental variability, preparation of the paired samples from all of the patients was always performed at the same time by the same investigator.

Data analysis. Detailed protocols for microarray data analysis and extensive documentation of the sensitivity and quantitative aspects of the methodology have been described (11, 20). Briefly, the CHP file from each post-LVAD support specimen was first normalized to the corresponding CHP file obtained from the same subject before LVAD support (using pre-LVAD support as the baseline comparison file), and the mean signal intensities of all GeneChips were normalized to a targeted total fluorescence intensity of 300 with Affymetrix Microarray Suite 5.0. These data files were then transferred into a database with Affymetrix MicroDB and further analyzed with Affymetrix Data Mining Tool and Microsoft Excel. Representation of increases or decreases in gene expression with the microarray technique requires special consideration. If a change in signal intensity is always calculated as post-LVAD/pre-LVAD, this will result in a nonsymmetrical representation of the changes, depending upon whether gene expression has increased or decreased. This is because a fractional increase in signal intensity has no upper limit, whereas a fractional decrease in signal intensity can never be less than zero. Consequently, progressively greater decreases of signal intensity will be represented by progressively smaller decrements of the ratio. For example, a fivefold increase in signal intensity would result in a ratio of 5.0, whereas a similar decrease in signal intensity would have a ratio of 0.2; doubling these changes would result in ratios of 10 and 0.1, respectively, with changes of +5 and −0.1 for equivalent increases or decreases in gene expression. This nonsymmetrical presentation is especially troublesome when computing the average changes, since increases in signal intensity will be weighted more heavily than decreases in signal intensity of the same magnitude. To avoid this problem, and to give equal weighting to increases and decreases in gene expression, we calculated the relative change in signal intensity as the absolute ratio [higher intensity/lower intensity], and then assigned a “+” when the signal intensity was greater after LVAD support (i.e., gene expression increased) or a “−” when the signal intensity was greater before LVAD support (i.e., gene expression decreased). With this system, no change in signal intensity is indicated by a value of 1.0, whereas increases in signal intensity result in ratios greater than +1.0, and decreases of signal intensity result in ratios less than −1.0 (there are no ratios between −1.0 and +1.0). This results in equal weighting for positive and negative changes, so that meaningful averages can be obtained when one or more individuals show a change in gene expression that is opposite of the group as a whole.

Gene transcripts were defined as upregulated after LVAD support when the following conditions were met: 1) the mean signal intensity ratio of post-LVAD to pre-LVAD was greater than or equal to +1.5-fold, and the signal intensity ratio of post-LVAD to pre-LVAD was greater than +1.2-fold in at least five of the seven patients; 2) the gene transcript was present in the arrays of all post-LVAD samples, and the signal intensity of this gene transcript was greater than the maximum background value; 3) the absolute change of the mean signal intensity was more than 100 (at least 1/3 of the average signal intensity of the whole microarray); and 4) statistical analysis showed that the change of signal intensity was significant (P < 0.05). Gene transcripts were defined as downregulated when the following conditions were met: 1) the mean signal intensity ratio was less than or equal to −1.5-fold, and the signal intensity ratio of pre-LVAD to post-LVAD was less than −1.2-fold in at least five of the seven patients; 2) the gene transcript was present in the arrays of all pre-LVAD samples, and the signal intensity of

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age</th>
<th>Sex</th>
<th>LVAD Duration, days</th>
<th>LVDD, mm</th>
<th>LV Shortening Fraction, %</th>
<th>LV Ejection Fraction, %</th>
<th>PAP, mmHg</th>
<th>PCWP, mmHg</th>
<th>Cardiac Index, l/min/m²</th>
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<tr>
<td>1</td>
<td>42</td>
<td>F</td>
<td>89</td>
<td>60</td>
<td>15</td>
<td>10%</td>
<td>30/22</td>
<td>20</td>
<td>1.3</td>
</tr>
<tr>
<td>2</td>
<td>37</td>
<td>F</td>
<td>145</td>
<td>68</td>
<td>6</td>
<td>15%</td>
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</tr>
<tr>
<td>3</td>
<td>46</td>
<td>M</td>
<td>521</td>
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<td>20%</td>
<td>76/30</td>
<td>30</td>
<td>1.6</td>
</tr>
<tr>
<td>4</td>
<td>45</td>
<td>F</td>
<td>159</td>
<td>73</td>
<td>18</td>
<td>10%</td>
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<td>21</td>
<td>1.2</td>
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<td>5</td>
<td>64</td>
<td>M</td>
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<td>96</td>
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<tr>
<td>6</td>
<td>46</td>
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</tbody>
</table>

LVDD, Left ventricular diastolic dimension (echocardiography); PAP, pulmonary artery pressure; PCWP, pulmonary capillary wedge pressure; LVAD, left ventricular assist device; M, male; F, female.
this gene transcript was greater than the maximal background value; 3) the absolute change of the mean signal intensity was more than 100 (at least 1/3 of the average signal intensity of the whole chip); and 4) statistical analysis showed that the change of signal intensity was significant ($P < 0.05$). When gene expression was increased after LVAD support, the ratio of signal intensity of post-LVAD to pre-LVAD was used to indicate the fold change. When gene expression was decreased after LVAD support, a negative value of the ratio of pre-LVAD to post-LVAD signal intensity was used to indicate the change. Clustered expression patterns of genes were performed with Spotfire it! (UPGMA, unweighted average) (Spotfire, Göteborg, Sweden).

**Reverse transcription and real-time quantitative RT-PCR.**

One microgram of total RNA was reverse-transcribed using random hexamers and Moloney murine leukemia virus (MMLV) reverse transcriptase (Life Technologies). Oligonucleotide primers were designed according the corresponding human cDNA sequences in the National Institutes of Health GenBank. The primers are listed in Table 2. mRNA levels for the same patient before and after LVAD support were compared by quantitative real-time RT-PCR analysis, using the Light Cycler Thermocycler (Roche Diagnostics). Reactions were prepared in the presence of the fluorescent dye SYBR green I for specific detection of double-stranded DNA. Quantification was performed in the log-linear phase of the reaction, and cycle numbers obtained at this point were plotted against a standard curve prepared from serially diluted control samples. Results were normalized to GAPDH expression levels. Duplicates were performed for GAPDH, FOX3A, DDAH1, connexin 43, and Na⁺/K⁺-transporting α3 (β-actin; GAPDH and β-actin genes are strongly present in all microarrays). For these genes the reported values represent the mean of the duplicates.

**Western blot.**

Protein levels of DDAH1 (antibody provided by Dr. Masumi Kimoto, Department of Nutritional Science, Okayama Prefectural University, Okayama, Japan) (17), eNOS, iNOS, caveolin 1, and caveolin 3 (Transduction Laboratories), Cu/Zn-superoxide dismutase (Cu/Zn-SOD) and Mn-SOD (Santa Cruz Biotechnologies), phosphoinositol-3-kinase (PI3K) regulatory subunit 1 (p85α), total AKT and phosphorylated AKT (Transduction Laboratories) were determined by Western blot.

**RESULTS**

**Clinical information.** Myocardial specimens were obtained from seven patients diagnosed with idiopathic dilated cardiomyopathy (Table 1). Coronary angiography was performed in all patients, and none showed significant occlusive disease. Before LVAD support all patients were New York Heart Association (NYHA) class IV. Prior to LVAD implantation, patients had been treated with diuretics, angiotensin converting enzyme inhibitors, inotropic agents, and/or beta blockers. All patients were clinically markedly improved after LVAD implantation. Since LVAD implantation was performed as a bridge to transplant patients, no systematic attempt was made to assess function of the patient’s own heart during the period of LVAD support. Mean duration of LVAD support was 194 ± 58 (67–521) days.

**General information: gene expression.** The U133A array contains a total of 22,283 probe sets. Three probe sets specific for the 3′, middle, and 5′ regions of the GAPDH and β-actin genes are strongly present in all microarrays. The signal ratios for 3′ to 5′ probe of GAPDH and β-actin were 0.91 ± 0.02 and 0.96 ± 0.06, indicating good preparation of the microarrays (good quality sample and array preparations should have a ratio < 3; ideal array preparations should have a ratio

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>F</th>
<th>R</th>
<th>Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>F AGCA ACTGGCAGCCATCAGC</td>
<td>R TGGGCAAGGTTGTCTGTA</td>
<td>NM_002046</td>
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<tr>
<td>DDAH1</td>
<td>F GGACTGGTTCTCTGTG</td>
<td>R GCTTTTGGAGTGACCAGCAA</td>
<td>AL078459</td>
</tr>
<tr>
<td>Ankyrin repeat domain 2</td>
<td>F TAGAGGTTGTGAAACTCTG</td>
<td>R CTTGGGGGAGGTTATCAT</td>
<td>NM_02349</td>
</tr>
<tr>
<td>Growth arrest &amp; DNA-damage-inducible γ</td>
<td>F GTCTGATCGACGTGGTGATGG</td>
<td>R AAACGAAGCATTGCC</td>
<td>N25732</td>
</tr>
<tr>
<td>Forkhead box O3A</td>
<td>F ATTGGTCTTTTCAAGCTACTC</td>
<td>R AAATCAGTGCAGAACTTCTC</td>
<td>NM_005951</td>
</tr>
<tr>
<td>Metallothionein 1H</td>
<td>F TCCAGTCTCAGCTGCG</td>
<td>R CGGATTTTACGTGTCATTCT</td>
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<tr>
<td>Four-and-a-half LIM domains 1</td>
<td>F CTTACCAGGATCAGCCC</td>
<td>R AAACGAAGCATTGCC</td>
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<tr>
<td>Protein phosphatase 1</td>
<td>F ACAACACGTAGGGACT</td>
<td>R CGGCTCACTCTTGACCTGC</td>
<td>NM_005398</td>
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<td>Transmembrane 4 superfamily 1</td>
<td>F GGTATCACTGAGCTGAGG</td>
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<td>Connexin43</td>
<td>F TCTTGTGGAGTGACCAGCAA</td>
<td>R CGGATTTTACGTGTCATTCT</td>
<td>AI346835</td>
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<tr>
<td>ATPase, Na⁺/K⁺-transporting α3</td>
<td>F CGCGTTTCGGCTCTCAGTC</td>
<td>R GCTGGGAGGTTGTCTGTA</td>
<td>NM_000165</td>
</tr>
</tbody>
</table>

F, forward; R, reverse.

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**Table 2. Primers for real-time quantitative PCR**

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close to 1.0). The mean difference of the housekeeping genes GAPDH and β-actin were unchanged after LVAD support. A total of 10,402 ± 153 (46.7%), 392 ± 8 (1.76 ± 0.04%), and 11,489 ± 147 (51.6 ± 0.7%) gene transcripts were present, marginally present, or absent in all samples. Considering all 14 samples, a total of 14,010 different gene transcripts were detected, of which 7,595 (54.2%) were present in all samples.

**Genes differentially expressed after LVAD support.** A total of 10,428 ± 256 (46.8 ± 1.1%), 395 ± 12 (1.77 ± 0.05%), and 11,460 ± 245 (51.4 ± 1.1%) gene transcripts were present, marginally present, or absent in the samples pre-LVAD, and a total of 10,377 ± 194 (46.6 ± 0.9%), 389 ± 12 (1.75 ± 0.05%), and 11,513 ± 186 (51.7 ± 0.8%) gene transcripts were present, marginally present, or absent in the post-LVAD samples. A total of 130 gene transcripts achieved the strict criteria for upregulation and 49 gene transcripts achieved the strict criteria for downregulation between the pre-LVAD and post-LVAD specimens. To determine the gene expression patterns across all samples, an unsupervised hierarchical clustering method was used to generate a dendrogram (Fig. 1). Columns 1–7 illustrate gene expression patterns for subjects 1–7. These genes were divided into nine functional groups under the titles: transcription factors, metabolism, substrate/ion transport, cell growth/apoptosis/DNA repair, cell signaling/communication, myofibrillar/cytoskeletal/extra-cellular matrix, cell/organism defense, cytokines, and others (see Supplemental Table 3, available at the Physiological Genomics web site). The percent of genes in each functional category that were upregulated or downregulated following LVAD support is shown in Fig. 2.

A few general comments can be made regarding patterns of change in gene expression following LVAD support. A total of 24 genes that underwent significant change in expression following LVAD support were transcription factors; of these, 22 were upregulated. Twenty-five genes in the cell signaling/communication group were significantly changed after LVAD support; 14 of these were upregulated. A total of 21 genes in the cell growth/apoptosis/DNA repair group were upregulated, whereas four were downregulated. Several of the upregulated genes in the above groups are related to the PI3K-forkhead protein pathway, including genes for transcription factors (forkhead box O3A, NF-κB inhibitor-α), genes in the cell signaling/communication group [serine threonine kinase 39 (STE20/SP51 homolog), insulin receptor substrate 2, insulin receptor, PI3K-p85α], and genes in the cell growth/apoptosis/DNA repair group [growth arrest and DNA-damage-inducible γ (GADD45), and growth arrest and DNA-damage-inducible β]. The changes in expression following LVAD support of several of these genes were confirmed by real-time PCR (Fig. 3).

Genes for several closely related structural proteins were upregulated; most of these are costamere-related including spectrin-based membrane cytoskeleton (ankryin, spectrin, and four-and-a-half LIM domains), telothemion, enactin-1, laminin β1, vinculin, titin, and crystallin. Several genes for skeletal muscle contractile proteins tended to be increased, including skeletal muscle α-actin, skeletal muscle (slow) troponin T, skeletal muscle (fast-twitch) calsequestrin 1, and skeletal muscle troponin I, suggesting a possible contractile protein isoform shift during unloading of the failing ventricle, but these changes failed to meet our strict criteria for upregulation.

An interesting finding in the metabolism group was the upregulation of DDAH1. Since DDAH1 plays an important role in regulation of eNOS activity and NO production by catabolizing the endogenous NOS inhibitor ADMA, we confirmed the change in DDAH1 gene expression with real-time RT-PCR (Fig. 3) and measured the protein content of DDAH1 by Western blot (Fig. 4).

In the substrate/ion transport category we found that several metallothionein isoforms (2A, 1E, 1F, 1H-like protein 1X) were increased after LVAD, but efforts to detect the corresponding proteins by Western blot were unsuccessful. Downregulated genes in the substrate/ion transport group included connexin 43 (confirmed by PCR, Fig. 3), chloride channel 3, potassium inwardly rectifying channel J8, calcium channel voltage-dependent β2, soluble carrier family 5 (inositol transporters), Na-K transporting ATPase-α3 (confirmed by PCR, Fig. 3), transforming growth factor, crystallin-μ, and phosphodiesterases 4B and 4D (cAMP specific). Genes for several cytokines were downregulated including TNF superfamily 10, interferon-induced protein with tetratricopeptide repeats 1, small
Fig. 2. Hierarchical clustering for 7 subjects and 179 genes. Black indicates the signal change was less than 20%. Dark to bright red indicates the signal increased 1.2- to 4-fold (over 4-fold was considered as 4-fold). Dark green to bright green indicates the signal decreased 1.2- to 4-fold. From left to right are subjects 1–7. The GenBank accession number, gene name, and the mean fold change of these genes are listed beside the dendrogram.
inducible cytokine subfamily A21, and small inducible cytokine A2. (Several other cytokines were decreased but failed to reach our criteria for downregulation.)

Validation by real-time RT-PCR. To validate the microarray data, we used real-time RT-PCR on 10 gene transcripts that were significantly changed after LVAD. All 10 genes showed changes with PCR that were consistent with the microarray analysis (Fig. 3A). Specifically, in all of the subjects the changes (increased, decreased, or no change) of these 10 gene transcripts were consistent between the microarray data and the PCR data, although the fold change by PCR for each individual did not precisely match the fold change of the microarray data (Fig. 3B).

Western blotting. The genes for DDAH1 and p85 were upregulated, and Western blot also showed significant increases in protein expression for DDAH1 (1.00 ± 0.14 pre-LVAD vs. 1.49 ± 0.15 post-LVAD) and p85 (1.00 ± 0.16 pre-LVAD vs. 1.36 ± 0.14 post-LVAD) (Fig. 4). The microarray data showed that the genes for AKT, caveolin 1, caveolin 3, and Mn-SOD were unchanged after LVAD, and Western blotting showed that these proteins were also unchanged (Fig. 4). The CuZn-SOD gene was increased in only two subjects, but Western blotting showed that CuZn-SOD protein was significantly increased (1.00 ± 0.16 pre-LVAD vs. 1.23 ± 0.16 post-LVAD, P < 0.05). The microarray failed to detect a signal for either eNOS or iNOS, but the Western blotting showed that eNOS protein expression was significantly increased (1.00 ± 0.19 pre-LVAD vs. 1.58 ± 0.24 post-LVAD, P < 0.05) (Fig. 4). Interestingly, iNOS protein expression was unchanged after LVAD support (1.0 ± 0.19 pre-LVAD vs. 0.81 ± 0.19 post-LVAD) (Fig. 4).

DISCUSSION

Using the oligonucleotide microarray technique, we found that chronic unloading of the failing LV resulted
in significant upregulation of 130 genes and downregulation of 49 genes. Most of these genes have not been previously reported after LVAD support. These genes were grouped into nine functional categories representing transcription factors, metabolism, substrate/ion transport, protein modification/proteolysis, cell growth/apoptosis/DNA repair, cell signaling/communication, myofibrillar/cytoskeleton/extracellular matrix, cell/organism defense, and cytokines.

An interesting finding was that a large proportion of the genes undergoing significant change in expression were transcription factors, and most of these were upregulated after LVAD support. Many of these transcription factors are related to stress or control of cell growth/apoptosis/DNA damage (such as forkhead box 3A, basic transcription element binding protein 1, delta sleep-inducing peptide, Hif-1, neuropilin 1, cardiac-specific homeobox, TBP-like 1). The upregulation of these factors after LVAD support suggests that transcriptional regulation plays a role in the remodeling process in response to mechanical unloading of the failing heart. Approximately 13% of the genes undergoing significant change in expression are concerned with cell growth/apoptosis/DNA damage, consistent with the finding that LVAD support decreases LV mass and myocyte cell volume (34).

Recent reports have suggested that the PI3K pathway plays a critical role in the transition from cardiac hypertrophy to heart failure. Thus, in mice with pressure overload secondary to transverse aortic constriction, cardiac-specific deletion of a carboxyl terminal peptide of \( \Delta \gamma \) (TgGqI) resulted in ablation of PI3K activation in response to pressure overload and prevented the deterioration of LV function that occurred in wild-type animals (8). Furthermore, cardiac-specific expression of constitutively active PI3K resulted in mice with larger hearts, while hearts were smaller in dominant-negative PI3K mice (28). An increase of PI3K activity in myocardium, as well as an increase in myocardial insulin receptor and insulin receptor substrate 2, has been reported in animals with diabetes and ventricular hypertrophy (19). It was therefore somewhat unanticipated to find that removal of the pathologically increased load, which is known to result in reversal of myocyte hypertrophy in failing hearts (34), was associated with upregulation of several genes related to the PI3K-forkhead protein pathway including the PI3K-regulatory subunit (p85\( \alpha \)); three different gene transcripts of forkhead box O3A; growth arrest and DNA-damage-inducible \( \beta \) (3 isoforms); growth arrest and DNA-damage-inducible \( \gamma \) (GADD45G), a direct target of FOX3A on DNA repair; NF-\( \kappa \)B inhibitor \( \alpha \); gene transcripts of proteins which regulate CDK activity (ras homolog gene family-member E, ras-related associated with diabetes, RGC32 protein); insulin receptor; insulin receptor substrate 2 (2 isoforms), and connective tissue growth factor (insulin-like growth factor receptor binding protein). The unchanged mRNA and total AKT and phosphorylated AKT protein content suggest that AKT may not be critical for the decrease in cardiac mass following LVAD support.

Whether upregulation of inhibitory subunit p85 would contribute to a decrease in cardiac mass and myocyte size following LVAD support is unknown.

The effect of forkhead protein on cell growth and apoptosis is controversial. Several studies have demonstrated that forkhead protein is involved in apoptosis and control of cell growth, and recent reports have demonstrated that forkhead box 3A plays an important role in protecting against DNA damage through GADD45G (31), with increases in both free radical scavenging and resistance to oxidative stress (10, 24). Three different FOX3A genes were upregulated after LVAD support; one of these genes was clustered next to GADD45G, and one was clustered next to NF-\( \kappa \)B inhibitor \( \alpha \). Honda and Honda (14) and Taub et al. (30) reported that FOX3A acts as a transcriptional activator of antioxidant such as SOD and catalase, but our array data found no correlation between the alteration of the gene for FOX3A following LVAD support and the Mn-SOD, CuZn-SOD, or catalase genes. Our finding of lack of change in Mn-SOD protein content is consistent with the unchanged gene expression, while the increase of CuZn-SOD protein content indicates that RNA alterations are not necessarily correlated with protein content.

Metallothionein is a highly conserved, metal-binding, low-molecular-weight, thiol-rich protein (16) that recently has been reported to be a potent scavenger of free radicals; overexpression of metallothionein prevented the development of cardiomyopathy in the OVE26 transgenic murine model of diabetes (21) and protected the heart from ischemia-reperfusion injury (16). Metallothionein has been reported to be upregulated in the failing heart, and LVAD support has previously been reported to cause a decrease of metallothionein content in the heart, although metallothionein mRNA levels were not measured (1). We found that the gene transcripts of metallothionein 1H, metallothionein 1X, and metallothionein 1E were all significantly upregulated after LVAD support.

In the present study LVAD support resulted in changes of connexin 43, junction adhesion protein, LIM domains, and calponin, titin, myomesin, and vinculin that were the converse of those reported in the failing heart (15, 29, 35). The changes that we observed in the genes for ankyrin repeat domain 2, calponin, nidogen, and \( \gamma \)-filamin have not been previously reported in patients with heart failure undergoing LVAD support, and the effects of these genes in the hypertrophied or failing heart is unclear. The upregulation of high-abundance four-and-a-half LIM domains is an area for future study. The upregulation of insulin receptor substrate 2, solute carrier family 1 member 3, and pyruvate dehydrogenase kinase 4 might be associated with increased sensitivity to insulin and increased glucose uptake and metabolism. Cardiac failure is associated with a shift from fatty acid toward glucose metabolism by the heart (6), but the effect of LVAD support on glucose uptake has not been studied.

Increased expression of the gene for tissue inhibitor of metalloproteinase after LVAD support might play a
role in collagen remodeling, as failing hearts often have increased abundance of collagen I and increased metalloproteinase activity (4). Several genes related to protein glycosylation were decreased after LVAD support, but the physiological significance of this is not clear.

Previous studies using RT-PCR have identified several genes that were altered after LVAD support of the failing heart (2, 13, 26, 27, 32). Interestingly, only a few of these genes were found to be significantly altered using the microarray technique in the present study. Several reasons might account for our failure to find some of these alterations. First, because of the massive amount of data generated with the microarray technique, relatively strict criteria were used to accept a change in gene expression as significant. For example, only changes greater than 1.5-fold were accepted as significant. Consequently, lesser changes would not be detected even if they occurred consistently in all or most of the subjects. Moreover, the requirement of an absolute change of at least 1/3 of the mean signal intensity of the whole microarray would limit the ability to detect lower abundance genes, even if the signal intensity was increased severalfold. Furthermore, the relatively low sensitivity of the microarray technique, compared with real-time quantitative PCR, could impair the ability to detect low-abundance genes and changes in their expression. This is supported by our detection of several genes, including genes for iNOS and eNOS, with real-time PCR that were undetectable with the microarray. A recent study comparing data from Affymetrix GeneChips and real-time PCR concluded that microarray analysis is accurate and reliable but can underestimate differences in gene expression (33). An additional limitation of the present study is lack of detailed myocardial functional data both pre- and post-LVAD support for correlation with changes in gene expression.

In addition to these technical considerations, differences in the etiology of heart failure or in treatment regimens might cause differences in gene expression. For example, we found a markedly different pattern of change in gene expression after LVAD support in two paired samples from patients with ischemic cardiomyopathy (data not shown) compared with the subjects with idiopathic dilated cardiomyopathy analyzed in this report. We confined our analysis to patients with idiopathic dilated cardiomyopathy with the intent that a more uniform patient group would reduce the variability in response to ventricular unloading. Nevertheless, congestive cardiomyopathy likely encompasses a range of etiologies that could introduce variability into the response to LVAD support. There are likely to be several phases in the development of congestive cardiomyopathy; an initial insult (viral, toxic) or underlying genetic defect may result in contractile abnormalities of the myocyte or alterations of the extracellular matrix that result in ventricular dilatation and remodeling. The consequent increase in systolic load could then cause progressive contractile dysfunction with progression to end-stage cardiomyopathy. Mechanical unloading with an LVAD could reverse the abnormalities engendered by the increased load but would have no effect on the abnormality that initiated the dysfunction. Thus the response to mechanical unloading could well be influenced by the initiating cause of failure. Furthermore, unloading with an LVAD results in markedly decreased LV cavitary pressures that likely result in wall stresses that are far less than normal, and which might initiate stimuli for myocyte atrophy. As a result, the response to LV unloading is unlikely to result simply in a reverse sequence of the abnormalities that led to the development of heart failure, but is far more complex, combining the beneficial effects of ventricular unloading with the atrophy-inducing response to underloading. In addition, systemic responses to the improved cardiac output and tissue perfusion following LVAD placement might influence myocardial gene expression.

During our analysis, we noted prominent upregulation of the gene for DDAH1 after LVAD support. This finding was confirmed using PCR and was associated with a significant increase in myocardial DDAH1 protein expression. DDAH1 (the vascular dominant isoform of this enzyme) can regulate NOS activity by degrading asymmetric dimethylarginine (ADMA), an endogenous inhibitor of NOS (22, 23). This is of interest because substantial evidence indicates that NO bioavailability is decreased in the setting of heart failure (3), whereas circulating levels of ADMA are increased (25). (It should be noted that DDAH2, the dominant isoform in cardiac myocytes, was not significantly changed; the mean signal intensity of DDAH1 was almost twofold greater than DDAH2 across all of the samples, indicating a higher mRNA abundance of DDAH1.) To more fully explore mechanisms that could cause changes in NO bioavailability in response to LV unloading, we also examined the expression of eNOS. We found that eNOS gene and protein expression were increased after LVAD support, whereas iNOS was unchanged. The increases of DDAH1 and eNOS protein content could increase vascular NO production following LVAD support, changes that could potentially reverse the endothelial dysfunction in the failing heart. Binding to caveolin 3 has been shown to decrease eNOS activity, and increased caveolin 3 protein expression in canine heart failure appears to contribute to the augmented response to catecholamine simulation following NOS inhibition in the failing heart (12). Consequently, we also examined caveolin 1 and 3 before and after LVAD support; gene expression by microarray and RT-PCR, as well as protein content, for caveolin 1 and 3 were unchanged in our subjects.

Although the upregulation of DDAH and eNOS would be expected to enhance NO production, changes in iNOS expression could also alter NO availability. The microarray demonstrated downregulation of a group of genes for cytokines, consistent with previous reports that TNF-α and/or IL-6 were decreased after LVAD support (6, 26). Because cytokines can increase iNOS expression, we anticipated that the decreased cytokine-related genes might be associated with a de-
crease of iNOS expression. However, the decreased gene expression of cytokines after LVAD was not associated with a decrease of iNOS gene or protein expression, suggesting that factors other than cytokines play a role in iNOS expression.

Since heart failure is associated with increased oxidative stress, and because superoxide reacts avidly with NO to decrease NO bioavailability, we also examined genes for SOD. Neither the array data nor RT-PCR showed significant change in expression of SOD genes in response to LVAD support. However, Western analysis demonstrated an increase in CuZn-SOD after ventricular unloading, whereas Mn-SOD protein expression was unchanged. Since superoxide tends to react with the compartment in which it is generated, this finding suggests that cytosolic superoxide effects would be decreased following LVAD support, while mitochondrial effects (where Mn-SOD resides) would be unchanged.

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DISCLOSURES

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