Mechanical unloading increases caveolin expression in the failing human heart

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Abstract

Objective: Implantation of a left ventricular assist device (LVAD) in the failing human heart initiates structural and functional changes termed reverse remodeling. Mechanical unloading improves cardiac adrenergic responsiveness and lipid metabolism, processes regulated by caveolar function. We tested the hypothesis that mechanical unloading alters the expression of caveolins and these changes are linked to altered expression of markers of reverse remodeling.

Methods: Paired human myocardial samples were obtained from patients who received an LVAD as a bridge to cardiac transplantation. Transcript levels were measured using real-time Q-RT-PCR in RNA prepared from 34 pairs of formalin-fixed myocardial tissue blocks. Caveolin-1 and -3 protein levels were determined from frozen tissue (n=5) by Western blots. Caveolin-3 localization was demonstrated by immunohistochemistry.

Results: Caveolin-1 protein levels were upregulated in all LVAD-patients after mechanical unloading (P<0.002). Caveolin-1 mRNA was increased in 76% of the patients (n=34, P<0.001). Larger induction of caveolin-1 was associated with greater suppression of ANF. Caveolin-3 transcript levels increased in 82% of the cohort, along with a 2.5-fold induction of caveolin-2. Sarcolemmal caveolin-3 staining was increased after LVAD-support, although no change in total caveolin-3 protein was detected. The mRNA levels of the caveolin-associated CD36 also increased with unloading. Patients with ischemic cardiomyopathy showed greater induction of CD36 (P<0.05) than non-ischemic cases, as well as highly correlated changes in the expression of caveolin isoforms.

Conclusion: Mechanical unloading induces the expression of caveolins and CD36. The induction of caveolin-1 and the reciprocal suppression of ANF suggest that the changes in the expression of both genes are linked to decreased hemodynamic load. Enhanced caveolin expression during mechanical unloading of failing human hearts may be a part of the reverse remodeling of lipid metabolism, nitric oxide production and adrenergic signaling.

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1. Introduction

Mechanical unloading of the failing heart can lead to improvement of myocardial structure and function through a process termed ‘reverse remodeling’ [1]. Comparing tissue samples at the time of implantation and explantation of left ventricular assist devices (LVAD) has made it possible to track changes due to mechanical unloading, in the expression of genes linked to myocardial contractility, calcium handling, extracellular matrix, cell death and survival, and humoral signaling [2–6]. Using DNA microarrays we have profiled the effects of ventricular unloading on the expression of a large panel (>9000) of human genes (Uray, unpublished results). Prominent among the genes whose expression was altered were the genes encoding caveolins.

As members of a family of scaffolding proteins, caveolins concentrate in membrane microdomains a num-
ber of receptors and coupling proteins involved in signal transduction pathways and lipid metabolism. These include the GPI-linked fatty acid transport protein CD36, the α1- and β-adrenergic receptors, membrane tyrosine kinases, and hetero-trimeric G proteins [7–10]. Two isoforms, caveolin-1 and -2, are expressed ubiquitously and at highest levels in endothelial cells, fibroblasts and adipocytes, whereas the third, caveolin-3, is expressed selectively in muscle cells of all types [11]. Loss of caveolin-3 expression results in the activation of a program of progressive hypertrophy in cardiac myocytes, and deletion of both caveolin-1 and -3 results in severe cardiomyopathy [12,13]. On the other hand, both the induction of ischemia or chronic catecholamine administration, in experimental animals, suppresses myocardial caveolin expression [14,15]. The loss of caveolin expression has been linked to alterations in adrenergic signaling and lipid and carbohydrate metabolism [8,15,16]. Heart failure induced by pacing results in selective caveolin-3 upregulation [17]. Thus, perturbations in caveolin-3 expression can result in cardiomyopathy and cardiac disease can lead to altered caveolin expression. All the preceding observations have been drawn from studies in experimental animals. The goal of our study has been to determine whether interventions that change mechanical loading of the human heart also result in alterations in caveolin expression. Furthermore, we want to know whether these changes are linked to changes indicated by markers of reverse remodeling and altered myocardial function, as assessed by expression of the atrial natriuretic factor.

We demonstrate that both myocardial expression of caveolin-1 and sarcolemmal abundance of caveolin-3 increase during ventricular unloading. In a larger panel of patients, transcripts for all three caveolin isoforms were elevated following LVAD-implantation. Induction of caveolin-1 was associated with hemodynamic unloading, as reflected by the suppression of atrial natriuretic factor (ANF) expression. The expression of caveolins was changed in a highly coordinated way and there was marked induction of CD36 in the subset of LVAD-patients with ischemic cardiomyopathy. The upregulation of caveolin expression following mechanical unloading of failing human hearts does not represent a simple reversal of the changes associated with advanced heart failure, but rather is consistent with the remodeling of lipid metabolism and adrenergic signaling.

2. Methods

2.1. Samples

Total protein was extracted from five pairs of freshly frozen left ventricular tissue taken before and after LVAD-implantation (mean age 41±20 years, 40% ischemic cardiomyopathies, mean duration of unloading 261±139 days). Paired tissue blocks obtained from another six hearts before and after mechanical unloading were immunostained for caveolin-3 expression. For RNA analysis, fixed tissue blocks from all available (n=34) paired transmural left ventricular samples were obtained from patients (mean age 49.5±13 years, 65% ischemic cardiomyopathies), who had undergone LVAD-implantation at the Texas Heart Institute (duration of treatment 4–314 days, mean 132±86). Clinical and demographic data on these patients were reported previously [6]. The investigation conformed with the principles outlined in the Declaration of Helsinki.

2.2. Western-blot analysis

Proteins extracted with Tri-reagent (Sigma) were solubilized and fractionated by 12% SDS–PAGE and transferred to PVDF membrane. After blocking with 5% milk, the membrane was probed with anti-caveolin-1 antibody (Santa Cruz, 1:250) and subsequently with peroxidase-conjugated secondary antibody (Bio-Rad). After stripping (30 min at 50°C in 2% SDS and 100 mM β-MEA) the same membrane was re-probed with anti-caveolin-3 antibody (Transduction Laboratory, 1:4000), and again by anti-GAPDH antibody (RDI, 1:2000). Signals were detected by ECL detection kit (NEN).

2.3. RNA extraction and quantitation

RNA was extracted from formalin-fixed and paraffin-embedded tissue blocks and quantitated as described previously [6]. Each 10-μm section of ventricular tissue yielded ~200 ng of 18S RNA, each microlitre of extract containing ~1.7×10^{11} 18S RNA transcripts. Every sample was assayed in triplicate, with a ‘non-RT control’ lacking reverse transcriptase in parallel, to control amplifications due to genomic DNA contamination. Transcript quantitation based on real-time monitoring of amplification was carried out using an ABI 7700 performing 40 cycles of 95°C for 12 s and 60°C for 30 s. Values of transcripts in unknown samples were obtained by interpolating their Ct (PCR cycles to threshold) values on a standard curve derived from known amounts of cognate, amplicon-specific synthetic oligonucleotides. Transcript levels were normalized to the level of cyclophilin. Sequences of the primers and TaqMan probes used for quantitation of transcript levels, inter- and intra-assay coefficient of variation data are given in Table 1.

2.4. Immunohistochemistry

Deparaffinized sections were treated sequentially with a monoclonal anti-caveolin-3 antiserum (1:200), a biotinylated anti-mouse antibody and avidin-containing labeling agent and diaminobenzidine substrate (Vectastain ABC labeling reagent), and counterstained with Richard-Allen hematoxylin, and blueing reagent.
Table 1
Primers and TaqMan probes used for transcript quantitation

<table>
<thead>
<tr>
<th>Transcripts</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Fluorogenic probe</th>
<th>Variation coefficient (%)</th>
<th>Intra-assay</th>
<th>Inter-assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclophilin</td>
<td>(52+) ACGCGAGGCCCTTG</td>
<td>(117-) TTTCTGCTGTTCCTTTGGGACCT</td>
<td>(69+) FAM–CGCGCTCTCTTTTGAGCTGTTTGCA–TAMRA</td>
<td>9.7</td>
<td>16.7</td>
<td></td>
</tr>
<tr>
<td>Caveolin-1</td>
<td>(921+) GTAACCCGGACCCCTAAAC</td>
<td>(1001-) TGTCCCCCTGGGTCTTGGCA</td>
<td>(941) FAM– CCTCAACGATGACGTTGCAAGATTG–TAMRA</td>
<td>12.7</td>
<td>14.6</td>
<td></td>
</tr>
<tr>
<td>Caveolin-2</td>
<td>(143+) TCCCCACCGGCTCAAC</td>
<td>(205-) GTCACCGGTCTGCGAT</td>
<td>(162+) FAM–GTCACCGGTCTGCGAT–TAMRA</td>
<td>22</td>
<td>25.2</td>
<td></td>
</tr>
<tr>
<td>Caveolin-3</td>
<td>(347+) GGGCCATGCCCAAGACTA</td>
<td>(417-) TGCCAGATGAGATGAGGTAG</td>
<td>(374+) FAM–CGAGATCCAGTCATCGCCACA–TAMRA</td>
<td>27.9</td>
<td>32.9</td>
<td></td>
</tr>
<tr>
<td>CD36</td>
<td>(969+) TGGGAAAGTCACTGCCACAT</td>
<td>(1048-) TGCATAACGTGCTTCTCTCA</td>
<td>(990+) FAM–ATTAATGGTGACAGGCCTCATTTCCAC–TAMRA</td>
<td>16.5</td>
<td>29.6</td>
<td></td>
</tr>
<tr>
<td>ANF</td>
<td>(178+) CCCATGTACAATGCGGTG</td>
<td>(245-) TCTTCAAAATGTCACAGCA</td>
<td>(198) FAM–CAACGAGCTGTGATGTTCAAGAT–TAMRA</td>
<td>13.9</td>
<td>41.2</td>
<td></td>
</tr>
</tbody>
</table>

FAM, 6-carboxy-fluorescein; TAMRA, 6-carboxy-tetramethyl-rhodamine.
2.5. Statistical analyses

Differences in protein and transcript levels between pre- and post-LVAD implantation tissues were tested by Student’s paired t-tests; \(P<0.05\) was considered statistically significant. Cluster analysis was performed on the full set and subsets of patients by creating complete linkage of the changes of mRNA levels based on Euclidean distances, and forming clusters by \(K\)-means clustering based on changes of transcript levels (Statistica, StatSoft, Tulsa, OK, USA).

3. Results

To assess the effect of mechanical unloading on caveolin expression, we compared the level of caveolin-1 protein, as detected by Western-blot analysis, in extracts prepared from five pairs of human myocardial samples taken before and after implantation of a left ventricular assist device (LVAD; mean duration of unloading 261±139 days). In all instances, mechanical unloading resulted in increased levels of caveolin-1 (1.7-fold increase of median, \(P=0.002\), paired t-test; Fig. 1A). In addition, we measured the level of caveolin-1 mRNA in a large series (\(n=34\)) of paired formalin-fixed, paraffin-embedded left ventricular samples from LVAD-patients (mean duration of treatment 132±86 days). There was a 1.9-fold increase in mean caveolin-1 transcript levels in post-compared to pre-LVAD samples (\(P<0.001\), paired t-test, Fig. 1A). To assess the relationship between the induction of caveolin-1 and ventricular loading, we compared the
change in caveolin-1 and ANF mRNAs in individual subjects. We have previously reported that the level of ANF expression in pre-LVAD myocardia from these subjects is elevated 12-fold with respect to non-diseased control hearts [6]. Following mechanical support, caveolin-1 was induced in 76% of the LVAD-recipients and ANF transcript levels were suppressed in 74%. The suppression of ANF was significantly greater (P=0.01) in patients showing higher than 1.4-fold (50th percentile or above) caveolin induction compared to those with either lower than 1.4-fold upregulation or suppression (49th percentile or below) (Fig. 1B). Other parameters, including the age of patients, duration of LVAD-support and the type of cardiomyopathy, were not correlated with the degree of caveolin-1 induction. Caveolin-2 transcript levels were also upregulated (68% of subjects, increase in median 2.5-fold, P<0.001, Fig. 1C) but the induction of caveolin-2 and the suppression of ANF in individual subjects were not correlated.

Caveolin-3 transcript levels also increased in the majority (82%) of cases following LVAD-implantation (increase in median=2.3-fold, P<0.001, paired t-test, Fig. 2A and B). There was no significant correlation between caveolin-3 mRNA induction and ANF suppression (data not shown). Unlike caveolin-1, there was no corresponding increase in total caveolin-3 protein levels (Fig. 2B). We did, however, detect enhanced caveolin-3 immunostaining concentrated in the sarcolemma in four of six post-LVAD samples, compared to their pre-implantation controls (representative micrographs Fig. 2C and D). The distribution of caveolin-3 immunostaining was noticeably more heterogeneous in the pre-LVAD samples than in the samples obtained after mechanical unloading. In the vicinity of sites of ischemic tissue injury, marked by fibrotic transformation, the immunostaining of caveolin-3 in the sarcolemmal membranes was much stronger than in the non-fibrotic areas (Fig. 2E and F).

CD36 is a caveolin-associated membrane glycoprotein that is responsible for a major fraction of fatty acid uptake by muscle [18]. Comparison of the levels of CD36 mRNA
Fig. 2. (continued)

in the ‘explant’ versus ‘implant’ samples revealed a moderate, but significant (median 1.5-fold, \( P<0.05 \), paired \( t \)-test) increase in the post-LVAD tissues. However, the induction of CD36 mRNA was significantly greater in the subset of patients with ischemic cardiomyopathy (\( n=22 \)) than in those with non-ischemic disease (\( P<0.05 \), Fig. 3A).

We used clustering algorithms to examine relationships among the changes in multiple transcripts that occur in response to ventricular unloading in individual subjects. For this analysis, we used changes in the expression of the three caveolins, CD36, and three cytokine receptor genes (Her2/neu, Her4 and gp130) that we had measured in a previous study [6]. The pattern of changes was dependent on the type of cardiomyopathy present in the individual subjects. In patients with ischemic cardiomyopathy, mechanical unloading resulted in coordinated alterations in the expression of the three caveolin isoforms. There was also coordinated change in the three receptor genes, while the changes in CD36 were not linked to either the changes in the caveolins or the cytokine receptor genes (Fig. 3B). Fig. 3C and D shows the tight correlation in the level of expression of the three caveolins in individual subjects. In the subset of patients with non-ischemic cardiomyopathy the changes in the expression of the membrane receptors were linked, but there was no correlation in either the level of expression or the changes of expression of the three caveolins (data not shown).

4. Discussion

Our results demonstrate that mechanical unloading of patients with end-stage (NYHA Class IV) heart failure induces expression of all three caveolin isoforms. The upregulation of caveolins indicates that unloading does not simply reverse changes in gene expression related to heart failure towards normal. The restoration of the expression of caveolins and CD36 following mechanical unloading is consistent with ‘reverse remodeling’ of lipid metabolism and adrenergic signaling observed in patients following LVAD-implantation [19,20]. The alterations in gene expression that follow LVAD implantation are most likely associated with hemodynamic unloading of the ventricle, but we recognize that other non-hemodynamic consequences associated with implantation of a ventricular assist
device could also contribute to the changes we have observed.

The induction of caveolin-1 is associated with the reciprocal suppression of ANF, suggesting that the changes in the expression of both genes are linked to decreased hemodynamic load. There were, however, substantial differences in these responses depending on the type of the underlying cardiomyopathy. In patients with ischemic...
cardiomyopathy the change of expression of all caveolin isoforms were tightly correlated and alterations in CD36 levels were more pronounced than in patients with non-ischemic disease. While there is no information available on the effects of alterations of caveolin expression in human hearts, there is extensive evidence in experimental animals that changes in caveolin expression are associated with a number of perturbations in cardiac function. Hypoxia, elevated catecholamines and genetic perturbations in alpha1-adrenergic signaling are associated with suppression of caveolin gene expression [8,14,15]. Genetic ablation of caveolin-3 results in activation of a progressive hypertrophic response in cardiac myocytes, and combined deletion of both caveolin-1 and -3, causes severe cardiomyopathy [12,13]. On the other hand, pacing-induced heart failure in dogs results in selective induction of caveolin-3, and pressure-overload cardiac hypertrophy was recently found to cause upregulation of caveolin-3 (Martina Schinke, personal communication). At the time of LVAD-implantation the patients in this study have also had significantly increased caveolin-3 expression ($P<0.02$), compared to a limited set of non-diseased human heart tissue (Table 2). Taken together, the studies in experimental animals and in LVAD-recipients suggest an association between alterations in caveolin-3 expression and cardiac dysfunction.

Mechanical unloading of the failing human heart can lead to improved cardiac function [21], but not necessarily to normalization in the patterns of cardiac gene expression. Studies in experimental animals have demonstrated that unloading results in activation of a program of gene expression associated with cardiac hypertrophy. In human heart failure we have previously reported that unloading results in increased expression of hypertrophy-associated genes such as Her4 (erbB4), that are expressed at elevated levels in the failing myocardium. The expression of caveolin-3 appears to be very similar, namely its expression is elevated in the failing human heart and further increased by unloading. The induction of caveolin-3 associated with unloading suggests that its expression may be regulated in parallel with genes associated with cardiac hypertrophy.

In congestive heart failure the decline in cardiac function and deterioration of tissue structure is accompanied by a reactivation and induction of the atrial natriuretic factor [6,22]. ANF expression is upregulated in the hypertrophied myocardium and is downregulated in response to reduction in hemodynamic overload [23,24]. Therefore, we have used the embryonic gene ANF as a surrogate marker for ventricular load. There was significantly greater suppression of ANF during LVAD-support in the group of patients with marked induction of caveolin-1. This relationship is indicative of a functional link between caveolin-1 induction and the molecular adaptation of the myocardium to altered hemodynamic load. The question of how these changes may be related to cardiac function is more complex. In a limited number of patients ($n=6$) for whom cardiac index data are available only one third showed improved cardiac index between pre-LVAD and during LVAD-support (temporary turn-off). These data are very similar to reports by Razeghi of improved cardiac indices in a subset of LVAD recipients [25]. These discordant results may be due to the fact that the cardiac index is not necessarily an accurate marker of the contractile properties of the myocytes.

The fact that molecular indices of ventricular unloading do not translate directly into improved cardiac function, highlights the complicated relationship between ventricular load and cardiac contractility. For instance, improvements in cardiac function following LVAD function have been shown to have non-linear temporal kinetics, with optimal improvements between 4 and 12 weeks of unloading. Expression of the receptor tyrosine kinase Her2/neu also follows a similar biphasic pattern, with the greatest change in patients who had an LVAD for more than 6 and less than 24 weeks. Similarly, the median increase in caveolin-1 transcript levels was greatest in patients with medium duration of unloading (6 to 24 weeks) and declined thereafter. It appears, that in the failing human heart, reprogramming of cardiac gene expression and improved cardiac function in response to ventricular unloading may take several weeks to attain optimal effects. The cellular and molecular basis for the deterioration that seems to be associated with prolonged unloading is not well understood.

One of the most consistent findings in our studies on the effects of ventricular unloading in the failing human heart has been the concerted difference in the response to unloading of patients with ischemic versus non-ischemic cardiomyopathy [6]. The response of individuals with a non-ischemic cardiomyopathy both in terms of induction of receptor tyrosine kinases and caveolins is very heterogeneous whereas the subset of patients with ischemic cardiomyopathy shows a much more uniform response. In

### Table 2

Transcript levels expressed as percent of cyclophilin in non-failing, failing and unloaded human heart tissue

<table>
<thead>
<tr>
<th></th>
<th>Caveolin-1</th>
<th>Caveolin-2</th>
<th>Caveolin-3</th>
<th>CD36</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>7.238 (1.0)</td>
<td>23.95 (4.0)</td>
<td>0.2129 (0.12)</td>
<td>33.16 (17.0)</td>
</tr>
<tr>
<td>Pre-LVAD</td>
<td>7.271 (9.4)</td>
<td>25.61 (3.4)</td>
<td>0.5926 (0.68)</td>
<td>58.61 (21.0)</td>
</tr>
<tr>
<td>Post-LVAD</td>
<td>12.230 (5.1)</td>
<td>31.75 (11.2)</td>
<td>0.8799 (1.77)</td>
<td>86.24 (44.0)</td>
</tr>
</tbody>
</table>

Median values of controls ($n=11$) and LVAD group ($n=34$); values in parentheses represent S.E.M.
particular, in these patients there is a coordinated upregulation of all three caveolin isoforms. Combined induction of caveolin-1 and caveolin-3 may result in beneficial cardiac effects. In canine pacing-induced heart failure, expression of caveolin-3 and of sarcomemmal caveolae is increased. This increase was shown to be associated with augmented contractile activity and agonist-stimulated nitric oxide signaling [17]. While in myocytes reduced nitric oxide levels resulting from increased caveolin-3 expression may cause augmentation of the inotropic properties, it has been shown that in fibroblasts and endothelial cells caveolin-1 attenuates cardiac dysfunction after ischemia–reperfusion by maintaining nitric oxide release from the endothelium through the inhibition of protein kinase C [26]. Based on these studies in experimental animals we speculate that concerted upregulation of caveolins in endothelial and myocardial cells of the heart could result in altered nitric oxide signaling that is associated with improved cardiac performance.

In cardiac myocytes, several components of the adrenergic signal transduction pathways, including the β2-adrenergic receptor, are sequestered in caveolin-rich membrane microdomains [9]. Some studies have shown that caveolin-dependent sequestration improves the coupling between the β2-adrenergic receptor and adenylate cyclase [27]. On the other hand, pharmacologic manipulations that deplete myocardial membrane cholesterol and disrupt caveolae have also been reported to increase β-adrenergic-dependent cyclic AMP accumulation [9]. Mechanical unloading in patients similar to those in this study, however, resulted in marked improvements in β-adrenergic signaling [19]. Thus, our results suggest, that increased expression of caveolins in human myocardium may be a factor in improving β-adrenergic responsiveness. Further studies in animal models will be required to determine whether the induction of caveolins, comparable to the changes shown in failing human hearts, is directly linked to improved adrenergic signaling.

CD36 (FAT/fatty acid translocase), the major myocardial fatty acid transport protein, is also sequestered in caveolae [7]. Loss of CD36 results in both insulin resistance and impaired fatty acid transport [28,29]. CD36 deficiency in humans can lead to the development of hypertrophic cardiomyopathy [18,30]. The upregulation of CD36 expression following mechanical unloading may contribute to improvements in both myocardial fatty acid transport and insulin sensitivity. The finding that upregulation of CD36 primarily occurs in patients with ischemic cardiomyopathy is especially intriguing in the light of recent experimental data that reveal increased tolerance to ischemia in CD36 overexpressing hearts [18].

In conclusion, our data suggest that changes linked to reverse remodeling are associated with consistent transcriptional upregulation of caveolin expression and of CD36. The restoration of caveolin expression following mechanical unloading may result not only in normalization and improvements in myocardial fatty acid transport and insulin sensitivity, but also modulation of β-adrenergic responsiveness.

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