TRANSLATIONAL PHYSIOLOGY

Attenuation of the unfolded protein response and endoplasmic reticulum stress after mechanical unloading in dilated cardiomyopathy

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NEW & NOTEWORTHY

Our study shows that mechanical unloading by left ventricular assisted devices can improve cardiac markers of endoplasmic reticulum stress and cell death in advanced heart failure. Improvement of endoplasmic reticulum stress with LVAD support may be mediated by the improvement of calcium handling in patients with advanced heart failure.

Advanced heart failure (HF) is a complex progressive disease that leads to pump failure, ventricular dilation, and cardiac death. Mechanical unloading with left ventricular (LV) assist devices (LVAD) is increasingly used in patients with HF awaiting heart transplantation (HTx) or as destination therapy in cases where HTx is contraindicated. LVAD therapy decreases LV dimensions, reduces inflammation, and increases LV ejection fraction (EF) during mechanical support (11). As a consequence of pressure-volume unloading, the abnormalities in cardiac and peripheral hormonal and cytokine levels seen after HF (such as elevated cardiac TNF-α and circulating creatinine and bilirubin) may be reversed, and these changes can promote myocardial recovery (38), and even LVAD removal. However, weaning of LVAD is uncommon and no patients in the current study achieved myocardial recovery sufficient for device weaning. Several studies have reported very different incidences of weaning, ranging from 4.5% to 73% (9).

In the current study, we focused on the effect of mechanical unloading by LVAD support on HF-induced endoplasmic reticulum (ER) stress and its subsequent adaptive unfolded protein response (UPR), the associated altered calcium (Ca2+) cycling, and ER stress-related cell death. Impaired Ca2+ cycling is a pathologic mechanism central to the development of contractile dysfunction in ischemic and nonischemic cardiomyopathy. In addition to its function regulating protein folding, the ER also controls normal Ca2+ cycling, which is the basis of contractile function. A complex series of channels and proteins fine-tune cardiomyocyte Ca2+ homeostasis, and disruption of the normal levels of these proteins may lead to a compensatory response of the ER known as the UPR. If UPR mechanisms are unable to lessen ER stress, apoptotic pathways are activated leading to cardiomyocyte death (27). Although studies have reported improvement of Ca2+ handling after LVAD support...
(7, 33), the effect of mechanical unloading on ER stress and UPR has not been studied to date. We hypothesize that LVAD therapy improves cardiac calcium handling and subsequently reduces downstream UPR-related signaling and apoptosis activation. These findings may correlate with clinical markers of myocardial recovery improvement and reverse remodeling.

MATERIALS AND METHODS

Study design. To test the effect of mechanical unloading on UPR we aimed to analyze the changes in protein levels of UPR, ER stress markers, and apoptosis in paired cardiac samples before and after LVAD support and in comparison with healthy human hearts. To confirm deregulated calcium cycling as a causal event leading to UPR and apoptosis in cardiac cells we used in vitro studies using AC16 cardiomyocyte-like cells.

Patient population. All subjects were recruited at the New York Presbyterian Hospital-Columbia University campus, between 2010 and 2012. All patients met standard criteria for LVAD implantation. Patients in cardiogenic shock at the time of implantation, possible viral etiology, surgical complications after LVAD, chronic infection, or right ventricular failure were excluded from the analysis. Paired myocardial samples from patients with end-stage, idiopathic dilated or right ventricular failure were excluded from the analysis. Paired myocardial samples from patients with end-stage, idiopathic dilated cardiomyopathy (DCM) (n = 10) were obtained at the time of LVAD implantation and explantation/HTx. LVAD type consisted of both pulsatile (n = 4) and axial flow devices (n = 6). Samples from healthy donors were used as controls (n = 4). These samples were obtained from healthy hearts offered for donation by the United Network for Organ Sharing for heart transplantation that ended up not being used for logistic reasons or size, not due to any problems with the heart. At the time of LVAD implantation and explantation, a specimen was obtained from the apex of the LV. Samples were immediately flash frozen in liquid nitrogen and stored at −80°C. Demographic and clinical data for subjects were collected. LV end-diastolic dimension (LVEDD) was measured by echocardiography. LV EF was calculated by the simplified Quinones equation, and LV mass was calculated using the Devereux formula. Serum brain natriuretic peptide (BNP) was measured at the New York Columbia Presbyterian Hospital clinical laboratories by using the Architect BNP assay (Abbott, Abbott Park, IL). This study met all guidelines of the Institutional Review Board of Columbia University and conforms to the Declaration of Helsinki. All patients in the study were given informed consent upon enrollment.

Protein analysis. Western blot was performed as previously described (12) in all samples. Lysates of heart tissue were made by homogenization in RIPA buffer (Boston BioProducts, Boston, MA) in the presence of protease and phosphatase inhibitors (Roche, Indianapolis, IN). The protein concentration in each sample was determined by the bicinchoninic acid assay method using BSA as a standard. Samples (50 μg) were diluted in Laemmli’s SDS sample buffer (No. BP-110R; Boston BioProducts, Ashland, MA), denatured at 95°C for 10 min and loaded in 12% Tris-glycine polyacrylamide gels, and SDS-PAGE was performed under reducing conditions. Electrophoresis (1 h at 100 V) was performed in a Mini-Protean II cell (Bio-Rad, Hercules, CA) followed by wet transfer of proteins (4°C for 1 h at 100 V) onto PVDF membranes in a mini trans-blot transfer cell (Bio-Rad) filled with Tris-Glycine transfer buffer (Bio-Rad) with 20% methanol. Blots were blocked for 1 h in 5% nonfat milk diluted in Tris-buffered saline with 0.1% Tween-20 (TBST). For phosphorylated proteins, 5% BSA was used for blocking instead of milk. Blots were then incubated overnight at 4°C with primary antibody diluted 1:1,000 in 5% milk/BSA TBST. After being washed in TBST, blots were incubated in the presence of a peroxidase-labeled secondary antibody diluted 1:5,000 for 1 h at room temperature. Blots were washed again and incubated with H2GLO Quick Spray Chemiluminescent HRP Antibody Detection Reagent (Denville Scientific, Denville, NJ) for 1 min, followed by autoradiography. The following proteins were analyzed using the indicated antibodies: 1) Ca2+-handling proteins: ryanodine receptor type 2 (RyR2), and serine 2808 phosphorylated RyR2 (P-RyR2) (mouse monoclonal, No. ab2827 and rabbit polyclonal No. ab59225; Abcam, Cambridge, MA), Na+-Ca2+- exchanger type 1 (NCX1, rabbit polyclonal, No. π11–13; Swant, Bellinzona, Switzerland), SERCA type 2a (SERCA 2a, rabbit polyclonal, No. A010-20; Badrilla, Leeds, UK), phospholamban (PLB), and serine 16 phosphorylated PLB (P-PLB) (mouse monoclonal, No. ab2866 and No. ab15000; Abcam); 2) Ca2+- binding proteins: calreticulin (goat polyclonal, No. sc6467; Santa Cruz Biotechnology, Santa Cruz, CA) and calsequestrin (rabbit polyclonal, No. NB12-3516; Novus Biologicals, Littleton, CO); 3) UPR proteins: binding immunoglobulin protein (BiP) (rabbit polyclonal, No. 3177; Cell Signaling, Danvers, MA), eIF2α and phosphorylated eIF2α (serine 51 P-eIF2α) (rabbit polyclonal, No. 9722 and No. 721; Cell Signaling, Danvers, MA), total X-box binding protein (XBP1) (rabbit polyclonal, No. ab37152; Abcam) and XBP1 isoform s (XBPIs, rabbit polyclonal, No. sc7160; Santa Cruz Biotechnology); and 4) apoptosis marker: C/EBP homologous protein (CHOP) (mouse monoclonal, No. 2895; Cell Signaling). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, mouse monoclonal, No. sc32233; Santa Cruz Biotechnology) was used as a loading control. Secondary antibodies (anti-rabbit, anti-mouse and anti-goat, No. sc2030, No. sc2005) were purchased from Santa Cruz Biotechnology. Western blots for calsequestrin, RyR2/P-RyR2, and PLB/P-PLB were modified as follows. For calsequestrin, extracts were mixed with equal volumes of 10% SDS and sample buffer. For PLB/P-PLB (25 KDa), samples were incubated with loading buffer at 37°C for 1 h, electrophoresis was performed under nonreducing conditions, and wet transfer time was reduced to 20 min at 100 V. For RyR2/P-RyR2 (565 KDa) wet transfer was performed overnight at 4°C at 20 V with reduced methanol transfer buffer (7%).

Real-time PCR. To confirm the results obtained by Western blot of XBPI s isoform, we analyzed the mRNA levels of XBPIs using the primers described by Hirota et al. (16). Total mRNA was isolated using the RNeasy mini kit (Qiagen, Valencia, CA). Total RNA concentration and purity were measured on a NanoDrop spectrophotometer (NanoDrop technologies; Wilmington). cDNA synthesis was performed with Maxima H Minus First Strand Kit (Thermo Scientific, Waltham, MA). Real-time PCR for quantification of mRNA was performed in a Piko Real time system (Eppendorf Scientific) by using a SYBR-Green protocol. Results were expressed as fold changes in expression when compared with healthy hearts using the cycle threshold 2(ΔΔC_t) method with 18S as reference gene.

Fibrosis and nuclear DNA fragmentation. Paired pre- and post-LVAD cardiac tissue samples were stored in formalin and subsequently embedded in paraffin. Five-micrometer sections were prepared and triple stained. Masson’s Trichrome staining was performed to assess fibrosis according to the guidelines of the agent kit from Sigma (HT15, St. Louis, MO). To quantify the proportion of cells with DNA fragmentation, an in-situ TUNEL assay (Roche, Indianapolis, IN) was performed. DNA fragmentation events were visualized under fluorescent light (Nikon DMS510 filter) with excitation wavelengths of 488 and 555 nm. The proportion of collagen fibers to myocyte area and the number of TUNEL positively labeled cardiomyocytes was counted in 30 fields in each myocardial sample.

In vitro cell culture. To test the hypothesis that UPR activation and subsequent apoptosis can occur directly due to dysregulated ER Ca2+ and not just as a secondary effect of chronic HF, we used in vitro studies using the human adult left ventricular cardiomyocyte-like cell line, AC16 cells, which were gifted from Dr. Constantinos Drosatos (Temple University). The AC16 cell line contains cardiac- and muscle-specific markers, which reliably replicate the human cardiac transcription program (6). Characterization of AC16 was performed as described by Davidson et al. (6) by confirming expression levels of cardiac markers (BNP, cardiac actin) and the absence of a fibroblast marker (FSP1) and a skeletal muscle marker (MyoD), in comparison with fresh human septal cardiomyocytes. The cell division capacity of the
AC16 cells is absent in primary human cardiomyocytes, making AC16 a useful tool for in vitro studies: levels of all the cardiac markers studied were comparable in AC16 and primary human cardiomyocytes. Cells were used between passages 4 and 9. AC16 cells were grown in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin in 5% CO₂ atmosphere at 37°C. When cells reached ~80% confluence, they were removed by trypsinization (0.25% trypsin in PBS) and seeded into 10-cm culture plates in the presence of 10% FBS until they reached ~60% confluence. Cells were treated for 16 h with 0.5 μM of the calcium ionophore A23187 (Sigma-Aldrich, St. Louis, MO), plus 0.5 μM of the SERCA inhibitor thapsigargin (Sigma-Aldrich). In a separate experiment, AC16 cells were treated for 16 h with 0.5 μM A23187 and 0.5 μM thapsigargin plus 10 μM of the eEF2-α inhibitor salubrinal (Sigma-Aldrich). Control cardiomyocyte-like cells were treated with solvent (0.1% DMSO). The concentration of drugs used here was based on previous reports (19, 39), and the A23187 and thapsigargin concentration corresponded with a dose that effectively increased intracellular Ca²⁺, which was analyzed by imaging techniques as explained below. A shorter treatment time (4 h) resulted in the absence of effects, whereas a longer treatment (24 h) had toxic effects in the cells. Protein extracts were made by scraping and homogenization in RIPAbuffer followed by sonication using a Model 100 Sonic Dismembrator (Fisher Scientific, Asheville, NC). Extracts were kept at 4°C during the entire procedure. Protein analysis was performed by Western blot as described above.

**Calcium imaging.** Ratiometric Ca²⁺ imaging was used to confirm that the selected concentrations of thapsigargin and A23187 increased intracellular Ca²⁺ levels in AC16 cells. Cells were loaded with 5 μM Fura-2 AM for 30 min at room temperature. Coverslips were mounted on a gravity-fed perfusion chamber and were washed with Ringer's solution (Boston BioProducts) for 10 min. Images were acquired at 1 Hz using a Nikon Eclipse TE3500 inverted microscope equipped with a 40× 1.30 NA objective (Nikon), a pco.EDGE CMOS camera (pcO, Kelheim, Germany), a Lambda LS light source and a Lambda LS-2 filter wheel with 340 nm and 380 nm filters (Sutter, Novato). For the control period, cells were imaged for 10 s in the absence of drugs. Following this period, cells were perfused with 0.5 μM A2318, 0.5 μM thapsigargin, or vehicle (0.1% DMSO) for 20 min. Cells were then imaged for 10 s in the presence of these drugs. Following acquisition, images were ratioed on a pixel-by-pixel basis using FIJI software version 1.48. Changes in intracellular Ca²⁺ before and after drug addition were assessed from the average pixel intensities of manually drawn regions of interest encompassing cells.

**Data analysis.** The adequacy of our sample size was tested before by power analysis. Based on a preliminary experiment, we defined cardiac SERCA2a protein level mean and standard deviation in healthy and DCm hearts. With the assumption of a power of 0.8 with and a significant level of 0.05, a sample size of four healthy hearts and 10 DCm patient samples provides a 80–90% chance to detect a 20% difference in cardiac SERCA2a protein levels using one-way ANOVA. Descriptive statistics were used to describe patient characteristics. Continuous variables were presented as means ± SD, and categorical data are presented as counts and percentages. Categorical data were represented as frequency distributions and percentages. Continuous variables were compared using independent two-tailed t-testing or Wilcoxon-Mann-Whitney nonparametric testing, paired t-testing, and one-way ANOVA with post hoc Bonferroni analysis, whereas categorical variables were compared using χ² test of homogeneity and independence in contingency tables. Pearson’s product-moment correlation coefficient was used to determine the correlation between UPR measurements and markers of reverse remodeling. For all analyses, P values were two-sided and a P < 0.05 was considered significant. Following univariate correlation, a linear regression model was used to assess hazard ratios (HRs) with 95% confidence intervals for independent predictors of ER stress reduction after LVAD support. Significant associations in univariate analysis, defined as possible multivariable associations with P values less than 0.2, clinically relevant baseline covariables (body mass index, history of coronary artery disease, hypertension, and LVEDD), and elevated ER stress at pre-LVAD were entered into a multivariable linear regression model. All data were analyzed using SPSS 22 (SPSS, Chicago, IL).

**RESULTS**

**Demographic and clinical data.** None of the patients analyzed in this study demonstrated heart myocardial recovery significant enough for LVAD explantation without HTx. Table 1 shows patient demographic and clinical data. HF medical regimens during LVAD support included diuretics, and neuro-hormonal blockade with angiotensin converting enzyme-inhibitors or angiotensin receptor blockers. Doses were increased to maximally tolerated doses to achieve maximal neurohormonal blockade. Diuretics were used to maintain volume status. Patients were monitored closely in follow up. The duration of LVAD support ranged from 48 to 595 days (mean = 270.9 ± 53.8). The availability of a matching donor conditioned the duration of LVAD support. The LVEF of the patients was 13.8 ± 1.1% before and 27.2 ± 3.9% during LVAD (P < 0.05), suggestive of partial recovery of contractile function (Fig. 1A). Geometric reverse remodeling was evident by a reduction in LVEDD demonstrating effective unloading of the LV (7.1 ± 0.2 cm at baseline vs. 5.9 ± 0.3 cm during LVAD support; see Fig. 1B). Serum BNP, a marker strongly correlated with clinical HF events and volume overload, was drastically decreased from 1,693 ± 59 to 346 ± 40 pg/ml during LVAD support.

Table 1. Patient data: patient demographics and changes after LVAD support

<table>
<thead>
<tr>
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<th>Before LVAD</th>
<th>After LVAD</th>
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<tr>
<td>n</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Age, years</td>
<td>53.7 ± 5.0</td>
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<tr>
<td>Weight, kg</td>
<td>85.7 ± 4.6</td>
<td>85.2 ± 4.7</td>
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<td>27.8 ± 1.5</td>
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<td>LVAD duration, days</td>
<td>270.9 ± 53.8</td>
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<tr>
<td>Duration of chronic heart failure, days</td>
<td>1787.7 ± 345.8</td>
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<td>Bridged to orthotopic heart transplant, n</td>
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<tr>
<td>Pulmonary capillary wedge pressure, mmHg</td>
<td>27.4 ± 3.4</td>
<td>22.9 ± 2.9</td>
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<td>Mean pulmonary artery pressure, mmHg</td>
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<td>Posterior wall thickness, mm</td>
<td>0.98 ± 0.1</td>
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<tr>
<td>Creatinine, mg/dl</td>
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<td>1.4 ± 0.1</td>
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<td>Comorbidities</td>
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<tr>
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<tr>
<td>Chronic obstructive pulmonary disease</td>
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<td>—</td>
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<tr>
<td>Coronary artery disease</td>
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<td>—</td>
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<tr>
<td>Stroke</td>
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<td>—</td>
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<tr>
<td>Medication during LVAD</td>
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<tr>
<td>Angiotensin converting enzyme inhibitor</td>
<td>5</td>
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</tr>
<tr>
<td>Amiodarone</td>
<td>—</td>
<td>4</td>
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<tr>
<td>β-Blocker</td>
<td>—</td>
<td>7</td>
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<tr>
<td>Ca²⁺ blocker</td>
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<tr>
<td>Dipyridamole</td>
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<td>6</td>
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<tr>
<td>Diuretic</td>
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<td>9</td>
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<tr>
<td>Statin</td>
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<td>Warfarin</td>
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Values are means ± SE. LVAD, left ventricular assist devices.

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 supports (Fig. 1C), consistent with improved heart failure state. LV mass was decreased from 407 ± 47 to 235 ± 24 g following LVAD support (P < 0.001; Fig. 1D), as expected after pressure-volume unloading.

Calcium handling. Alterations of RYR2, NCX1, SERCA2a, and PLB are shown in Fig. 2. Before contraction, extracellular Ca\(^{2+}\) enters the cytosol through L-type channels; this influx of Ca\(^{2+}\) induces Ca\(^{2+}\) release from the ER into the cytosol through the RyR2. Although total RYR2 protein levels were increased in DCM patients and increased even further after LVAD support (data not shown), increased P-RyR2 was apparent pre-LVAD and reduced after LVAD support (Fig. 2A).

Hyperphosphorylation of RyR2 channels at S0828 contributes to diastolic Ca\(^{2+}\) leak, which can deplete ER Ca\(^{2+}\) stores (36). Normalization of the P-RyR2/RyR2 levels represents an important mechanism to restore calcium handling (Fig. 2A).

When high levels of cytosolic Ca\(^{2+}\) released from L-type channels and RyR2 interact with troponin, contraction (systole) occurs (23). Once Ca\(^{2+}\) dissociates from the fiber, it is cleared from the cytosol by being pumped outside the cell through the NCX1 exchanger by using a sodium antipporter mechanism, and back into the ER through SERCA2a, which uses ATP. SERCA2a and NCX1 levels were significantly reduced in DCM patients at the time of LVAD implant, which would result in an excessive amount of cytosolic Ca\(^{2+}\) during diastolic relaxation in HF. LVAD implantation restored SERCA2a and NCX1 to levels comparable with those of healthy hearts. SERCA2a activity is partially regulated by P-PLB, which inhibits SERCA2a activity in its serine 16 phosphorylated state. P-PLB/PLB was unchanged before and after LVAD support.

In the ER lumen, calsequestrin and calreticulin buffer Ca\(^{2+}\). Calsequestrin binds to Ca\(^{2+}\) to reduce the ER Ca\(^{2+}\) gradient that SERCA2a has to pump against. Calreticulin stores and releases Ca\(^{2+}\) with high affinity and can also bind to misfolded proteins to prevent them from being released to the cytosol. Figure 3A shows calreticulin levels decreased in DCM patients, in contrast with calsequestrin (Fig. 3B), which was increased. LVAD implant did not affect the levels of either Ca\(^{2+}\) chaperone; thus, Ca\(^{2+}\) buffering is not a likely factor regulated by LVAD.

Markers of ER stress. Disruption of ER Ca\(^{2+}\) cycling may induce ER stress and activate the UPR. When ER stress is sensed by the chaperone binding immunoglobulin protein (BiP, also known as Grp78), three different compensatory UPR pathways are activated: serine/threonine-protein kinase/endoribonuclease IRE1 (IRE1), eukaryotic translation initiation factor 2-α kinase 3 (PERK), and activating transcription factor 6 (ATF6) are simultaneously activated by the dissociation of BiP, which is bound to the hydrophobic regions of these proteins in basal conditions. IRE1 and ATF6 signaling branches converge by activating XBP1 and its target genes. UPR induces the switch from the regular XBP1 mRNA unspliced version (XBP1u), to a shorter spliced version that translates into a different XBP1 isoform, (XBP1s), which activates ER protein degradation and production of chaperones. LVAD support blocked BiP increase (Fig. 4A) and reverted the increased XBP1s/XBP1 protein ratio to levels similar to healthy hearts (Fig. 4B), an effect that was confirmed at the gene expression level. XBP1s/XBP1 ratio improvement correlated with the changes in SERCA2a levels induced by LVAD support (P = 0.024, R\(^2\) = 0.70; Fig. 4E).

Activated PERK phosphorylates eIF2α, which attenuates translation initiation and protein synthesis, and activates apoptosis via CHOP. Increased levels of total and P-eIF2α were evident in failing hearts, and LVAD support partially restored P-eIF2α and CHOP levels (Fig. 4, C and D). There was a significant correlation between P-eIF2α/eIF2α and CHOP (P = 0.03, R\(^2\) = 0.46; Fig. 4F). We analyzed a possible correlation between UPR markers and clinical data and found a significant correlation between improvement in LVEDD and P-eIF2α/eIF2α (P = 0.01, R\(^2\) = 0.54; Fig. 4G).

Preoperative predictors of elevated ER stress [as reflected by (P-eIF2α/eIF2α)] included elevated body mass index (HR = 14.29; P = 0.025), hypertension (HR = 4.42; P = 0.07), and hyperlipidemia (HR = 6.65; P = 0.06). Change in LVEDD (P = 0.002) was independently associated with a reduction in...
P-eIF2α/eIF2α stress of 15% of greater after LVAD support on multivariate regression analysis.

Fibrosis and apoptosis. ER stress directly induces apoptosis and DNA fragmentation. TUNEL staining (Fig. 5, A and B) confirmed the activation of apoptosis suggested by CHOP upregulation, showing a significant decrease after LVAD support (12 ± 0.8 post-LVAD vs. 15 ± 0.9 pre-LVAD nuclear DNA fragment events/1,000 cardiomyocytes, P < 0.05). Moreover, reduced apoptosis in LVAD supported patients as reflected by TUNEL staining correlated with improvement in LVEDD (P = 0.015; R² = 0.54; Fig. 5C), and a decrease of P-eIF2α/eIF2α (P = 0.071; R² = 0.47; Fig. 5D) and CHOP (P = 0.032; R² = 0.48; Fig. 5E). Fibrosis was not modified by LVAD support (130.3 ± 8.9 pre-LVAD vs. 128.3 ± 7.6 normalized units post LVAD, P = NS; Fig. 5, F and G).

In vitro activation of UPR. To test whether UPR activation can occur directly as a result of specifically low intraluminal ER Ca²⁺ levels, we studied the effect of blocking SERCA2a on UPR activation and apoptosis. The calcium ionophore A23187 increases intracellular Ca²⁺-levels; the SERCA2a blocker thapsigargin prevents Ca²⁺ from being pumped into the ER. Cotreatment with A23187 and thapsigargin was used to recreate a similar homeostatic environment as the one found in the hearts of HF patients by 1) increasing intracellular
calcium and 2) decreasing SERCA2a activity. Stimulation by the calcium ionophore A23187 together with inhibition of SERCA2a activity by thapsigargin resulted in increased intracellular calcium as seen by Fura-2 AM imaging (Fig. 6A). High intracellular Ca$^{2+}$ was accompanied by increases in markers of UPR: BiP, P-eIF2α/eIF2α, and XBP1s/XBP1 (Fig. 6, B–D). The functional effects of ER stress included cell death, as demonstrated by increased levels of CHOP (Fig. 6E). This suggests a causative effect of reduced ER Ca$^{2+}$ content on UPR and apoptosis. Treatment with A23187 or thapsigargin individually resulted in partial increase intracellular calcium and activation of the studied markers (data not shown). To determine whether UPR blockade can prevent increased intracellular Ca$^{2+}$-induced-activation of apoptosis, we cotreated AC16 cells with A23187 and thapsigargin plus the eIF2-α inhibitor salubrinal. Salubrinal partially attenuated eIF2α phosphorylation and prevented A23187/thapsigargin-induced activation of CHOP (Fig. 6E), suggesting that direct inhibition of the UPR can block the activation of apoptosis induced by states of high intracellular Ca$^{2+}$.

**DISCUSSION**

The results of this study show that mechanical unloading with an LVAD is associated with ameliorated UPR in end-stage HF patients. Our primary findings were as follows: 1) UPR is triggered in failing myocardium as shown by elevated BiP, P-eIF2α, and XBP1s; 2) LVAD support partially restores SERCA2a, NCX1, and P-RyR2 levels, suggesting an improvement of overall Ca$^{2+}$ homeostasis; 3) mechanical unloading is associated with reduced UPR as shown by improved P-eIF2α levels and normalized BiP and XBP1s/XBP1 ratio; 4) reduced UPR activation correlates with a decrease in apoptosis and an improvement in LVEDD post-LVAD; and 5) UPR activation may occur as a direct consequence of increased intracellular Ca$^{2+}$ and decreased SERCA2a activity.

Intracellular Ca$^{2+}$ homeostasis alteration and subsequent contractile dysfunction is a common finding in HF (24), as outlined in Fig. 7. A and B. Figure 7 summarizes potential healthy (Fig. 7A) and impaired (Fig. 7B) intracellular Ca$^{2+}$ homeostasis. Activation of the UPR can occur when primary ER functions are altered (27); in the current study, we confirm that increased intracellular Ca$^{2+}$ is enough to activate UPR response by using in vitro studies. The adaptive UPR response slows down ER machinery to allow recovery, but when the response is strong or sustained, apoptosis and cell death can result. UPR has been reported in human ischemia (13) and animal models of HF (34), but its role in human HF remains to be defined. After mechanical unloading, we found remission of the UPR markers studied together with a decrease in apoptosis rate: BiP is a master switch that activates the different UPR branches, whereas eIF2α activates apoptosis, and XBP1s transcriptionally induces ER stress genes. Although cardioprotective actions of activated eIF2α and XBP1 have also been reported (22, 35), our results indicate that UPR normalization by LVAD may be preventing ongoing apoptosis and cell loss. This deactivation of the UPR is probably a direct consequence of an improvement in Ca$^{2+}$ cycling in cardiomyocytes and the ER, as suggested by correlation between SERCA2a and XBP1s/XBP1 improvement and supported by the effect of SERCA2a inactivation in AC16 cells.

In HF, systolic and diastolic abnormalities are associated with altered cytoplasmic protein levels and function regulating Ca$^{2+}$ intake (RyR2) and release (SERCA2a, PLB, NCX1) (1). Impaired SERCA2a function is considered central to Ca$^{2+}$ deregulation in CHF, and decreased SERCA2a levels have been found in DCM and ischemic heart disease (1). Overall, a net decrease in the Ca$^{2+}$ clearance from the cytoplasm in DCM patients with failing hearts is suggested by decreased SERCA2a, decreased NCX1, and Ca$^{2+}$ leakage from ER through hyperphosphorylated RyR2. LVAD support is known to reverse these changes, which may be partially stretch related (38). Heerdt et al. (15) found that restored contractile function after LVAD correlated with SERCA2a improvement, which has led to novel therapeutic avenues supplementing SERCA2a to the failing myocardium (40). A recent study in sheep with myocardial infarction confirmed preserved SERCA2a levels, calcium cycling, and cardiac function with short-term mechanical support (37). NCX1 levels, which act to compensate

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**Fig. 3. LVAD support does not affect levels of cardiac calcium chaperones.** Calreticulin (Calret; A) and calsequestrin (Calseq; B) in healthy hearts (n = 4) and samples from failing hearts before (pre-LVAD; n = 10) and after (post-LVAD; n = 10) LVAD implantation (*P < 0.05 vs. healthy by 1-way ANOVA) are shown.

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**Fig. 7.** Schematic representation of how mechanical unloading improves intracellular Ca$^{2+}$ homeostasis. A, B, C, and D depict levels of BiP, eIF2α, XBP1s/XBP1, and CHOP in healthy and failing hearts (HF) before (pre-LVAD) and after (post-LVAD) LVAD support. The left panel shows increased levels of BiP, eIF2α, XBP1, and CHOP in HF patients compared with healthy individuals. LVAD support decreases these levels in failing hearts, suggesting a reversal of Ca$^{2+}$ homeostasis. The right panel shows normalized BiP, P-eIF2α, XBP1s, and CHOP in HF patients after LVAD support.
decreased cytoplasmic clearance of Ca\textsuperscript{2+} by SERCA2a (18), were also restored by LVAD support, consistent with animal and human models of HF (8). Based on the improvement of SERCA2a, NCX1, and P-RyR2, which acts to leak Ca\textsuperscript{2+} during diastole (2), we conclude that net available Ca\textsuperscript{2+} in the ER is likely increased after LVAD support. Our results on NCX1 contrast with previous findings that have reported increased NCX1 at the mRNA (31), protein (10), and activity (26) levels. Previous results from our group in a canine model of LVAD support also reported increased NCX in HF (12). It has been

Fig. 4. LVAD support reduces activation of cardiac unfolded protein response. Binding immunoglobulin protein (BiP; A), total and isoform s X-box binding protein (XBP1) and Xbp1s mRNA (B), total and phosphorylated eukaryotic initiation factor (eIF2\textalpha; C), and C/EBP homologous protein (CHOP; D) in healthy hearts (n = 4) and samples from failing hearts before (pre-LVAD, n = 10) and after (post-LVAD, n = 10) LVAD implantation (*P < 0.05 vs. healthy; #P < 0.05 vs. pre-LVAD by 1-way ANOVA) are shown. AU, arbitrary units. Graphical representation of the correlation between XBP1s/XBP1 ratio change and SERCA2a protein level change with LVAD support (E), CHOP protein level change and P-eIF2\textalpha/eIF2\textalpha ratio change with LVAD support (F), and LVEDD change with P-eIF2\textalpha/eIF2\textalpha ratio change (G) is shown. *P < 0.05 significant correlation.
postulated that increased NCX1 may act as a compensatory mechanism to reduced SERCA2a activity to clear excessive Ca^{2+} from the ER, although causing an increased risk of arrhythmias (30). Our present results show decreased NCX1 in HF, whereas LVAD support reversed this effect. Other human studies have similarly reported no change in NCX1 levels in HF (25, 28) and suggest that diastolic tension could potentially be responsible for alterations in NCX1 levels (14).

UPR activation occurs as a result of an accumulation of misfolded proteins in the ER lumen; adequate levels of Ca^{2+} in the ER are necessary for UPR activation (58). We observed increased apoptosis in Pre LVAD compared to Post LVAD. Fibrosis was not improved by LVAD support. Apoptosis was significantly reduced by LVAD support, although fibrosis did not improve (Fig. 5). Graphical representation of the correlation between apoptosis change and LVEDD change with LVAD support (C), apoptosis change and P-eIF2α/eIF2α ratio change with LVAD support (D), and apoptosis change and CHOP protein level change with LVAD support (E) is shown. *P < 0.05 significant correlation. F: fibrosis before and after LVAD support. G: representative Masson’s Trichrome staining. Scale bar = 100 μm.

Fig. 5. LVAD support reduces cardiac apoptosis and does not improve fibrosis. A: apoptosis as reflected by DNA damage before and after LVAD support. #P < 0.05 vs. pre-LVAD by 1-way ANOVA. B: representative double staining of transferase dUTP nick end labeling (TUNEL) and 4',6-diamidino-2-phenylindole (DAPI) staining. DAPI shows the localization of the nuclei. TUNEL positive nuclei are indicated by arrowhead. Scale bar = 100 μm. Graphical representation of the correlation between apoptosis change and LVEDD change with LVAD support (C), apoptosis change and P-eIF2α/eIF2α ratio change with LVAD support (D), and apoptosis change and CHOP protein level change with LVAD support (E) is shown. *P < 0.05 significant correlation. F: fibrosis before and after LVAD support. G: representative Masson’s Trichrome staining. Scale bar = 100 μm.
the ER are necessary for successful protein folding. Inappropriate nutrient levels (4) or disturbed redox balance (20) can also lead to ER stress and activation of the UPR. In our study, correlation between SERCA2a and XBP1 activation, together with our results showing increased UPR markers in AC16 in response to high intracellular Ca\(^{2+}\)/H\(_{11001}\), suggest that impaired Ca\(^{2+}\)/H\(_{11001}\) cycling is the main event triggering UPR in HF patients. Although it has also been suggested that ER stress can increase SERCA2a expression and activity (17) we did not find changes in SERCA2a protein levels in response to the treatments in AC16 cells (data not shown).

Sen et al. (29) have previously suggested an attenuation of the UPR by mechanical unloading in HF patients. These authors reported a decrease in the ER chaperones Grp72, Grp94, and BiP in HF patients after LVAD support. LVAD-induced reverse remodeling is accompanied by improvement in β-adrenergic signal transduction, lower natriuretic peptide levels, increased cell viability, and reduced inflammation (3). It is

Fig. 6. Increased intracellular calcium activates unfolded protein response (UPR) and apoptosis; UPR inhibition can prevent increased intracellular calcium-induced activation of apoptosis. Intracellular Ca\(^{2+}\) levels (A), BiP (B), and total and isoforms XBP1 (C) in AC16 cultured cardiomyocytes treated for 16 h with 0.5 μM of the calcium ionophore A23187 (A) and 0.5 μM of the SERCA inhibitor thapsigargin (T) are shown. Total and phosphorylated eIF2α (D) and CHOP levels (E) in AC16 cultured cardiomyocytes treated for 16 h with 0.5 μM of A23187 (A) and 0.5 μM of thapsigargin (T) in the absence or presence of the eIF2α inhibitor salubrinal (S) are shown. Results are expressed as percentage of control. *P < 0.05 vs. control; #P < 0.05 vs. A + T by 1-way ANOVA, n = 4/group.
likely that these effects are partially mediated not only by an improvement in Ca$^{2+}$ cycling but also improvement in UPR.

Restoration of Ca$^{2+}$ handling and reduction of ER stress is an active process that may impact reverse remodeling due to LVAD implant. Despite clinical evidence of reverse remodeling with decreased pulmonary congestion, reduced volume overload, improved cardiac geometry, and partially restored Ca$^{2+}$ homeostasis, no patients achieved myocardial recovery sufficient for LVAD removal. Although remission of the UPR correlated with improved LVEDD, it did not correlate with clinical recovery. This supports the known fact that a great number of HF patients are refractory to any improvement due to device support, likely due to the end-stage nature of the disease stage at LVAD implantation (21). The reduction of intracellular Ca$^{2+}$- induced apoptosis, as seen by blocked CHOP by salubrinal, suggests that blocking UPR could be a successful approach to prevent cell loss. Moreover, even in the absence of clinical recovery that allows weaning from the mechanical device, recovery of cardiac remodeling markers due to LVAD support may still improve overall functional capacity, provide protection to the right ventricle and pulmonary circulation, and maintain peripheral organ function; these factors may subsequently influence the outcome of subsequent transplantation. It has been proposed that adjuvant therapies may be the key to improving the recovery success of LVAD therapy (21). Previously, we found that treatment with the β-antagonist metoprolol improved Ca$^{2+}$ cycling, ER stress, and systolic function in dogs with HF (12). Treatment of LVAD patients with drugs that prevent ER stress or reduce UPR may be an approach to improve LVAD success.

Our study has several limitations. First, direct measurement of intracellular Ca$^{2+}$ levels was not performed in human tissue due to the difficulty in viably extracting and growing cells from explanted tissue; thus changes in homeostasis can only be inferred by its reflection in protein content. This limitation was partially offset by direct measurement of intracellular Ca$^{2+}$ in our cell studies. We did not measure activity of the different enzymes and activity can be modified differently than protein levels. Patients were not stratified based on their medical regimen, which could confound the interpretation of results. A broader study with patients taking similar medications as well as comparison with patients that are effectively weaned from LVAD support and confirmatory data in animal models would provide vital information into the role of ERS on myocardial recovery. We did not find any significant differences between the types of devices in our study, although the sample size for each device was small. Finally, previous reports have shown various effects of LVAD on fibrosis (5), and ER stress has been proposed to be profibrotic (32), but further study is needed to make definitive conclusions regarding the impact of ERS and LVAD support on fibrosis.

In summary, patients with advanced HF show activation of the UPR. Mechanical unloading with LVAD implantation was associated with improved Ca$^{2+}$ cycling protein levels and decreased UPR, which may prevent apoptosis and cell loss. These findings suggest that restoration of normal ER metabolism is an important parameter for myocardial homeostasis in HF and during cardiac mechanical unloading.

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