Contractile Reserve and Calcium Regulation Are Depressed in Myocytes From Chronically Unloaded Hearts

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Background—Chronic cardiac unloading of the normal heart results in the reduction of left ventricular (LV) mass, but effects on myocyte contractile function are not known.

Methods and Results—Cardiac unloading and reduction in LV mass were induced by heterotopic heart transplantation to the abdominal aorta in isogenic rats. Contractility and [Ca\(^{2+}\)]\(_i\) regulation in LV myocytes were studied at both 2 and 5 weeks after transplantation. Native in situ hearts from recipient animals were used as the controls for all experiments. Contractile function indices in myocytes from 2-week unloaded and native (control) hearts were similar under baseline conditions (0.5 Hz, 1.2 mmol/L [Ca\(^{2+}\)]\(_o\), and 36°C) and in response to stimulation with high [Ca\(^{2+}\)]\(_o\), (range 2.5 to 4.0 mmol/L). In myocytes from 5-week unloaded hearts, there were no differences in fractional cell shortening and peak-systolic [Ca\(^{2+}\)]\(_i\) at baseline; however, time to 50% relengthening and time to 50% decline in [Ca\(^{2+}\)]\(_i\) were prolonged compared with controls. Severe defects in fractional cell shortening and peak-systolic [Ca\(^{2+}\)]\(_i\) were elicited in myocytes from 5-week unloaded hearts in response to high [Ca\(^{2+}\)]\(_o\). However, there were no differences in the contractile response to isoproterenol between myocytes from unloaded and native hearts. In 5-week unloaded hearts, LV protein levels of phospholamban were increased (345% of native heart values). Protein levels of sarcoplasmic reticulum Ca\(^{2+}\) ATPase and the Na\(^+\)/Ca\(^{2+}\) exchanger were not changed.

Conclusions—Chronic unloading of the normal heart caused a time-dependent depression of myocyte contractile function, suggesting the potential for impaired performance in states associated with prolonged cardiac atrophy. (Circulation, 2003;107:1176-1182.)

Key Words: calcium ▪ myocytes ▪ contractility ▪ transplantation

The cardiovascular system of normal animals and humans undergoes multiple changes during prolonged space-flight and microgravity, including cardiac unloading and loss of left ventricular (LV) mass.\(^1\)\(^-\)\(^2\) Mechanical unloading of failing human hearts with LV-assist devices (LVADs) reduces excess LV load and mass and improves cardiac performance in some patients.\(^3\)\(^-\)\(^4\) However, the effect of severe and chronic unloading of the normal heart on myocyte contractile function is unclear.

To study the effect of chronic and severe cardiac unloading on myocyte contractile function, we used the rat model of heterotopic transplantation. This model has been used to study the effect of mechanical unloading on LV mass, structure, and gene expression of the nonfailing heart.\(^5\)\(^-\)\(^11\) Previous studies showed that the size of the transplanted heart in this model is determined by cardiac workload such that the decrease in the size of unloaded hearts is mainly due to decreased mechanical stress rather than the loss of cells, the denervation of the transplanted hearts, or slower heart rate.\(^5\)\(^-\)\(^10\) In addition, this model of chronic unloading is associated with the induction of fetal genes that are expressed during pressure overload hypertrophy, including atrial natriuretic peptide (ANP) and β-myosin heavy chain (β-MHC).\(^5\)\(^-\)\(^10\) To elucidate the functional and molecular effects of chronic unloading on contractile function, we tested the hypothesis that severe cardiac unloading leads to time-dependent changes in Ca\(^{2+}\)-regulatory gene expression and myocyte contractility.

Methods

Animal Model

LV unloading was induced in Lewis rats (12 weeks old; male, Charles River, Portage, Mich) by heterotopic heart transplantation to the abdominal aorta of isogenic recipients (n=51).\(^12\) Briefly, the ascending aorta of the donor is anastomosed end-to-side on the abdominal aorta of the recipient, and the pulmonary artery of the donor is anastomosed end-to-side on the inferior vena cava of the donor.

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recipient. The donor heart is perfused through the coronary arteries and beats, but the left ventricle is unloaded and flaccid. Therefore, both the native and transplanted hearts are perfused with the same blood and subjected to similar circulatory neurohumoral environments until euthanization. Native hearts from recipient animals were used as controls in these experiments. Animals were studied at either 2 weeks (n=17) or 5 weeks (n=34) after transplantation.

Gene Expression and Protein Levels in LV Tissue
LV tissues of both native (control) hearts and transplanted unloaded hearts from the same recipient animals were frozen and stored at −80°C until use. LV tissues from native hearts of nonoperated and operated age- and body weight–matched animals were also analyzed at 2 and 5 weeks after operation (n=6 hearts per group). Total RNA was extracted from frozen LV tissue. Northern blot analyses were performed to assess mRNA levels of ANP and β-MHC and were normalized to GAPDH (n=4 to 5 animals per group). Western blot analyses were performed to assess protein levels of sarcoplasmic reticulum Ca2+ ATPase (SERCA2a), phospholamban (PLB), and the Na+/Ca2+ exchanger and were normalized to GAPDH (n=3 to 6 animals per group).

Simultaneous Measurement of Cell Motion and [Ca2+]i
LV myocytes were dissociated from both control and transplanted unloaded hearts from animals both 2 weeks (5 animals, n=15 myocyte experiments per group) and 5 weeks (7 animals, n=18 myocyte experiments per group) after transplantation. Cell motion and [Ca2+]i-sensitive fluorescence with fluo-3 were measured simultaneously. The procedures of cell dissociation, instrumentation, and calibration of [Ca2+]i, are described elsewhere. At baseline, myocytes were paced with field stimulation at 0.5 Hz with 1.2 mmol/L [Ca2+]i at 36°C. After baseline measurements, contractile reserve was then studied in the isolated myocytes 2 weeks (n=11 to 12 myocyte experiments per group) and 5 weeks (n=11 myocyte experiments per group) after transplantation. To study contractile reserve at high work states, the [Ca2+]i was increased to 2.5 and 4.0 mmol/L at a constant pacing frequency of 0.5 Hz. Measurements were made after 3 minutes at each level of elevated [Ca2+]i. In separate experiments, the dose response to isoproterenol (10−5 to 10−6 mol/L) was measured. Studies were performed both 2 weeks (4 animals, n=6 to 9 myocyte experiments per group) and 5 weeks (5 animals, n=9 to 10 myocyte experiments per group) after transplantation.

Statistical Analysis
Values are expressed as mean±SEM. Comparisons among the groups were analyzed using ANOVA followed by a post hoc test using the Dunnett multiple-comparisons test. Two-way ANOVA with repeated measures was used to assess the response (intervention term) to the sequential stepped increases in [Ca2+]i and to compare any differences between the transplant and control groups (group term). Statistical significance was accepted at the level of P<0.05.

Results
LV Remodeling in the Normal Unloaded Heart
The LV-to–body weight ratio was lower in transplanted unloaded hearts than in native controls at 2 weeks (1.6±0.1 versus 3.1±0.2 mg/g, P<0.001; n=17 hearts per group) and 5 weeks after transplantation (1.2±0.1 versus 2.6±0.2 mg/g, P<0.001; n=34 hearts per group). Corroborating prior observations in the heterotopic transplant model, LV message levels of ANP and β-MHC, which are known to be differentially expressed during the development of hypertrophy, were higher (P<0.05) in unloaded than in control hearts both 2 and 5 weeks after transplantation (data not shown).

Myocyte Function
The baseline characteristics of cell morphology, contraction, and [Ca2+]i, transients in myocytes from both control and unloaded hearts are shown in Table 1 (2-week unloading) and Table 2 (5-week unloading). Myocyte area and length were smaller in myocytes from unloaded compared with control hearts after both short-term and chronic unloading. At 2 weeks after transplantation, there were no differences in baseline contractile parameters between myocytes from unloaded and control hearts (Table 1). At 5 weeks after transplantation, fractional cell shortening and levels of peak-systolic and end-diastolic [Ca2+]i, under baseline conditions were similar in myocytes from unloaded and control hearts. However, time to 50% relengthening and time to 50% decline in [Ca2+]i, were prolonged in myocytes from 5-week unloaded hearts compared with controls (Table 2).

Contractile function at high work states was studied by increasing [Ca2+]i, to 2.5 and 4.0 mmol/L in myocytes from transplanted unloaded hearts.

| TABLE 1. Baseline Characteristics of Myocyte Contraction and the [Ca2+]i, Transients (2 Weeks) |
|-----------------------------------|-------------------|----------|
| Myocyte area, μm²                | Control (2 Weeks) | Unloaded (2 Weeks) |
|                                  | 3285±269          | 2438±141 |
| Diastolic cell length, μm        | 117±5             | 95±3     |
| Fractional cell shortening, %    | 7.1±0.6           | 7.0±0.7  |
| Time to peak shortening, ms      | 79±2              | 84±3     |
| Time to 50% relengthening, ms    | 34±2              | 40±5     |
| Peak-systolic [Ca2+]i, nmol/L    | 549±48            | 532±55   |
| End-diastolic [Ca2+]i, nmol/L    | 74±6              | 77±9     |
| Amplitude of [Ca2+]i transients, nmol/L | 475±50 | 455±55   |
| Time to peak [Ca2+]i, ms         | 34±2              | 38±3     |
| Time to 50% decline in [Ca2+]i, ms | 70±3             | 77±6     |

Values are mean±SEM. N=15 myocytes obtained from 5 hearts per group. Control indicates LV myocytes from native hearts of animals 2 weeks after receipt of the heterotopic transplanted hearts. Unloaded indicates LV myocytes from the transplanted unloaded hearts.

| TABLE 2. Baseline Characteristics of Myocyte Contraction and the [Ca2+]i, Transients (5 Weeks) |
|-----------------------------------|-------------------|----------|
| Myocyte area, μm²                | Control (5 Weeks) | Unloaded (5 Weeks) |
|                                  | 3206±123          | 1905±128 |
| Diastolic cell length, μm        | 120±3             | 102±3    |
| Fractional cell shortening, %    | 5.9±0.6           | 5.9±0.6  |
| Time to peak shortening, ms      | 80±4              | 90±4     |
| Time to 50% relengthening, ms    | 40±3              | 53±4     |
| Peak-systolic [Ca2+]i, nmol/L    | 426±28            | 428±25   |
| End-diastolic [Ca2+]i, nmol/L    | 79±4              | 78±3     |
| Amplitude of [Ca2+]i transients, nmol/L | 346±28 | 351±24   |
| Time to peak [Ca2+]i, ms         | 35±1              | 39±2     |
| Time to 50% decline in [Ca2+]i, ms | 69±4             | 85±3     |

Values are mean±SEM. N=18 myocytes obtained from 7 hearts per group. Control indicates LV myocytes from native hearts of animals 5 weeks after receipt of the heterotopic transplanted hearts. Unloaded indicates LV myocytes from the transplanted unloaded hearts.
dissociated at 2 weeks (Figure 1; n=11 to 12 myocyte experiments per group) or 5 weeks (Figure 2; n=11 myocyte experiments per group). Diastolic cell length in myocytes from both control and unloaded hearts decreased slightly, but there were no differences in the responses after 2-week and 5-week unloading. In myocytes from both 2-week unloaded and control hearts, fractional cell shortening and peak-systolic \([Ca^{2+}]\) increased in response to the increased \([Ca^{2+}]_o\) (Figure 1A and 1B). These findings indicate that \(Ca^{2+}\)-dependent contractile reserve was preserved in myocytes from 2-week unloaded hearts compared with native controls. In contrast, in myocytes from 5-week unloaded hearts compared with controls, fractional cell shortening was severely depressed (11.4±1.1% versus 14.7±1.0% at 4.0 mmol/L \([Ca^{2+}]_o\), \(P<0.005\)), in association with impaired augmentation of peak-systolic \([Ca^{2+}]\) (725±54 versus 1026±77 mmol/L at 4.0 mmol/L \([Ca^{2+}]_o\), \(P<0.005\) (Figure 2A and 2B). The relationships between peak-systolic \([Ca^{2+}]\), and fractional cell shortening are shown after 2 weeks (Figure 1C) and 5 weeks (Figure 2C) of unloading. These data suggest that chronic cardiac unloading by heterotopic transplantation for 5 weeks results in (1) slower kinetics of myocyte relaxation and \([Ca^{2+}]\), transients and (2) depressed contractile reserve at higher work states.

The response to isoproterenol was also assessed to determine whether impaired contractile reserve was in part related to defects in \(\beta\)-adrenergically mediated phosphorylation of multiple proteins, the integrated function of which modulates contractility. Studies were performed after 2 weeks (4 animals, n=6 to 9 myocyte experiments per group) and 5 weeks of unloading (5 animals, n=9 to 10 experiments per group). Isoproterenol stimulation (10\(^{-6}\) mol/L) corrected the prolonged time to 50% relengthening (48±4 versus 38±1 ms \(P=0.01\)) to 35±4 versus 30±2 ms \(P=0.26\)) and the prolonged time to 50% decline in \([Ca^{2+}]_i\), (100±3 versus 82±7 ms \(P=0.03\)) to 69±5 versus 60±6 ms \(P=0.29\)) in myocytes from 5-week unloaded hearts compared with controls. In response to isoproterenol, there were no differences in the augmentation of contractility or peak-systolic \([Ca^{2+}]\), between myocytes from unloaded and native hearts at 2 and 5 weeks after transplantation (data not shown). These findings imply that impaired \([Ca^{2+}]\), handling, evident under conditions of an increased work state, can be partially overcome via increased \(\beta\)-adrenergic stimulation in myocytes from unloaded hearts.

### Protein Levels in LV Tissue

We studied the levels of \(Ca^{2+}\)-regulatory proteins to address the mechanisms potentially responsible for depressed contractile function in 5-week, but not 2-week, unloaded hearts. Protein levels were also compared between hearts of unoperated animals and in situ native hearts subjected to the acute and chronic stress of operation and heterotopic transplantation (n=6 hearts per group). There was a slight increase in PLB and reduction in SERCA2a levels in the in situ native hearts resulting in a decrease in SERCA2a-to-PLB ratio of 40% at 2 weeks and 46% at 5 weeks after operation \((P<0.05)\), with no change in Na\(^+/Ca^{2+}\) exchanger levels. In unloaded transplanted hearts compared with native hearts

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**Figure 1.** \(Ca^{2+}\)-dependent contractile reserve in isolated myocytes from unloaded hearts 2 weeks after transplantation. A, Relationship between \([Ca^{2+}]_o\) and fractional cell shortening. B, Relationship between \([Ca^{2+}]_o\) and peak-systolic and end-diastolic \([Ca^{2+}]_i\). C, Relationship between peak-systolic \([Ca^{2+}]_i\) and fractional cell shortening. There was no difference in contractile reserve between myocytes from 2-week unloaded hearts and those from native control hearts.
exposed to the identical neurohormonal circulatory environment, there was marked dysregulation of PLB 5 weeks after transplantation. Figure 3 shows that protein levels of PLB were increased in 5-week unloaded versus native hearts (3.67±0.88 versus 1.09±0.29 densitometric units, P<0.05). Protein levels of SERCA2a were not depressed in 5-week unloaded versus native hearts (1.07±0.11 versus 0.74±0.10 densitometric units, P=0.47). The SERCA2a-to-PLB ratio, which is a major determinant of cardiac contractility, was severely depressed in 5-week unloaded versus native hearts (0.38±0.08 versus 0.89±0.17 densitometric units, P<0.05; n=6 hearts per group) but not in 2-week unloaded hearts (0.66±0.05 versus 0.81±0.08 densitometric units, P=0.2; n=6 hearts per group). Protein levels of Na+/Ca2+ exchanger were not changed in either 2-week (0.84±0.01 versus 0.74±0.04 densitometric units) or 5-week (0.85±0.01 versus 0.88±0.03 densitometric units) unloaded hearts compared with native hearts (n=3 hearts per group).

Discussion

Cardiac unloading of normal hearts by heterotopic transplantation causes a sustained reduction in LV mass and myocyte size. The present study reports the novel observation that chronic cardiac unloading of normal hearts results in a time-dependent depression of myocyte contractile reserve and an impaired [Ca2+]i handling in association with a reduced SERCA2a-to-PLB protein ratio.

Contractile Reserve in Myocytes From Unloaded Hearts

At 2 weeks after transplantation, the kinetics of both cell motion and [Ca2+]i transients under baseline conditions were similar in myocytes from unloaded and control hearts. At 5 weeks after transplantation, however, both relengthening and decay in [Ca2+]i, were slower in myocytes from unloaded compared with control hearts. These observations are consistent with previous reports. Welsh et al16 reported that contractile function was intact in isolated myocytes and papillary muscles from 2-week unloaded rat hearts after transplantation. Kolar et al17 reported that the relaxation was prolonged in isolated perfused rat hearts unloaded by transplantation for 4 weeks while systolic function was maintained compared with native hearts. Assessment of contractile reserve at high work states was not analyzed.

Under baseline conditions in the present study, fractional cell shortening and levels of peak-systolic and end-diastolic [Ca2+]i, were similar between myocytes from both 2-week and 5-week unloaded and control hearts. To study myocyte contractile reserve, we subjected myocytes to a high workload challenge by stepped increases in perfusate Ca2+ concentration. This approach discriminates differences in contractile reserve in myocytes from normal and hypertrophied hearts, as well as myocytes from wild-type and transgenic hearts overexpressing the SERCA2a transgene.14,18 In the present study, this challenge clearly showed an impaired capacity to augment myocyte shortening and peak-systolic [Ca2+]i, in myocytes from 5-week unloaded hearts compared with controls, whereas contractile reserve was preserved in myocytes from 2-week

Figure 2. Ca2+-dependent contractile reserve in isolated myocytes from unloaded hearts 5 weeks after transplantation. A, Relationship between [Ca2+]o and fractional cell shortening. B, Relationship between [Ca2+]o and peak-systolic and end-diastolic [Ca2+]i. C, Relationship between peak-systolic [Ca2+]i and fractional cell shortening. Ca2+-dependent contractile reserve is depressed in myocytes from 5-week unloaded hearts compared with native control hearts.
unloaded hearts. Although we did not study myofilament sensitivity to Ca\(^{2+}\), the relationship between peak-systolic [Ca\(^{2+}\)]\(_i\) and fractional cell shortening was similar in myocytes from native and unloaded hearts over the range of [Ca\(^{2+}\)]\(_i\), that was studied (Figures 1C and 2C). This suggests that the depressed contractile reserve in myocytes from 5-week unloaded hearts was in part related to an impaired capacity to augment peak-systolic [Ca\(^{2+}\)], at higher work states.

Factors That Contribute to Depressed Contractile Reserve

The expression of ANP is increased in the unloaded compared with control hearts, and ANP has a negative inotropic effect in normal rat myocytes.\(^{19}\) This is unlikely to be the mechanism of depressed contractile reserve in myocytes from 5-week unloaded hearts because ANP was upregulated after both 2-week and 5-week unloading. The SERCA2a-to-PLB protein ratio was markedly decreased in 5-week unloaded hearts, but not in 2-week unloaded hearts, compared with native hearts. We also observed that there was a slight reduction in the SERCA2a-to-PLB protein ratio in native hearts from animals undergoing heterotopic transplant surgery compared with same-litter hearts from unoperated animals. This underscores the importance of utilizing in situ native hearts as the controls such that both the native and transplanted hearts are chronically exposed to the same circulatory environment of the recipient animal.

An extensive body of experiments has shown that increased levels of PLB in its unphosphorylated state inhibit sarcoplasmic reticulum (SR) Ca\(^{2+}\) uptake function of SERCA2a.\(^{15}\) Korecky et al\(^{20}\) reported that SR Ca\(^{2+}\) uptake activity in membrane fractions enriched with SR vesicles was decreased in 8-week unloaded hearts compared with native hearts in this heterotopic transplant model. We recently showed that a depressed SERCA2a-to-PLB protein ratio in hypertrophied myocytes is associated with impaired capacity to augment SR Ca\(^{2+}\) load at high work states, demonstrating the functional importance of dynamic changes in SR Ca\(^{2+}\) load on contractile reserve.\(^{14}\) Taken together, the slow relaxation and decay in [Ca\(^{2+}\)], and depressed contractile reserve in myocytes from 5-week unloaded hearts are likely to be related to depressed SR Ca\(^{2+}\) uptake capacity. The contractile response to isoproterenol is similar in myocytes from unloaded and native hearts, implicating preserved capacity for phosphorylation of multiple regulatory proteins, including PLB. Because regulation involves many different molecules working in concert, we cannot exclude the possibility that other mechanisms contribute to the depressed contractile reserve, including the changes in action potential duration and Ca\(^{2+}\) release mechanisms from SR\(^{21,22}\) and alterations of myocardial energy generation.\(^{5}\)
**Differences in Unloaded Versus Pressure-Overloaded Hearts**

The reduced Ca\(^{2+}\)-dependent contractile reserve in myocytes from 5-week unloaded hearts is similar to the observations of depressed Ca\(^{2+}\)-dependent contractile reserve and depressed SERCA2a-to-PLB protein ratio in hypertrophied myocytes.\(^{14}\) However, there are differences in alterations of Ca\(^{2+}\)-cycling proteins between unloaded hearts and pressure-overloaded hypertrophied hearts. First, the reduced SERCA2a-to-PLB ratio in the present study was mainly due to the increase in PLB protein levels and was not accompanied by a decrease in SERCA2a protein levels. In hypertrophied failing hearts, we and others have observed both a decrease in SERCA2a and upregulation of the PLB protein levels and was not accompanied by a decrease in SERCA2a protein levels. In hypertrophied failing hearts, we and others have observed both a decrease in SERCA2a and upregulation of the PLB protein levels and was not accompanied by a decrease in SERCA2a protein levels.\(^{14,18,23}\) Second, protein levels of the Na\(^+/Ca\(^{2+}\) exchanger were not changed in the unloaded hearts, whereas upregulation of the Na\(^+/Ca\(^{2+}\) exchanger may partially compensate for reduced SR function in hypertrophied and failing hearts.\(^{14,23,24}\)

**Implications for Humans With Excess Cardiac Unloading**

The findings of depressed Ca\(^{2+}\) regulation and contractile function after chronic unloading of normal hearts may have implications for humans that undergo cardiac unloading during very long-term microgravity in space and prolonged extreme unloading in patients with LVADs. We recognize the limitations of earth-based models to simulate sustained microgravity of prolonged spaceflight. Nonetheless, the data in the present study are consistent with the observations of cardiac atrophy obtained from rodents subjected to prolonged microgravity.\(^{1,25,26}\) The limited imaging data available from prior\(^{2,27}\) and recent\(^{28}\) studies of human astronauts immediately after long-term spaceflight also suggest a reduction in both LV mass and stroke volume as well as ejection fraction.

The present study did not address the effects of heterotopic transplantation on contractile function of failing hearts with excess load and pathological increase in mass. Unloading of failing rodent hearts by heterotopic transplantation\(^{29}\) and failing human hearts by LVAD has the potential to contribute to functional recovery.\(^{3,4}\) Mechanical LVAD unloading in a subset of patients with heart failure can improve dysregulated cardiac gene expression\(^{3,4,30,31}\) as well as contractile properties and \([Ca^{2+}]_i\), handling.\(^{32,33}\) It is speculated that cardiac unloading by LVAD for a very long duration in heart failure patients may result in partial cardiac atrophy and depressed contractile performance. Hetzer et al\(^{34}\) reported that there is an individual optimum of recovery that cannot be further improved by prolonged unloading and that ventricular function gradually declines beyond that point. The present study, which shows a time-dependent depression of contractile reserve in normal hearts subjected to prolonged unloading, supports the hypothesis that there are limited boundaries of load for optimal cardiac performance and normal Ca\(^{2+}\)-regulatory gene expression.

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