Does the $\beta_2$-Agonist Clenbuterol Help to Maintain Myocardial Potential to Recover During Mechanical Unloading?

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Objective—Chronic mechanical unloading induces left ventricular (LV) atrophy, which may impair functional recovery during support with an LV-assist device. Clenbuterol, a $\beta_2$-adrenergic receptor (AR) agonist, is known to induce myocardial hypertrophy and might prevent LV atrophy during LV unloading. Furthermore, $\beta_2$-AR stimulation is reported to improve Ca$^{2+}$ handling and contribute to antiapoptosis. However, there is little information on the effects of clenbuterol during LV unloading.

Methods and Results—We investigated LV atrophy and function after LV unloading produced by heterotopic heart transplantation in isogenic rats. After transplantation, rats were randomized to 1 of 2 groups (n=10 each). The clenbuterol group received 2 mg·kg$^{-1}$·d$^{-1}$ of the drug for 2 weeks; the control group received normal saline. The weight of unloaded control hearts was 48% less than that of host hearts after 2 weeks of unloading. Clenbuterol significantly increased the weight of the host hearts but did not prevent unloading-induced LV atrophy. Papillary muscles were isolated and stimulated, and there was no difference in developed tension between the 2 groups. However, the inotropic response to the $\beta_2$-AR agonist isoproterenol significantly improved in the clenbuterol group. The mRNA expression of myocardial sarco(endo)plasmic reticulum Ca$^{2+}$-ATPase 2a (SERCA2a) and fetal gene shift (myosin heavy chain [MHC] mRNA isozyme) was also significantly improved by clenbuterol treatment. There was no difference in $\beta_1$-AR mRNA expression between the 2 groups. In contrast, $\beta_2$-AR mRNA was significantly decreased in the clenbuterol-treated, unloaded heart. This indicates that clenbuterol may downregulate $\beta_2$-ARs. In the evaluation of apoptosis, mRNA expression of caspase-3, which is the central pathway for apoptosis, tended to be better in the clenbuterol group.

Conclusions—During complete LV unloading, clenbuterol did not prevent myocardial atrophy but improved gene expression (SERCA2a, $\beta$-MHC) and $\beta$-adrenergic responsiveness and potentially prevented myocardial apoptosis. However, chronic administration of clenbuterol may be associated with downregulation of $\beta_2$-ARs. (Circulation. 2005;112[suppl I]:I-51–I-56.)

Key Words: cardiomyopathy ■ heart-assist device ■ heart failure ■ inotropic agents ■ receptors, adrenergic, beta

Recent clinical observations in some patients with end-stage heart failure have demonstrated that a left ventricular-assist device (LVAD) can improve cardiac function and be used as a “bridge to recovery” without heart transplantation. However, its success rate as a bridge to recovery is low, and it is difficult to predict which patients will show functional improvement. In addition, the mechanisms of functional recovery, if any, are unknown. Chronic mechanical unloading induces cardiac atrophy, and this may impair the recovery of function. Recently, to prevent cardiac atrophy and improve myocardial function during mechanical unloading, administration of a $\beta_2$-adrenergic receptor (AR) agonist, clenbuterol, was advocated in combination with mechanical unloading. Clenbuterol is known to induce muscle hypertrophy in animal hearts without adverse effects, similar to physiologic hypertrophy. Furthermore, $\beta_2$-AR stimulation is reported to improve Ca$^{2+}$ handling and reduce apoptosis. However, antiatrophic effects or other beneficial effects of clenbuterol during mechanical unloading are not fully understood. Thus, we tested the hypothesis that clenbuterol induces beneficial changes in heart size and biophysiologic characteristics of the unloaded heart by using a rat heterotopic heart transplantation model. The heterotopically transplanted hearts were mechanically unloaded in a manner similar to an LVAD and underwent cardiac atrophy. We evaluated histologic changes, gene expression, and contractile function of isolated papillary muscles in both host and transplanted (ie, unloaded) hearts.
Experimental Design
All experimental procedures were conducted according to Kyoto University’s guidelines for animal care. Inbred male Lewis rats (weighing 150 to 200 g) were used in this study. Heterotopic heart transplantation was performed as previously described.\(^5\) In brief, the ascending aorta of the donor heart was anastomosed end-to-side on the recipient’s abdominal aorta, and the pulmonary artery of the donor was anastomosed end-to-side on the recipient’s inferior vena cava. Thus, the donor heart continued to beat because of perfusion through the coronary arteries, but the LV was unloaded. Transplanted hearts spontaneously resumed beating within a few seconds of reperfusion. The total procedure time was <40 minutes. After heterotopic transplantation, surgical mortality was <5%. The transplanted rats were randomly divided into 2 groups (n = 10 each). The control group consisted of rats that were injected with subcutaneous saline (0.5 mL) once daily for 2 weeks. The treatment group was injected with subcutaneous clenbuterol (MP Biomedicals, Inc) once daily at a dose of 2 mg/kg body weight for 2 weeks.

Methods

Papillary Muscle Function
Papillary muscle function was examined at experimental end points. The animals were anesthetized with pentobarbital sodium (100 mg/kg) via intraperitoneal injection and heparinized (500 U) via intravenous infusion. After the chest or abdomen was opened, the heart was rapidly removed. The heart was placed into normal Tyrode’s solution containing (in mmol/L) Na\(^+\) 142, K\(^+\) 5.6, Mg\(^2+\) 1.1, Cl\(^-\) 154.6, HEPES 5, and glucose 11. The entire heart, right ventricle, and LV were weighed. The posterior papillary muscle was ligated, dissected free, and mounted in a tissue bath containing Krebs-Henseleit solution (in mmol/L) Na\(^+\) 152, K\(^+\) 3.6, Mg\(^2+\) 0.6, Ca\(^2+\) 2.5, Cl\(^-\) 135, HCO\(_3^-\) 25, H\(_2\)PO\(_4^-\) 1.3, SO\(_4^-\) 0.6, glucose 5.6, and 2.3-butane-dione monoxime 30, pH 7.4. The bath was maintained at a constant temperature of 37°C and bubbled with 95% O\(_2\) and 5% CO\(_2\). The bath was maintained at the maximum tension position, and papillary muscle diameter was measured with a calibrated eyepiece. Developed tension was normalized to cross-sectional area.

After baseline measurements were completed, developed tension was recorded with an intravenous infusion. After the chest or abdomen was opened, the heart was placed into normal Tyrode’s solution containing (in mmol/L) Na\(^+\) 142, K\(^+\) 5.6, Mg\(^2+\) 1.1, Cl\(^-\) 154.6, HEPES 5, and glucose 11. The entire heart, right ventricle, and LV were weighed. The posterior papillary muscle was ligated, dissected free, and mounted in a tissue bath containing Krebs-Henseleit solution (in mmol/L) Na\(^+\) 152, K\(^+\) 3.6, Mg\(^2+\) 0.6, Ca\(^2+\) 2.5, Cl\(^-\) 135, HCO\(_3^-\) 25, H\(_2\)PO\(_4^-\) 1.3, SO\(_4^-\) 0.6, glucose 5.6, and 2.3-butane-dione monoxime 30, pH 7.4. The bath was maintained at a constant temperature of 37°C and bubbled with 95% O\(_2\) and 5% CO\(_2\). The papillary muscle was stimulated at 1 Hz with impulses of 5-ms duration and current ~20% above threshold. The muscle was stretched to the length at which maximum tension development occurred. After stabilization, isometric tension was recorded digitally at the maximum tension position, and papillary muscle diameter was measured with a calibrated eyepiece. Developed tension was normalized to cross-sectional area.

After baseline measurements were completed, developed tension was recorded during exposure to the β-adrenergic agonist isoproterenol. Isoproterenol HCl was dissolved in distilled water and added to the bath to produce cumulative concentrations of 10\(^{-8}\) and 10\(^{-7}\) mol/L. Developed tension was measured when the response was maximal (5 to 10 minutes after each addition of isoproterenol).

After removal of the papillary muscle from the heart, the LV myocardium was transversely sliced into 2-mm-diameter sections at the base of the papillary muscles and fixed in 10% buffered formalin. After slices were taken, the remaining LV myocardium was frozen at −80°C until analyzed.

Pathologic Studies
Transverse sections of LV myocardium were stained with hematoxylin-eosin to evaluate myocyte size. Mean myocyte diameter was calculated by measurement of 50 cells in the myocardium under microscopy (magnification ×400). Cells were accepted for measurement if they met the following criteria: (1) cross sections of cardiomyocytes were present; (2) cardiomyocytes had a visible nucleus; and (3) their cellular membranes were intact. Other transverse sections were used for in situ detection of apoptosis by terminal dUTP nick end-labeling (TUNEL) assay. The TUNEL assay was performed in accordance with the manufacturer’s protocol (Takara Biomedicals). TUNEL-positive cells were counted, in a blinded manner (400 to 500 cells), in 30 randomly chosen fields for each experiment.

Analysis of mRNA Expression
Total mRNA was prepared from the frozen LV pieces with TRIzol (Life Technologies Inc) reagent, reverse-transcribed, and amplified with an ABI Prism 7700 sequence detector (Applied Biosystems). Polymerase chain reaction (PCR) conditions were 40 cycles of denaturing at 94°C for 20 seconds and primer annealing/extension at 62°C for 60 seconds. The nucleotide sequences of PCR primers and TaqMan probes were as follows: α-myosin heavy chain (MHC) forward primer, 5’-TGACAAATCGCTACCTCATG-3’ and reverse primer, 5’-TGACATACTCGTTACACCTTA-3’; TaqMan probe 5’-CTGCTCAGGGTTCTGTGTACCCCTCG-3’; β-MHC forward primer, 5’-CAATGCTACCTCATGACGTGG-3’ and reverse primer, 5’-TGACTGAGGTGCGCCACAA-3’; TaqMan probe 5’-CTGACCTCGCTGACCTGCTCAA-3’; caspase-3 forward primer, 5’-AATTCAAGGGTCGGTGCT-3’ and reverse primer, 5’-GGTTCGGTGCTACAGTTTCCC-3’; and TaqMan probe 5’-TTCATCCACCTTACCTTGCCCATG-3’. The PCR sequences of sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase2a (SERCA2a), β-AR, and β-AR were previously reported in our studies.\(^9,10\) The TaqMan rodent glycolaldehyde 3-phosphate dehydrogenase (GAPDH) control reagent was used to detect rat GAPDH as the internal standard. Expression levels of the target gene were normalized to the GAPDH level in each sample.

Statistical Analysis
All data are expressed as the mean±SEM. Statistical differences of paired data between host and unloaded hearts were evaluated by a paired t test, and an unpaired t test was used for comparing unpaired data. Two-way ANOVA with repeated measures was used to assess the inotropic response to isoproterenol. Statistical analyses were performed with Statview for Windows, version 5.0 (SAS Institute Inc). A value of P<0.05 was considered statistically significant.

Results

Effects of Clenbuterol on Heart Size
As shown in Table 1, there was an 18% increase in LV weight (from 0.60±0.04 to 0.71±0.08 g) in the clenbuterol-treated, host hearts compared with the control host hearts. In contrast, the size of the unloaded hearts in control animals decreased by 48%, from 0.60±0.04 to 0.31±0.04 g, and myocyte diameter decreased from 25.3±1.6 to 21.5±2.1 μm after 2 weeks of unloading; these changes were not affected by clenbuterol treatment.

| TABLE 1. Effect of Clenbuterol on Heart Size |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | Control         | Clenbuterol     |                 |                 |
|                 | Host Heart      | Unloaded Heart  | Host Heart      | Unloaded Heart  |
| LV weight, g    | 0.60±0.04       | 0.31±0.04*      | 0.71±0.08†      | 0.32±0.06*      |
| Myocyte diameter, μm | 25.3±1.6       | 21.5±2.1*       | 26.5±1.9        | 21.7±2.5*       |

Abbreviations are as defined in text.

*P<0.05 vs host heart; †P<0.05 vs control group.

Myocardial Expression of mRNAs Encoding SERCA2a, α-MHC, and β-MHC
As shown in Figure 1, mRNA expression of SERCA2a significantly decreased in the unloaded heart compared with
the host heart in the control group. However, this impaired expression of SERCA2a improved in the clenbuterol-treated animals. The isoforms of α- and β-MHC mRNAs are shown in Figure 2. There was no significant difference in α-MHC mRNA between the 2 groups. In the unloaded heart, β-MHC mRNA expression significantly increased compared with the host heart. By contrast, clenbuterol treatment significantly reduced β-MHC mRNA expression induced by mechanical unloading. Therefore, clenbuterol can prevent the adverse changes in SERCA2a gene expression and the fetal gene shift of MHC isoforms.

Evaluation of Myocardial Apoptosis
To evaluate the degree of myocardial apoptosis, we evaluated TUNEL staining and the expression of caspase-3 mRNA (Figure 3). The unloaded heart showed significant increases in caspase-3 mRNA expression and TUNEL-positive cells. In the clenbuterol-treated group, caspase-3 expression of the unloaded heart showed a nonsignificant decrease compared with the control group ($P<0.08$). TUNEL-positive cells were significantly increased in unloaded hearts in the control group. However, there was no significant difference in the clenbuterol-treated group.

Papillary Muscle Function
As shown in Table 2, papillary muscle cross-sectional area in the unloaded heart was significantly smaller than in the host heart. Although papillary muscles in unloaded hearts showed atrophic changes, developed tension was maintained even after mechanical unloading. There was no significant difference in developed tension between the clenbuterol- and the saline-treated groups. The inotropic response to isoproterenol is shown in Figure 4. Although isoproterenol produced a dose-related increase in developed tension in the normal heart, the unloaded heart revealed a diminished inotropic response. Furthermore, administration of clenbuterol improved the inotropic response to isoproterenol in the unloaded heart.

Expression of β-AR mRNA
To assess the effect of inotropic response in the clenbuterol-treated group, we measured the expression of β1- and β2-AR mRNAs. There was no significant difference in β1-AR mRNA among the 4 groups, as shown in Figure 5. However, clenbuterol reduced the expression of β2-AR mRNA in the unloaded heart compared with the host heart.

Discussion
In the clinical setting, excessive or prolonged ventricular unloading with an LVAD causes disuse cardiac atrophy. These atrophic changes are thought to impede the functional recovery associated with an LVAD. To prevent atrophy during mechanical unloading, clenbuterol was administered in a recent study. Clenbuterol is a selective β2-adrenergic agonist known to produce muscle hypertrophy in the rat heart. The hypertrophied muscle shows normal histology and normal gene expression. In contrast, isoproterenol, a β1- and β2-adrenergic agonist, produces cardiac hypertrophy with myocyte necrosis, fibrosis, and increased expression of fetal
In the present study, the dose of clenbuterol given by subcutaneous injection was selected on the basis of a prior study, which showed that clenbuterol induced hypertrophy of the normal rat heart. Although clenbuterol induced hypertrophy, as shown by an 18% increase in weight of the host heart, clenbuterol treatment did not alter the weight of the unloaded heart. If clenbuterol influences heart weight during mechanical unloading, then the response should occur similarly in both the hemodynamically unloaded heart and the working host heart. These results suggest that clenbuterol does not prevent cardiac atrophy during mechanical unloading in this model. Atrophy of the heart is strongly related to hemodynamic loading. Experimental studies have reported that heart size and growth are determined in large part by the volume loading status of the heart. Moreover, atrophy is a complex and highly regulated phenomenon involving the interplay of multiple signaling pathways and the simultaneous activation of protein synthesis and protein degradation. Although the mechanism of atrophy was unclear, this study revealed that stimulation of β2-ARs could not prevent cardiac atrophy during mechanical unloading.

Administration of clenbuterol did not affect LV size in the unloaded heart. However, the results showed other beneficial effects on molecular markers. The mRNA expression of β-MHC was significantly lower and SERCA2a higher in the clenbuterol-treated, unloaded heart compared with the control, unloaded heart. In small animals, where the predominant MHC isoform is the α-type, a switch to the β-MHC isoform is often seen in the failing and hypertrophic heart. SERCA2a activity was also decreased in these hearts. Down-regulation of SERCA2a is thought to impair intracellular Ca2+ handling, which plays an important role in functional recovery with an LVAD. In this heterotopic transplantation model, the LV cavity was completely unloaded, and these unloaded normal hearts underwent significant alterations in SERCA2a and MHC isoforms. Prior studies reported an increase in β-MHC in heterotopically transplanted hearts that included a switch from the "adult" to the "fetal" isoform. Despite significant atrophy of the unloaded heart, the molecular changes in SERCA2a and MHC isoforms were similar to those observed in the hypertrophied heart. Thus, opposite hemodynamic changes in unloaded and hypertrophic hearts induce a similar pattern of gene response. In the normal heart, clenbuterol treatment induced cardiac hypertrophy without alterations in SERCA2a and α,β-MHC. This means that clenbuterol induces a physiologic hypertrophy in the normal heart. However, it is unknown whether clenbuterol induces molecular alterations in the mechanically unloaded heart. This study is the first report to resolve these issues. There are some studies that showed evidence of beneficial effects of β2-adrenergic agonists in only failing and hypertrophied hearts. Wong et al reported that clenbuterol increased myocardial SERCA2a levels in a rat model of hypertrophy induced by pressure overload. Ahmet et al reported that β2-adrenergic agonists could prevent LV remodeling and improve LV function in rat hearts with dilated cardiomyopathy. In the present study, clenbuterol-induced hypertrophy was not observed in the unloaded hearts; however, clenbuterol can prevent the adverse changes in SERCA2a gene

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**TABLE 2. Papillary Muscle Contractions**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Clenbuterol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Host Heart</td>
<td>Unloaded Heart</td>
</tr>
<tr>
<td>Papillary muscle area, mm²</td>
<td>0.39±0.1</td>
<td>0.27±0.06*</td>
</tr>
<tr>
<td>Developed tension, g</td>
<td>0.28±0.09</td>
<td>0.22±0.06</td>
</tr>
<tr>
<td>Developed tension/area, g/mm²</td>
<td>0.70±0.1</td>
<td>0.75±0.40</td>
</tr>
</tbody>
</table>

Abbreviations are as defined in text.

*P<0.05 vs host heart.

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**Figure 4.** Inotropic response to isoproterenol (expressed as the increase in developed tension) as a percentage of baseline. All values are mean±SEM. *P<0.05 vs host hearts in the same treatment group; †P<0.05 vs control in unloaded hearts. Abbreviations are as defined in text.

**Figure 5.** Expression of β1- and β2-AR mRNAs. All values are mean±SEM. *P<0.05 vs host hearts in the same treatment group; †P<0.05 vs control host hearts or unloaded hearts. Abbreviations are as defined in text.
expression and MHC isoform shifts not only in the hypertrophied heart but also in the unloaded heart. These results suggest that administration of clenbuterol can improve molecular changes in both hypertrophied and unloaded hearts.

Myocardial apoptosis, which is 1 of the factors that can deteriorate LV function, was evaluated in the unloaded heart. Schena et al18 reported that ventricular unloading increased myocardial apoptosis in the heterotopic heart transplantation model, as indicated by TUNEL staining and caspase-3 activity. TUNEL staining is commonly used for detection of apoptosis by labeling the DNA-free 3′OH ends of DNA fragments. Caspase-3, which is 1 of the interleukin-converting enzyme families of cysteine protease, is known to mediate apoptosis in cardiomyocytes. The process of apoptosis has many pathways, but caspase-3 is thought to be central in the apoptotic pathway.19 Therefore, caspase-3 activity can be used as a molecular marker of myocardial apoptosis. The present study showed significantly increased caspase-3 mRNA and TUNEL-positive cells in the heterotopically transplanted heart. In addition, caspase-3 expression in the unloaded heart tended to decrease in the clenbuterol group compared with the control group. However, there was no significant difference in TUNEL-positive cells in the heterotopic heart transplantation. The results of the present study suggest that clenbuterol may have blocked the apoptosis pathway via caspase-3 during mechanical unloading. Prior studies8 support these findings. β2-AR stimulation is reported to protect cardiac myocytes from apoptosis, whereas β1-AR stimulation is proapoptotic. In fact, Ahmet et al8 reported that administration of a β2-AR–selective agonist improved LV function and attenuated apoptosis in a rat ischemic model. They suspected that the effect of the β2-AR agonist to reduce apoptosis might be mediated through β2-AR–inhibitory G(Gi)-protein coupling. Gi opposes the action of Gs, which appears to be central to β1-AR–stimulated apoptosis. Moreover, a recent study20 demonstrated that a β2-AR agonist inhibited caspase-3 activity via direct inhibition of p38 mitogen-activated protein kinase. Activation of the β2-AR pathway might play an important role in the prevention of apoptosis during mechanical unloading.

The contractile function of papillary muscles was maintained in the unloaded heart. However, β-adrenergic responsiveness deteriorated in the unloaded heart compared with the host heart. Clenbuterol did not change contractile function in either the host or unloaded heart but improved β-adrenergic responsiveness in the unloaded heart. The decrease in β-adrenergic responsiveness is due mainly to a decrease in β-AR density and impairment of the β-adrenergic signaling pathway, including Gi protein, β-adrenergic kinase, and intracellular Ca2+ homeostasis.21,22 In this study, we measured β-AR density, as indicated by the expression of β1- and β2-AR mRNAs. The β1-AR was not altered in either the host heart or the unloaded heart. In contrast, clenbuterol induced downregulation of β2-ARs in the unloaded heart. Isoproterenol is a nonselective β-adrenergic agonist, which stimulates both β1- and β2-ARs, and mainly affects myocardial contractility through β1-AR stimulation. These findings suggest that the improvement of β-adrenergic responsiveness in the clenbuterol-treated, unloaded heart may not have been caused by an increase in β1-AR density. Although the present study does not clarify the specific mechanism by which clenbuterol improves β-adrenergic responsiveness, these beneficial effects may be related to the improvement in intracellular homeostasis. The molecular data on SERCA2a, fetal gene shifts of MHC, and apoptosis support this mechanism.

Downregulation of β2-ARs is an important issue, because β2-AR signaling may play a significant role in functional recovery during mechanical unloading. β2-AR signaling is valuable for maintaining heart function and distinctly differs from that of β1-AR. However, β2-AR is easier to downregulate than β1-AR.21 In this study, β2-AR was downregulated in the clenbuterol-treated, unloaded heart. Previous studies have also shown that chronic administration of clenbuterol decreased total β-AR density, mostly β2-AR.23 Downregulation of β2-ARs may deteriorate heart function. Recently, experimental studies have reported that cardiac β2-AR overexpression increases contractile function in chronic heart failure models.24 Tevaeaarai and Koch24 delivered the β2-AR transgene during heterotopic heart transplantation, and this resulted in higher levels of β2-AR expression in the heart. In the present study, β2-AR stimulation by clenbuterol demonstrated beneficial effects during mechanical unloading but was accompanied by downregulation of β2-ARs. Before application of these results to the clinical setting, future studies are needed to focus on the optimal dose of clenbuterol during mechanical unloading to minimize receptor downregulation of β2-ARs.

The current study has some limitations. First, clenbuterol was systemically given to animals. This drug may influence the hemodynamics of the host heart and contribute to the beneficial effects observed in the unloaded heart. In our preliminary studies, administration of clenbuterol induced cardiac hypertrophy but did not change hemodynamics such as blood pressure. Petrou et al25 also reported that clenbuterol did not alter hemodynamics in an ovine model. Thus, the hemodynamic influences of clenbuterol on the host heart should be minimal. Second, the LV cavity of the heterotopically transplanted heart was completely unloaded, and this may mimic the full unloading that occurs during support with an LVAD in the clinical setting. Although partial unloading of the LV cavity may be desirable, this rat model is convenient and can provide much information about the functional recovery associated with mechanical unloading.

Conclusions
During mechanical unloading by heterotopic heart transplantation, clenbuterol did not prevent myocardial atrophy but improved gene expression (SERCA, βMHC) and β-adrenergic responsiveness and potentially prevented myocardial apoptosis. These effects of clenbuterol may contribute to the recovery of LV function after mechanical unloading. However, chronic administration of clenbuterol may be associated with downregulation of β2-ARs. Future studies are needed to focus on the optimal dose and duration of clenbuterol therapy in mechanical unloading.

References


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