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·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 $\beta_2$-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.$^{1,2}$ However, its success rate as a bridge to recovery is low,$^3$ 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.$^4$ 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.$^5$ Clenbuterol is known to induce muscle hypertrophy in animal hearts without adverse effects,$^6$ similar to physiologic hypertrophy. Furthermore, $\beta_2$-AR stimulation is reported to improve Ca$^{2+}$ handling and reduce apoptosis.$^7,8$ 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 biophysicologic 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.
Methods

Experimental Design
All experimental procedures were conducted according to Kyoto University’s guidelines for animal care. Inbred male Lewis rats (weighing 150 to 200g) were used in this study. Heterotopic heart transplantation was performed as previously described. 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.

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 saline (0.5 mL) once daily for 2 weeks. The treatment group was loaded with subcutaneous clenbuterol (MP Biomedicals, Inc) once daily at a dose of 2 mg/kg body weight for 2 weeks.

Pathologic Studies
Transverse sections of LV myocardium were stained with hematoxylin-eosin to evaluate myocyte size. Mean cardiomyocyte 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 deoxynucleotidyl transferase 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 s and primer annealing/extension at 62°C for 60 s. The nucleotide sequences of PCR primers and TaqMan probes were as follows: α-myosin heavy chain (MHC) forward primer, 5'-TGACAAAATCTGCTACTCTAGTG-3' and reverse primer, 5'-TGACATACCTGTTCACCCTTCA-3'; TaqMan probe 5'-CTGCTCAAGGTTCTGTTGACCTACCTCG-3'; β-MHC forward primer, 5'-CAAGTAGCCTACCTCATTG-3' and reverse primer, 5'-TGACCTAGGTTGGCAACA-3'; TaqMan probe 5'-CTGAACCCTGAGCTGAGCAG-3'; caspase-3 forward primer, 5'-ATTTCAAGGGGTAATGATG-3' and reverse primer, 5'-GCTGTGCGCGTACATT-3'; and TaqMan probe 5'-TTCACATCGTACCTTGGCCATG-3'. The PCR sequences of sarco(endo)plasmic reticulum Ca²⁺-ATPase2a (SERCA2a), β-AR, and β-AR were previously reported in our studies. The TaqMan rodent glyceraldehyde-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.

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 control.
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 Ca^{2+} 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 isozymes. 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 isozymes 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 αβ-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 expression.

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<th>Table 2: Papillary Muscle Contractions</th>
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<td>Papillary muscle area, mm²</td>
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<td>Developed tension/area, g/mm²</td>
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Abbreviations are as defined in text.

*P<0.05 vs host heart.
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 al. 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. 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 clenbuterol group. The results of the present study suggest that clenbuterol may have blocked the apoptosis pathway via caspase-3 during mechanical unloading. Prior studies support these findings. β2-AR stimulation is reported to protect cardiac myocytes from apoptosis, whereas β1-AR stimulation is proapoptotic. In fact, Ahmet et al. 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 is proapoptotic. In fact, Ahmet et al. reported that cardiac β2-AR overexpression increases contractile function in chronic heart failure models. Tevaearai and Koch 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 al. 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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