Clenbuterol Induces Hypertrophy of the Latissimus Dorsi
Muscle and Heart in the Rat With Molecular and Phenotypic
Changes

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Abstract

Background Skeletal muscle assistance of the circulation for patients in end-stage heart failure requires electrical training of the latissimus dorsi flap to produce fatigue resistance. This process of electrical transformation and the development of postmobilization atrophy results in a profound loss in peak power generated. The β2-adrenoceptor agonist clenbuterol was used to investigate its potential to selectively induce skeletal muscle hypertrophy, particularly the latissimus dorsi muscle (LDM), independent of adverse effects on cardiac muscle.

Methods and Results Forty-one male Sprague-Dawley rats were divided into four groups and used in this study. Clenbuterol 2 µg · g body wt⁻¹ · d⁻¹ was administered subcutaneously for a period of either 5 weeks (group A) or 2 weeks (group A1). Groups B and B1 (controls) were injected with 0.5 mL normal saline once daily. At the end of the experimental period, all rats were weighed and terminally anesthetized for removal of the left LDM, left gastrocnemius-plantaris-soleus (GPS) muscles, and heart.

The results showed that the increase in body weight did not differ significantly between the clenbuterol-treated and control groups (P>.5). The ratio of LDM to tibial length (hypertrophic index) for groups A and A1 was significantly greater than controls (P<.01), which represented a 20% to 29% increase. The hypertrophy was more pronounced for hindlimb skeletal muscle (21% to 35% for GPS), and the effects of this relatively high dose of clenbuterol on the heart were less marked (18% to 20% hypertrophy).

RNA analyses indicate that ventricles of clenbuterol-treated rats express elevated levels of mRNA to
atrial natriuretic factor without a concomitant increase in skeletal α-actin and β-myosin heavy chain, consistent with a "physiological" form of cardiac hypertrophy. 

**Conclusions** Clenbuterol induces significant hypertrophy of the LDM associated with specific changes in cardiac gene expression. 

**Key Words:** clenbuterol • muscles • hypertrophy • rats

### Introduction

The LDM has been used for skeletal muscle assist both experimentally and clinically during the past 10 years. Results have been variable, however, particularly with respect to hemodynamic parameters. Part of this may be due to the significant reduction in the absolute power generated by LDM after electrical transformation, which is a necessary precursor for the acquisition of fatigue resistance. Other factors leading to impaired performance include atrophy secondary to reduced stretch and loading conditions that occur after mobilization and translocation from the chest wall into the mediastinum for cardiomyoplasty. In addition, patients with severe heart failure are known to have structural and functional changes in their skeletal muscles that result in muscle weakness and fatigue. It is desirable, therefore, to inhibit atrophy or induce hypertrophy to improve the power of the transformed LDM and thus increase the magnitude of cardiac assist. Clenbuterol is a relatively selective β2-adrenoceptor agonist with known anabolic effects specific to skeletal muscle, although most studies to date have focused on its effects on hindlimb muscles. This study investigates the effects of clenbuterol in the rodent, with particular reference to the LDM. Furthermore, the question of specificity is addressed more closely in an attempt to determine whether cardiac hypertrophy is induced concomitantly, and if so, whether at the molecular level any changes in RNA expression might be indicative of any potentially detrimental effects on cardiac function. In rat heart, two MHC (α and β-MHC) and two sarcomeric actin iso-mRNAs (cardiac and skeletal α-actins) are expressed. In the adult rat heart, cardiac α-actin represents the vast majority of sarcomeric α-actins, but after either constriction of the aorta or injection of thyroid hormone, skeletal α-actin expression is rapidly and transiently induced. Likewise, MHC expression in the rat heart is known to be regulated by pressure overload, thyroid hormone, and adrenergic agonists. Since changes in their gene expression are so
well characterized in rat models of cardiac hypertrophy, they are often used as molecular markers of changes in gene expression linked with either "physiological" or "pathological" forms of cardiac growth.\textsuperscript{9, 20} We therefore analyzed the RNA expression of sarcomeric α-actins, cardiac MHCs, and ANF, currently the best molecular markers for cardiac hypertrophy.

### Methods

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<th>Animals and Treatment</th>
<th>Regimen</th>
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<td>Forty-one male Sprague-Dawley rats (Charles River) were divided into four groups with an equal mean body weight of 100 g and fed rat chow and water ad libitum. They were housed individually and subjected to a 12-hour light-and-dark cycle. After a settling-in period of 1 week, groups A (n=12) and A1 (n=10) were started on once-daily subcutaneous injections of clenbuterol (a gift from Boehringer Ingelheim, UK) 2 µg/g body wt, and groups B (n=11) and B1 (n=8) were injected with an equivalent volume (0.5 mL) of the vehicle (normal saline). At the end of the experimental period, which was 5 weeks for groups A and B and 2 weeks for A1 and B1, all animals were weighed and anesthetized with diazepam 0.2 mL IM and fentanyl citrate 0.315 mg/mL+fluanisone 10 mg/mL 0.5 mL IP. The left LDM, heart, GPS, and the left perinephric fat pad were dissected free and weighed fresh. Care was taken to avoid atrial contamination by taking the left ventricular samples close to the apex (including interventricular septum as well as free wall) and well away from the atrioventricular groove. Samples were snap-frozen in liquid nitrogen for total RNA extraction or mounted in embedding medium and stored at -70°C for histochemistry. The left tibia was also removed and its length recorded for standardizing the weight of individual muscles.\textsuperscript{22} Cardiac samples from hyperthyroid and hypothyroid rats were used as positive controls for physiological and pathological forms of cardiac hypertrophy, respectively. Samples of liver from control animals were used as negative controls.</td>
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**Histology**

The presence of interstitial fibrosis was determined on 6-µm sections of LDM and left ventricle (from control and clenbuterol-treated rats) that were soaked in van Gieson solution (1 part 1% aqueous acid fuchsin, 9 parts saturated aqueous picric acid, and 10 parts distilled water) for 5 minutes. Excess stain was rinsed off before rapid dehydration by immersion in an alcohol series, clearing, and permanent
mounting. Slides were surveyed for any areas of fibrosis under the light microscope (Zeiss), and representative areas were photographed.

**Cell Culture**

Primary cultures of neonatal rat heart cells were established with modifications as previously described. Briefly, after trypsinization and preplating for 45 to 60 minutes to limit the number of contaminating noncardiocytes, cells were plated at a density of $3.75 \times 10^6$ cells per 75-cm$^2$ culture dish ($5 \times 10^4$ cells/cm$^2$) and allowed to attach for 24 to 36 hours in DMEM supplemented with penicillin/streptomycin and 5% FCS. Nonmuscle cell growth was inhibited by the addition of 0.1 mmol/L bromodeoxyuridine, and the cells were maintained in a 5% CO$_2$ atmosphere. On the morning of day 3, the cells were switched to serum-free DMEM/Medium 199 (4:1) supplemented with penicillin/streptomycin/insulin/transferrin (Sigma). Adrenergic agonists or their diluent (100 µmol/L ascorbic acid) were added to the dishes 6 hours after transfer to serum-free medium. Medium was changed thereafter each 24 hours with the addition of fresh agonist or vehicle. For these studies, 4 µmol/L concentrations of norepinephrine, isoproterenol, or clenbuterol were used.

**RNA Analyses**

Total RNA was extracted from tissue samples weighing 0.2 to 0.6 g or from plated neonatal cardiocytes using the guanidinium thiocyanate–phenol-chloroform procedure. The concentration was measured by optical density, and 15 µg of total RNA was loaded per well on a denaturing agarose gel. After electrophoretic separation, overnight transfer was performed onto a nylon membrane (Hybond-N, Amersham), and prehybridization in a 50-mL solution containing 50% deionized formamide, Denhardt's solution (0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% gelatin), 1% SDS, and 200 µL of 10 mg/mL herring sperm DNA (denatured at 95°C before inclusion) was carried out at 42°C for 4 hours. A $^{32}$P-labeled cDNA probe complementary to ANF mRNA (plasmid kindly provided by K. Knowlton, San Diego) was then added to the solution for hybridization overnight. Nonspecifically bound probe was removed by a series of washes at a final stringency of 1xSSPE/0.1xSDS at 55°C for 15 minutes before the membrane was subjected to autoradiography for 24 to 72 hours at -70°C with an intensifying screen. Membranes were subsequently dehybridized for 60 minutes with 0.1% SDS heated to 100°C before rehybridization with a $^{32}$P-labeled oligonucleotide complementary to 18S ribosomal RNA as previously described. The 18S hybridization was used to standardize the transferred quantity
of total RNA and thus validate any differences in intensity seen on the final autoradiograph for ANF mRNA expression.

'Hot' RT-PCR Analysis of the Actin and MHC Iso-mRNAs

Complementary DNA was synthesized from 1 µg of total RNA extracted from the left ventricles of control, clenbuterol-treated, hypothyroid, and hyperthyroid rats by use of a first-strand cDNA synthesis kit and as primer, oligo (dT)$_{18}$ according to the manufacturer's instructions (Pharmacia). Incubations were at 37°C for 1 hour, followed by denaturation at 95°C for 10 minutes before amplifications. PCR amplifications were achieved with the following oligonucleotides: forward primer, ACC AGG GTG TCA TGG, and reverse primer, GTG AGC AGG GTC GGG. An aliquot of each RT reaction was taken for PCR and amplified with Taq polymerase in the presence of 5' end-labeled forward primer, dNTPs, and a standard buffer mix (Promega). The α-actin isoforms were distinguished by digestion of an aliquot of the PCR reaction mix with 15 U of Sac I (10 U/µL, Boehringer) to yield fragments of 202 bp for skeletal α-actin and 161+39 bp for cardiac α-actin. Fragments were separated on a 6% urea/polyacrylamide gel after addition of the appropriate loading buffer. Quantification was achieved by densitometric analysis of the resulting autoradiograms.

We have developed a similar rapid technique for differentiating between the cardiac iso-mRNAs of MHC using a "hot" RT-PCR amplification technique. The same cDNAs generated for the actin analyses above were used to distinguish between the MHCs. Two oligonucleotides of 20 or 19 bases in length (forward, GAG GCG GTG CAG GAG TGT AG, and reverse, GTT GGC CTG TTC CTC CGC C) identical to sequences for both α- and β-MHC were identified and used in these amplifications. The reaction mix contained 50 mmol/L KCl, 10 mmol/L Tris-HCl, pH 9.0 (25°C), 0.1% Triton X-100, 1.5 mmol/L MgCl$_2$, 18 pmol forward primer, 20 pmol reverse primer, 0.8 mmol/L dNTPs, and 2.5 U of Taq DNA polymerase. The reaction was supplemented with 2 pmol radioactively labeled forward primer (using T4 polynucleotide kinase in the presence of [γ$^{32}$P]ATP (5000 Ci/mmol). Amplifications were as follows: program 1, 95°C, 3 minutes; 63°C, 30 seconds; 72°C, 30 seconds (1 cycle); program 2, 95°C, 45 seconds; 63°C, 30 seconds; 72°C, 30 seconds (15 cycles); and program 3, 95°C, 45 seconds; 63°C, 30 seconds; 72°C, 5 minutes (1 cycle). The MHC iso-RNAs were distinguished by digestion of 10 µL of the PCR reaction mix with 10 U of Tru91 (10 U/µL, Boehringer) in a standard reaction buffer at 65°C for 90 minutes. Fragments were separated on an 8% urea/polyacrylamide gel after addition of loading buffer. The gel was run overnight, dried, and exposed to x-ray film at -70°C for 12 to 72 hours.
The resultant bands on the autoradiograms were then quantified densitometrically, and ratios between complementary isoforms were calculated.

Statistics
A one-tailed Student's $t$ test for unpaired data was performed for the statistical analyses of muscle hypertrophy based on preliminary studies showing that clenbuterol led to an increase in the wet weights of the skeletal muscle and heart. For the densitometric analyses, a two-tailed $t$ test was used. All data are presented as mean±SD, and results were considered significant at values of $P<.05$.

Results

In Vivo Experiments

Mean body weight and left perinephric fat pad weight. Rats in all four groups continued to grow normally during the experimental period. The absolute mean body weights for the clenbuterol-treated rats were higher than their respective controls but did not reach statistical significance: mean body weights for groups A and B (5 weeks) were 417.3±63.7 and 406.6±29.7 g, respectively ($P=.6$), and for groups A1 and B1 (2 weeks), 203.9±22.3 and 197.4±10.4 g, respectively ($P=.5$). For groups A1 and B1, the weights of the left perinephric fat pads were 0.91±0.18 and 1.21±0.59 g, respectively ($P=.012$). This reduction in body fat could not be attributed to malnutrition secondary to inappetance (which may occur for the first few days of treatment with clenbuterol\textsuperscript{11}), since the mean body weight remained normal.

Wet weight of skeletal muscle and heart. Hypertrophy was seen in the GPS ($P<.01$), LDM ($P<.01$), and heart ($P<.01$) in both groups A (5 weeks of treatment) and A1 (2 weeks of treatment). The degree of this hypertrophy is summarized in Fig 1a and 1b; it follows the order GPS (21% to 35%) >LDM (20% to 29%) >heart (18% to 20%) and was greater in each case after 5 weeks compared with 2 weeks of treatment with clenbuterol. There was a good correlation between heart-to-tibia ratio and LDM-to-tibia ratio ($r=.81$).
Figure 1. Histograms showing the hypertrophic response in rats treated with clenbuterol vs controls for 5 weeks (a) or 2 weeks (b). The degree of hypertrophy is greatest in the GPS (21% to 35%), followed by the LDM (20% to 29%), with a modest but significant degree of cardiac hypertrophy (18% to 20%).

Histology. To determine whether clenbuterol-induced hypertrophy was associated with any interstitial fibrosis of LDM or left ventricle, sections from control and clenbuterol-treated rats were analyzed after van Gieson staining. The van Gieson–stained sections of left ventricle and LDM from control and clenbuterol-treated rats were similar in appearance (see Fig 2A and 2B). The yellow-staining muscle fibers had a normal morphology without evidence of necrosis. Pink-staining collagen was seen mainly in the adventitia of the blood vessels and in the perimysium of the LDM. No areas of heavy interstitial fibrosis were seen in either skeletal or cardiac muscle.

Figure 2. Van Gieson staining of a transverse section of the LDM (A) and left ventricle (B) taken from a clenbuterol-treated rat showing the absence of interstitial fibrosis with collagen limited to the perimysium and arteriolar adventitia.
RNA analyses in heart. The level of ANF expression in the adult left ventricle of the rat is generally only 2% to 3% of the atrial level; however, enhanced ventricular expression of ANF mRNA is one of the best molecular markers for all forms of cardiac hypertrophy. To determine whether clenbuterol-treated rats exhibit molecular changes in their myocardium in addition to their increase in ventricular mass, ANF mRNA levels were measured and normalized to 18S RNA (Fig 3). The analyses demonstrate that the levels of ANF mRNA are threefold greater in the ventricles of clenbuterol-treated animals (0.97±0.24 AU) compared with the controls (0.38±0.17 AU) (P<.01).

![Figure 3. Autoradiogram showing the expression of ANF and 18S ribosomal RNA in total RNA isolated from ventricles of clenbuterol-treated (Clen) and control (CTL) rats. Hybridizations to total RNA isolated from liver and atrium are also shown as a negative and positive control, respectively.](72K)

Distinction between the mRNA isotypes for rat cardiac and skeletal α-actin is also a useful marker for cardiac hypertrophy. We used a "hot" RT-PCR to distinguish between these iso-mRNAs. Results from RNA isolated from a clenbuterol-treated ventricle, control ventricle, and liver after electrophoretic separation of the amplified fragments are shown in Fig 4. No bands are detectable in the liver sample. A single band is detectable before digestion (u) for clenbuterol and control, whereas after Sac I digestion (c), two bands are detectable. The data demonstrate conclusively that skeletal α-actin...
expression was not induced with clenbuterol treatment at the time points studied. It is possible that a transient increase in expression may have occurred, but we have no data to support this possibility. It was therefore important to look at MHC iso-mRNA expression to see whether there were any changes in gene expression that could be equated with the development of a pathological form of cardiac hypertrophy.

Figure 4. Autoradiogram showing the results of a "hot" RT-PCR reaction performed on RNA isolated from liver and ventricles treated with vehicle or clenbuterol (Clen) after electrophoresis to distinguish between the amplified fragments of different sizes. No bands are detectable in liver samples. A single band is detectable in RNA isolated from ventricles before digestion (u), and two bands after Sac I digestions (c), one at 202 and one at 168 bp for skeletal and cardiac α-actin, respectively.

Simultaneous and unambiguous distinction between the mRNA isotypes for rat cardiac MHCs is difficult by classic means, necessitating an S1 nuclease or an exonuclease VII digestion. To simplify the assay, we developed a sensitive and rapid technique capable of differentiating between them. In the establishment of this protocol, several criteria had to be met. These included (1) identification of two oligonucleotides that hybridized equally to both MHCs that would yield fragments of identical lengths and nearly identical sequences and (2) identification of an internal restriction site in one of the MHC sequences that could be used to distinguish between the two isoforms. One such sequence and one restriction site were identified.

After amplification of cardiac cDNAs, a single PCR fragment of 443 bp is detectable on polyacrylamide gels that yields a second smaller band after Tru91 digestion. Liver samples are always negative for both bands. Fig 5 shows the results of such an experiment after digestion by the restriction enzyme Tru91. To validate this technique, we performed experiments using dysthyroid samples in which hypothyroid samples (100% β-MHC) and hyperthyroid samples (100% α-MHC) were mixed, the results of which are shown in Fig 5. In lane a (100% hypothyroid), a single band is detectable that
corresponds to β-MHC, whereas in lane e (100% hyperthyroid), the single band seen corresponds to α-MHC. Both bands are seen in lanes b, c, and d, with the intensity of the α-MHC band increasing and that of the β-MHC decreasing as the proportion of RNA from the hyperthyroid ventricle increases relative to that of the hypothyroid ventricle. When the relative quantities were plotted against the optical densities (arbitrary units), we found a linear increase in α-MHC expression ($r = .96$) and a linear decrease in β-MHC expression ($r = .98$), indicating that this technique could be used to distinguish simultaneously and unambiguously between the two MHC iso-mRNAs. Analyses of the clenbuterol- and vehicle-treated rats (Fig 5) all contained equivalent amounts of α-MHC, with only trace amounts of β-MHC present with longer exposure times. Therefore, the hypertrophied ventricles from clenbuterol-treated rats have a much greater abundance of α-MHC compared with β-MHC mRNA, which is similar to other models of physiological hypertrophy, e.g., hyperthyroidism and swimming rats. 

**Figure 5.** Results after an MHC "hot" RT-PCR reaction after digestion with the restriction enzyme *Tru*91. To validate the relative expression of α- and β-MHC iso-mRNA, experiments were performed in which hypothyroid samples (100% β-MHC) and hyperthyroid samples (100% α-MHC) were mixed: a, 100% hypothyroid; b, 75% hypothyroid + 25% hyperthyroid; c, 50% hypothyroid + 50% hyperthyroid; d, 25% hypothyroid + 75% hyperthyroid; and e, 100% hyperthyroid. The results are shown in lanes a through e and indicate a clearly definable transition from β- to α-MHC. Identical experiments on total RNA isolated from clenbuterol-treated and control ventricles indicate that α-MHC expression remains predominant despite drug treatment. Liver is included as a negative control.

**In Vitro Experiments**

The next question addressed was whether cardiac hypertrophy might be due to direct actions of clenbuterol. To test this possibility, we used a system of neonatal cardiocytes in culture and measured ANF expression.
A photograph of the cardiocytes in culture is shown in Fig 6. Addition of a nonselective adrenergic agonist, norepinephrine, or a β-selective adrenergic agonist, isoproterenol, rapidly altered the morphology of the cardiocytes (Fig 6B and 6C), similar to what has been described previously. These changes are characterized by long cytoplasmic projections between adjacent myocytes and rapid beating. Only the norepinephrine-treated cells hypertrophied. These changes are not, however, apparent in cells treated with ascorbate alone. In fact, these cells appeared quiescent. Addition of the β2-selective agonist clenbuterol for 3 days to the cultured cells produced no remarkable effects on morphology, and the cells did not appear to beat any differently from those treated with ascorbate alone. Analyses of total RNA isolated from the neonatal cardiocytes indicated that ANF expression normalized to 18S RNA increased only after administration of norepinephrine- (0.52±0.21 AU) relative to ascorbate-treated cells (0.24±0.07 AU). No significant change in ANF expression was seen with isoproterenol (0.29±0.19 AU) or with clenbuterol (0.29±0.27 AU).

**Figure 6.** Neonatal rat myocardial cells were plated at 5.0x10⁴ cells/cm². Thirty-six hours after plating, the cells were transferred to serum-free medium supplemented with insulin and transferrin. Six hours later, ascorbate (A), norepinephrine (B), isoproterenol (C), or clenbuterol (D) was added. After an additional 60 hours in culture, the cells were photographed on a Nikon inverted phase-contrast microscope. The cells treated with norepinephrine or isoproterenol formed long cytoplasmic projections, whereas those treated with vehicle (ascorbate) or clenbuterol have minimal intercellular contacts. Magnification x20.

**Discussion**

This study shows that clenbuterol induces a specific pattern of hypertrophy in rat striated muscle with
significant hypertrophy of the LDM, on the order of 20% to 30%. This anabolic effect is associated with a reduction in body fat content, consistent with its repartitioning properties described in previous studies.\textsuperscript{11,12,13}

Skeletal muscle circulatory support is a potentially useful modality for the treatment of end-stage heart failure,\textsuperscript{1,2,3,4,5} but several limitations need to be addressed before it can be established. One of these relates to the known structural and functional changes that occur in patients with severe heart failure,\textsuperscript{9,10} which may mean relying on an LDM that is abnormal from the outset to provide cardiac assist. Furthermore, the peak power generated by skeletal muscle is known to decline by more than eightfold when it is electrically transformed into a fatigue-resistant slow-twitch muscle.\textsuperscript{3} In addition to this drop in specific power (watts per kilogram muscle weight), there is a reduction in total power generated resulting from atrophy that is secondary to reduced resting length and tension after mobilization. There is also an element of disuse atrophy that occurs during the "vascular delay period" when the muscle remains inactive. All these factors sum to produce progressive atrophy estimated to be on the order of 40% and may be a major reason for the reported lack of consistent improvement in central hemodynamics after cardiomyoplasty.\textsuperscript{6,2} It is important to emphasize, however, that the exact mechanism of skeletal muscle assistance of the heart is still unknown and may involve factors that are independent of size and power of the LDM (eg, chronic "girdling" of the heart). Our hypothesis of using clenbuterol ultimately to improve the degree of cardiac assistance is based on the assumption that the beat-to-beat synchronous systolic assist provided by the muscle wrap is of greater importance. The effects of clenbuterol on the size, power, and fatigue resistance of electrically transformed skeletal muscle need to be investigated.

Anabolic steroids have been used experimentally in an attempt to compensate for loss in muscle bulk, although in general, any hypertrophy induced by these agents does not seem to translate into an increase in power.\textsuperscript{35,36} This may be due to the mineralocorticoid effect causing hypertrophy simply by increased water and salt retention without any significant upregulation of contractile elements. A further limitation with anabolic steroids is their sex specificity and important side effects. Another potential agent is clenbuterol, which is a synthetic analogue of epinephrine, not a steroid, thus lacking sex specificity and known to induce skeletal muscle hypertrophy selectively in a variety of animal species, with minimal adverse effects.\textsuperscript{11,12} The response is a true hypertrophy without hyperplasia and produces a transition toward fast twitch (and thus greater peak tension) fibers.\textsuperscript{37} Moreover, clenbuterol has been
shown to inhibit and reverse denervation-\textsuperscript{,38 \textsuperscript{39}} disuse-\textsuperscript{,40} endotoxemia-\textsuperscript{,41} and cachexia-related\textsuperscript{42} skeletal muscle atrophy.

Our study has shown a significant hypertrophy of the intact nontransformed LDM in the rat and is an important finding in view of the known wide variability in clenbuterol responsiveness that different skeletal muscles in the same organism show. The reason for this variability is unknown, but it may be linked to differences in $\beta_2$-receptor density in different skeletal muscle fibers.\textsuperscript{43} Although the growth-promoting effects of clenbuterol are generally believed to be $\beta_2$-adrenoceptor mediated, antagonist studies with propranolol\textsuperscript{44 \textsuperscript{45 \textsuperscript{46}}} have given conflicting results, and other as yet unidentified mechanisms (eg, involving the putative $\beta_3$-receptor) may be involved.

The fact that an 18\% to 20\% hypertrophy of the heart was observed confirms other reports\textsuperscript{11} and has implications for any future use of clenbuterol in patients with heart failure treated by skeletal muscle assist. The exact mechanism of cardiac hypertrophy in this model is not known, particularly with regard to whether it is due to a direct effect of the drug or secondary to changes in skeletal muscle. The molecular changes seen in our study, in which ANF mRNA reexpression (a nonspecific molecular marker of hypertrophy) in the clenbuterol-treated ventricle occurs without any transition in the contractile protein iso-m RNAs, are similar to the physiological hypertrophy seen in the hyperthyroid model.\textsuperscript{47 \textsuperscript{48}} In both cases, cardiac $\alpha$-actin and $\alpha$-MHC m RNAs are the predominant iso-m RNAs transcribed, in contrast to the pathological hypertrophy that occurs in pressure and volume overload, hypothyroidism, and catecholamine infusions, in which there is reexpression of several iso-m RNAs found more abundantly in the fetal heart, ie, skeletal actin (transient increase) and $\beta$-MHC.\textsuperscript{19 \textsuperscript{20}} Our findings of a physiological cardiac hypertrophy that increases in magnitude at an equal rate with the degree of skeletal muscle hypertrophy might be explained in terms of a secondary or indirect response by the myocardium to an increased demand made by a greater skeletal muscle bulk (as with athletic training). The absence of any significant necrosis or interstitial fibrosis in the hearts of clenbuterol-treated rats supports the idea of an indirect effect on the heart. This is in contrast to isoproterenol, another synthetic $\beta$-agonist known to have a direct action, and causes myonecrosis and fibrosis even after a few days of treatment in rats.\textsuperscript{49} These data are further supported by our initial findings that clenbuterol treatment of neonatal cardiocytes in culture for almost 3 days has no remarkable effect on the cell morphology, as evidenced by the lack of cytoplasmic projections or induction of ANF expression. Isoproterenol treatment of the cardiocytes does not induce ANF expression, but it does lead to prominent changes in cell morphology. Previous data have indicated that isoproterenol treatment
does in fact induce skeletal \(\alpha\)-actin expression in neonatal cardiocytes,\(^{24,33,34}\) but in preliminary data, it is apparent that under these same conditions, clenbuterol does not (M.P. and K.R.B., unpublished data). A more complete understanding of the effects of clenbuterol on cardiomyocytes in culture, including a complete dose-response curve and changes in the plating density, will help elucidate whether a direct action exists.

We believe that the findings in this study could have important clinical implications. In particular, the potential effect of clenbuterol on the electrically transformed LDM to result in a fatigue-resistant muscle that is larger and more powerful may be of great value and needs to be investigated. It could be argued, however, that any potential use of clenbuterol to induce hypertrophy of the LDM in patients with end-stage heart failure undergoing cardiomyoplasty would be limited if concomitant cardiac hypertrophy also occurs. Although the observation of clenbuterol-induced hypertrophy in the normal rat heart is difficult to extrapolate to the failing human heart, the molecular changes of physiological hypertrophy would suggest that such a response may not necessarily be harmful and could possibly be beneficial. Further studies are needed to investigate the functional characteristics of the clenbuterol-enlarged heart as well as to look at the acute and chronic effects of the drug in large-animal models of heart failure.

There is some evidence that skeletal muscles undergoing atrophy are more sensitive to clenbuterol\(^{39}\) and therefore respond to doses low enough not to produce generalized hypertrophy of normally innervated muscles or, indeed, any cardiac hypertrophy. Furthermore, Palmer et al\(^{50}\) showed in the rat that treatment with fenbufen (a nonsteroidal anti-inflammatory agent that specifically inhibits synthesis of prostaglandin \(F_{2\alpha}\)) completely inhibits the clenbuterol-induced cardiac hypertrophy without blunting the skeletal muscle response. There is a need for a concentration-response study to be undertaken to identify the dose required to induce skeletal muscle hypertrophy without any concurrent cardiac hypertrophy.

In conclusion, clenbuterol was found to induce a 20% to 30% hypertrophy of the LDM in the rat. This was associated with an 18% to 20% hypertrophy of the heart that was shown at the molecular level to be a physiological hypertrophy, ie, a more than threefold increase in ANF mRNA expression occurring in the ventricle without any transition in the contractile protein iso-mRNAs. The action of clenbuterol on electrically transformed skeletal muscle remains unknown and needs to be investigated to define its potential role, if any, in skeletal muscle assist.
Selected Abbreviations and Acronyms

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<th>Abbreviation</th>
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<tr>
<td>AU</td>
<td>arbitrary units, relative quantities plotted against optical densities</td>
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<td>ANF</td>
<td>atrial natriuretic factor</td>
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<td>GPS</td>
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<td>LDM</td>
<td>latissimus dorsi muscle</td>
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<td>MHC</td>
<td>myosin heavy chain</td>
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<td>RT-PCR</td>
<td>reverse transcriptase–polymerase chain reaction</td>
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