Effects of chronic administration of clenbuterol on function and metabolism of adult rat cardiac muscle


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Clenbuterol (Clen), a β2-agonist, is known to produce skeletal and myocardial hypertrophy. This compound has recently been used in combination with left ventricular assist devices for the treatment of end-stage heart failure to prevent or reverse adverse consequences of unloading-induced myocardial atrophy. However, the mechanisms of action of Clen on myocardial cells have not been fully elucidated. In an attempt to clarify this issue, we examined the effects of chronic administration of Clen on cardiac muscle. We chronically treated male Lewis rats with either 2 mg·kg⁻¹·day⁻¹ Clen or saline (Sal) for 4 wk with the use of osmotic minipumps. Ventricular myocytes were enzymatically dissociated. Cells were field stimulated at 0.5, 1, and 2 Hz, and cytoplasmic Ca²⁺ transients were monitored with the use of the fluorescent indicator indo-1 acetoxymethyl ester. Two-dimensional surface area and action potentials in current clamp were also measured. We found that in the Clen group there was a significant hypertrophy at the organ and cellular levels compared with Sal. In Clen myocytes, the amplitude of the indo-1 ratio transients was significantly increased. Sarcoplasmic reticulum Ca²⁺ content, estimated by rapid application of 20 mM caffeine, was significantly increased in the Clen group. The action potential was prolonged in the Clen group compared with Sal. Carbohydrate contribution to the tricarboxylic acid cycle (Krebs cycle) flux was increased several times in the Clen group. The action potential was prolonged in the Clen group compared with Sal. Carbohydrate contribution to the tricarboxylic acid cycle (Krebs cycle) flux was increased several times in the Clen group.

METHODS

Insertion of Osmotic Minipumps

Male Lewis rats (Charles River) 210–310 g (260.4 ± 7.57 g) were treated with either Clen (2 mg/kg body wt, Sigma) or vehicle only [0.9% saline (Sal), 154 mM Na⁺, and 154 mM Cl⁻] using continuous infusion by osmotic minipumps (model-2ML4, Alzet). All animals were anesthetized with an intraperitoneal injection of fentanyl + fluanisone (Hypnorm) and midazolam (Hypnovel). Under asceptic conditions, the scruff of the neck was shaved and skin incised. The skin was then dissected from the muscle layer by blunt dissection to prevent the adverse effects of unloading-induced myocardial atrophy. However, the mechanisms of action of Clen on myocardial cells have not been adequately investigated. In this study, we chronically treated male Lewis rats with Clen and found that this treatment induces 1) development of organ and cellular hypertrophy, 2) increased Ca²⁺ transients and SR Ca²⁺ content without changes in the rate of Ca²⁺ decline in isolated ventricular myocytes, and 3) increased oxidative carbohydrate utilization in the heart. These results suggest that Clen may have beneficial effects on Ca²⁺ regulation and energy substrate preference while inducing mild hypertrophy with preserved ventricular function. This could be beneficial in counteracting possible disuse atrophy in completely unloaded hearts.
create a subcutaneous pocket into which the osmotic minipump was placed. The skin was closed with interrupted sutures and a single dose of antibiotic (amoxycillin) given. All the animals were monitored daily and weighed weekly. At the end of 4 wk, Clen levels in the blood were measured using liquid chromatography-tandem mass spectrometry followed by blood extraction using a methanol-zinc sulfate procedure. The sample was separated on a 2 mm × 15 cm chromatographic column packed with 3 μm BDS Hypersil. The mass detector was operated in a selective reaction-monitoring mode. This new procedure has been validated for linearity, specificity, and recovery with satisfactory results. Using this procedure in blood samples collected at the end of the experiments, we found that the Clen level was maintained between 0.1 and 0.2 μM. All animal procedures were in compliance with the Home Office UK regulations.

Assessment of Hypertrophy and LV Function

After 4 wk of treatment, the animals were anesthetized and underwent transthoracic echocardiography (Sequoia 512, Siemens and 15 MHz probe) for assessment of hypertrophy and cardiac function. M-mode images were acquired and used for calculation of chamber dimensions and LV function using calculation procedures provided by the manufacturer. The chest was then opened, and the heart was excised and weighed after being blotted on paper. Left tibial length was measured with the use of a caliper. The heart weight-to-body weight ratio and the heart weight-to-tibial length ratio were calculated.

Cardiomyocyte Studies

Cell isolation. Cells were isolated using a method described in detail elsewhere (30). Briefly, LV myocytes were enzymatically dissociated using hyaluronidase (0.5 mg/ml, Sigma) and collagenase (0.75 mg/ml, Worthington). The cells were filtered, centrifuged, resuspended, and then stored in enzyme solution (in mM: 120 NaCl, 5.4 KCl, 5 MgSO4, 5 sodium pyruvate, 20 glucose, 20 taurine, 10 HEPES, and 200 μl of 1 mM CaCl2; pH 6.96) at room temperature. After isolation, 75–90% myocytes were rod shaped and Ca2+ tolerant.

Cell size measurements. Cells were selected as randomly as possible, and digital images were recorded using a video camera. The images were analyzed offline to assess cell dimensions (cell area, width, and length) using Image J software (NIH).

Functional measurements. Cells were placed on a superfusing chamber (volume, ~60 μl) positioned on the stage of an inverted microscope (Nikon TE200). A thin coating of laminin (Sigma) was applied to the bottom of the chamber to improve cell adhesion. Cells were superfused at 37°C with normal Tyrode solution (in mM: 140 NaCl, 6 KCl, 1 MgCl2, 2 CaCl2, 10 glucose, and 10 HEPES, pH to 7.4 with 2 M NaOH) at a rate of 1–2 ml/min. Stimulation of the cells was achieved with either field stimulation using a pair of platinum wires or via the microelectrode.

Action potential measurements. The electrophysiological experiments were performed with the use of an Axoclamp-2B system (Axon Instruments). To avoid dialysis of the cells and to minimize the effects of changing the intracellular environment, high-resistance (15–30 MΩ) microelectrodes were used. The microelectrode filling solution contained 2 M KCl, 0.1 mM EGTA, and 5 mM HEPES (pH 7.2). Action potentials were elicited by a 1.2-nA current pulse (5 ms duration) at 1 Hz and measured in current clamp mode.

Monitoring of cytoplasmic [Ca2+]. Cytoplasmic [Ca2+] was determined using the Ca2+-sensitive, single-excitation, dual-emission fluorescent dye indo-1. Cells were loaded with 10 μM of the AM form of the indicator (indo-1 AM, Molecular Probes) for 20 min at room temperature. The supernatant was then discarded and replaced by fresh ES solution. Cells were stored in the dark at room temperature and used within 6–7 h. Myocytes were field stimulated at different frequencies. For the assessment of SR Ca2+ content, cells were field stimulated to a steady-state contraction, stimulation was stopped, and 20 mM caffeine in Na+-free/Ca2+-free solution (in mM: 140 LiCl, 6 KOH, 1 MgCl2, 10 glucose, 10 HEPES, 0.1 EGTA; pH to 7.4 with 1 M LiOH) was rapidly applied for 5 s as previously described (29).

Metabolic Studies

Analysis of the rate of cardiac carbohydrate utilization was performed as previously described (19) except that rats were kept anesthetized during the experiment and that tandem mass spectrometry was applied for identification of 13C enrichments. [1-13C]glucose was infused as a 20% solution at the rate of 2 mg·kg−1·min−1 for 180 min. After 3 h of glucose infusion, rats were connected to a ventilation system, the chest was opened, and the hearts were freeze clamped in situ. Perchloric acid extracts were prepared as previously described (25), and the extracts were analyzed by liquid chromatography mass spectrometry. Separation procedure followed ion-pairing method for nondervitized amino acids described previously (Petritis, 2002 1168/1178) using a LCQ Deca XP ion-trap tandem mass detector linked to the surveyor chromatography system. Mass detection was carried out in fragmentation mode (Tandem MS), and 13C isotopic enrichment of fragments containing C3 of alanine or C4 of glutamate was monitored. Carbohydrate contribution to the tricarboxylic cycle (Krebs cycle) was calculated from the ratio of glutamate to alanine after correction for natural abundance of 13C. This is a simplified approach that does not take into account the anaplerotic entry into the Krebs cycle or secondary labeling of the other cardiac substrates, such as lactate, that could reach as much as 50% of glucose enrichment (37). Our results therefore represent carbohydrate contribution rather than glucose contribution to the Krebs cycle flux. During infusion of [1-13C]glucose, blood samples were collected for measurement of free fatty acid.

Table 1. Echocardiographic measurements

<table>
<thead>
<tr>
<th></th>
<th>Sal (n = 8)</th>
<th>Clen (n = 9)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVAWs, μm</td>
<td>0.19±0.009</td>
<td>0.22±0.011</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>LVAWd, μm</td>
<td>0.31±0.02</td>
<td>0.37±0.02</td>
<td>NS</td>
</tr>
<tr>
<td>LVPWd, μm</td>
<td>0.32±0.01</td>
<td>0.34±0.01</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>LVEDD, μm</td>
<td>0.76±0.03</td>
<td>0.76±0.02</td>
<td>NS</td>
</tr>
<tr>
<td>LVESD, μm</td>
<td>0.35±0.04</td>
<td>0.31±0.03</td>
<td>NS</td>
</tr>
<tr>
<td>LVAW% thickening</td>
<td>69.5±8.8</td>
<td>68.04±5.07</td>
<td>NS</td>
</tr>
<tr>
<td>LVPW% thickening</td>
<td>70.04±1.12</td>
<td>54.98±8.95</td>
<td>NS</td>
</tr>
<tr>
<td>%FS</td>
<td>54.73±2.12</td>
<td>59.36±2.48</td>
<td>NS</td>
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<tr>
<td>LVAW/LVPW</td>
<td>0.995±0.077</td>
<td>1.2±0.08</td>
<td>&lt;0.01</td>
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<td>LV mass, μg</td>
<td>1.44±0.04</td>
<td>1.69±0.05</td>
<td>&lt;0.01</td>
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<td>LVEF (M-mode, %)</td>
<td>87.73±2.62</td>
<td>91.69±1.4</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, number of rats. Sal, saline; Clen, clenbuterol; LVAWs, left ventricular (LV) anterior wall thickness in diastole; LVAWd, LV anterior wall thickness in systole; LVPWs, LV posterior wall thickness in diastole; LVPWd, LV posterior wall thickness in systole; LVEDD, LV end-diastolic diameter; LVESD, LV end-systolic diameter; LVAW% thickening, LV anterior wall percent thickening; LVPW% thickening, LV posterior wall percent thickening; %FS, percent fractional shortening; LVAW/LVPW, ratio of LV anterior and posterior wall thickness; LVEF, LV ejection fraction; NS, not significant.

Table 2. Morphometric measurements

<table>
<thead>
<tr>
<th></th>
<th>Sal (n = 8)</th>
<th>Clen (n = 10)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart weight, g</td>
<td>1.13±0.04</td>
<td>1.37±0.04</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>367±4</td>
<td>427±4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Tibial length, mm</td>
<td>45±0.18</td>
<td>45±0.1</td>
<td>NS</td>
</tr>
<tr>
<td>Heart weight/body weight (g/g × 1,000)</td>
<td>3.1±0.1</td>
<td>3.2±0.08</td>
<td>NS</td>
</tr>
<tr>
<td>Heart weight/tibial length, g/mm</td>
<td>0.025±0.0008</td>
<td>0.03±0.0008</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, number of rats.
glucose, and lactate concentrations at hourly intervals. Analysis has been performed with enzymatic methods using diagnostic kits from Wako Chemicals according to manufacturer recommendations.

**RNA Analysis**

Peroxisome proliferator-activated receptor-α (PPAR-α) and muscle carnitine palmitoyl transferase-1 (MCPT-1) mRNA expression were quantified by real-time RT-PCR using the iCycler iQ Real Time Detection System (Bio-Rad). Total RNA was extracted from the frozen tissue specimens using the Qiagen RNeasy minicolumn procedure according to the manufacturer’s instructions (Hilden, Germany). RNA quality and quantity was assessed by agarose gel electrophoresis and by relative absorbance at 260 versus 280 nm. Primers were designed with sequence analysis software package (Informagen) from gene sequences obtained from Ensembl Genome Browser (www.ensembl.org). A BLAST (2) search for each primer

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**Fig. 1.** Morphometric assessment of isolated cardiac myocytes from the left ventricle showed increased cell area and cell width in the clenbuterol (Clen)-treated group compared with control. *Top:* examples of Clen-treated myocytes; *middle:* control myocytes; *bottom:* despite variability in cell dimension in both groups, in the Clen-treated myocytes larger dimensions were more frequent than in the control group, suggesting cellular hypertrophy. No difference was found in cell length between the groups. *\( P < 0.05 \) vs. saline; NS, not significant.

**Fig. 2.** *Left:* action potentials recorded from cardiac myocytes isolated from Clen-treated and control animals (saline). Cells were stimulated at 1 Hz using a pulse of 1.2 nA current (5 ms). Action potentials were recorded using high-resistance microelectrodes in current clamp. *Right:* action potential duration was increased in myocytes from the Clen-treated group.
confirmed homologous binding to the desired mRNA. cDNA was synthesized from 150 ng of total RNA with the use of AMV reverse transcriptase (Roche Molecular Biochemicals, Mannheim, Germany). Reactions were diluted to 100 μl. Two microliters of each RT reaction were amplified in a 25-μl PCR mix containing 0.3 μM of each primer and QuantiTect SYBR Green PCR Master Mix. Samples were incubated for an initial denaturation and polymerase activation at 95°C for 15 min, followed by 40 PCR cycles each consisting of 95°C for 30 s, 55°C for 30 s, and 72°C for 90 s. The following oligonucleotide primer pairs were used: PPAR-α, 5'-CAT CGA GTG TCG AAT ATG TGG-3' (sense) and 5'-GCA GTA CTG GCA TTT GTT CC-3' (antisense); MCPT-1, 5'-CAT GGT GAA CAG CAA CTA TTA CG-3' (sense) and 5'-CAT CGT GGA GCA CAT GG-3' (antisense); and 36B4, 5'-TCT GCT GCA TCT GCT TGG-3' (antisense). SYBR green I fluorescence emission data were captured, and mRNA levels were quantified using the critical threshold value. Analysis was performed with iCycler software. Controls without RT and with no template cDNA were performed with each assay, and all samples were run in triplicate. To compensate for variations in input RNA amounts and efficiency of RT, ribosomal protein 36B4 mRNA was quantified, and results were normalized to these values. Relative gene expression levels were obtained as previously described (36). Amplification of specific transcripts was further confirmed by obtaining melting curve profiles and subjecting the amplification products to agarose gel electrophoresis.

**Western Blotting**

Tissue samples were immediately frozen in liquid nitrogen and stored at −80°C. Samples were then homogenized in buffer containing 10 mM Tris-HCl (pH 8.0), 0.3% Triton X-100, 1 mM EDTA, and protease inhibitor mixture (Roche). Protein concentrations were assessed by using a Bradford assay, and 40 μg of total protein were loaded per lane. Proteins were separated by 10% SDS-PAGE for most proteins. The proteins were then blotted onto a nitrocellulose membrane (Hybond C-Super, Amersham). Membranes were blocked in 3% (wt/vol) nonfat, dried milk in phosphate-buffered saline containing 0.05% Tween 20 for 1 h. Blots were then incubated separately in primary antibodies [1:500 sarco(endo)plasmic reticulum Ca2+-
ATPase (SERCA2a), 1:20,000 phospholamban, 1:100 calsequestrin (Affinity Bioreagents), 1:500 Na\(^+\)/Ca\(^{2+}\) exchanger (Swant, Switzerland), and 1:50 Ca channel V1.2 (α1C) (BD Biosciences) against the above proteins followed by exposure to horseradish peroxidase-conjugated, species-specific, secondary antibody (1:1,000; 1:5,000 for the CaV-α1c subunit). Positivity was detected using enhanced chemiluminescence (Amersham), and densitometry was performed using GENES tools (Syngene) software. The level of expression of each protein was standardized to tubulin reactivity on the same blot.

**Data Acquisition and Statistical Analysis**

The data obtained from the epifluorescence apparatus and the Axoclamp-2B system were recorded on a computer using pCLAMP 8 (Axon Instruments). The rate of sampling was between 0.5 and 3 kHz. Time to peak was measured between the point before the initial increase of the trace and the peak of the signal. Time to 50% decay (\(T_{50}\)) was measured between the peak of the signal and the point on the declining phase corresponding to half of the total size of the Ca\(^{2+}\) transient. To assess statistical differences between means, the Student’s \(t\)-test was used. \(P < 0.05\) was considered significant. Unless otherwise specified, the results are expressed as means ± SE. In cellular studies, \(n\) is the number of cells. Each set of experiments was performed in cells isolated from a minimum of five different animals.

**RESULTS**

**Clen Induces Hypertrophy Without Altering LV Function**

Echocardiography was performed in Clen- and Sal-treated animals. Clen induced a significant increase in diastolic anterior and posterior wall thickness and LV mass compared with control. However, the ejection fraction and fractional shortening were unchanged, indicating preserved LV function. The results obtained are summarized in Table 1.

For a direct assessment of cardiac mass, we weighed the excised hearts and normalized the values to body weight or tibial length because Clen affects skeletal muscle mass (23, 34). Rats treated with Clen gained more body weight at the end of 4 wk compared with control (Sal) rats (weight change

between week 0 and week 4: Clen, 111 ± 10.49 g; \(n = 10\); Sal, 89.38 ± 10.16 g; \(n = 8\); \(P < 0.0001\)). Total heart weight (Clen, 1.37 ± 0.123 g; \(n = 10\); Sal, 1.13 ± 0.115 g; \(n = 8\); \(P < 0.001\)) and heart weight-to-tibial length ratio (Clen, 0.031 ± 0.003 g/mm; \(n = 10\); Sal, 0.025 ± 0.003 g/mm; \(n = 8\); \(P < 0.001\)) were increased confirming cardiac hypertrophy (Table 1). There was no change in the tibial length (Clen, 45.01 ± 0.325 mm; \(n = 10\); Sal, 45.08 ± 0.512 mm; \(n = 8\); \(P = \text{not significant}\)) between the two groups (Table 2).

LV myocytes from Clen-treated animals showed an increase in cell width (Clen, 39.972 ± 1.098 μm; \(n = 45\); Sal, 37.23 ± 0.806 μm; \(n = 40\); \(P < 0.05\)) and surface area (Clen, 3930.08 ± 107.16 μm\(^2\); \(n = 45\); Sal, 3,607.78 ± 108.72 μm\(^2\); \(n = 40\); \(P < 0.05\)) without any change in cell length (Clen, 132.28 ± 1.9 μm; \(n = 45\); Sal, 128.81 ± 2.46 μm; \(n = 40\); \(P = \text{not significant}\)) (Fig. 1).

**Clen Affects Excitation-Contraction Coupling**

Functional studies performed on isolated myocytes from Clen-treated animals showed differences in action potential morphology and Ca\(^{2+}\) regulation compared with control. Action potential duration was increased in the Clen myocytes compared with control (time to 90% repolarization, Clen: 204 ± 30 ms, \(n = 11\); Sal: 117 ± 14 ms, \(n = 7\); \(P < 0.05\)) (Fig. 2). Figure 3, top, shows that in Clen myocytes the amplitude of the indo-1 ratio transients was significantly increased compared with control at all frequencies (0.5 Hz: Clen, 1.18 ± 0.08, \(n = 38\); Sal, 0.8 ± 0.04, \(n = 36\); \(P < 0.05\); 1 Hz: Clen, 1.14 ± 0.08, \(n = 38\); Sal, 0.8 ± 0.06, \(n = 36\); \(P < 0.05\); 2 Hz: Clen, 0.9 ± 0.07, \(n = 38\); Sal, 0.6 ± 0.02, \(n = 36\); \(P < 0.01\)). However, indo-1 fluorescence decay (\(T_{50}\)) was not statistically different. The indo-1 fluorescence-frequency relationship, normally negative at these frequencies in rat myocardium, remained negative after the administration of Clen (Fig. 3). Finally, the SR Ca\(^{2+}\) content assessed by rapid application of
20 mM caffeine was significantly increased in Clen myocytes compared with control (indo-1 ratio Clen: 2.87 ± 0.2, n = 23; Sal: 1.88 ± 0.2, n = 20; P < 0.05) (Fig. 4).

Western blotting analysis showed that there was a significant increase in SERCA2a (Clen: 0.11 ± 0.02 OD units, n = 10; Sal: 0.04 ± 0.007 OD units; n = 8; P < 0.02), in phospholamban (Clen: 2.14 ± 0.18 OD units, n = 10; Sal: 0.6 ± 0.1 OD units; n = 8; P < 0.0001), and in Na+/Ca2+ exchanger (Clen: 0.68 ± 0.11 OD units, n = 10; Sal: 0.2 ± 0.03 OD units, n = 8; P < 0.002) protein levels in the Clen group compared with control. There was no change in the expression of calsequestrin or the cardiac α-subunit of the Ca2+ channels (Fig. 5).

Clen Increases Carbohydrate Oxidation in Hearts

Analysis of the heart extracts for [13C]glutamate and [13C]alanine isotopomers after [1,13C]glucose infusion indicated that carbohydrate contribution to the Krebs cycle increased in Clen-treated hearts compared with control. According to isotopomer ratio calculations for alanine and glutamate in heart extracts, carbohydrate contribution to the Krebs cycle was 17.7 ± 7.5% in control hearts, whereas in Clen-treated hearts, it increased to 55.9 ± 10.6% (Fig. 6). There were no differences in the levels of free fatty acid, lactate, and glucose between control and Clen groups at any time point during the infusion of [1-13C]glucose. At the end

![Fig. 5. Top: immunoblots for Ca2+ handling proteins. Bottom: there was a 277% increase in sarco(endo)plasmic reticulum Ca2+-ATPase (SERCA2a), 357% in phospholamban, and 342% in Na+/Ca2+ exchanger, protein levels in the Clen group compared with control. There was no change in the expression of calsequestrin. *P < 0.05 vs. saline; **P < 0.01 vs. saline; ***P < 0.001 vs. saline.](image-url)
of the 3-h infusion, free fatty acid concentrations were 498.8 ± 73.4 and 509.4 ± 103.8 μmol/l, glucose concentrations were 6.15 ± 0.48 and 6.89 ± 0.97 mmol/l, and lactate concentrations were 535 ± 86 and 521 ± 93 μmol/l in Clen and control groups, respectively. To study potential mechanism of metabolic switch in Clen-treated hearts, expression of mRNA for PPAR-α and MCPT-1 was evaluated. PPAR-α mRNA expression decreased to about 60% of the control value in the Clen group (Fig. 7A). Consistently with this finding, expression of MCPT-1 decreased to 70% of the control value in Clen group (Fig. 7B).

**DISCUSSION**

This study has shown that chronic administration of Clen induces changes in Ca²⁺ regulation and energy metabolism of rat hearts that accompany organ and cellular hypertrophy. Cardiac hypertrophy in Clen-treated rats was observed by echocardiography and confirmed by the presence of increased heart weight. Heart weight was normalized to tibial length and to body weight. The ratio of heart weight to tibial length significantly increased in Clen-treated animals. However, the ratio of heart weight to body weight did not change after Clen administration, presumably because of the increase in body weight due to skeletal muscle hypertrophy (3, 7, 16). It could be argued that after the increase in body mass, a proportional increase in cardiac output and blood flow is expected; the myocyte hypertrophy observed could therefore be the consequence of pressure-volume overload rather than a direct effect of Clen. However, studies in cultured cardiomyocytes have shown that Clen can directly induce hypertrophy (4). It is therefore likely that cardiac hypertrophy in our study is also generated by a direct mechanism of induction of hypertrophy mediated by Clen.

This study was designed to investigate the effects of chronic, rather than acute, Clen administration, and a single time point (4 wk) was chosen. However, studies designed to investigated the onset, the time course, and the reversibility of these effects need to be performed.

**Ca²⁺ Regulation in Cardiomyocytes From Clen-Treated Animals**

We found that Clen induces several changes in Ca²⁺ regulation. We observed increased Ca²⁺ transient amplitude, increased SR Ca²⁺ content, maintained rate of Ca²⁺ removal, maintained indo-1 fluorescence-frequency relationship, and increased expression of SERCA2a, phospholamban, and Na⁺/Ca²⁺ exchanger.

The increased Ca²⁺ transients support larger contractions and can be explained by the increased SR Ca²⁺ content. The mechanisms involved in the increased SR Ca²⁺ content without increased diastolic [Ca²⁺] levels are unclear. Increased SERCA function associated with prolonged action potentials (31) may be responsible for this effect. Na⁺/Ca²⁺ exchanger and phospholamban overexpression observed in Clen-treated hearts can also be involved (24, 30, 42).

Increased Ca²⁺ entry can be another reason for larger SR Ca²⁺ content. The L-type Ca²⁺ current was not directly measured. We did not observe changes in the expression of the α₁c-subunit of the Ca²⁺ channels. However, the Ca²⁺ current may be increased on application of Clen because of a cAMP-
dependent activation of the channels, possibly via a stimulatory G protein, as shown for other β2-adrenergic agonists (43).

Concomitant effects on the mechanisms involved in Ca2+ release from the SR may also be present but were not investigated in this study. The maintained rate of Ca2+ decline is consistent with β2-adrenergic stimulation (39) and could be useful to maintain diastolic function.

The increased contractility observed at a cellular level was not observed at organ level. This could be because an optimal cardiac performance, controlled by cardiac and extracardiac factors, was already present in normal animals. Further studies in diseased hearts (e.g., heart failure) are required to assess whether cardiac function in vivo would be affected by Clen administration.

In heart failure, Ca2+ transients are reduced in size and have a slower decline, offering the basis for systolic and diastolic dysfunction (5). Restoring SR Ca2+ content has been shown to reverse Ca2+ dysregulation in heart failure (14). In this context, the findings from the present study suggest that the association of Clen treatment with mechanical unloading in heart failure could be beneficial.

It has been reported that administration of a single, larger dose of Clen (5 vs. 2 mg/kg used in this study) causes cardiac necrosis in rats (6). We did not perform direct quantification of cellular necrosis in our study, but we did not observe functional impairment of the heart nor was this observed in previous studies where Clen was used chronically (38). This suggests that the extent of Clen-induced necrosis, if present in the conditions used in our study, is not relevant for cardiac function.

**Glucose Metabolism in Clen-Treated Hearts**

In this study, we found changes in carbohydrate contribution to cardiac oxidative metabolism after chronic treatment with Clen. Although our data could not provide exact details, such as the relative contribution of glucose, pyruvate, and lactate, general finding of increased contribution of carbohydrates to cardiac energy metabolism is a solid and important observation. This finding may represent a beneficial adaptive change to a different metabolic environment because both theoretical consideration and experimental data suggest that glucose or pyruvate are better substrates for cardiac cells under stress (26). The mechanism of this change could involve either direct effect of Clen on the regulation of enzymes or expression of genes related to substrate preference, e.g., increased expression of glucose transporters and decrease of enzymes of fatty acid transport and metabolism. PPAR-α is considered to be a major transcriptional regulator of cardiac substrate preference (35). We provide evidence here that a possible mechanism involves downregulation of PPAR-α expression. In parallel to the decreased PPAR-α mRNA level, we observed downregulation of muscle MCPT-1 mRNA—an enzyme that controls the rate of cardiac fatty acid utilization and is transcriptionally regulated by PPAR-α.

**Relevance for Use of Clen in Patients With Dilated Cardiomyopathy Undergoing LVAD Treatment**

Although LVAD support could produce beneficial effects on the myocardium (“reverse remodeling”), one potential problem is the consequent heart muscle atrophy (15, 20, 38, 40). This could be an important impediment to the removal of the LVAD when the reverse remodeling has been achieved, limiting the efficacy of the treatment. Pharmacological agents that prevent atrophy or induce “physiological” hypertrophy, such as Clen, have been considered of value in this regard (40), but little is still known about the effects of these drugs on cardiac function. This study shows that Clen induces cardiac hypertrophy without impaired cardiac function in vivo and with increased Ca transient amplitude and carbohydrate utilization. These findings, together with the recent observation that β2-adrenergic agonists have beneficial effects in heart failure (1), support the hypothesis that Clen can be used in combination with LVADs to treat heart failure. However, the present study was carried out in normal hearts, and further studies to test this hypothesis in the presence of both heart failure and mechanical unloading need to be performed. In addition, further studies to investigate the time course of the observed effects are required.

In conclusion, chronic administration of Clen in rats induces cardiac hypertrophy without deterioration of function, increased Ca2+ transient amplitude associated to increased SR Ca2+ content, expression of SERCA2a, phospholamban, and Na+/Ca2+ exchanger, and increased carbohydrate over fatty acid utilization to provide energy. These findings suggest that Clen could have beneficial effects in preventing or reversing cardiac atrophy, but more studies, particularly using unloaded hearts with heart failure are required.

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**GRANTS**

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**REFERENCES**

10. Doenst T, Goodwin GW, Cedars AM, Wang M, Stepkowski S, and Taegtmeyer H. Load-induced changes in vivo alter substrate fluxes and


