Myotoxic effects of clenbuterol in the rat heart and soleus muscle

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Burniston, Jatin G., Yeelan Ng, William A. Clark, John Colyer, Lip-Bun Tan, and David F. Goldspink. Myotoxic effects of clenbuterol in the rat heart and soleus muscle. J Appl Physiol 93: 1824–1832, 2002; 10.1152/japplphysiol.00139.2002.—Myocyte-specific necrosis in the heart and soleus muscle of adult male Wistar rats was investigated in response to a single subcutaneous injection of the anabolic β2-adrenergic receptor agonist clenbuterol. Necrosis was immunohistochemically detected by administration of a myosin antibody 1 h before the clenbuterol challenge and quantified by using image analysis. Clenbuterol-induced myocyte necrosis occurred against a background of zero damage in control muscles. In the heart, the clenbuterol-induced necrosis was not uniform, being more abundant in the left subendocardium and peaking 2.4 mm from the apex. After position (2.4 mm from the apex), dose (5 mg clenbuterol/kg), and sampling time (12 h) were optimized, maximum cardiomyocyte necrosis was found to be 1.0 ± 0.2%. In response to the same parameters (i.e., 5 mg of clenbuterol and sampled at 12 h), skeletal myocyte necrosis was 4.4 ± 0.8% in the soleus. These data show significant myocyte-specific necrosis in the heart and skeletal muscle of the rat. Such irreversible damage in the heart suggests that clenbuterol may be damaging to long-term health.

anabolic adrenergic agonist; cardiomyocytes; sympathomimetic; necrosis; immunohistochemistry; β-adrenergic antagonists

The anabolic and lipolytic effects of the β2-adrenergic receptor (AR) agonist clenbuterol have been widely investigated, principally at doses ranging from 1 to 5 mg/kg in a variety of sedentary laboratory and livestock animals (6, 12, 25, 29). In response to such doses, the size of the heart, skeletal muscle, bone, lung, and kidney all increase, whereas the liver (35) and adipose tissue decrease in mass (6, 30). However, not all investigations have been able to demonstrate clenbuterol’s anabolic effects, particularly when low doses are used (5), which suggests a dose-response relationship between clenbuterol and muscle hypertrophy.

Clenbuterol administration has been shown to be beneficial in some animal models of Duchenne muscular dystrophy (14, 15) but not in others (21). It has yet to be proven, however, that clenbuterol-induced muscle hypertrophy is of any functional significance in normal populations, whether the dose administered is low (5) or high (20). More recent animal studies using doses (1–5 mg/kg) known to promote anabolism and investigating the combined effects of clenbuterol administration and exercise have shown a decrease in exercise performance (17, 26) and a high incidence of sudden cardiac failure (11). This suggests that clenbuterol administration may be antagonistic to the muscular and/or cardiovascular adaptation to exercise, although the mechanism by which this occurs is not yet understood.

It is conceivable that, like the less selective β-AR agonist isoproterenol (3, 27), clenbuterol may induce cell death and necrosis in the heart. Indeed, clenbuterol has previously been shown to induce general histological damage in the soleus muscle of the rat in response to a dose of 2 mg/kg administered via drinking water (36). We have, therefore, tested the hypothesis that clenbuterol administration may induce myocyte damage in the heart as well as the soleus. This may then provide a possible mechanism for the adverse effects of clenbuterol on the adaptation to exercise (17, 26) and for the increased collagen deposition found in the heart after its long-term administration (11).

Not surprisingly, the aforementioned anabolic and lipolytic (i.e., repartitioning) effects of clenbuterol have attracted the attention of many athletes, despite clenbuterol being banned by the World Anti-Doping Agency. Body builders in particular take high doses of this β2-AR agonist (28). The protocol for determining an individual’s optimal dose is crude and involves ever-increasing daily doses until the side effects can no longer be tolerated (10). The doses employed during clenbuterol abuse, therefore, vary widely, with men being better able than women to tolerate the side
effects, which include tachycardia, hypokalemia, arrhythmia, muscle cramps, and muscle tremors (19). An average daily dose for males can be eight tablets or ~2 μg clenbuterol/kg body wt. In addition, because of its lack of androgenic side effects, clenbuterol is also popular among sedentary as well as athletic women for use as a repartitioning agent. Scientific investigations into the effects of clenbuterol in humans are far less numerous than those pertaining to livestock or laboratory animals. Nonetheless, clenbuterol has been shown to have some therapeutic potential in speeding up the rehabilitation of postoperative muscle wasting in humans (23) and has been proposed for the pharmacological amelioration of cachexia in chronic diseases such as cancer (4) and Duchenne muscular dystrophy (15). The present finding that even a single administration of clenbuterol induces necrosis in cardiac and skeletal myocytes demonstrates that, before clenbuterol can be properly considered for clinical use, its potential myotoxic effects need to be more closely investigated.

The pharmacokinetics of chronic clenbuterol administration are likely to be complex, with the possibility of tachyphaxis or the accumulation of unmetabolized clenbuterol in the plasma. To avoid these complications, we have used only a single administration (enteral or parenteral) of clenbuterol and investigated the incidence of myocyte-specific necrosis in response to this in the heart and soleus muscle of the rat.

Clenbuterol is lipophilic and is known to have direct intracellular actions (1). Although the anabolic effects of clenbuterol administration have previously been shown to be β2-AR mediated (6), the same cannot be assumed for clenbuterol’s myotoxic effects. To investigate whether clenbuterol-induced necrosis was receptor mediated or a direct action of clenbuterol, selective β-AR antagonists have been used. In addition, the noradrenaline (NE) depleting agent reserpine was also used to investigate the possible neuromodulation of the sympathetic nervous system (SNS) by clenbuterol.

The present study presents data on clenbuterol-induced necrosis in the heart and soleus muscle of the rat at doses commonly employed to demonstrate its anabolic effects. The dose dependency and time course of cardiac and skeletal myocyte necrosis has been investigated in detail. To achieve meaningful quantification in the heart, a study of the topographical distribution of this cellular damage was crucial. Furthermore, by scaling the data provided in the current rat model, it was found that doses commonly abused by athletes fall within the range capable of inducing myocyte death and loss.

**METHODS**

**Animal care and tissue harvesting.** All experimental procedures were carried out under the British Home Office Animal (Scientific Procedures) Act 1986. Male Wistar rats weighing 298 ± 22 g were bred in-house in a conventional colony, housed in controlled conditions of 25°C, 50% relative humidity, and a 12-h light (0600–1800) and 12-h dark cycle, with water and food (containing 18.5% protein) available ad libitum. After the appropriate experimental procedures, rats were killed by cervical dislocation, and the heart and soleus muscles were quickly isolated. The atria were dissected from the ventricles and mounted with a piece of liver as a support. The remaining great vessels were removed directly superior to the coronary sulcus, and the ventricles were mounted apex uppermost. Soleus muscles were mounted in transverse section and supported with liver. Tissues were immediately snap frozen in super-cooled isopentane and stored at −80°C before cryosectioning (5 μm) and storage at −20°C.

**Detection and quantification of myocyte-specific necrosis.** Necrosis was detected in skeletal and cardiac myocytes by using an anti-myosin monoclonal antibody (Ab) in vivo (3, 27). This Ab, administered before clenbuterol challenge, can only permeate the disrupted sarcolemmal membranes of irreversibly damaged necrotic myocytes. All animals (experimental and control) received an intraperitoneal injection of Ab (0.9 mg/kg) 1 h before either administration of clenbuterol (experimental groups) or saline vehicle (control group). The anti-myosin Ab was then immunohistochemically detected on the sections of the harvested tissues by using a horseradish peroxidase-conjugated secondary Ab and visualized with 3,3′-diaminobenzidine. Sections were then counterstained with hematoxylin and permanently mounted before being examined by using light microscopy (×100 magnification). To quantify the necrosis in the soleus, randomized fields of view across each traverse section taken at four points about the midbelly of the muscle were investigated. Both necrotic and viable myocytes were counted (>500), and the number of necrotic fibers was expressed as a percentage of the total. In the heart, randomized fields of view within discrete areas of the ventricles (i.e., subendocardium, subepicardium, etc.) were digitized, and image analysis was used to measure the percent area of positive staining (cardiomyocyte damage) within each region.

**Administration of clenbuterol.** To precisely control the dose of clenbuterol received by each animal and the time of its administration, a single dose of clenbuterol (ICN Biomedical) was subcutaneously administered in a saline vehicle. The only exception to this was a comparative investigation into the effect of the route of administration, in which a precisely controlled dose of clenbuterol was administered either by gavage or by subcutaneous injection.

**Dose dependency of clenbuterol-induced myocyte necrosis.** Clenbuterol was administered over the range 1 μg/kg to 5 mg/kg (n = 5 in each group). Animals were killed 18 h after clenbuterol challenge, according to the procedure described above. The incidence of myocyte necrosis was then quantified in the heart and soleus muscle from each of these animals.

**Time course of clenbuterol-induced myocyte necrosis.** The peak-damaging dose of clenbuterol (5 mg/kg) was administered to seven independent groups (n = 3 in each group) of rats. Each group was then killed at selected time points from 0 (saline vehicle controls) to 24 h after the administration of clenbuterol, and the incidence of myocyte necrosis was then quantified in the heart and soleus muscles.

**Topographical distribution of clenbuterol-induced cardiomyocyte necrosis.** Throughout the investigations into the dose dependency and time course of clenbuterol-induced necrosis, the myocyte necrosis in the ventricles was consistently sampled at a point 2 mm from the apex (based on preliminary studies). To further strengthen the model and characterize the clenbuterol-induced necrosis, its distribution was investigated along the longitudinal and transverse planes of the ventricles. Rats (n = 3) were administered a peak damaging dose of 5 mg/kg, and the hearts were harvested 12 h (peak time) later. Each heart was sampled at 400-μm intervals.
along the longitudinal axis from apex to base, and the incidence of cardiomyocyte necrosis was quantified in the subendocardium and subepicardium of the left ventricles.

In the atria and papillary muscles of the left and right ventricles of the same hearts, random samples were taken, and the incidence of clenbuterol-induced necrosis was quantified.

**Effect of route of administration on clenbuterol-induced myocyte necrosis.** Rats were randomly assigned into four independent groups: parenteral clenbuterol, enteral clenbuterol, parenteral control, and enteral control (n = 5–6 rats in each group). The parenteral clenbuterol group received a single subcutaneous injection of 5 mg clenbuterol/kg body wt, whereas the parenteral control group received an equivolume subcutaneous administration of the saline vehicle only. Rats in the enteral clenbuterol and enteral control groups received 5 mg clenbuterol/kg body wt in 1 ml of saline or the saline vehicle, respectively, and both were administered by gavage. All animals were killed 12 h after clenbuterol (experimental groups) or saline (control groups) administration, and the incidence of myocyte-specific necrosis was investigated in the heart and soleus muscles.

**Receptor-mediated pathway of clenbuterol-induced necrosis.** Rats were randomly assigned to the following groups: negative or positive controls, and those undergoing β2-AR blockade, β1-AR blockade, or NE depletion (n = 4–10 rats in each group). The negative control group received a subcutaneous injection of the saline vehicle only; the positive control group received a subcutaneous injection of 5 mg clenbuterol/kg body wt. The β2-AR blocked group received 10 mg IC1 118,551/kg body wt, the β1-AR blocked group received 10 mg bisoprolol/kg body wt, and each β-AR antagonist was administered subcutaneously 1 h before a peak damaging dose of 5 mg clenbuterol/kg body wt. The NE-depleted group received an intraperitoneal injection of 2 mg reserpine/kg body wt 24 h (8, 32) before subcutaneous administration of a peak damaging dose of 5 mg clenbuterol/kg body wt. All animals were killed 12 h (peak time) after administration of clenbuterol, and the heart and soleus muscles were harvested and analyzed.

**Statistical analyses.** All data are presented as means ± SE. Experiments were analyzed by using either one-way analysis of variance with multiple post hoc analyses or Student’s independent t-test. P values of <0.05 were taken to indicate statistical significance.

**RESULTS**

**Dose dependency of clenbuterol-induced myocyte necrosis.** No necrotic damage was evident in either cardiac or skeletal muscle from control animals receiving the myosin Ab and saline vehicle only (Fig. 1, A and C). Clearly discernible myocyte necrosis was found in both the heart (Fig. 1B) and soleus (Fig. 1D) after clenbuterol administration. The onset of this myocyte necrosis in the left ventricular subendocardium of clenbuterol-treated rats occurred at a dose of 0.1 mg clenbuterol/kg body wt (Fig. 2A). In the soleus from the same animals, a dose of only 0.01 mg clenbuterol/kg body wt was sufficient to initiate similar damage (Fig. 2B). Necrosis in the left ventricular subendocardium seemed positively correlated to the dose administered, whereas the degree of necrosis in the soleus appeared maximal and was maintained throughout the dose range of 0.01 to 5.0 mg clenbuterol/kg body wt (Fig. 2).

**Time course of clenbuterol-induced myocyte necrosis.** The time course of necrosis was investigated in detail over 0–24 h in response to a single injection of 5 mg clenbuterol/kg body wt (Fig. 3). No necrosis was found in the zero time controls, which received the myosin Ab and saline vehicle only. In both the subendocardium (Fig. 3A) and soleus (Fig. 3B), necrosis was first detected at 4 h, with peak necrosis reached at 12 h in the subendocardium and 15 h in the soleus.

**Topographical distribution of clenbuterol-induced cardiomyocyte necrosis.** Necrosis in response to a single dose of clenbuterol was heterogeneously distributed throughout the heart. To investigate this in detail and permit the standardization and quantification of peak necrosis, the damage was sampled along the entire length of the ventricles. Necrosis in the left ventricular subendocardium was followed and found to peak 2.4 mm from the apex, i.e., approximately one-quarter of the way along the axis from the apex to the base (Fig. 4). In any given cross section at this point of maximal injury, more damage was found in the left ventricular subendocardium (0.87 ± 0.05%), with damage in the right ventricular subendocardium (0.5 ± 0.1%) being greater than the right and left subepicardia (0.2 ± 0.03%).

Although not studied in the same degree of detail, significant (P < 0.05) necrosis was also found in the papillary muscles of the left ventricle (1.1 ± 0.2%) and left (0.17 ± 0.04%) and right (0.08 ± 0.03%) atria.

Fig. 1. Immunohistochemical identification of myocyte necrosis in the heart and soleus muscle. Control sections from animals receiving only the myosin antibody and saline vehicle in left ventricular (LV) subendocardium (×400 magnification; a) and soleus muscle (×200 magnification; c) cross sections are shown. No damage was found in these tissues. Typical examples of myocyte necrosis 12 h after being exposed to a single in vivo administration of 5 mg of clenbuterol/kg body wt in LV subendocardium (b) and soleus (d) cross sections are also shown. Diaminobenzidine brown stain represents secondary (in vitro) immunoperoxidase detection of the primary myosin antibody administered in vivo and, therefore, represents necrosis. Tissue preparation: 5-μm cryosections with hematoxylin counterstain.
Effect of route of administration on clenbuterol-induced myocyte necrosis. The subcutaneous administration of the saline vehicle only (parenteral control group) did not induce any necrosis in the myocytes of either the heart or the soleus (Fig. 5). In contrast, administration of the saline vehicle by gavage (enteral control group) did induce some necrosis in both the heart and soleus, presumably due to the increased stress of this procedure. Both parenteral and enteral administration of 5 mg clenbuterol/kg body wt induced myocyte-specific necrosis in the heart (Fig. 5A) and soleus (Fig. 5B); in both cases (parenteral and enteral), this necrosis was significantly greater than that found in their respective control groups. In the heart, there was no significant difference in the incidence of the clenbuterol-induced necrosis by either route of administration (Fig. 5A), whereas in the soleus the enteral administration of clenbuterol induced significantly more myocyte necrosis than parenteral administration (Fig. 5B). Subcutaneous injection was therefore retained as the most precise means of administering clenbuterol in the investigation into the receptor pathway mediating clenbuterol-induced necrosis.

Receptor-mediated pathway of clenbuterol-induced necrosis. The effects of prior β-AR antagonism are shown in Table 1. No necrosis was found in the negative control group, which received the myosin Ab and saline vehicle only. In the soleus, only prior β2-AR

Fig. 2. Dose dependency of myocyte necrosis in response to a single parenteral administration of clenbuterol. Myocyte-specific necrosis was quantified as percentage area in the LV subendocardium (A) and percent number of fibers in the soleus (B) 18 h after subcutaneous administration of clenbuterol. No necrosis was found in the control tissues receiving saline vehicle only. Data are presented as means ± SE of n = 5 samples. *P < 0.05.

Fig. 3. Time course of myocyte-specific necrosis in response to a single subcutaneous administration of clenbuterol. Incidence of myocyte necrosis in the LV subendocardium (A) and soleus muscle (B) at specific time points after a single subcutaneous administration of 5 mg clenbuterol/kg body wt is shown. No necrosis was found in the control group receiving the myosin antibody and saline vehicle only. Data are presented as means ± SE of n = 3 samples.
antagonism had any significant effect, whereas in the heart both \( \beta_1 \)- and \( \beta_2 \)-AR antagonism significantly reduced the clenbuterol-induced necrosis compared with the positive control group. Reserpine was administered to investigate clenbuterol’s neuromodulation of the SNS and concomitant release of NE. Depletion of the NE-releasing capacity of the SNS by reserpine significantly prevented clenbuterol-induced necrosis in the heart but not in the soleus.

**DISCUSSION**

The use of a high avidity anti-myosin Ab has allowed us to investigate myocyte-specific necrosis. This, in combination with the carefully controlled in vivo protocol, ensured that this technique only identifies those myocytes with a ruptured sarcolemmal membrane, a key indicator of the transition from reversible (oncosis) to irreversible (necrosis) cell death (22, 34). Using this model, we have demonstrated that clenbuterol administration induces necrosis in the heart and soleus muscle of the rat.

The finding that clenbuterol induces myocyte-specific necrosis in the heart is novel. It may be speculated that, in the absence of a functional satellite cell system, all necrosis in the heart will lead to reparative fibrosis. Our acute data may therefore provide etiological support to those of Duncan et al. (11), who showed an increase in collagen infiltration (possibly reparative fibrosis) in the heart after chronic clenbuterol administration. A possible mechanism for clenbuterol’s cardiotoxicity is its adverse effect on taurine levels in the heart (9, 36). This amino acid is known to have a protective role in some tissues, particularly in the heart and lungs, with one of its possible roles being the modulation of calcium levels (16). Doheny et al. (9) showed that taurine levels in the heart are depressed in response to a single subcutaneous administration of clenbuterol. Furthermore, the dose of clenbuterol (125 \( \mu g/kg \) body wt) used and the time point (5 h) after clenbuterol administration at which the taurine levels in the heart become significantly depressed almost exactly match those found for the onset of necrosis in the heart in our investigation (Figs. 2A and 3A).

![Fig. 4. Topographical distribution of necrosis along the longitudinal axis of the left ventricle. Incidence of cardiomyocyte-specific necrosis was investigated at 400-\( \mu m \) intervals along the longitudinal axis of the heart. Peak cardiomyocyte necrosis (0.87 ± 0.05%) in the LV subendocardium was found 2.4 mm from the apex. Data are presented as means ± SE of \( n = 3 \) samples.](image)

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![Fig. 5. Effect of route of administration on the incidence of clenbuterol-induced myocyte necrosis. Myocyte-specific necrosis in the heart (A) and soleus (B) in response to 5 mg clenbuterol/kg body wt administered by either subcutaneous injection (closed bars) or gavage (open bars) is shown; all tissues were harvested 12 h after clenbuterol administration. All data are presented as means ± SE (\( n = 5–6 \) samples). *\( P < 0.05 \), **\( P < 0.01 \). †Experiment group significantly different from respective control; ‡administration by gavage significantly different from subcutaneous administration.](image)
Doheny et al. (9) did not investigate taurine levels in the soleus but only in the gastrocnemius muscle, where taurine levels increased 6 h after clenbuterol administration. In light of the present findings, it may also be of interest to investigate changes in the levels of taurine in the soleus after controlled administration of clenbuterol.

This is the first time that myocyte-specific damage has been rigorously investigated and quantified in either the heart or the soleus in response to controlled doses of this $\beta_2$-AR agonist. Waterfield et al. (36) have previously demonstrated generalized histological damage in the soleus in response to a dose of 2 mg clenbuterol/kg body wt given via drinking water. Although our data clearly support those of Waterfield et al., they further our knowledge by revealing that clenbuterol-induced necrosis occurs in the myocytes and may therefore directly affect muscle function. In addition, by rigorously controlling clenbuterol administrations, we have also been able to advance our knowledge of the dose dependency and time course involved in clenbuterol-induced necrosis.

Throughout our initial investigations (dose dependency, time course, and topographical distribution) of clenbuterol-induced necrosis, clenbuterol was administered parenterally. Many of the previous studies investigating the effects of clenbuterol have administered clenbuterol in the drinking water. Such an approach, although easy to use and effective in inducing anabolism, has several possible shortcomings that cannot always be sufficiently well controlled. Clenbuterol is readily oxidized and needs to be protected from light. The practice of making up fresh solutions on a daily or more often a weekly basis means that the actual dose received by each animal in a communal cage cannot be measured with any precision. This problem is further complicated by the clenbuterol-induced increase in thirst, which we have found to be dependent on the dose administered (unpublished observation). To establish that ingestion of clenbuterol is also myotoxic, we administered a single dose of clenbuterol enterally, the only controllable way to accurately achieve this is by gavage administration. Although this method does not exactly match that of administration via the drinking water, it does replicate the method of administration chosen by most humans, i.e., ingestion of clenbuterol in tablet form. The data (Fig. 5) clearly demonstrate clenbuterol's myotoxic effects when administered enterally. Necrotic damage in the heart was virtually the same whether clenbuterol was administered parenterally or enterally (Fig. 5A). Interestingly, in the soleus, enteral administration of clenbuterol appeared even more damaging than parenteral administration (Fig. 5B). The finding that enteral administration of the saline vehicle control also induced necrosis is presumed to be stress related, although the animals were compliant and apparently relaxed during administration. Hence, we have favored parenteral administration of clenbuterol. This route of administration provides certainty of the dose received by each animal and the time at which the dose was administered. It is quick and achievable under stress-free conditions such that no necrosis is found in the tissues from control animals.

We have shown that cardiomyocyte-specific necrosis in the myocardium is not uniformly distributed. Of the possible factors mediating this heterogeneity, the principal ones may be regional differences in $\beta$-AR distribution, taurine metabolism, other metabolic requirements, or hemodynamic stresses. Unfortunately, present data relating to the distribution of $\beta$-ARs and in particular $\beta_2$-ARs in the heart are scarce. Beau et al. (2) found the transmural distribution of $\beta$-ARs to be uniform in the nonfailing human heart. Although generally true for the rat heart, in some cases a greater density of $\beta$-ARs has been found in the papillary muscles and subendocardium (33). This is consistent with the pattern of damage found in the present investigation (Fig. 4). A possible explanation for this disparity is that Tofukkji et al. (33) only sampled the heart at three points (base, midventricular, and apex). If the same sampling frequency had been applied to the data in Fig. 4, then it is easy to see how important information would have been missed. These data (Fig. 4) serve to reaffirm the dangers of random sampling and the absolute requirement of in-depth topographical knowledge and standardized procedures when quantifying cell death, or anything else, in a complex organ such as the heart.

In support of a possible hemodynamic contribution to the pattern of damage, we consistently found a greater degree of necrosis in the left, rather than right, side of the heart, with damage being most extensive in the left ventricular subendocardium. The inherently higher energy demands of the subendocardium may make this

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Table 1. Selective $\beta$-AR blockade of clenbuterol-induced necrosis

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<thead>
<tr>
<th>Group</th>
<th>Myocyte Necrosis</th>
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<tr>
<td></td>
<td>Heart, % area</td>
</tr>
<tr>
<td>Positive control (n = 10)</td>
<td>0.99 ± 0.2</td>
</tr>
<tr>
<td>$\beta_2$-AR blockade (n = 5)</td>
<td>0.08 ± 0.03(91%)</td>
</tr>
<tr>
<td>$\beta_1$-AR blockade (n = 5)</td>
<td>0.03 ± 0.02(96%)</td>
</tr>
<tr>
<td>NE depletion (n = 4)</td>
<td>0 ± 0.00(100%)</td>
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Values are means ± SE. Positive control represents clenbuterol-induced myocyte necrosis in response to the peak damaging dose (5 mg/kg) administered subcutaneously and sampled at the optimum time (12 h) and peak topographical region (2.4 mm from the apex) in the heart. $\beta_2$-adrenergic receptor (AR) blockade, 10 mg/kg ICI 118,551; $\beta_1$-AR blockade, 10 mg/kg bisoprolol; norepinephrine (NE) depletion, 2 mg/kg reserpine. Values in parentheses (as percent) indicate the level of protection provided by each intervention. *P < 0.01 for differences between the positive control and intervention groups.
region of the heart more sensitive to hemodynamic perturbations. This compounded with clenbuterol-induced tachycardia and concomitant reductions in diastolic interval, and therefore blood supply, may explain the increased susceptibility of the subendocardium to clenbuterol-induced necrosis.

Also of particular importance within the present investigation is the novel finding that clenbuterol administration induced a significant amount of necrosis in papillary muscles, with possible effects on valve function. It is conceivable that this damage, in combination with the irreversible loss of myocytes from the ventricular walls (and hence a reduction in the pumping capacity of the heart; Refs. 7, 37), may play an etiologic role in the reduction in exercise capacity, and even cardiac failure, seen in clenbuterol-treated animals subjected to exercise (11).

It was found that a significant ($P < 0.01$) proportion (89%) of clenbuterol-induced necrosis in the soleus was mediated through the $\beta_2$-AR pathway. Therefore, only a small percentage of clenbuterol-induced necrosis can be attributed to the passage of clenbuterol through the lipid membrane. In contrast, in the heart, both prior $\beta_1$- or $\beta_2$-AR antagonism was highly effective in preventing clenbuterol-induced necrosis with little residual damage attributable to any direct intracellular action (Table 1). Because of clenbuterol’s greater potency over many other common $\beta_2$-AR selective agonists, it is difficult to extrapolate the findings of the present study to other agents. However, the finding that clenbuterol-induced myotoxicity is mediated through the $\beta_2$-AR system suggests that over stimulation of this pathway per se would be toxic.

The finding that clenbuterol induces damage in the heart through both the $\beta_1$- and $\beta_2$-AR is not in agreement with our previous findings that the less selective $\beta_1$- and $\beta_2$-AR agonist isoproterenol induces necrosis in the myocardium through the $\beta_1$-AR pathway only (27). This, coupled with the knowledge that NE itself can be cardiotoxic (24), led us to investigate the possibility that clenbuterol may have a neuromodulatory effect over the SNS. Clenbuterol acting on the $\beta_2$-AR of the sympathetic varicosities could facilitate the release of NE, which could then preferentially act on $\beta_1$-ARs to induce necrosis through overstimulation of this pathway. Reserpine was administered to block the uptake of NE from the cytosol into the transport vesicles of the sympathetic varicosities. Thus, after a period of basal neuronal activity, the NE-releasing capacity of the neuron is depleted, effectively blocking this pathway. The results (Table 1) support this hypothesis, with the prior administration of reserpine significantly ($P < 0.01$) preventing clenbuterol-induced necrosis in the heart but not in the soleus. These data clearly show that the myotoxic effects of clenbuterol on the heart ($\beta_1$-AR mediated) can be separated from its anabolic ($\beta_2$-AR mediated) effects on the heart and skeletal musculature. This information may be of great value when proposing clenbuterol administration as a pharmacological aid for the amelioration of muscle wasting in chronically ill patients. The discovery that clenbuterol-induced necrosis in the heart is indirectly mediated through the SNS and $\beta_1$-ARs, whereas that in the soleus is directly mediated through $\beta_2$-ARs, may also account for the differences found between the two muscle types in the dose-dependency experiments (Fig. 2). It appears that the heart is not simply less sensitive to clenbuterol, but rather the indirect route of action of clenbuterol on the heart (i.e., $\beta_2$-AR stimulated NE release, which then acts on cardiomyocyte $\beta_1$-ARs) requires a higher dose to elicit comparable damage (Fig. 2). In the soleus, the data (Fig. 2B) suggest a threshold response with doses $>10 \mu g/kg$ body wt possibly inducing receptor desensitisation and, hence, no further increase in the incidence of necrosis.

The present finding that clenbuterol-induced necrosis is mediated through the $\beta$-AR pathway in vivo lends support to previous work in vitro showing that $\beta$-AR stimulation reduces the viability of cultured cardiomyocytes (24). Although the intracellular mechanisms of clenbuterol-induced myotoxicity have not been investigated here, the aforementioned work (24) in vitro elegantly demonstrated that loss of cardiomyocyte viability was preceded by an increase in intracellular cAMP followed by an increase in intracellular $Ca^{2+}$. This is consistent with earlier work demonstrating that an increase in intracellular $Ca^{2+}$ is a final common pathway in cell death (31), leading to the activation of proteases and phospholipases (18).

The findings of this investigation show that the doses commonly employed to elicit clenbuterol’s anabolic properties also induce significant myocyte necrosis in the heart and soleus muscle. It is surprising, therefore, that so little information exists on the myotoxic effects of clenbuterol. A possible explanation for this is that the anabolic effects of clenbuterol have been predominantly investigated in sedentary populations of livestock or caged laboratory animals whose daily activity levels do not make full use of their cardiac functional reserve (7, 37). It is conceivable that cumulative clenbuterol-induced cardiomyocyte necrosis would gradually reduce an animal’s cardiac reserve. This could remain asymptomatic until such time when the animal is stressed or required to do work, i.e., vigorous exercise. This could explain the seminal finding of Duncan et al. (11) of a reduction in exercise capacity and a high incidence of sudden cardiac failure in swim endurance-trained rats when receiving clenbuterol. Although the anabolic or hypertrophic effects of clenbuterol have been quite widely demonstrated, studies investigating the functional significance of this anabolism in the form of increased isometric force (5, 20) or exercise capacity (17, 26) in normal populations have been ambiguous. The results from the current investigation could help to resolve this uncertainty, particularly in those studies that have used high doses of clenbuterol (17, 20). It is conceivable that the concomitant loss of myocytes incurred during such administration protocols would reduce the muscles’ capacity to do work despite their increased cross-sectional area and wet weight. For example, some of the extra mass could be attributable to reparative fibrosis. Unfortunately, mus-
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REFERENCES


