The $\beta_2$-agonist fenoterol has greater effects on contractile function of rat skeletal muscles than clenbuterol

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Short title: Effects of fenoterol and clenbuterol on muscle function

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Potential treatments for skeletal muscle wasting and weakness ideally possess both anabolic and ergogenic properties. Although the $\beta_2$-adrenoceptor agonist clenbuterol has well-characterised effects on skeletal muscle, less is known about the therapeutic potential of the related $\beta_2$-adrenoceptor agonist, fenoterol. We administered an equimolar dose of either clenbuterol or fenoterol to rats for 4 weeks to compare their effects on skeletal muscle and tested the hypothesis that fenoterol would produce more powerful anabolic and ergogenic effects. Clenbuterol treatment increased fiber cross sectional area (CSA) by 6% and maximal isometric force ($P_o$) by 20% in extensor digitorum longus (EDL) muscles, whereas fiber CSA in soleus muscles decreased by 3% and $P_o$ was unchanged, compared with untreated controls. In the EDL muscles, fenoterol treatment increased fiber CSA by 20% and increased $P_o$ by 12% above values achieved following clenbuterol treatment. Soleus muscles of fenoterol-treated rats exhibited a 13% increase in fiber CSA and a 17% increase in $P_o$ above that of clenbuterol-treated rats. These data indicate that fenoterol has greater effects on the functional properties of rat skeletal muscles than clenbuterol.

Keywords: $\beta$-adrenoceptor, fiber type, skeletal muscle, hypertrophy, muscle wasting, plasticity, muscle contraction
A severe loss of muscle mass is a risk factor for mortality in a number of conditions and disease states. The loss of protein from skeletal muscle can lead to severe and progressive muscle fiber wasting (atrophy) and weakness including that responsible for the death of boys with Duchenne muscular dystrophy (DMD), but also for other conditions including prolonged sepsis, surgical trauma, sarcopenia, cancer cachexia, AIDS, radiotherapy, chemotherapy, burn injury, and chronic renal failure (5, 21, 42) where the ability of an individual to carry out the tasks of daily living is impaired dramatically (22, 23). Therapies to alleviate the symptoms of muscle wasting are directed toward preserving existing muscle fibers, enhancing muscle fiber regeneration and promoting muscle fiber growth. Agents that stimulate an increase in muscle size (hypertrophy), by either increasing protein synthesis, decreasing protein degradation, or both, have the potential to be applied clinically to combat muscle wasting conditions (21-23).

Synthetic β2-adrenoceptor agonists (β2-agonists) were developed primarily to facilitate dilation of the bronchiolar smooth muscle in asthma patients (2). However, it became apparent that at high doses β2-agonists were causing an increase in body mass, which was later attributed to an increase in skeletal muscle mass (11). Not surprisingly, β2-agonists such as clenbuterol, were examined for possible application in the livestock industry with the aim of promoting muscle growth, and hence improving the efficiency of meat production (26, 39-41). Clenbuterol has been described as one of the most potent synthetic β2-agonists for producing increases in skeletal muscle mass (6, 10, 19, 34), and has been examined extensively as a treatment for a number of animal models of muscle wasting disorders in an attempt to ameliorate muscle atrophy (5, 7, 21-23). In addition to causing significant increases in muscle mass in animals (23), clenbuterol can also cause a dynamic shift in fiber proportions within skeletal muscle, from
slow, fatigue-resistant type I fibers towards the faster, and more fatigue susceptible type IIa, IIb and IIId/x isoforms (14, 44).

The potential for β2-agonists to improve the size and strength of muscles of human patients affected by neuromuscular diseases has received only limited attention (20, 27). Orthopaedic patients administered 20μg clenbuterol twice daily for 4 weeks did not exhibit any improvement in the absolute strength of their knee extensor muscles compared with patients given placebo (27). A three month pilot trial of albuterol (16 mg/day) given to 15 patients with facioscapulohumeral muscular dystrophy led to improved maximum voluntary isometric contractile performance and this was followed by a year long randomised, double-blind, placebo-controlled trial where patients were treated with up to 16 mg of albuterol twice daily (20). Although maximum voluntary isometric strength was not higher after treatment, there were improvements in other measures, such as muscle mass and grip strength, indicating that treatment strategies involving β2-agonists have clinical merit (20).

Studies on animals have shown that β2-agonists such as clenbuterol affect skeletal muscles, but they also affect the heart deleteriously, as evidenced by tachycardia, cardiac hypertrophy and decreased cardiac performance (10, 23, 25). At present, the dose required to ameliorate skeletal muscle wasting exceeds the estimated safe limit in humans (6) and adverse effects such as cramps, tremors, insomnia and nervousness, as reported in the clinical trial of Kissel and colleagues (20), clearly have to be minimised if β2-agonists are to have greater therapeutic application for treating muscle wasting disorders. Our purpose was to examine whether other β2-agonists have greater clinical potential than the most well characterised, clenbuterol. If another β2-agonist produced a greater anabolic effect on muscle at a similar or lower dose then such a compound would have significant clinical application.
Fenoterol is a synthetic β₂-agonist which is a full agonist at the β₂-adrenoceptor, unlike clenbuterol which is a partial agonist and has been shown to be a full β₁-adrenoceptor agonist capable of producing a maximum β-adrenoceptor activated cAMP response (4, 29). In isolated human papillary muscle preparations fenoterol exerts a direct positive inotropic effect in the heart mediated by β₁- and β₂-adrenoceptors (30). Acute intravenous administration of fenoterol to anesthetized dogs did not affect diaphragm muscle contractility in the resting state but it did improve contractility during fatigue (43). Fenoterol given to lambs in their food for 8 weeks did not increase the mass of their infraspinatus or pectoralis profundus muscles (13). Daily subcutaneous injections of fenoterol (1 mg/kg) for 19 days increased the gastrocnemius muscle mass in rats by ~12.5% (11). However, no studies have compared the efficacy of fenoterol and clenbuterol treatment on skeletal muscle function in rats. To this end, we tested the hypothesis that, due to the involvement of both β₁- /β₂-adrenoceptors and a greater cellular response, fenoterol would produce greater anabolic and ergogenic effects on skeletal muscle than an equimolar dose of clenbuterol.

METHODS

All experiments were approved by the Animal Experimentation Ethics Committee of The University of Melbourne and were conducted in accordance with the guidelines for the care and use of experimental animals as outlined by the National Health and Medical Research Council of Australia and in accordance with the American Physiological Society’s guiding principles for research involving animals and human beings. (1)
Animals

Young adult (4 month old) male Sprague Dawley (SD) rats (290-300g), were randomly allocated into one of three groups that were housed in standard cages with a 12 hour light/dark cycle and provided with food (rat chow) and water *ad libitum*. Treated rats received equimolar doses of clenbuterol (2 mg·kg\(^{-1}\) day\(^{-1}\)) or fenoterol (2.8 mg·kg\(^{-1}\) day\(^{-1}\)) administered via intraperitoneal (*i.p.*) injections in 1 ml of isotonic saline every day for 4 weeks. Control rats received a daily injection of an identical volume of saline vehicle. This concentration of clenbuterol produces significant increases in muscle mass with minimal β-adrenoceptor desensitisation (10, 14), and therefore provided a standard treatment dose for comparison with fenoterol. Clenbuterol and fenoterol were obtained from Sigma-Aldrich (Castle Hill, NSW, Australia).

Tissue harvest

At the completion of the four-week treatment period rats were anesthetized with sodium pentobarbitone (Nembutal, Rhone Merieux, Pinkenba, QLD, Australia; 60 mg/kg, *i.p.*), with supplemental doses administered to maintain an adequate depth of anesthesia, such that there was no response to tactile stimulation. The EDL (fast-twitch) and the soleus (slow-twitch) muscles from the left hindlimb were surgically exposed and a length of silk suture (3/0, Pearsalls Suture, Somerset, UK) was tied to the proximal and distal tendons of the muscles. The associated nerve and vessel supplies were cut last to ensure optimum condition of muscles before entering the organ bath. The excised muscles were blotted once on filter paper and immediately placed into a custom built plexiglass chamber filled with Krebs-Ringer solution [composition (in mM); NaCl 1.37, NaHCO\(_3\) 24, d-glucose 11, KCl 5, CaCl\(_2\) 2, NaH2PO\(_4\)·H2O 1, MgSO\(_4\)·7H2O 0.487, d-tubocurarine chloride 0.293] oxygenated with 95% O\(_2\) and 5% CO\(_2\) (BOC gases,
Preston, Victoria, Australia) and thermostatically maintained at 25°C, which is optimal for maintaining the viability of the muscles in vitro for the duration of the experimental protocol (37).

Rats were euthanased by excision of the heart, which was trimmed of connective tissue, weighed and frozen in thawing isopentane, and stored at -80°C pending measurement of β₁- and β₂-adrenoceptor density. The left and right adrenal glands were excised, trimmed, blotted and then weighed on an analytical balance.

**Muscle contractile measurements**

Contractile properties of the left EDL and soleus were assessed in vitro according to methods described in detail previously (14, 24, 33). Briefly, the distal tendon of the muscle was tied to a fixed pin in the organ bath, while the proximal tendon was attached to the lever arm of a dual mode servomotor (305-LR, Aurora Scientific Inc., Aurora, Ontario, Canada). Platinum plate electrodes flanked the muscle on either side. Muscles were field stimulated by supramaximal square wave pulses (0.2 ms duration) that were amplified (EP500B Power Amplifier, Audio Assemblers, Campbellfield, Victoria, Australia) to produce a current intensity sufficient to produce a maximum isometric tetanic contraction (P₀). The servomotor and stimulation operations were controlled by custom-built applications (D.R. Stom Software Solutions, Ann Arbor, MI, USA) of LabView software (National Instruments, Austin, TX, USA) driving a personal computer with on-board controller (PCI-MIO-16XE-10, National Instruments, USA) interfaced with the servomotor control/feedback position controller hardware (6650LR Dual-Mode Lever System, Aurora Scientific, Canada). Optimum muscle length (L₀) was determined from maximum isometric twitch force (P₁). Optimum fiber length (Lᵢ) was
determined by multiplying $L_o$ by the previously determined fiber length to muscle ratio of 0.44 for the EDL and 0.71 for the soleus muscle (14, 33).

A frequency-force curve was established following successive stimuli at 10-150 Hz for EDL muscles and 5-120 Hz for soleus muscles, with 2 min rest between stimuli. $P_o$ was determined from the plateau of the frequency-force relationship, after which the muscle was subjected to a 4 minute stimulation protocol to induce muscle fatigue. Muscles were stimulated once every 5 s at optimum length, voltage and frequency, with a stimulation duration of 350 ms for the EDL and 1,200 ms for the soleus muscles. $P_o$ was also determined 5 and 10 min after the completion of the fatigue protocol as a measure of the ability of the muscles to recover their maximum force producing capacity following repeated intermittent contractions.

Following all measurements, the muscle was trimmed of connective tissue and weighed. Specific force (kN/m$^2$) was determined for each muscle according to standard procedures taking into account cross-sectional area; muscle mass divided by the product of $L_f$ and 1.06 mg/mm$^3$, the density of mammalian skeletal muscle (28). Muscles were frozen in thawing isopentane for later histological/histochemical examination and radioligand binding assays.

*Histology and histochemistry*

A portion of each frozen muscle sample was cryosectioned transversely through the midbelly region on a cryostat microtome at -20°C (CTI cryostat, IEC, Needham Heights, Massachusetts, USA). Eight 8 μm thick serial sections, from each of EDL and soleus muscle, were cut and placed onto uncoated glass microscope slides. Two EDL and two soleus muscle cross-sections were placed onto each labelled slide (total of four sections per slide) and stored in an airtight container at -20°C overnight.
One slide was stained with hematoxylin and eosin (H & E) to determine muscle fiber cross-sectional area and general muscle architecture, whilst another was used to determine succinate dehydrogenase (SDH) activity (14), based upon a modified version of the method by Blanco and colleagues (3). Briefly, sections were incubated (in mM; sodium succinate 33, phosphate buffer 33 (pH 7.6), sodium azide 1, nitroblue tetrazolium 0.612) for 60 min at 37°C to produce a colored nitroblue-diformozan precipitate indicator according to increasing SDH activity within the muscle fibers in the sections. The reaction was terminated by multiple rinses in distilled water, and then air-dried and coverslipped. Individual muscle fibers were classified according to their SDH activity (high or low reactivity in EDL muscles; high or very high reactivity in soleus muscles) by interactive determination of the histochemical reaction intensity of individual fibers (3, 14).

The remaining slides were used to determine muscle fiber type proportions according to myosin ATPase (mATPase) reactivity, utilising the method of Hämäläinen and Pette (15). Serial sections were pre-incubated at pH 4.3 and 4.55, which identifies four individual muscle fiber types (type I, IIa, IIb, and IId/x). At the completion of the procedure, muscle sections were dehydrated in serial concentrations of ethanol and coverslipped. Muscle fibers were classified according to their mATPase activity by interactive determination of fiber histochemical reaction intensity (15).

Images of all sections were acquired using a digital imaging camera (Spot model 1.3.0, Diagnostic Instruments, Sterling Heights, MI) attached to an upright microscope (BH-2, Olympus, Tokyo, Japan). A field of view (900 x 750 μm) was chosen at random from the H & E stained section and the same field was acquired from the SDH and the myosin ATPase reacted sections. Image files were analysed using Image Pro (v4.0, Media Cybernetics, Silver Spring,
Effects of fenoterol and clenbuterol on muscle function

MD, USA) in a double-blinded manner. The mean cross sectional area (CSA) of individual muscle fibers was calculated by interactive determination of the circumference of no less than 120 adjacent fibers from the centre of every muscle section.

\(\beta\)-adrenoceptor density

Frozen EDL and soleus muscle samples were placed in 5 ml of ice-cold buffer A (in mM: Tris (pH 7.0) 50, sucrose 250, EGTA 1; pH 7.4 at 4°C) and homogenised (Polytron PT 2100, Kinematica AG, Luzernerstrasse, Switzerland) separately for 30 s. Cell membrane fragments were prepared by centrifugation at 4°C, based upon the previous work of Sillence and colleagues (38, 39). Briefly, the homogenates were centrifuged for 10 min at 1,000 \(\text{g}\) (Avanti J-25I centrifuge, JA-17 rotor, Beckman, CA, USA). The supernatant was filtered through three layers of surgical gauze (Smith & Nephew, Victoria, Australia) and centrifuged for a further 15 min at 10,000 \(\text{g}\). The supernatant was ultracentrifuged for 30 min at 100,000 \(\text{g}\) (L7 Ultracentrifuge, SW41TI rotor, Beckman, CA, USA) and the pellets resuspended in 1 ml of ice-cold buffer B (in mM: Tris (pH 7.6) 50, MgCl\(_2\) 10, NaCl 150; pH 7.4 at 4°C) using a Pasteur pipette. The resuspended pellet was stored at -80°C for 4 days prior to analysis.

The procedure used for the radioligand binding assay utilised the methodology of Sillence and colleagues (41). Frozen cell membrane pellets were thawed, resuspended in buffer and then vortexed for 30 s. Protein concentration was determined by the Bradford protein assay (Bio-Rad, Richmond, VA, USA) with bovine serum albumin standards. The membrane suspension was used at an assay concentration of 0.2 mg/ml since previous studies had shown that binding of the radioligand to \(\beta_2\)-adrenoceptor sites was linear over the protein concentration range of 0.05 to 0.3 mg/ml (41).
Because of the limited quantity of membrane protein obtained from these small muscles, single point saturation assays were performed by incubating 400 µl cell membrane suspension with 50 µl [125I]-iodocyanopindolol (135 pM; ICYP, the radioligand), and 50 µl of either buffer B (to determine the total counts of ICYP bound to β2-adrenoceptors), or DL-Propranolol (2 µM; a non-selective β-adrenoceptor antagonist that determines non-specific binding of ICYP to the membrane) in polyethylene tubes (12 mm x 75 mm). Assays were initiated with the addition of cell membranes, and tubes were incubated for 90 min in a shaking water bath set at 37ºC (130 cycles/min). Separation of bound ligand from free ligand was achieved by filtering the contents of each tube through Whatman GF-C glass fiber filter papers (Whatman GF-C filter paper, Maidstone, England, UK) with 21 ml of ice-cold buffer B using a cell harvester (Brandel M-48R cell harvester, Biomedical Research and Development Labs Inc., Gaithersburg, MD, USA). Radioactivity remaining on the filters was determined in a gamma counter (1470 Wizard-automatic gamma counter, Wallac OY, Turku, Finland) at a counting efficiency of 78%. Results were obtained as γ-radiation counts per minute (cpm) for all tubes, and then converted into [β-adrenoceptor] per mg of protein (40, 41). Previous experiments have shown that rat muscle contains a predominant population of β2-adrenoceptors, with β1-adrenoceptors usually undetectable by this technique (41). Hence the β-adrenoceptors measured were designated β2-adrenoceptors.

The left ventricle and associated atrium from each animal was used to determine the concentration of β1- and β2-adrenoceptors in cardiac muscle tissue. Samples within each treatment group (control, clenbuterol and fenoterol) were pooled such that there were 4 samples per group (cardiac tissue from 2 rats per sample). Cardiac muscle tissue membranes were prepared in the same way as described for the EDL and soleus muscles. The resuspended pellet
was stored overnight at -80°C before the radioligand assay was performed. Incubation tubes for the cardiac tissue assay contained: 150 μl cell membrane (working concentration of 0.3 mg/ml), 50 μl ICYP (120 nM) and one of the following: 50 μl buffer B (total counts of bound ICYP), 50 μl dl-propranolol (5 μM; non-specific binding), or 50 μl CGP 20712A (1 μM; a β1-selective antagonist) (40, 41). Total β-adrenoceptor binding was measured as the difference between total binding and non-specific binding (determined using propranolol). β1-adrenoceptor binding was measured as the total binding minus binding determined using CGP 20712A. β2-adrenoceptor binding was calculated as the difference between total binding and β1-adrenoceptor binding.

Statistical Analyses

Individual variables were compared between groups using separate one-way analyses of variance with Fisher’s LSD post hoc multiple comparison procedure used to determine significance between groups. Significance was set at $P < 0.05$. All values are expressed as mean ± SEM unless otherwise specified.

RESULTS

Skeletal muscle mass

After the four-week experimental period, body mass (BM) was not different between any of the three groups (Table 1). The mass of the EDL muscle in clenbuterol-treated rats was 20% greater than in the control rats, and in fenoterol-treated rats was 27% greater than in control rats. The EDL mass/BM ratio in clenbuterol-treated rats was 13% greater than in control rats, and 21% greater in fenoterol-treated rats compared with controls (Table 1).
Soleus muscle mass was not different after clenbuterol treatment, but fenoterol-treated rats had a 26% and 18% greater muscle mass than control rats and clenbuterol-treated rats, respectively. The soleus mass/BM ratio in clenbuterol-treated rats was also not different from control values, but fenoterol-treated rats had a 22% greater soleus mass/BM ratio than control and clenbuterol-treated rats (Table 1).

Muscle Fiber Cross Sectional Area

The increased EDL muscle mass in clenbuterol-treated rats was associated with a 6% increase in the average muscle fiber cross-sectional area (CSA), compared with untreated controls. Fenoterol treatment, however, increased the average EDL muscle fiber CSA by 27% above control, and 20% above that in clenbuterol-treated rats. The increased soleus muscle mass in fenoterol-treated rats was also associated with a 9% increase in average muscle fiber CSA compared with control rats and a 12% increase above that in clenbuterol treated-rats (Table 1).

Fiber type transitions

Fiber type proportions in the EDL muscles of clenbuterol treated rats were not different from those in control rats, but in fenoterol-treated rats there was a 20% reduction in the proportion of type IIa fibers compared with controls (Figure 1).

Soleus muscles from clenbuterol-treated rats had a reduced proportion (10%) of type I fibers and an increased proportion (6%) of type II d/x fibers compared to control rats, but no change in type IIa fibers. Fenoterol-treated rats also had a reduced proportion (10%) of type I fibers, compared with control rats, but unlike in clenbuterol-treated rats, they had an increased proportion (11%) of type IIa fibers compared with control rats, but no change in the proportion of type II d/x fibers (Figure 1).
Cross sectional area of fast and slow muscle fibers

The CSA of type I, IIa and IIb fibers from EDL muscles of clenbuterol-treated rats was larger (12%, 7% and 10%, respectively) than in control rats (Figure 1). Fenoterol treatment increased the CSA of type I and IIa fibers by 34%, type IIb fibers by 29% and type IId/x fibers by 31% compared with EDL muscles from untreated control rats. The CSA of type I, IIa, IIb and IId/x fibers were also 19%, 26%, 17%, and 28% greater, respectively, in EDL muscles of fenoterol- than clenbuterol-treated rats (Figure 1).

The CSA of type I and IId/x fibers in the soleus muscles of clenbuterol-treated rats were 6% and 12% smaller than fibers from muscles of untreated control rats (Figure 1). The CSA’s of type IIa and IId/x fibers in the soleus muscles of fenoterol-treated rats were 25% and 18% larger than those from muscles of untreated control rats. The CSA of type I, IIa and IId/x fibers were also 7%, 33%, and 38% greater, respectively, with fenoterol than clenbuterol treatment (Figure 1).

Skeletal muscle function

Peak twitch force (Pt) of EDL and soleus muscles was not affected by clenbuterol treatment, but fenoterol treatment increased Pt of EDL muscles by 40% above control and 30% above clenbuterol-treated rats. Similarly, fenoterol treatment increased soleus Pt by 16% above that for control and clenbuterol-treated rats. Time to peak twitch tension (TPT), one-half relaxation time (½RT) and peak rate of twitch force generation (dPt/dt) were not altered by clenbuterol, in either the EDL or soleus muscles. Fenoterol treatment increased dPt/dt by 27% in the EDL muscle compared with muscles from control and clenbuterol-treated rats, but it had no effect on twitch contraction time. In the soleus muscle, fenoterol dramatically reduced TPT and
Effects of fenoterol and clenbuterol on muscle function

½RT, and increased dP/dt compared with values for both control and clenbuterol-treated rats (Table 2).

Clenbuterol treatment increased maximum isometric force (P₀) of EDL muscles by 20% compared with control values, but P₀ of soleus muscles were not affected. Fenoterol treatment increased P₀ of EDL muscles by 35% above control values and 12% above values for clenbuterol-treated rats. Fenoterol treatment also increased P₀ of soleus muscles by 15% compared with both control and clenbuterol-treated rats (Table 2). When P₀ was corrected for muscle CSA, sP₀ was not different in EDL or soleus muscles among groups. The ratio of Pₒ/P₀ was higher in EDL muscles after fenoterol compared to clenbuterol treatment (0.38 ± 0.01 vs. 0.32 ± 0.01; P < 0.05), but the ratio was unchanged in soleus muscles.

After four minutes of repeated intermittent stimulation P₀ of EDL muscles from control, clenbuterol and fenoterol-treated rats was reduced to 24%, 25% and 17% of initial P₀ values, respectively (Figure 2). P₀ (expressed as a percentage of initial P₀) of EDL muscles from fenoterol-treated rats was lower than both control and clenbuterol-treated rats after 180 s of intermittent maximal stimulation and remained lower for the duration of the fatigue protocol. After 5 min recovery (i.e. no stimulation), P₀ of the EDL muscles of control rats was restored to 34% of initial values, and remained at this level after 10 min recovery time. Recovery of P₀ in EDL muscles from clenbuterol-treated rats was less than that of controls and in fenoterol-treated rats recovery of P₀ was impaired even further (Figure 2).

Following the same stimulation protocol, P₀ of the soleus muscles from control, clenbuterol and fenoterol-treated rats was reduced to 45%, 39% and 33% of initial P₀ values, respectively (Figure 2b). P₀ (expressed as a percentage of initial) was not different between any group, at any time point during the fatigue protocol. After 5 and 10 min recovery, P₀ was
restored to 66% and 70% of initial, in control rats. $P_o$ of soleus muscles from fenoterol-treated rats did not recover to the same extent as clenbuterol-treated rats (Figure 2).

**Adrenal and cardiac mass**

Clenbuterol treatment did not alter the mass of the adrenal glands, compared to control rats, but in fenoterol-treated rats adrenal mass was increased by 13% compared to control rats (Table 3). The adrenal mass/BM ratio was not altered following either treatment.

In clenbuterol-treated rats, heart mass was 22% greater and the ratio of heart mass to body mass (BM) was 13% greater than control values. In fenoterol-treated rats, heart mass was 38% greater than that of control rats and 13% greater than that of clenbuterol-treated rats. The heart mass/BM ratio in fenoterol-treated rats was 28% and 13% greater than that of control and clenbuterol-treated rats, respectively (Table 3).

**Skeletal and cardiac muscle $\beta$-adrenoceptor densities**

In control rats, $\beta$-adrenoceptor density was greater in the soleus (19 ± 2 fmol/mg protein) than in EDL muscles (9 ± 1 fmol/mg protein). $\beta$-adrenoceptor density was decreased by fenoterol in EDL muscles (51%) compared to control rats, whereas the apparent decrease by clenbuterol treatment (34%) did not reach statistical significance (Figure 3). $\beta$-adrenoceptor density in the soleus muscles of clenbuterol- and fenoterol-treated rats was reduced by 42% and 44%, respectively.

The hearts of control rats contained a population of ~70% $\beta_1$-adrenoceptors and 30% $\beta_2$-adrenoceptors. There was no significant difference in the density of total $\beta$-adrenoceptors observed in the hearts of clenbuterol-treated rats. Total $\beta$-adrenoceptor density in the hearts of fenoterol-treated rats was reduced by 37% compared with control rats, with $\beta_1$-adrenoceptor
density reduced by 27% and $\beta_2$-adrenoeceptor density reduced by 66% (Figure 3). $\beta_1$-adrenoeceptor density in the hearts of fenoterol-treated rats was reduced by 25% compared with clenbuterol-treated rats, but $\beta_2$-adrenoeceptor density was not different from that in clenbuterol-treated rats. The hearts of fenoterol-treated rats also had an altered proportion of $\beta_1$- and $\beta_2$-adrenoeceptors compared with control rats, with $\beta_1$-adrenoeceptors comprising ~90% and $\beta_2$-adrenoeceptors comprising ~10% of the total $\beta$-adrenoeceptor population.

**DISCUSSION**

The most important finding of this study was that fast-twitch EDL and slow-twitch soleus muscles of rats treated with fenoterol for four weeks had a greater force producing capacity than muscles from rats that received an equimolar dose of clenbuterol. The increase in mass of the EDL muscles was similar following either treatment, but fenoterol produced greater increases in soleus muscle mass. Average fiber size in the EDL and soleus muscles was also greater following fenoterol treatment. Based on these findings, the hypothesis that fenoterol would have a greater effect on skeletal muscle structure and function than an equimolar dose of clenbuterol, was supported.

The lack of a significant anabolic effect of clenbuterol in rat soleus muscle compared to EDL muscle is not unprecedented. Differences in the sensitivity and responsiveness to $\beta$-agonists of various muscles in the rat have been reported (28, 35). The mechanism of this differential response is not understood. An obvious explanation would relate to differences between the muscles in their proportion of fast type II fibres (hypertrophied by $\beta$-agonists), but this is inconsistent with our present observation that clenbuterol showed muscle selectivity,
whereas fenoterol did not. Indeed, the increase in muscle mass caused by fenoterol in soleus (26%) and EDL muscles (27%) was strikingly similar. Differences in muscle responsiveness to clenbuterol do not simply reflect β-adrenoceptor density, as this was greater in soleus than in EDL muscles. Nor have we found a close negative association between the rate of receptor downregulation in various muscles, and their responsiveness to β-agonists. Recent evidence indicates that the mechanism of desensitization of β-adrenoceptors involves phosphorylation by specific receptor kinases, the G-protein-coupled receptor kinases (GRKs). This phosphorylation is followed by binding of arrestins to the receptors, which causes uncoupling of receptors and G-proteins and thus results in a loss of receptor function (12). Long-term desensitization often involves a significant reduction in receptor numbers, or receptor downregulation (8, 9). We propose that different skeletal muscles of the rat differ in the extent to which they contain other β-adrenoceptor subtypes, and (or) in the efficiency with which their β-adrenoceptors are coupled to the second messenger system.

In addition to there being physiological differences among skeletal muscles, there is a clear difference in the pharmacological properties of the two drugs used in this study. The lack of an anabolic effect of clenbuterol compared with fenoterol in soleus muscles, is unlikely to be due to a difference in β2-adrenoceptor binding affinity, as clenbuterol binds to β2-adrenoceptors with an affinity five times greater than that of fenoterol (18). Instead, the difference could be accounted for by a different level of efficacy and/or a difference in sub-type selectivity for the two drugs.

Our observation that unlike clenbuterol, fenoterol treatment caused comparable responses in the EDL and the soleus muscles, coupled with the knowledge that fenoterol acts at both β1- and β2-adrenoceptors (4), suggests that fenoterol might cause skeletal muscle hypertrophy by actions at both adrenoceptors. Previous studies have shown that when β2-adrenoceptors in
bovine skeletal muscle are selectively blocked using ICI 118551, it is still possible to obtain a maximum cAMP response following stimulation with the non-selective β-adrenoceptor agonist, isoprenaline. The isoprenaline response could be blocked by the β₁-adrenoceptor-selective antagonist CGP 20712A, providing evidence for the existence of a population β₁-adrenoceptors in skeletal muscle that is small, but efficiently coupled to the second-messenger (30, 38). Hence, the greater effects observed in rat skeletal muscles following fenoterol administration in the present study, could be due to its actions at both β₁- and β₂-adrenoceptors, resulting in a greater cellular response.

Fenoterol is said to be a full agonist at both adrenoceptors, that is, stimulation of a β-adrenoceptor by fenoterol mediates a greater cellular response (production of cAMP, via the stimulatory G-protein complex) than stimulation by clenbuterol (a partial agonist). Accordingly, fenoterol could be expected to cause a greater increase in protein accretion (31). The mechanism for protein accretion following chronic β-agonist administration has been described recently by Navegantes and colleagues (32). Briefly, an increase in adenylate cyclase production activates cAMP-dependent protein kinase (PKA) which in inhibit the calpain and calpastatin pathways regulating proteolysis. Additionally, these authors point out that the anabolic effects of catecholamines on skeletal muscle could also be due to stimulation of protein synthesis (32). Although the mechanism of action of fenoterol and clenbuterol on skeletal muscle is likely to be similar (32), the difference in efficacy may set these two agonists apart as much as differences in their subtype selectivity.

Clenbuterol treatment did not alter the fatigue resistance of the EDL or the soleus muscles, but it did impair the recovery of force production in the fast-twitch EDL muscles. In contrast, fenoterol treatment increased the fatiguability of EDL muscles and impaired the recovery of
force in both the EDL and soleus muscles. The impaired recovery of force in the soleus muscles from fenoterol treated rats is likely mediated by the shift in fiber proportions from slow type I, towards the faster type IIa and IIId/x fibers that are less resistant to fatigue. This notion, however, was not supported by observations in the EDL muscles, where the fast-twitch fiber proportions were unaffected by clenbuterol treatment and only altered to a minor extent by fenoterol treatment.

Both clenbuterol and fenoterol treatment mediated a shift in fiber type proportions towards the faster type IIa and II d/x forms in soleus muscles, but only fenoterol treatment decreased the time course of contraction. No change in the TPT or ½RT despite significant changes in fiber proportions, supports the notion that there may be other factors (such as sarcoplasmic reticulum function) contributing to these changes in contractility following fenoterol treatment.

Whilst the results of these experiments demonstrate unequivocally that fenoterol has greater effects on structure and function of skeletal muscle than an equimolar dose of clenbuterol, its effects on the heart were similar to those of clenbuterol. Previous studies indicate that clenbuterol administration is associated with cardiac hypertrophy (10) and adrenal gland hypertrophy (19). In contrast to previous studies, in the present study the mass of the adrenal glands from clenbuterol treated rats was not different from control rats, possibly due to the shorter duration of treatment; 4 weeks in this study compared with 7 weeks reported previously (19). The ratio of adrenal mass to body mass was not different in fenoterol treated rats suggesting that the increase in absolute adrenal mass can be attributed solely to the overall increase in body mass following treatment.

Both clenbuterol and fenoterol treatment caused a large increase in absolute heart mass and heart mass relative to body mass, compared with control values. These results suggest that both
agonists also mediate effects at β-adrenoceptors in the heart. Our results indicate that fenoterol binds to both β₁- and β₂-adrenoceptors in the heart (with a greater affinity for the β₂-adrenoceptor), and thus mediates a greater cardiac hypertrophy than that following clenbuterol treatment, which likely occurs via stimulation of β₂-adrenoceptors alone (40).

Whilst these experiments demonstrate gross morphometric changes in heart mass, further studies are required to fully determine whether fenoterol administration deleteriously affects cardiac function. If fenoterol binds both β₁- and β₂-adrenoceptors in the heart, then co-administration of a selective β₁-antagonist with fenoterol might reduce cardiac hypertrophy. As such, the anabolic effect of fenoterol in the heart would be reduced, whilst the effects on skeletal muscle structure and function would be maintained. This combined treatment would also uncover whether the co-activation of β₁- with β₂-adrenoceptors contributes to the anabolic effects of fenoterol. Therefore, the combination of fenoterol with a selective β₁-antagonist is worth exploring and further studies are warranted.

This experiment examined the effects of an equimolar dose of fenoterol or clenbuterol on muscle function, and the significantly greater effect of fenoterol is deserving of further investigation. The concentration of fenoterol chosen for these experiments was based on the optimal dose of clenbuterol that we have used previously to produce significant changes in muscle structure and function (10, 14). An increase in muscle functional capacity and muscle fiber size might be achieved at lower concentrations of fenoterol, with only minimal concomitant cardiac hypertrophy. If a lower dose of fenoterol is combined with a selective β₁-antagonist, then it may be possible to eliminate the unwanted side effects associated with cardiac hypertrophy yet maintain a physiologically significant effect on skeletal muscle function. Only then will the full therapeutic potential of fenoterol treatment be realised.
ACKNOWLEDGMENTS

This work was supported by the Muscular Dystrophy Association (USA), the Australian Research Council, and the National Health & Medical Research Council (Australia).
REFERENCES


Table 1. **Selected morphometric parameters of rats following treatment with clenbuterol, fenoterol or saline vehicle.**

<table>
<thead>
<tr>
<th></th>
<th>Control (n=10)</th>
<th>Clenbuterol (n=9)</th>
<th>Fenoterol (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial body mass (g)</td>
<td>292 ± 3</td>
<td>292 ± 8</td>
<td>295 ± 1</td>
</tr>
<tr>
<td>Final body mass (g)</td>
<td>439 ± 13</td>
<td>468 ± 13</td>
<td>462 ± 15</td>
</tr>
<tr>
<td>EDL mass (mg)</td>
<td>211 ± 11</td>
<td>254 ± 8 *</td>
<td>268 ± 15 *</td>
</tr>
<tr>
<td>EDL mass/body mass (mg/g)</td>
<td>0.48 ± 0.01</td>
<td>0.54 ± 0.02 *</td>
<td>0.58 ± 0.02 *</td>
</tr>
<tr>
<td>EDL fiber CSA (μm²)</td>
<td>2083 ± 27</td>
<td>2206 ± 35 *</td>
<td>2653 ± 50 *†</td>
</tr>
<tr>
<td>Soleus mass (mg)</td>
<td>163 ± 4</td>
<td>174 ± 7</td>
<td>206 ± 8 *†</td>
</tr>
<tr>
<td>Soleus mass/body mass (mg/g)</td>
<td>0.37 ± 0.01</td>
<td>0.37 ± 0.02</td>
<td>0.45 ± 0.02 *†</td>
</tr>
<tr>
<td>Soleus fiber CSA (μm²)</td>
<td>2161 ± 21</td>
<td>2096 ± 25 *</td>
<td>2373 ± 25 *†</td>
</tr>
</tbody>
</table>

EDL, extensor digitorum longus; CSA, cross-sectional area. * Different from control rats ($P < 0.05$); † Different from clenbuterol treated rats ($P < 0.05$)
Table 2. Isometric contractile measurements of rat EDL and soleus muscles, following treatment with clenbuterol, fenoterol or saline vehicle.

<table>
<thead>
<tr>
<th></th>
<th>Control $n = 10$</th>
<th>Clenbuterol $n = 9$</th>
<th>Fenoterol $n = 9$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>EDL</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$L_0$ (mm)</td>
<td>34.3 ± 0.4</td>
<td>35.3 ± 0.7</td>
<td>34.9 ± 0.5</td>
</tr>
<tr>
<td>$P_t$ (mN)</td>
<td>1185 ± 74</td>
<td>1278 ± 29</td>
<td>1654 ± 77</td>
</tr>
<tr>
<td>TPT (ms)</td>
<td>24.9 ± 0.7</td>
<td>26.0 ± 0.7</td>
<td>25.5 ± 2.6</td>
</tr>
<tr>
<td>$\frac{1}{2}RT$ (ms)</td>
<td>25.3 ± 1.0</td>
<td>22.5 ± 1.0</td>
<td>21.8 ± 2.8</td>
</tr>
<tr>
<td>$dP_t/dt$ (mN/ms)</td>
<td>113 ± 6</td>
<td>115 ± 4</td>
<td>157 ± 9</td>
</tr>
<tr>
<td>$P_o$ (mN)</td>
<td>3254 ± 146</td>
<td>3912 ± 114</td>
<td>4386 ± 171</td>
</tr>
<tr>
<td>$sP_o$ (kN/m²)</td>
<td>250 ± 12</td>
<td>257 ± 8</td>
<td>269 ± 7</td>
</tr>
<tr>
<td>$P/P_o$</td>
<td>0.35 ± 0.01</td>
<td>0.32 ± 0.01</td>
<td>0.38 ± 0.01</td>
</tr>
<tr>
<td><strong>Soleus</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$L_0$ (mm)</td>
<td>30.4 ± 0.5</td>
<td>32.1 ± 0.5</td>
<td>31.4 ± 0.6</td>
</tr>
<tr>
<td>$P_t$ (mN)</td>
<td>351 ± 22</td>
<td>348 ± 20</td>
<td>408 ± 15</td>
</tr>
<tr>
<td>TPT (ms)</td>
<td>66 ± 3</td>
<td>58 ± 3</td>
<td>46 ± 1</td>
</tr>
<tr>
<td>$\frac{1}{2}RT$ (ms)</td>
<td>93 ± 6</td>
<td>89 ± 5</td>
<td>63 ± 3</td>
</tr>
<tr>
<td>$dP_t/dt$ (mN/ms)</td>
<td>34 ± 2</td>
<td>35 ± 1</td>
<td>46 ± 4</td>
</tr>
<tr>
<td>$P_o$ (mN)</td>
<td>1768 ± 77</td>
<td>1745 ± 97</td>
<td>2039 ± 69</td>
</tr>
<tr>
<td>$sP_o$ (kN/m²)</td>
<td>249 ± 9</td>
<td>244 ± 7</td>
<td>235 ± 4</td>
</tr>
<tr>
<td>$P/P_o$</td>
<td>0.20 ± 0.01</td>
<td>0.20 ± 0.01</td>
<td>0.20 ± 0.01</td>
</tr>
</tbody>
</table>

EDL, extensor digitorum longus; $L_0$, optimum length of muscle for maximum twitch response; $P_t$, peak twitch tension; TPT, time to peak twitch; $\frac{1}{2}RT$, one-half relaxation time; $dP_t/dt$, rate of twitch force development; $P_o$, maximum isometric tetanic force; $sP_o$, specific or normalized $P_o$ (force per
Effects of fenoterol and clenbuterol on muscle function

cross-sectional area); $P_t/P_o$, ratio of twitch/tetanic force. * Different from control rats ($P < 0.05$); † Different from clenbuterol treated rats ($P < 0.05$)

Table 3. Morphometric parameters of adrenal and cardiac tissue from rats treated with clenbuterol, fenoterol or saline vehicle.

<table>
<thead>
<tr>
<th></th>
<th>Control (n=10)</th>
<th>Clenbuterol (n=9)</th>
<th>Fenoterol (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrenal mass (mg)</td>
<td>25.4 ± 0.9</td>
<td>26.4 ± 1.0</td>
<td>28.7 ± 1.4 *</td>
</tr>
<tr>
<td>Adrenal mass/body mass (mg/g)</td>
<td>0.059 ± 0.002</td>
<td>0.057 ± 0.003</td>
<td>0.063 ± 0.004</td>
</tr>
<tr>
<td>Heart mass (mg)</td>
<td>1118 ± 41</td>
<td>1368 ± 53 *</td>
<td>1542 ± 24 *†</td>
</tr>
<tr>
<td>Heart mass/body mass (mg/g)</td>
<td>2.61 ± 0.08</td>
<td>2.96 ± 0.06 *</td>
<td>3.34 ± 0.08 *†</td>
</tr>
</tbody>
</table>

* Different from control rats ($P < 0.05$); † Different from clenbuterol treated rats ($P < 0.05$)
Figure 1.

Proportion of fiber types and cross-sectional area of individual fiber types, in the EDL and soleus muscles of control, clenbuterol- and fenoterol-treated rats. Muscles of fenoterol treated rats comprised larger fibers than control or clenbuterol treated rats. * Different from control rats (P < 0.05); † Different from clenbuterol-treated rats (P < 0.05)
Figure 2.

Relative $P_o$ of the EDL, and soleus muscles during and after a four-minute fatigue protocol. Note that for fenoterol treated rats there was a reduced ability to recover from fatigue in both muscles.

* Clenbuterol different from control rats ($P < 0.05$); † fenoterol different from control and clenbuterol-treated rats ($P < 0.05$); ‡ Fenoterol different from clenbuterol-treated rats ($P < 0.05$)
Effects of fenoterol and clenbuterol on muscle function

**EDL**

- Control
- Clenbuterol
- Fenoterol

**Soleus**

% of initial P₀ vs. Time (s)

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* † ††
** ‡‡‡

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* † ††
** ‡‡‡
Figure 3.

β-adrenoceptor density in skeletal muscle (EDL and soleus), and cardiac muscle following treatment with clenbuterol, fenoterol or saline vehicle. Note that only total β-adrenoceptor density is shown for skeletal muscle, while both β1- and β2-adrenoceptor density is shown for cardiac tissue. * Different from untreated control rats (P < 0.05); † Different from clenbuterol-treated rats (P < 0.05); ‡ Different between EDL and soleus muscles (P < 0.05); § Different between β1- and β2-adrenoceptors (P < 0.05)
Effects of fenoterol and clenbuterol on muscle function

**Skeletal Muscle**

- **EDL SOL**
- **-adrenoceptor density (fmol/mg protein)**
- **Control**
- **Clenbuterol**
- **Fenoterol**

**Cardiac muscle**

- **Total**
- **-adrenoceptor density (fmol/mg protein)**
- **Control**
- **Clenbuterol**
- **Fenoterol**