Metabolic and physiologic characteristics of skeletal muscle determine its response to clenbuterol treatment

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β-Adrenoceptor agonists are reported to induce skeletal muscle hypertrophy and hence serve as valuable adjunct to the treatment of wasting disorders. In the present study, we attempted to find out whether metabolic and physiologic characteristics of fibres are important in determining skeletal muscle response to clenbuterol (an adrenergic receptor agonist) therapy, as proposed in the treatment of wasting disorders. The treatment of mice with clenbuterol (2 mg/kg body wt for 30 days) resulted in skeletal muscle hypertrophy, more common amongst fast-twitch glycolytic fibres/muscle, with increase in body mass and a parallel rise in muscle mass to body mass ratio. Measurement of fibre diameters in soleus (rich in slow-twitch oxidative fibres), ALD or anterior latissimus dorsi (with a predominance of fast-twitch glycolytic fibres) and gastrocnemius (a mixed-type of muscle) from clenbuterol-treated mice for 30 days revealed noticeable increase in the percent population of narrow slow-twitch fibre and a corresponding decline in white-type or fast-twitch glycolytic fibres in gastrocnemius and ALD. As revealed by counting of muscle cells in soleus, narrow red fibres declined with corresponding increase in white-type glycolytic fibres population. A significant decline in the succinic dehydrogenase activity was observed, thereby suggesting abnormality in oxidative activity of skeletal muscles in response to clenbuterol therapy.

Keywords: Clenbuterol, Metabolic and Physiologic characteristics, Skeletal muscles

Clenbuterol, a selective β-adrenergic receptor agonist is widely used in clinical practice to treat asthma and allergic conditions. Like other β-receptor agonists, it is also known to stimulate skeletal muscle hypertrophy, reduce body fat content and improve functional capacity of skeletal muscle by increasing contractile properties. Skeletal muscle fibre-specific protein anabolic effects of these drugs, including clenbuterol reportedly result, following an accelerated protein turnover coupled with decreased degradation rate. Rats and mice muscles comprising fast type II motor units, for example, extensor digitorum longus (EDL) or latissimus dorsi, exhibit significant increase in protein content and tissue mass in response to β-adrenoceptor agonist treatment. β-Agonists also possess remarkable ability to induce fiber conversions in skeletal muscle. Clenbuterol transforms slow-twitch oxidative fibers into fast-twitch oxidative and glycolytic types in EDL muscle. Rat gastrocnemius undergoes a conversion of fast-twitch into slow-twitch oxidative types. A differential effectiveness of clenbuterol in different muscles and regional, dose-dependent and muscle fibre-specific effects of clenbuterol have also been documented.

Succinate dehydrogenase (SDH) is a marker enzyme of the respiratory chain complex embedded in inner mitochondrial membrane. It has frequently been used as a qualitative histochemical marker in skeletal muscle for histopathological assessment of muscle tissue. The present study was undertaken to confirm muscle fiber type-specific effects of repeated administration of a β-adrenoceptor agonist clenbuterol for 30 days in mice. We used SDH to study the metabolic and physiological characteristics of different skeletal muscles. More specifically, we tested the hypothesis whether or not predominant fibre type constitution (slow-twitch oxidative, fast-twitch glycolytic and fast-twitch oxidative glycolytic) of a skeletal muscle is important in determining tissue response to clenbuterol.

Materials and Methods

Animals
Sexually mature male Swiss albino mice were procured from Central Research Institute, Kasauli (H. P.) and kept under appropriate hygienic conditions in the animal house of the department providing feed and water ad libitum. Mice were divided into two
groups. Group I served as control animals and group II received clenbuterol (Sigma Aldrich, USA) as under.

**Administration of drug**

Mice were orally administered clenbuterol daily at 2 mg/kg body wt for 1 month. The above dose was chosen, as clenbuterol in dose range of 1-2 mg/kg body wt was effective in producing hypertrophy and protein accumulation. Also, the effectiveness of clenbuterol administration via drinking water was well established. A stock solution (10 mg/ml) of clenbuterol was prepared in double-distilled water and diluted further at the time of actual administration. Control animals received corresponding volume of vehicle daily.

Animals were sacrificed by cervical dislocation after clenbuterol (30 days) treatment was over. Three muscles viz., soleus, gastrocnemius and anterior latissimus dorsi (ALD) were excised, weighed and employed for both qualitative and quantitative studies.

**Histochemical localization of SDH**

Histochemical localization of SDH was accomplished as described previously, with slight modifications in incubation time at 37°C, employing fresh frozen thin tissue sections. Tissue sections were washed in cold (4°C) phosphate buffer (pH, 7.6) and transferred to an incubation medium containing sodium succinate.

**Preparation of muscle mitochondria**

A homogeneous pure preparation of skeletal muscle mitochondria was prepared as described previously. Purity of preparation was confirmed by transmission electron micrography (TEM).

**SDH activity assay**

SDH activity of mitochondrial homogenates was assayed in a reaction mixture containing 50 mM phosphate buffer (pH 7.4), 40 mM sodium succinate, 0.4 mg/ml neo-tetrazolium chloride (NTC) and 0.01% gelatin. The 200 μl mitochondrial homogenate was added to start the reaction. Reaction mixture was incubated for 30 min at 37°C and optical density (OD) of reaction product (formazan extracted in acetone) measured at 420 nm in a Hitachi double-beam spectrophotometer (model 150-20). Per cent change in OD was finally plotted against control.

**Histometric analysis**

Tissue sections stained for SDH activity were employed for measuring the per cent populations and mean fibre diameter in different muscle cells to calculate the degree of fibre transition and hypertrophy. At least 100-150 individual muscle fibres were randomly selected from 4 different muscle preparations and employed for histometric analysis. Three principal fibre types viz., slow-twitch oxidative or narrow red fibres, slow-twitch glycolytic oxidative or intermediate fibres and fast-twitch glycolytic or broad white fibres were counted as per the details described elsewhere.

**Statistical analysis**

The results were obtained as mean ± SEM. The differences between control and treated groups of mice were considered significant at *P<0.05 and **P<0.01 by using student’s ‘t’ test.**

**Results**

Clenbuterol induces skeletal muscle hypertrophy in all fibre types

The mean fibre diameters indicated a significant hypertrophy in all the three muscles albeit at different rates (Fig. 1). Mean fibre diameter of muscles viz., gastrocnemius, ALD and soleus of control mice were 67.8 ± 0.79 μm, 50.7 ± 0.50 μm, and 46.5 ± 0.75 μm, respectively, which increased to 74.9 ± 1.09 μm, 67.2 ± 0.78 and 52.8 ± 0.73 μm, on clenbuterol administration. Thus, ALD, a white type of muscle underwent maximum hypertrophy, as compared to other muscle types.

![Fig. 1—Mean fibre diameters of mice gastrocnemius, ALD and soleus muscles on clenbuterol administration (2 mg/kg body wt/day; maximum 30 days) [Values are mean ± SEM; *P<0.05 and **P<0.01, n = 4 each].](image-url)
Equal number of fast-twitch glycolytic (white), slow-twitch oxidative (red) and slow-twitch oxidative glycolytic (intermediate) fibres was included in mean fibre measurements. Mean fibre diameters of principle fibre types in three muscles demonstrated a characteristic variability. Fig. 2 shows changes in the mean fibre diameters of three muscle fibre types, after clenbuterol treatment. In control gastrocnemius muscle, mean fibre diameters of slow-twitch oxidative (46.52 ± 0.6 μm), slow-twitch oxidative glycolytic (66.88 ± 0.59 μm) and fast-twitch glycolytic fibres (78.1 ± 0.9 μm) increased to 54.02 ± 0.66 μm, 78.25 ± 0.65 μm and 92.58 ± 2.3 μm, respectively. Changes in mean fibre diameters were most significant in ALD (Fig. 2B). The mean diameter of slow-twitch oxidative fibres (red) increased from 39.68 ± 0.62 μm to 50.82 ± 0.89 μm, whereas intermediate or slow-twitch oxidative glycolytic and fast-twitch glycolytic (white) fibres exhibited an increase from 48.94 ± 0.34 μm and 63.64 ± 0.56 μm to 69.82 ± 0.67 μm and 82.0 ± 0.79 μm, respectively.

Interestingly, white and intermediate types of muscle cells exhibited maximum stimulation of hypertrophying response. Soleus muscle (Fig. 2C), on the other hand, exhibited 12 to 14% increase in mean fibre diameters of three fibre types, compared to control muscle. An average diameter of 41.47 ± 0.77 μm and 51.30 ± 0.74 μm in slow-twitch oxidative fibres and slow-twitch oxidative glycolytic fibres increased to 47.7 ± 0.5 μm and 57.9 ± 0.96 μm respectively in treated group. Whereas white or fast-twitch glycolytic fibres demonstrated an increase from 60.08 ± 3.89 μm in control group to 72.37 ± 4.88 μm in treated group.

Counting of fibres in eight muscle bundles selected randomly from different muscle preparations revealed that gastrocnemius muscle in clenbuterol-treated mice displayed (Fig. 3A) a significant decline in per cent

Fig. 2—Effect of clenbuterol administration on hypertrophy of fibre sub-types of gastrocnemius (a), ALD (b) and soleus (c) muscles, as evidenced from an increase in respective fibre diameters [Clenbuterol resulted in a significant hypertrophy of all fibre sub-types, however it was more common in white and intermediate fibres as compared to red fibres. Values are mean ± SEM; *P<0.05 and **P<0.01, n = 4 each]

Fig. 3—Muscle-specific shifts in respective fibre population, following clenbuterol treatment [Gastrocnemius (a) and ALD (b) showed a shift from fast glycolytic types to slow oxidative category and soleus (c) exhibited opposite response [Values are mean ± SEM; *P<0.05 and **P<0.01, n = 4 each]
population of white or fast-twitch glycolytic (from 38 to 15.5%) and slow-twitch oxidative glycolytic or intermediate fibres (from 30 to 26.66%). Per cent population of red or slow-twitch oxidative fibres correspondingly underwent an increase from 31.7% in control group to more than 57% in treated group. ALD muscle too showed (Fig. 3B) a parallel increment in the population of slow-twitch oxidative fibres from 28% in control group to 43% in treated group and a simultaneous decline in fast-twitch glycolytic fibres from 43.94 to 29.62%. Soleus showed a clenbuterol-induced decline in red or slow-twitch oxidative fibres from 49.5 to 31.9% (Fig. 3C). The broad white or fast-twitch glycolytic fibres underwent a marginal increase in number from 21.6 to 24.4%. However, intermediate fibres increased significantly in their number (28.8 to 43.6%), following clenbuterol administration.

**Effect of clenbuterol on tissue weight**

Repeated administration of clenbuterol resulted in an increase in the wet weight of different muscles (Fig. 4). Mean muscle weight of gastrocnemius, ALD and soleus muscles from control mice increased from 89 ± 5.16 mg, 20.17 ± 1.8 mg and 4.87 ± 0.37 mg to 103.7 ± 2.05 mg, 24.77 ± 0.86 mg and 5.75 ± 0.28 mg, respectively (n = 4 each) in treated group. ALD muscle demonstrated maximum stimulation in growth.

**Muscle weight (mg) to body weight (g) ratio**

Mean body weight of control mice (24.12 ± 0.39 g; n = 6) registered a 13.89% increase on day 30 (27.62 ± 0.53 g; n = 6). Ability of clenbuterol to increase muscle mass was further confirmed from stimulated muscle weight to body weight ratio (Fig. 5). Gastrocnemius, ALD and soleus muscles to body weight ratios of 3.6 × 10^3, 8.3 × 10^4 and 1.8 × 10^4 in control mice showed slight increase to 3.7 × 10^3, 8.9 × 10^4 and 2.0 × 10^4, respectively.

**SDH [EC 1.3.99.1] activity**

Microscopic examination of tissue sections stained for SDH activity revealed a heterogeneous cell population, distinguished on the basis of both cell size and distribution of enzyme activity within individual fibres of different muscles (Fig. 6A). Tissue sections from control mice showed three types of cells viz., (i) broad, fast-twitch glycolytic or white fibres, poor in SDH activity, (ii) slow-twitch oxidative fibres with narrow diameters, extremely rich in SDH activity/duffarmazan distribution, and, (iii) slow-twitch oxidative glycolytic or intermediate fibres with a moderate distribution of SDH were invariably observed in tissue sections.

Muscle sections from all three tissues treated with clenbuterol for a month exhibited a small population of hypertrophying fibres. These cells not only underwent hypertrophy, but also demonstrated other pathological abnormalities including disfigured outlines and other variable shapes, especially in areas,
Fig. 6—(A): Photomicrograph of the transverse section (T.S) of gastrocnemius muscle showing fiber heterogeneity, both on the basis of size and SDH distribution in control (X 300); (B): Administration of clenbuterol resulted in a selective hypertrophy of fast-twitch glycolytic (white) or fast-twitch oxidative glycolytic intermediate fibers of the muscle (X 300); (C): ALD muscle showing clenbuterol-induced depletion of enzyme from white and intermediate fibers. A small population of red fibers, however, became overladden with excessive SDH activity (X 250); (D): A regional clustering of red fibers in selected areas of section characteristically noticed (X 250); (E): Soleus muscle showing normal distribution of SDH in control (X 250); (F): Photomicrograph from soleus (T.S) showing drug induced difarmazan-deficient fibers with increased white fiber population in comparison to control (X 250).

where myonecrosis was also common. Major population of white and intermediate cells appeared almost enzyme-free (Fig. 6C). On the other hand, slow-twitch oxidative or narrow red fibres demonstrated decrease in SDH activity (Fig. 6B), in comparison to control which were extremely rich in difarmazan (Fig. 6A). However, clenbuterol-treated muscles viz., gastrocnemius and ALD were conspicuous by their more frequent distribution of red oxidative fibres. A random sampling of tissue sections from treated muscles revealed regional grouping of red and intermediate fibres, and a large number of slow-twitch oxidative fibres found clustered at many places in the form of groups (Fig. 6D).

Tissue sections gave a false impression of selective increment in expression levels of SDH in these regionally clustered fibre groups. Such regional groups were scattered throughout and suggested a possible transformation of constituent cells into slow-twitch oxidative type in these areas only. A significant overall decline in the SDH activity was observed in all fibre types. Quantitative measurement of SDH activity, expressed as mean absorbance for difarmazan at 420 nm, demonstrated a decline in enzyme activity in all muscles from clenbuterol-treated mice (Fig. 7); the decline in enzyme activity ranged from 18.81% in gastrocnemius muscle to as high as 42.39% in ALD.

Fig. 7—Effect of clenbuterol treatment on tissue SDH activity [A significant decline was observed in SDH activity, in sharp contrast to regionally clustered group of enzyme rich fibres (Fig. 6D). The activity was determined in mitochondrial preparations. [Values are mean ± SEM; * P<0.05 and ** P<0.01, n = 4 each]

Discussion
In this paper, we report a dual and differential fibre-specific response of skeletal muscle to the repeated clenbuterol treatment in mice. Most visible effect of clenbuterol appeared an onset of a variable degree of fibre hypertrophy in all principal fibre types. A significant decline in SDH activity, as a result of the mitochonarging enzyme deficiency, amongst red fibres may also be correlated to influence fibre specific response to clenbuterol.
types. The cumulative response of all the fibre types appears to be a major contributor to stimulated growth as a result of β-adrenergic therapy, as is evidenced from the microscopic examination of individual fibres and histometric analysis of cellular diameters of individual fibre type. Different β-adrenergic agonists are reported to produce growth-stimulating effects in skeletal muscle, primarily by inducing hypertrophy3,5,30-32. But, whether the hypertrophy is limited to a specific fibre category or embraces all constituent fibre types, remains unclear. Hyperplasia has, however, been ruled out in drug-induced growth of skeletal muscle3,5,30-32, though this may be a common feature accompanying amelioration of denervation atrophy in adult chick skeletal muscle as a result of β-adrenoceptor agonist treatment34.

Increments in respective fibre diameters, stimulation in muscle weight and muscle weight to body weight ratios in different muscle types suggest that maximum growth occurred in ALD; it showed as much as 23% increase in muscle weight, followed by 16.5% by gastrocnemius. Soleus, a predominantly slow-twitch oxidative type of muscle revealed least stimulation in its growth (13.5%).

Metabolic and/or physiologic response of the tissue to clenbuterol is mainly restricted to narrow red or slow-twitch oxidative and intermediate of slow-twitch oxidative glycolytic fibres. This is evident from decline in levels of SDH activity, both qualitatively and quantitatively in all three muscles. Decline in SDH activity in soleus is well supported by decrease in red, oxidative fibres and presence of pale, and diformazan-deficient fibres in the photomicrographs. However, narrow red fibres population increased in ALD and gastrocnemius, but appeared to be SDH deficient in comparison to control red fibres. Thus, observed anatomical changes and overall decline in SDH activity confirmed a drug-induced inhibition and a consequent defect in oxidative activity. Partial SDH deficiency (15 to 50%) from normal reference enzyme activity in skeletal muscle commonly causes mitochondrial myopathy in cardiomyopathy and exercise15-38.

Fibre-specific differences in response to clenbuterol treatment although intriguing may perhaps be correlated with differences in receptor distribution amongst different muscle types. This, in turn, is likely to influence the sensitivity of different muscles or muscle fibres to β-adrenergic agonist application. Just as normal innervated and denervated muscles have widely different sensitivities to β-adrenoceptor agonists39, the same may perhaps hold true to the different response shown by all three muscles after treatment. Whether or not red and white types of muscle fibres in the muscles under study have differential distribution of β-adrenoceptors is merely a speculation right now. However, it should be interesting to ascertain in quantitative terms, relative distribution of these receptors in these physiologically and metabolically distinct cells. This is more interesting in light of previous report30, indicating that β2-adrenoceptor agonists clearly affect fast-twitch muscle fibres by inducing hypertrophy and that their effects on slow-twitch oxidative fibres are less consistent. Further, fast-twitch oxidative fibres are also known to exhibit a decrease in sensitivity to calcium in EDL and soleus muscles in response to long-term treatment of clenbuterol.

In view of therapeutic potential of clenbuterol in improvement of strength and power-related functions (work-load) in experimental animals, it became imperative to investigate its long-term effects on characteristics and properties of constituent muscle cells, especially because of metabolic and physiological division into structural and functional sub-types. Clenbuterol caused a slow to fast-twitch fibre conversions within soleus, accompanying increases in contractile speed39. It also significantly altered the contractile properties of intact fast and slow-twitch muscles of mouse40. The results of present study, however, suggested transformation of white and intermediate fibres into red types in gastrocnemius and ALD muscles, while soleus showed transformation in opposite direction.

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References
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