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Changes in tissue blood flow and β-receptor density of skeletal muscle in rats treated with the β_2-adrenoceptor agonist clenbuterol

Nancy J. Rothwell, Michael J. Stock[1] & Deepak K. Sudera

Department of Physiology, St George's Hospital Medical School, Tooting, London SW17 0RE

1 Rats injected with the β_2-adrenoceptor agonist clenbuterol (2 mg kg^{-1} per day) for 18 days gained significantly more weight than controls.

2 Tissue blood flow assessed 24 h after the last injection from the distribution of radiolabelled microspheres was increased in white (5 fold) and brown (3 fold) adipose tissue of clenbuterol-treated rats but was unaffected in kidney, brain and diaphragm, and was reduced by about 80% in skeletal muscle.

3 Acute injection of clenbuterol one hour before measuring blood flow, increased blood flow to brown fat (20 fold) in both treated and control groups. Blood flow to skeletal muscle increased more in the rats treated chronically with clenbuterol (6 fold increase) than in control rats (2 fold increase), but absolute flow rates were still significantly lower in the rats treated chronically with clenbuterol.

4 Skeletal muscle β-adrenoceptor density and subtype were assessed from ligand binding and displacement studies using [^{3}H]-dihydroalprenolol. Rats treated with clenbuterol for 18 days showed a 50% reduction in β-receptor density, but the ratio of β_1/β_2-receptors was unaffected (15% β_1/85% β_2).

5 The results indicate that, although clenbuterol produces acute increases in muscle blood flow, chronic treatment results in lower flow rates immediately (1 h) and 24 h after the previous injection. The attenuated response following chronic treatment is associated with a marked reduction in skeletal muscle β-adrenoceptor density.

6 The data suggest that any anabolic effects of clenbuterol on muscle which may require, or may be mediated by increases in blood supply, cannot be sustained by chronic treatment. Conversely, blood flow to white and brown adipose tissue would appear to be potentiated by chronic treatment, possibly reflecting increases in lipolytic and/or thermogenic activity.

Introduction

β-Adrenoceptor agonists are noted for their catabolic effects since acute administration stimulates metabolic rate and lipolysis, and chronic treatment is usually associated with loss of body weight and fat (see Rothwell *et al.*, 1982; Stribling, 1983; Rothwell & Stock, 1984 for reviews). However, a novel anabolic effect of some β_2-selective agonists has been observed recently, and one in particular, clenbuterol, has been found to stimulate growth and protein deposition (see Stock & Rothwell, 1986 for review). In young rats, for example, daily treatment with clenbuterol for 18 days produces 27 and 24% increases in body weight and protein content, respectively, and fenoterol, another β_2-agonist, has similar but slightly less marked effects (Emery *et al.*, 1984). At the same time, there are decreases in the rate of fat deposition, and these effects on body composition and carcass quality have also been observed in commercial livestock (Dalrymple *et al.*, 1983; Ricks *et al.*, 1983; 1984a,b; Baker *et al.*, 1984). These, and similar compounds are currently under investigation as potential growth promoters for agricultural production.

The mechanisms involved in the anabolic effects of these β_2-agonists have not yet been properly identified, but in laboratory rodents clenbuterol appears to affect mainly skeletal muscle mass, and usually produces only minor changes in the mass of other organs such as heart (Winter, 1983; Emery *et al.*, 1984). The larger muscle mass would appear to be due to increases (up to 34%) in protein synthesis (Emery *et al.*, 1984), although there is also more recent evidence that protein degradation is reduced (Reeds *et al.*, 1986). The

[1] Author for correspondence

anabolic actions of β_2-agonists are not dependent on the presence of gonadal steroids (Rothwell & Stock, unpublished data) and clenbuterol does not appear to affect circulating levels of insulin, triiodothyronine or growth hormone (Winter, 1983). The compound could have a direct action on muscle itself since this tissue is known to possess β_2-adrenoceptors (Reddy & Engel, 1979; Williams *et al.*, 1984), but the proportions of β_1- and β_2-receptors and the effects of clenbuterol on receptor density and subtype have not been determined. Related to this is the possibility that enhanced muscle protein deposition could result indirectly from increases in muscle blood flow following β-adrenoceptor-induced vasodilatation.

In the present study we have attempted to address some of these questions by measuring the effects of acute and chronic clenbuterol treatment on blood flow in skeletal muscle compared to other tissues (brain, kidney, white and brown adipose tissue). In addition, muscle β-adrenoceptor density and subtype were assessed from radioligand binding and displacement studies.

Methods

All animals used were male, Sprague-Dawley (SPF) rats (aged 30 days, 110 g body weight, obtained from Charles River, Kent). They were divided into two groups and injected subcutaneously with either clenbuterol (2 mg kg^{-1} per day in two equal doses at 08 h 00 min and 18 h 00 min) or vehicle alone (medium chain triglyceride, 0.2 ml) for 18 days. The animals were housed in pairs in a metabolism room at 24°C with a 12 h light/dark cycle.

Experiment 1

After 18 days' treatment and 18 h after the last drug injection, the animals were anaesthetized with urethane (0.12 g 100 g^{-1} body weight) and then maintained without direct heating in a warm room (rectal temperatures were always between 36 and 38°C). The right carotid and right femoral arteries were cannulated with heparin-filled cannulae (100 u heparin ml^{-1} saline) of external diameters 1.02 and 0.6 mm, respectively. The femoral cannula was inserted about 1 cm into the artery and the carotid cannula was passed down into the left ventricle. Half the animals which had been receiving vehicle or clenbuterol were injected with 1 mg kg^{-1} clenbuterol (s.c.) 60 min before they were anaesthetized (i.e. 90 min before measurements of blood flow), while the remainder were injected with vehicle only.

^{57}Co-labelled microspheres (15 ± 3 μm in diameter; NEN TRAC, New England Nuclear, Boston, U.S.A.) were suspended in saline containing 0.2% Tween 80 to prevent aggregation, and were washed and resuspended every few days to remove any label that had leached from the spheres; 0.5 ml of a suspension of microspheres containing approximately 250,000 spheres and 5 μCi was injected via the carotid cannula into the left ventricle over a period of 15 s. A reference blood flow sample was collected from the femoral artery as described by Foster & Frydman (1978a). The femoral cannula was opened, and blood collected into pre-weighed vials over a period of 90s, commencing 10s before injection of the microspheres. The flow rate of this reference sample was calculated from the weight of blood collected and its specific gravity (1.046). The reproducibility of this method has previously been verified (Rothwell & Stock, 1981), and flow rates of 0.4–1.0 ml min^{-1} were observed in the present study. The radioactivity of this sample was determined at the same time as the other tissues (see below).

At least 5 min after injection of the microspheres, the animals were killed by exsanguination and the following tissues were dissected, washed, blotted and weighed: epididymal fat pads, interscapular and dorsal-cervical brown fat depots, kidneys, brain, diaphragm, soleus, gastrocnemius and back muscles. The radioactivity of all tissues and the reference blood flow sample was determined in a Beckman (Gamma 55000) counter. No correction was made for isotope decay since ^{57}Co has a half-life of 280 days and samples from each rat were counted on the same day. Tissue blood flow (ml min^{-1}) was calculated as follows:

$$\text{Blood flow} = \frac{\text{Counts min}^{-1}\text{ in tissue sample}}{\text{Counts min}^{-1}\text{ in reference blood}} \times \text{reference flow rate (ml min}^{-1}\text{)}$$

Experiment 2

After 18 days of treatment, rats were killed by decapitation and the gastrocnemius, plantaris and soleus muscles dissected, pooled, minced and then homogenized (Polytron, Mark 6, 7s) in 0.25 M sucrose buffer, pH 7.4. The microsomal (membrane) fraction was isolated by centrifugation, as described previously (Rothwell *et al.*, 1985) to produce a final suspension of 100–200 μg protein ml^{-1}. β-Adrenoceptor number and affinity were determined from the binding of 0.5–10 nM [^{3}H]-dihydroalprenolol ([^{3}H]-DHA, 83 Ci $mmol^{-1}$, Amersham International) to isolated membranes (for details see Rothwell *et al.*, 1985). Non specific binding was assessed from parallel incubations containing 5 μM (−)-propranolol. Binding data were subjected to Scatchard analysis and the maximum number of binding sites (B_{max}) and dissociation constant (K_D) calculated from regression analysis of Scatchard plots.

The relative proportions of β_1 and β_2-adrenoceptors were estimated from Eadie-Hofstee plots of the displacement of [^{3}H]-DHA-binding to membranes by the selective β_2-receptor antagonist ICI 118551 (erythro-(±)-1-(7-methylindan-4-xyloxy)-3-isopropylamino-butan-2-ol, see Minneman & Molinoff, 1980; Rothwell *et al.*, 1985, for details of method and validation of data). Curvilinear Eadie-Hofstee plots were resolved into two component lines by iterative computer analysis, and the dissociation constant for inhibition of binding to each site (K_D') was calculated from the slope of these lines. The percentage of each receptor subtype was determined from the ordinate intercept (% inhibition of binding) of each line. Membranes were also prepared from heart ventricles and the number of β-adrenoceptors calculated from Scatchard analysis of [^{3}H]-DHA binding, but β-receptor subtype was not estimated.

Values presented are means ± s.e.mean, and differences between groups were tested by means of Student's *t* test for unmatched data.

Results

In both experiments, clenbuterol-treated rats gained more weight than controls and were significantly heavier at the end of the experiment (final body weight: control 280 ± 6 g, clenbuterol-treated 301 ± 7 g, $P<0.05$). The mass of the heart (control 0.904 ± 0.038 g, clenbuterol 1.517 ± 0.068 g), gastrocnemius (control 1.141 ± 0.042 g, clenbuterol 1.363 ± 0.066 g) and soleus (control 95 ± 16 mg, clenbuterol 113 ± 4 mg) muscles were all significantly greater ($P<0.05$) in animals treated chronically with clenbuterol compared to controls. The weight of the epididymal fat pads was significantly ($P<0.05$) reduced in the former group (control 2.583 ± 0.0800 g, clenbuterol 1.640 ± 0.120 g). The size of other organs was unaffected by chronic injections of clenbuterol, and the acute injection of this β_2-agonist (Experiment 1) did not affect organ weight.

Experiment 1

Tissue blood flows per g of tissue are shown in Table 1, and were found to be reproducible between animals from the same treatment group and comparable to those obtained previously in normal laboratory rats (e.g. Foster & Frydman, 1978b; Rothwell & Stock, 1981). Rats treated chronically with clenbuterol, but given only vehicle on the morning of the study, showed large increases in blood flow to white and brown adipose compared to vehicle-tested controls, but lower blood flows to soleus, gastrocnemius and back muscles. The average flow to these three skeletal muscle groups was depressed by 80% compared to controls (Table 2).

The acute effects of clenbuterol on blood flow in control animals and in those which had received the drug chronically are also shown in Table 1. In animals which had received chronic treatment with vehicle alone, a single injection of clenbuterol stimulated blood flow to brown adipose tissue, diaphragm, soleus and gastrocnemius muscles, but reduced flow to kidney. One hour after clenbuterol injection, the rats which had been previously treated for 18 days with the drug showed increased blood flows to skeletal muscles, diaphragm and brown fat compared to chronically-treated rats given saline before the

Table 1 Tissue blood flow (ml min^{-1} g^{-1}) after acute or chronic treatment with saline or clenbuterol

Chronic	*Vehicle*		*Clenbuterol*	
Acute	*Saline* (1)	*Clenbuterol* (2)	*Saline* (3)	*Clenbuterol* (4)
Tissue				
(1) WAT	0.06 ± 0.01	0.07 ± 0.01	0.34 ± 0.10*	0.20 ± 0.03***
(2) BAT	0.36 ± 0.06	7.00 ± 1.31	1.03 ± 0.21*	21.41 ± 1.59***†††
(3) Kidney	5.24 ± 0.43	2.81 ± 0.16†††	5.55 ± 0.81	6.01 ± 0.28***
(4) Brain	0.96 ± 0.07	0.83 ± 0.07	0.94 ± 0.07	0.87 ± 0.07
(5) Diaphragm	0.52 ± 0.05	1.47 ± 0.13†††	0.55 ± 0.09	1.19 ± 0.10†††
(6) Soleus	0.51 ± 0.09	1.29 ± 0.08†††	0.08 ± 0.02**	0.66 ± 0.11***†††
(7) Gastrocnemius	0.40 ± 0.01	0.70 ± 0.09†	0.06 ± 0.01***	0.52 ± 0.06††
(8) Back muscle	0.40 ± 0.07	0.44 ± 0.04	0.11 ± 0.01**	0.39 ± 0.04†††
Average of (6), (7) & (8)	0.44 ± 0.05	0.80 ± 0.05†	0.09 ± 0.01***	0.52 ± 0.03***†††

Mean values ± s.e.mean are shown. $^{\dagger}P<0.05$; $^{\dagger\dagger}P<0.01$; $^{\dagger\dagger\dagger}P<0.001$ vs respective saline-treated group.
$^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ vs respectable vehicle-treated group.
WAT and BAT are white and brown adipose tissue, respectively.

Table 2 Relative changes in tissue blood flow (ml min^{-1} g^{-1}) in animals treated acutely or chronically with clenbuterol

	Clenbuterol saline[1] / *Control saline*	*Control clenbuterol*[2] / *Control saline*	*Clenbuterol clenbuterol*[3] / *Clenbuterol saline*
Tissue			
WAT	5.4 †	1.05	0.59
BAT	2.86†	19.4†	20.8
Kidney	1.06	0.54	1.09
Brain	0.98	0.86	0.93
Diaphragm	1.06	2.83†	2.16†
Soleus	0.16†	2.53†	7.95†
Gastrocnemius	0.16†	1.75†	8.25†
Back muscle	0.28†	1.1	4.7†
Skeletal muscle	0.2†	1.82†	6.60†

[1] Acute effect of clenbuterol in controls.
[2] Chronic effect of clenbuterol.
[3] Acute effect of clenbuterol in rats given the drug chronically.
† Value significantly different from unity. For abbreviations see Table 1.

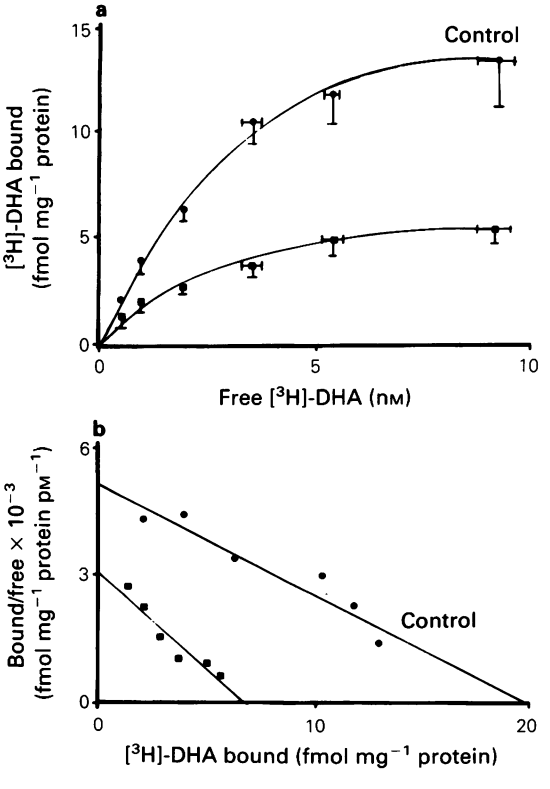

Figure 1 (a) Specific binding of [^{3}H]-dihydroalprenolol ([^{3}H]-DHA) to muscle membranes isolated from control (●) and clenbuterol-treated (■) rats at different concentrations of radioligand. Values shown are means with bars representing s.e.means. (b) Scatchard analysis of above data – see Table 3 for B_{max} and K_D values.

measurement. When the acute effects of clenbuterol were compared in control and chronic clenbuterol-treated groups, higher blood flows to white and brown fat, kidney and testes were seen in the latter group, but blood flow to soleus muscle was significantly lower than in animals receiving clenbuterol for the first time, as was the average flow to soleus, gastrocnemius and back muscles.

Experiment 2

The total membrane protein isolated from skeletal muscle (μg g^{-1} tissue) was 3.3 ± 0.3 μg g^{-1} for control and 4.3 ± 0.6 μg g^{-1} for clenbuterol-treated rats, and 1.4 ± 0.2 and 1.7 ± 0.1 μg g^{-1} for heart ventricles from these animals, respectively. The data presented in Figure 1 show that the binding of [^{3}H]-DHA to muscle membranes exhibited saturation at about 5–6 nM of ligand. Non-specific binding ranged from 40–60% of total binding, and in earlier studies (Rothwell *et al.*, 1985) we have confirmed that binding is reversible and stereospecific. Hill coefficients were close to unity in all cases and Scatchard analysis produced linear regressions (Figure 1). The low tissue density of β-receptors in muscle necessitated pooling of tissue from several animals for analysis, but nevertheless, reproducible values were obtained and these are shown in Table 3.

The maximum number of binding sites was reduced by over 60% in membranes isolated from the muscles of clenbuterol-treated rats; the dissociation constants for binding did not differ significantly. Linear regressions were also obtained for Scatchard analysis of binding data using heart ventricles (not shown) with dissociation constants of 2.7 ± 0.1 μM for control and 2.6 ± 2 μM for clenbuterol-treated rats (NS, $n = 5$). The maximum number of binding sites (B_{max}) was

Table 3 β-Adrenoceptor density and subtype in membranes isolated from skeletal muscle after 18 days of treatment with clenbuterol or vehicle.

	Vehicle	*Clenbuterol*	(n)
β-Receptor number			
B_{max} (fmol mg^{-1} protein)	20 ± 4	7 ± 3*	(6)
K_D (μM)	4.0 ± 0.8	2.2 ± 0.4	
r	0.96	0.96	
β-Receptor subtype			
% β_1	17 ± 4	15 ± 6	(3, 4)
% β_2	85 ± 5	86 ± 6	
K_D' β_1	2.0 ± 0.5	0.02 ± 0.04	
K_D' β_2	1.4 ± 0.3	0.03 ± 1.10	
r	0.96	0.96	
Density (fmol mg^{-1} protein)			
β_1	3 ± 4	1 ± 3	
β_2	17 ± 4	6 ± 2*	

Values derived from Scatchard analysis and Eadie-Hofstee plots of [^{3}H]-dihydroalprenolol binding and displacement measurements.
Each observation (*n*) was made in pooled tissue from 3–4 animals.
Mean values ± s.e.mean are shown. *$P < 0.05$ vs control.

reduced significantly in heart ventricles of clenbuterol-treated (110 ± 8 fmol mg^{-1} protein) compared to control rats (154 ± 13 fmol mg^{-1}, $P < 0.05$).

Curvilinear Eadie-Hofstee plots of the displacement of radioligand binding of ICI 118551 were resolved into two lines, and indicated a predominance of β_2-receptors in muscle from both groups of animals (Table 3). The proportion of each subtype and the K_D' for the receptors were unaffected by clenbuterol treatment, and ICI 118551 showed 50 to 100 fold selectivity for the β_2-receptor in each case. The density of β_2-receptors in muscle membranes was significantly decreased by 65% in clenbuterol-treated rats.

Discussion

As in previous studies (Winter, 1983; Emery *et al.*, 1984), daily treatment with clenbuterol stimulated growth in young rats and, although body composition was not measured in the present experiment, we have shown previously that this is due to enhanced protein deposition, particularly in skeletal muscles (Emery *et al.*, 1984). The greater mass of soleus and gastrocnemius muscles seen here is consistent with this, and the decreased weight of epididymal fat pads also accords with the earlier observations of lower rates of fat deposition. The present experiment was designed to test whether the enhanced muscle growth in clenbuterol-treated rats was associated with changes in blood flow and adrenoceptor density or subtype in skeletal muscle.

In all skeletal muscles studied (apart from diaphragm), blood flow was reduced after chronic treatment with clenbuterol, which contrasts with the large increases in flow seen one hour after acute injection in both treated and untreated rats. The blood flow response to acute injection in treated rats was considerably greater (6 fold increase) than in untreated controls (2 fold). However, the greater acute response in treated rats was not sufficient to increase muscle blood flow to the level seen in untreated rats given an acute injection of clenbuterol. Thus, it would appear that muscle perfusion in both the unstimulated and clenbuterol-stimulated condition is reduced by chronic treatment with the β_2-agonist, and this appears to be reflected in the changes in β_2-adrenoceptor density.

The methods used to estimate β-adrenoceptor density and subtype in this study have been described in detail and validated previously by ourselves (Rothwell *et al.*, 1985) and others (e.g. Minneman & Molinoff, 1980). The β-receptor density of the microsomal fraction was relatively low in muscle from untreated rats (B_{max} = 20 fmol mg^{-1} protein) compared to heart (154 fmol mg^{-1} protein), and density was markedly reduced in both tissues by chronic clenbuterol treatment. A similar decrease in muscle β-receptor density has been observed in rats treated chronically with isoprenaline (Vallieres *et al.*, 1979). This reduction is consistent with the concept of 'down-regulation' of receptors in response to repetitive stimulation (for review see Hertel & Perkins, 1984), and suggests that clenbuterol can influence muscle metabolism and/or

blood flow through direct effects on β-adrenoceptors. These effects of adrenoceptor agonists contrast with the rise in β-receptor density and oxidative capacity of muscles in exercise-trained rats (Williams *et al.*, 1984). It would appear, therefore, that 'up-regulation' of receptors accompanies motoneurone activation of muscle metabolism, while 'down-regulation' results from humoral activation of metabolism by exogenous adrenoceptor agonists.

One question raised by the apparent down-regulation of receptors is whether this applies equally to receptors in the smooth muscle of the vascular bed and those in skeletal muscle and other cells within the tissue. The reduced muscle blood flow would suggest the former, but reductions in flow secondary to diminished receptor number and metabolic activation of skeletal muscle cells cannot be ruled out.

Whatever the explanation for the changes in blood flow, it now appears that increases in muscle blood flow are not the primary cause of the anabolic response to clenbuterol. One reason for suggesting this is that significant increases in gastrocnemius muscle protein synthesis *in vivo* are seen within one hour after acute injection of clenbuterol in rats treated chronically for 7 days with the β_2-agonist (Emery *et al.*, 1984). In the present study, muscle blood flow one hour after clenbuterol injection in chronically-treated rats was increased, but the flow rate was less than that seen in untreated rats given a single injection. In fact, the blood flow to gastrocnemius muscle was not significantly greater than values measured in untreated rats receiving only a saline injection.

In normal, untreated rats we have recently found (preliminary observations) that increases in protein synthesis are seen only 4–6 h after a single injection of clenbuterol, even though the blood flow measurements presented here would indicate a rapid, 2 fold increase in flow within 1 h after injection. Thus, it appears that one can observe increases in protein synthesis in the absence of high blood flow rates, and high blood flow rates in the absence of increases in protein synthesis. However, these conclusions need to be treated with a degree of caution, since (a) the acute increases in blood flow seen after 1 h could be sustained for up to 4–6 h and (b) the acute stimulation of protein synthesis seen in 7 day treated rats (Emery *et al.*, 1984) may have become attenuated by 18 days of treatment, when the blood flow measurements were made in the present study. One final possibility is that the reduced skeletal muscle blood flow may be related to the lower rates of protein degradation during chronic clenbuterol treatment described by Reeds *et al.* (1986).

The elevated blood flow to white and brown adipose tissue seen after chronic clenbuterol treatment probably reflects the lipolytic and thermogenic actions of this β_2-agonist. Clenbuterol has been shown to be a potent agonist for brown fat thermogenesis (Winter, 1983) and the tissue has a high density of β_2-adrenoceptors (Rothwell *et al.*, 1985). As well as the chronic increase in brown fat blood flow seen here, there was also a very large response to a single, acute injection of clenbuterol after chronic treatment, with flows reaching values as high as $20\,ml\,g^{-1}\,min^{-1}$. This suggests that the thermogenic (and perhaps lipolytic) effects of clenbuterol are potentiated by chronic treatment, and would help explain the repartitioning effects of the agonist on body composition. The repartitioning effects are seen as an increase in the lean : fat ratio of the carcass (see Stock & Rothwell, 1986 for review), and could result from decreases in fat deposition independently of any effects of clenbuterol on protein synthesis and deposition.

In conclusion, any effect of clenbuterol on muscle growth which is mediated by increases in blood flow can only be transient, and is not sustained during chronic treatment. Conversely, effects on blood flow to white and brown adopose tissue are sustained and would continue to influence fat and energy metabolism.

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