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Evidence that the hypertrophic action of clenbuterol on denervated rat muscle is not propranolol-sensitive

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1 The effect of propranolol on the clenbuterol-induced protein anabolism in innervated and denervated soleus and plantaris muscles of the rat was studied.

2 The response to the β-agonist, clenbuterol, in both innervated and denervated muscles, was not significantly inhibited by the β-antagonist, propranolol.

3 The results provide further evidence to suggest that the action of clenbuterol on skeletal muscle protein accretion may not be directly mediated by β-adrenoceptors.

Introduction

The novel protein anabolic action (Ricks *et al.*, 1984; Emery *et al.* 1984; Baker *et al.*, 1984) of the β-adrenoceptor agonist clenbuterol has aroused considerable debate as to whether the actions of the drug are directly mediated by β-receptors. The changes in muscle glycogen, body fat, and energy expenditure and the effects of the drug on smooth muscle are all fairly typical responses to a classical β-adrenoceptor agonist. Furthermore, Rothwell *et al.* (1987) suggested that the observed effect on blood flow as well as the apparent down regulation of receptors can be accounted for by direct effects of the drug on β-adrenoceptors.

In contrast, the use of a variety of adrenoceptor antagonists demonstrated that the typical β effects of clenbuterol (lipolysis, increased energy expenditure, and increased heart mass) were separable from the skeletal muscle growth response (Reeds *et al.*, 1988). In particular, propranolol blocked the clenbuterol-induced rise in cardiac muscle mass and significantly limited the reduction in body fat. However, propranolol did not reverse the protein anabolic effect of clenbuterol in skeletal muscle (Reeds *et al.*, 1988), although fibre hypertrophy was reduced (Maltin *et al.*, 1987a). These data therefore suggest that at least the protein anabolic effects of clenbuterol might not be β-receptor mediated.

We have already shown that clenbuterol can reverse or ameliorate the effects of denervation-induced atrophy and that innervation status influences the sensitivity of muscle to clenbuterol treatment (Maltin *et al.*, 1986a; 1987b). Denervation is associated with an up-regulation of β-receptors (Banerjee *et al.*, 1977), and this might be considered to be the basis for the apparent greater sensitivity of denervated muscle to clenbuterol. Thus the present study was designed to examine the effect of a mixed β-receptor antagonist, propranolol, on the amelioration of denervation-induced atrophy by clenbuterol.

Methods

Male Hooded Lister rats of the Rowett Research Institute strain were used throughout. The rats were weaned at 19 days of age and were divided into four groups of 12 animals of equal mean body weight. The animals were fed to appetite a standard laboratory rat chow (Labsure CRM nuts, K and K Greff, Croydon, U.K.) and water was freely available at all times. After 4 days the animals were re-weighed and, if necessary, re-grouped to ensure that all groups had the same mean body weight and mean growth rate over the preliminary period. The animals were then housed singly in plastic, flat bottomed cages and fed to appetite a semi-synthetic diet, PW3 (Pullar & Webster, 1977). After 4 days all the animals were anaesthetized with ether and a short (1 cm) piece of the left sciatic nerve was removed under aseptic conditions. The 4 groups were subsequently treated as follows:– control group – fed control diet PW3 for 7 days post-denervation; clenbuterol group – fed control diet PW3 for 4 days followed by PW3 containing clenbuterol ($2\,mg\,kg^{-1}$) for 3 days; propranolol group – fed control diet PW3 for 3 days followed by PW3 containing propranolol ($200\,mg\,kg^{-1}$) for 4 days; clenbuterol + propanolol group – fed control diet PW3 for 3 days, followed by PW3 containing propranolol ($200\,mg\,kg^{-1}$) for 24 h

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Table 1 Fibre size and frequency in innervated and denervated soleus muscles of rats from the four treatments groups

	Control	*Prop.*	*Clen.*	*Cl + P*	*Control*	*Prop.*	*Clen.*	*Cl + P*	
		Innervated limb				*Denervated limb*			
				Mean fibre cross-sectional area (μm^2)					
FOG	1001	980	1294*	1356*	239	280	504*	403	LSD 222.1
SO	1209	1205	1226	1354	404	485	932***	803***	LSD 194.0
				Mean percentage frequency					
FOG	44.5	43.9	47.4	45.0	48.1	51.4	49.5	48.4	LSD 5.8
SO	55.5	56.1	52.6	55.0	52.0	48.6	50.5	51.6	LSD 5.8
				Mean percentage area					
FOG	39.8	38,8	48.6**	44.8*	35.5	38.4	34.4	32.1	LSD 4.9
SO	60.2	61.2	51.4**	55.2*	64.5	61.6	65.6	67.9	LSD 4.9

Values represent means of groups. Least significant difference (LSD) values were derived from the standard error of the difference of the means and the t statistic. Using the LSD, comparison between treated groups and the control group is made for innervated and denervated limbs. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Prop = propranolol, Clen = clenbuterol, Cl + P clenbuterol in combination with propranolol. FOG = fast twitch oxidative glycolytic, SO = slow twitch oxidative.

and then PW3 containing propranolol (200 mg kg^{-1}) and clenbuterol (2 mg kg^{-1}) for 3 days. The dose chosen for propranolol was a 100 × weight excess to that of clenbuterol since this dose had been shown to inhibit the typical β-agonist effects of clenbuterol (Reeds *et al.*, 1988). From each group of 12 animals, 6 were assigned at random for biochemical analysis, and 6 for histochemical analysis. Body weights of all animals were recorded daily.

At the end of the experimental period the animals were killed by cervical dislocation and the muscles removed. Only the soleus muscles were taken for histochemical analysis and treated as described previously (Maltin *et al.*, 1986b). Fibre type composition was assessed from the staining reactions for Ca^{2+} activated myofibrillar ATPase at pH 9.4 after methanol-free formalin fixation (Hayashi & Frieman, 1966). Those fibres giving the most dense reaction product were identified as fast twitch oxidative glycolytic (FOG) fibres, those which gave no reaction product were designated slow twitch oxidative (SO) fibres, while those given an intermediate reaction product were characterized as fast twitch glycolytic (FG) fibres. However, FG fibres represented less than 2% and were omitted from measurements and calculations. Quantitative assessments were made directly from the stained muscle preparations using a computer based (Torch Computers Ltd, Cambridge, U.K.) image analysis system (Vision Dynamics, Hemel Hempstead, Herts, U.K.). The use of this system required slight modifications of the ATPase stain to enhance the contrast for imaging (M.I. Delday, personal communication). A minimum of 200 fibres per muscle was measured. Soleus and plantaris muscles taken for biochemical analysis were frozen and stored at −20°C until estimations of protein, RNA and DNA were made. The methods used for determinations of protein and RNA were the same as outlined previously (Maltin *et al.*, 1986a). DNA was determined following the method of Burton (1956).

Statistical analysis

A three way analysis of variance was carried out on the data using Genstat (Rothampstead Experimental Station, 1977) on a Prime 550 computer to examine the effects of innervation, clenbuterol and propranolol. The interactions were examined in the general linear model, followed by means comparison of either main effects (no interaction) or significant interactions.

Results

As in previous studies all the animals grew well on their respective diets despite the trauma of denervation. At the end of the experiment the body weights for the groups were as follows: control 116 ± 1, clenbuterol 120 ± 3, propranolol 114 ± 3, clenbuterol + propranolol 119 ± 2 (mean values (g) ± s.e.mean, $n = 12$ for each group).

Histochemical analysis

The results of the histochemical analysis of soleus muscle are presented in Table 1. The soleus comprised two main fibre types FOG and SO.

The results from this study were broadly in line with those from previous studies. Briefly, analysis of variance for the variate fibre area showed that there were two significant main effects, those of innerva-

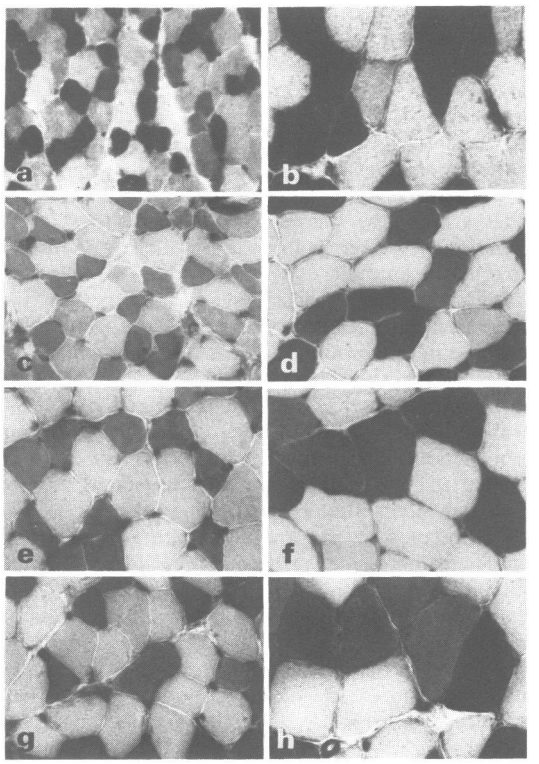

Figure 1 Transverse cryosections of soleus muscle from innervated and denervated limbs of rats from each of the four treatment groups. The tissue has been reacted to demonstrate the activity of Ca^{2+} myofibrillar ATPase. (a) Denervated muscle from control fed rat. (b) Innervated muscle from control fed rat. (c) Denervated muscle from propranolol fed rat. (d) Innervated muscle from propranolol fed rat. (e) Denervated muscle from clenbuterol fed rat. (f) Innervated muscle from clenbuterol fed rat. (g) Denervated muscle from clenbuterol + propranolol fed rat. (h) Innervated muscle from clenbuterol + propranolol fed rat. Bar = 50 μm.

tion and clenbuterol. Denervation resulted in a significant reduction in fibre size for both fibre types (Table 1). As found previously (Maltin *et al.*, 1986b) clenbuterol had an anabolic effect which was expressed as fibre hypertrophy. The effect was statistically significant in both fibre types in denervated muscles (Figure 1, Table 1) but was significant only in FOG fibres of innervated muscles (Figure 1, Table 1). Denervation, also significantly increased the frequency of FOG and decreased SO fibres as reflected in the grand means across the four groups ($P < 0.01$). There was also an interaction effect on percentage area ($P < 0.01$) between innervation status and the effect of clenbuterol. This changed in innervated muscles (Table 1), but not in denervated muscles. Hence this result provided further support for the contention that the innervation status of the muscle (for soleus at least) was important in determining the effect of clenbuterol (see also Maltin *et al.*, 1987b).

There were no significant main effects or interactions for the effects of propranolol alone or in combination with clenbuterol in either innervated or denervated muscles with respect to area, percentage frequency or percentage area.

Biochemical analysis

The results of the biochemical analysis of soleus and plantaris muscles are presented in Tables 2 and 3.

Soleus muscle (*Table 2*) Statistical analysis highlighted the significant ($P < 0.001$) main effects of innervation and clenbuterol treatment on muscle weight, protein, RNA and DNA contents. As described elsewhere (Maltin *et al.*, 1986a), denervation lead to the typical loss of muscle weight and protein and RNA content ($P < 0.001$). The presence of clenbuterol in the diet caused the typical increase ($P < 0.01$) in muscle weight irrespective of innervation status. The response in weight was accompanied by a simultaneous increase in protein and RNA

Table 2 Compositional changes in innervated and denervated soleus muscles of rats from the four treatment groups

	Control	*Prop.*	*Clen.*	*Cl + P*	
		Mean weight (mg)			
Inn.	57.9	56.1	66.2**	66.2**	
Den.	21.1	22.0	33.2***	35.8***	LSD 5.9
		Mean total protein content (mg)			
Inn.	8.6	8.7	9.9*	10.3**	
Den.	2.4	2.7	4.4**	4.8***	LSD 1.1
		Mean total RNA content (μg)			
Inn.	104.6	103.1	129.3***	126.9***	
Den.	40.2	49.9	70.9***	75.3***	LSD 10.7
		RNA/protein ($\mu g\,mg^{-1}$)			
Inn.	15.1	14.9	15.7	14.6	
Den.	24.9	23.9	20.3**	21.1*	LSD 3.0
		Mean total DNA content (μg)			
Inn.	73.7	76.3	72.7	81.0	
Den.	46.3	44.9	60.7*	64.0**	LSD 11.7
		RNA/DNA			
Inn.	1.7	1.7	2·2**	1.9	
Den.	1.2	1.5*	1.5	1.6*	LSD 0.3

Values represent means of groups. Least significant difference (LSD) values were derived as for Table 1. Using the LSD, comparison between treated groups and the control group is made for the innervated (Inn.) and denervated (Den.) limbs. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. For key to abbreviations used see legend of Table 1.

Table 3 Compositional changes in innervated and denervated plantaris muscles of rats from the four treatment groups

	Control	*Prop.*	*Clen.*	*Cl + P*	
		Mean weight (mg)			
Inn.	95.7	97.7	101.6	101.3	
Den.	37.7	41.2	52.7**	49.2*	LSD 10.6
		Mean total protein content (mg)			
Inn.	18.1	21.3*	23.0**	21.7**	
Den.	7.4	10.2*	11.4**	11.3**	LSD 2.3
		Mean total RNA content (μg)			
Inn.	171.6	164.7	201.7**	185.3	
Den.	94.2	108.8	140.7***	130.8***	LSD 17.9
		RNA/protein ($\mu g\,mg^{-1}$)			
Inn.	8.8	7.7	8.8	8.6	
Den.	10.1	10.8	12.4**	11.7*	LSD 1.3
		Mean total DNA content (μg)			
Inn.	120.2	116.0	106.8	104.3	
Den.	79.2	91.0	92.8	94.8	LSD 18.7
		RNA/DNA			
Inn.	1.4	1.4	1.9***	1.8***	
Den.	1.2	1.2	1.5***	1.4*	LSD 0.2

Values represent means of groups. Least significant difference (LSD) values and comparisons are as in Table 2. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. For key to abbreviations used see legend of Table 1.

content, which could be demonstrated in both denervated and innervated muscles. This response in denervated muscles correlated with the amelioration of atrophy as observed from the fibre sizes (Figure 1).

The sensitivity of the muscle to clenbuterol treatment was dependent on the innervation status, particularly with respect to the RNA/protein ratio and DNA content. The RNA/protein ratio was unchanged by clenbuterol in innervated muscles but was significantly ($P < 0.01$) reduced in denervated muscles and the DNA content was also largely unchanged in innervated muscles (although slightly increased in the presence of propranolol) but was significantly ($P < 0.05$) increased in denervated muscles.

Different sensitivities of innervated and denervated muscles were also evident in the effect of propranolol. Whilst for the majority of independent parameters measured propranolol could be found to have no significant effect alone or in combination with clenbuterol, with respect to RNA/DNA ratios this was not true. In denervated muscles, propranolol treatment alone was associated with an increase in RNA/DNA which was significantly ($P < 0.05$) augmented in the presence of clenbuterol. In contrast, propranolol alone had no effect on innervated muscles, whereas in combination with clenbuterol, propranolol reduced the clenbuterol-induced increase in RNA/DNA.

Plantaris muscle (*Table 3*) As described for soleus muscle, the effects of innervation and clenbuterol comprised the main significant influences on the parameters measured. Clenbuterol produced an anabolic effect in innervated muscle, and although the increase in weight was not statistically significant the anabolic effect was clearly evident as a significant ($P < 0.01$) increase in protein content. Typically, denervation gave rise to a significant ($P < 0.001$) reduction of protein and weight which was ameliorated by the anabolic effect of clenbuterol.

The difference in sensitivity of denervated and innervated muscles to treatment with clenbuterol was apparently confined to DNA for which the grand means showed that there was a significant ($P < 0.05$) interaction between the effects of clenbuterol and innervation. In innervated muscle clenbuterol produced a decrease in DNA whereas in denervated muscle the effects appeared to be opposite with clenbuterol increasing DNA.

Treatment with propranolol alone gave rise to a significant increase in protein content of both denervated and innervated muscles. In combination with clenbuterol there was significant interaction ($P < 0.05$) which was evident as a reduction in the clenbuterol-induced anabolic response in innervated but not denervated muscles due to propranolol. With regard to RNA a similar phenomenon was evident. There was the suggestion that propranolol also reduced the effect of clenbuterol but in this case independent of the innervation status of the muscle.

Discussion

The aim of this study was to assess whether the ameliorative effects of clenbuterol on denervation atrophy were inhibited by β-receptor antagonism and thereby to gain further insight into the mode of action of the drug. The results suggest that the anabolic effect of clenbuterol in denervated muscles is not significantly impaired by the administration of propranolol. In addition, the study has also produced some other important observations. First, the differences between the responses of the two muscles, and second the importance of innervation status in the response of the muscle.

The responses of both muscles to treatment with either drug alone or in combination were broadly similar to those described elsewhere for innervated soleus alone (Maltin *et al.*, 1987a). However, it must be noted that in the present experiment, the apparent separation of hypertrophic and compositional responses in innervated soleus muscles treated with the combination of drugs seen in the previous experiment was not observed. The reason for this is unclear, the only differences between this and the previous study (Maltin *et al.*, 1987a) were that in the

present study the animals were 4 days older and had been unilaterally denervated at the start of drug treatment. It is possible that denervation influenced the response of the innervated contralateral muscle by imposing an increased work load on the innervated limb. The anabolic effect of an increased work load has been shown to be additive to that of clenbuterol (Maltin *et al.*, 1987c).

Although overall the responses of plantaris and soleus were similar there were some important differences. Specifically, in plantaris muscle (but not soleus) there was a stimulation of protein accretion by propranolol alone. Furthermore in the innervated muscles, propranolol appeared to reduce the clenbuterol-induced protein anabolism. The explanation for this result is unclear. However, in other situations, such as in the myocardium (Maisel *et al.*, 1986) propranolol has been shown to up regulate β-adrenoceptors and it is possible that propranolol may do the same in skeletal muscle. Moreover in denervated muscle the 'up regulation' of receptors (Banerjee *et al.*, 1977) would also contribute to the response and might account for the greater increase in protein content in denervated plantaris muscles exposed to propranolol (38% for denervated cf 18% for innervated). Thus if the contention of Garber *et al.* (1976) that adrenaline increases amino acid retention was correct, then an up regulation of receptors available for endogenous catecholamine binding could lead to an increase in protein content of these muscles. However, this explanation is probably not satisfactory. Soleus muscle (which is often compared to cardiac muscle) does not exhibit this response, nor does this reasoning explain the observed reduction by propranolol of the clenbuterol-induced protein accretion in innervated muscle.

The changes in RNA, DNA and RNA/DNA were largely consistent with the clenbuterol-induced protein anabolic response and previous observations (Reeds *et al.*, 1986; 1988; Maltin *et al.*, 1987b). In the innervated muscles the increase in RNA and protein were not accompanied by increases in DNA, indicating that growth was expressed as hypertrophy rather than hyperplasia (see Maltin *et al.*, 1986b). In contrast, in the denervated muscles there was a tendency for increases in RNA and protein to be accompanied by increases in DNA content, implying some increase in cellularity or nucleation. The most likely source of such DNA would be satellite cells. In this context the greater increase of DNA content in denervated soleus is of interest since, in man at least, this muscle has been shown to have a large complement of satellite cells (Schmalbruch & Hellhammer, 1976). Preliminary morphological evidence suggests that these cells may contribute to nucleation of the regrowing fibres rather than to new fibres (Maltin, unpublished data).

Much of the data from the literature (see Stock & Rothwell, 1986 for review) would support the contention that the anabolic effect of clenbuterol is mediated through an action on the β-adrenoceptors. For example, Garber *et al.* (1976) demonstrated a reduction in amino acid release in incubated muscles treated with physiological levels of naturally occurring catecholamines. Interestingly this effect appeared to be mediated by the β-receptors and could be accounted for by a depression in muscle protein degradation. This might be considered analogous to the anabolic effects of clenbuterol, the action of which appeared to be mediated through a depression in protein degradation (Reeds *et al.*, 1986). Furthermore, the response of muscles to clenbuterol (slow muscles being more responsive than fast muscles (Festoff *et al.*, 1977; Maltin *et al.*, 1986a) and denervated muscles being more sensitive than innervated muscles (Maltin *et al.*, 1987b, and the present study)) is apparently consistent with the receptor densities in these muscles (Banerjee *et al.*, 1977; Williams *et al.*, 1984) and the β-receptor-mediated responses seen in innervated (Bowman & Nott, 1969) and denervated (Festoff *et al.*, 1977) muscles exposed to catecholamines.

However, there are several pieces of evidence to suggest that while the non-anabolic effects of clenbuterol are indeed mediated through β-adrenoceptors, the protein anabolic action is not. The work of Reeds *et al.* (1988) revealed that while the effects of clenbuterol on cardiac and fat mass and energy expenditure were inhibited by propranolol, the anabolic effects on muscle were unaltered. They concluded that the 'anabolic and anti-lipogenic actions' of clenbuterol were 'mechanistically distinct'. While this conclusion might be applied to the present study some caution should be used since in innervated plantaris, propranolol limited the effects of clenbuterol on protein and RNA, and furthermore in innervated soleus the effect of the drug on the RNA/DNA ratio was blocked. However, the remaining observations from the present study might lend some support to the contention of Reeds *et al.* (1988). It might be argued that the apparent inability of propranolol to block the protein response (in all muscles except innervated plantaris) was due to a variety of possibilities including different affinities of the appropriate receptors, an insufficient dose of propranolol or the relative pharmacokinetics of the two drugs. The animals were pre-fed with propranolol, (see Reeds *et al.* 1988) and the large dose of propranolol used was similar to that shown by Garber *et al.* (1976), to block the effects of adrenaline on amino acid release and was in excess of that required for 50% inhibition of isoprenaline stimulated adenylate cyclase activity (Maillet & Garber 1986). Concerning the pharmacokinetics of the two drugs, propranolol

is rather short acting (circa 2–4 h (Shand, 1974)) whereas clenbuterol has a half-life of 20–30 h in the rat. It is therefore possible that the effect of propranolol was wearing off during the periods when the rats were not feeding. If this were the case, however, it is difficult to explain the inhibition by propranolol of the clenbuterol-induced lipolysis which has been consistently observed in these experiments.

Consequently, the present observations that, in general, the anabolic response in both innervated and denervated muscles is not inhibited by propranolol may provide further evidence that the action of clenbuterol (or a metabolite) on protein accretion is not directly mediated by typical propranolol-sensitive β-adrenoceptors. This is in contrast to the typical β-receptor-mediated effects of the drug on fat, glycogen and energy expenditure.

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