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Effects of the cyclo-oxygenase inhibitor, fenbufen, on clenbuterol-induced hypertrophy of cardiac and skeletal muscle of rats

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1 When rats were fed with clenbuterol for 7 days skeletal muscle mass increased by 21% in the tonic soleus and phasic plantaris muscles and a 16% hypertrophy of the heart was also induced. Fenbufen, fed to rats for the same period, blocked the hypertrophy of the heart but not that of the skeletal muscles.

2 When feeding of fenbufen commenced 3 days before the administration of clenbuterol, plasma prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) was reduced by 79%; there was again no effect of fenbufen on clenbuterol-induced increases in the RNA or protein content of plantaris, nor in the increased area of fast or slow twitch fibres in the soleus. In the heart the clenbuterol-induced increases in the RNA (+21%) and protein content (+20%) were totally inhibited.

3 The effects of clenbuterol on heart muscle appear to be mediated by a cyclo-oxygenase metabolite of arachidonic acid whilst the effects on skeletal muscle are not.

Introduction

The mode of action of the β-adrenoceptor agonist, clenbuterol, in promoting muscle protein deposition remains unknown. The suggestion that muscle hypertrophy occurred principally as a result of a decrease in protein degradation (Reeds *et al.*, 1986) contrasts with the observation of a large increase in protein synthesis rates in denervated muscle (Maltin *et al.*, 1987). Even in the innervated muscle, increases in protein synthesis rates after 3 days have been observed (Maltin *et al.*, 1989), suggesting that a transient rise in synthesis is at least partly responsible for the hypertrophy in phasic muscles.

The β-adrenoceptor antagonist, propranolol, was shown to block the effects of clenbuterol on skeletal muscle in the study of MacLennan & Edwards (1989) whereas in other studies, β-adrenoceptor antagonists blocked effects of clenbuterol on the heart whilst having no effect on skeletal muscle anabolism (Reeds *et al.*, 1988). This latter observation has led to the suggestion that the skeletal muscle-directed effect of clenbuterol is separate from its β-mediated effects. Thus the possibility that the ability of clenbuterol to promote muscle hypertrophy was indirect and mediated by other hormones or growth factors has been considered. Interactions between β-adrenoceptor agonists and insulin binding have been reported in muscle (Webster *et al.*, 1986) but McElligott *et al.* (1987) demonstrated that clenbuterol was effective in diabetic rats, suggesting that the response was not insulin-mediated. The ability of clenbuterol to cause muscle hypertrophy in Snell dwarf mice (Pell *et al.*, 1987) suggests that its action is independent of prolactin, GH and thyroid hormones in which these animals are deficient.

Non-steroidal anti-inflammatory drugs (NSAIDs), such as indomethacin and meclofenamate, which reduce prostaglandin release by inhibition of cyclo-oxygenase, block increases in protein synthesis induced by stretch (Smith *et al.*, 1983) and by insulin *in vitro* (Reeds & Palmer, 1983) and *in vivo* (Reeds *et al.*, 1985). These observations have implicated cyclo-oxygenase metabolites of arachidonic acid, specifically prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (Smith *et al.*, 1983) in the control of protein synthesis. Another NSAID, fenbufen (a pro-drug, which is metabolized in the liver to 4-biphenyl acetic acid) has also been shown to affect rates of protein synthesis in rat muscle (McMillan *et al.*, 1987). The present work was undertaken to investigate the effects of inhibition of prostaglandin synthesis with fenbufen on clenbuterol-induced muscle hypertrophy.

Methods

Male hooded Lister rats of the Rowett strain were weaned at 19 days and treated prior to experiments as described previously (Maltin *et al.*, 1986). During the experiments the rats were housed individually and fed on PW3 diet (Pullar & Webster, 1977).

Clenbuterol was incorporated into the diet at a concentration of $2\,mg\,kg^{-1}$ diet, a concentration at which a significant hypertrophy of skeletal muscle occurred without any adverse effect on food consumption (Reeds *et al.*, 1986). Fenbufen (Lederle Laboratories, Gosport, Hants, U.K.) was fed at a concentration in the diet of $1200\,mg\,kg^{-1}$; this was the maximum dose which could be fed to rats without causing gastric ulceration whilst inducing a near maximal inhibition of prostaglandin release (McMillan, 1987).

In Experiment 1, the diets were fed for 7 days, after which the animals (4 groups of 6) were killed and tissues removed, frozen in liquid N_2 and stored at −20°C until analysed.

In Experiment 2, feeding of fenbufen started 3 days before the addition of clenbuterol to the diet; thus the rats were fed fenbufen for a total of 10 days and clenbuterol for 7 days. Ten rats were subjected to each dietary treatment; on the final day of the experiment 6 rats from each group were injected via a lateral tail vein with 150 μmol L-phenylalanine plus 75 μCi L-[2,6-^{3}H]phenylalanine per 100 g body weight (Garlick *et al.*, 1980). Precisely 10 min later the animals were killed and tissues were dissected and treated either for measurement of total protein and RNA and rates of protein synthesis (Reeds *et al.*, 1986), or for fibre area and frequency (Maltin *et al.*, 1986). The remaining 4 rats/group were not injected with phenylalanine. These rats were killed by decapitation, blood was collected from the neck and muscles removed, frozen in liquid N_2 and stored at −70°C for $PGF_{2\alpha}$ assay.

$PGF_{2\alpha}$ was measured in serum and muscle after extraction of 0.5 ml serum or homogenization of plantaris muscles in ethyl acetate: isopropanol: 0.2 M HCl (3 : 3 : 1, v/v/v) followed by the addition of 2 ml ethyl acetate and 3 ml H_2O. After mixing, 3 ml of the organic layer was evaporated to dryness,

Table 1 Effects of fenbufen on clenbuterol-induced tissue hypertrophy

Treatment	*Control*	*Clenbuterol*	*Fenbufen*	*Clenbuterol + fenbufen*
Weight				
Whole body (g)	111 ± 3	113 ± 2	108 ± 2	106 ± 1
Heart (mg)	467 ± 10	544 ± 13***	439 ± 12	489 ± 11##
Soleus (mg)	53.3 ± 2.2	64.3 ± 2.9*	52.3 ± 2.1	62.7 ± 1.4**
Plantaris (mg)	82.0 ± 2.5	99.2 ± 2.7**	81.2 ± 2.1	92.7 ± 1.8**

Rats were fed on clenbuterol (2 mg kg^{-1}, diet) and/or fenbufen (1200 mg kg^{-1}, diet) for 7 days. Values are means ± s.e.mean (n = 6).
By Student's t test: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ v control; ## $P < 0.01$ v clenbuterol alone.

resuspended and analysed by radioimmunoassay (Dade [^{3}H]-$PGF_{2\alpha}$ RIA kit; Steranti, St. Albans, Herts).

One way analysis of variance was used to assess the data; the significance of differences between groups was determined by Student's t test.

L-[2,6-^{3}H]phenylalanine was purchased from Amersham International (Amersham, Bucks, U.K.). All other reagents were from Sigma, or B.D.H. (both Poole, Dorset).

Results

In the first experiment feeding of clenbuterol and fenbufen was started simultaneously and continued for 7 days. There were no significant effects of any treatment on the body weight of the rats (Table 1). Gut and liver weights were also unaffected (data not shown). All 4 of the muscles examined (gastrocnemius, soleus, plantaris and extensor digitorum longus) showed significant hypertrophy in response to clenbuterol with no apparent effect of fenbufen on the hypertrophy; data for two of these muscles are presented in Table 1. The heart hypertrophied by 16% in response to clenbuterol alone and fenbufen appeared partially to reverse this effect: in the presence of fenbufen the clenbuterol-induced hypertrophy of the heart was significantly ($P < 0.01$) less than with clenbuterol alone. This effect of fenbufen was not apparent when the small and non-significant differences in final body weight were taken into account. Heart weight/100 g body weight was increased from 423 ± 5 mg (control) to 481 ± 6 mg, $P < 0.001$ (clenbuterol alone). In the presence of fenbufen the increase was from 410 ± 7 (fenbufen alone) to 464 ± 8 mg per 100 g body wt., $P < 0.001$ (fenbufen + clenbuterol).

The second experiment therefore examined the effect of pre-feeding the rats on fenbufen for 3 days to induce a reduction in prostaglandin synthesis before clenbuterol administration. Serum $PGF_{2\alpha}$ was reduced by 79% after fenbufen had been fed to the rats for 10 days but was not affected by clenbuterol; muscle $PGF_{2\alpha}$ was reduced by 50% (plantaris) (Table 2). RNA and protein accretion in both plantaris (Table 3) and soleus muscle (data not shown) were stimulated by clenbuterol; this response was unaffected by fenbufen. Clenbuterol also signifi-

Table 2 Effects of fenbufen and clenbuterol on prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) levels in serum and plantaris muscle

	$PGF_{2\alpha}$ concentration	
	pg per 50 µl serum	pg per muscle
Control	890 ± 108	118 ± 12
Clenbuterol	890 ± 78	108 ± 26
Fenbufen	190 ± 12**	64 ± 19
Clenbuterol + fenbufen	280 ± 96##	62 ± 21

Fenbufen (1200 mg kg^{-1}) was fed for a total of 10 days; clenbuterol (2 mg kg^{-1}) was fed for 7 days. Values are means ± s.e.mean (n = 4).
By Student's t test: ** $P < 0.01$ v control; ## $P < 0.01$ v clenbuterol alone.

Table 3 Effects of fenbufen and clenbuterol on the RNA and protein content and fractional rate of protein synthesis (k_s) of plantaris and cardiac muscle

	Total RNA (µg)	*Total protein* (mg)	*k_s* (%/day)
Plantaris			
Control	151 ± 3	16.6 ± 0.2	16.4 ± 0.8
Clenbuterol	202 ± 4***	20.0 ± 0.5***	17.1 ± 0.7
Fenbufen	150 ± 3	16.3 ± 0.4	15.3 ± 0.3
Clenbuterol + fenbufen	200 ± 14**	21.6 ± 0.8***	15.1 ± 0.6
Heart			
Control	1548 ± 71	59.2 ± 1.6	17.9 ± 0.3
Clenbuterol	1872 ± 69**	71.3 ± 1.7**	18.6 ± 0.6
Fenbufen	1476 ± 49	54.7 ± 1.1	16.6 ± 0.5
Clenbuterol + fenbufen	1426 ± 57##	52.1 ± 4.0##	17.0 ± 0.6

Fenbufen (1200 mg kg^{-1}) was fed for a total of 10 days; clenbuterol (2 mg kg^{-1}) was fed for 7 days. Values are means ± s.e.mean (n = 6).
By Student's t test: ** $P < 0.01$; *** $P < 0.001$ v control; ## $P < 0.01$ v clenbuterol alone.

Table 4 Effect of fenbufen on clenbuterol-induced increase in area and frequency of fast oxidative glycolytic (FOG) and slow oxidative (SO) fibres in soleus muscle

	Mean area (sq µm)		*Mean frequency* (%)		*Mean area* (%)	
Fibre type	FOG	SO	FOG	SO	FOG	SO
Control	973 ± 31	1226 ± 212	43.0 ± 0.8	57.0 ± 0.8	37.5 ± 0.7	62.5 ± 0.7
Clenbuterol	1425 ± 69***	1470 ± 77*	47.4 ± 1.6*	52.6 ± 1.6*	46.7 ± 1.6	53.3 ± 1.7
Fenbufen	983 ± 23	1212 ± 30	45.3 ± 1.3	54.7 ± 1.3	40.2 ± 1.4	59.8 ± 1.4
Clenbuterol + fenbufen	1427 ± 155*	1431 ± 144	43.5 ± 1.7	56.5 ± 1.7	43.3 ± 1.3	56.7 ± 1.3

Fenbufen (1200 mg kg^{-1}) was fed for a total of 10 days; clenbuterol (2 mg kg^{-1}) was fed for 7 days. Values are means ± s.e.mean for 6 observations except in the clenbuterol + fenbufen group where n = 5.
By Student's t test: * $P < 0.05$; $P < 0.001$ v control.

cantly increased the mean area of both fast-twitch oxidative glycolytic (FOG) and slow-twitch oxidative (SO) fibres in the soleus (Table 4) and this response too was unaffected by fenbufen. However, the mean fibre frequency, which was significantly changed by clenbuterol alone (a 10% increase in the frequency of FOG fibres) was apparently inhibited by fenbufen. In the heart the significant hypertrophy (increases of 21% in total RNA and 20% in total protein) was totally inhibited by fenbufen (Table 3).

Discussion

The mechanism of action of clenbuterol in increasing muscle mass remains poorly understood and has been claimed to result from changes in both rates of protein degradation and synthesis. A recent study has shown significant effects on protein synthesis, particularly in denervated phasic muscles (Maltin *et al.*, 1989). This increase in the rate of protein synthesis in normal, innervated muscle was transient which has led to the conclusion (Reeds *et al.*, 1986) that in the rat the effect of clenbuterol is mainly on protein degradation. The present study was undertaken to investigate the possibility that prostaglandin metabolism was involved in the clenbuterol-induced hypertrophy of muscle. Prostaglandins have been implicated in the control of both protein synthesis and protein degradation. One prostaglandin, PGE_2, has been shown to stimulate protein degradation (Rodemann & Goldberg, 1982) and is believed to be involved in some pathological states involving muscle wasting where a large increase in rates of protein degradation has been shown to be inhibited by NSAIDs such as indomethacin and naproxen (Ruff & Secrist, 1984; Tian & Baracos, 1989).

$PGF_{2\alpha}$ was shown to stimulate protein synthesis in rat (Rodemann & Goldberg, 1982) and rabbit muscle (Smith *et al.*, 1983) *in vitro*. The non-steroidal anti-inflammatory drug, indomethacin, which inhibits the action of cyclo-oxygenase and thus reduces the metabolism of arachidonic acid to prostanoids, was shown to inhibit the acute effects of insulin *in vivo* (Reeds *et al.*, 1985) and *in vitro* (Reeds & Palmer, 1983). Fenbufen, a pro-drug which is an active inhibitor of cyclo-oxygenase only after metabolism to 4-biphenyl acetic acid in the liver, is preferred in prolonged treatments since the gastric ulceration caused by feeding indomethacin and aspirin is prevented. Fenbufen inhibited prostaglandin release by 80% and reduced rates of protein synthesis in normal muscle and in muscle undergoing hypertrophy in response to tenotomy of a synergist (McMillan *et al.*, 1987). However, the ability of the muscle to hypertrophy in response to tenotomy was unimpaired in that experiment, suggesting that protein degradation had also been reduced. The results presented here are similar in that $PGF_{2\alpha}$ was reduced by fenbufen in plantaris muscle and in serum. However, the reduction in the fractional rate of protein synthesis in heart and plantaris muscle of the rats fed fenbufen was in no case statistically significant in the present study. Together with the ability of plantaris muscle to respond to clenbuterol in the presence of fenbufen, the data suggest that the response of skeletal muscle to the β-adrenoceptor agonist is not prostaglandin-mediated. They also provide further, albeit indirect evidence that the effect of clenbuterol is not insulin-mediated, since NSAIDs block both insulin action (Reeds & Palmer, 1983) and insulin secretion (Jepson & Millward, 1989).

Although the hypertrophy of the skeletal muscles was unaffected by fenbufen it is interesting to note that the effects of clenbuterol on fibre type were inhibited (Table 4). The clenbuterol-induced change in fibre frequency, consistent with previous data (Maltin *et al.*, 1986) and resulting in a significant shift (+10%) towards more fast-twitch oxidative glycolytic fibres, was not observed when clenbuterol and fenbufen were fed in combination. Thus it appears that the clenbuterol-induced change in fibre frequency can be separated from fibre hypertrophy by an agent which inhibits cyclo-oxygenase. The patho-physiological basis for this change in fibre frequency is not clear. Clenbuterol may impair the normal developmental fibre type conversion (Zeman *et al.*, 1988); alternatively the drug may interact directly or indirectly to alter muscle contractile and metabolic properties (Maltin *et al.*, 1986). Thus it may be speculated that since the clenbuterol-induced change in fibre frequency is sensitive to fenbufen, metabolites of cyclo-oxygenase may be involved in the determination of fibre frequency.

Noticeably different from the clenbuterol-induced hypertrophy of skeletal muscle in the presence of fenbufen was the effect on cardiac muscle, in which the hypertrophy induced by clenbuterol was completely abolished by the NSAID. This contrasts with work in the perfused rat heart where both insulin and pressure overload stimulated protein synthesis and 4-biphenylacetic acid (the active metabolite of fenbufen) failed to inhibit the effect of either stimulus (Smith & Sugden, 1987). Thus previous published data suggest that cardiac muscle is less likely to be sensitive to prostaglandins than skeletal muscle and the work presented here surprisingly suggests that the opposite is true in the case of clenbuterol-induced hypertrophy.

However, the results of the present study with fenbufen are remarkably similar to those obtained by Reeds *et al.* (1988). In that study a complete inhibition of the clenbuterol-induced hypertrophy of the heart was achieved with the β-adrenoceptor antagonists, propranolol and atenolol, which, like fenbufen had no effect on skeletal muscle hypertrophy. It has been proposed that the effects on the heart result from the increased blood flow, tachycardia or stretch of the heart muscle, that these effects are β-adrenoceptor-mediated and that the action of clenbuterol on skeletal muscle is separate from the β-adrenoceptor-mediated fat loss and cardiac hypertrophy (Reeds *et al.*, 1988; Maltin *et al.*, 1989). These proposals are not disputed in the present study. Clearly, increased stretch and work load are likely to be involved in the effects on the heart and less likely to be involved in the hypertrophy of the skeletal muscles. Although no direct effect of prostaglandins in the action of β-agonists has been proposed previously, the involvement of prostaglandins in secondary, stretch-related effects of increased blood flow is perhaps not unexpected.

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