Action and interaction of growth hormone and the β-agonist, clenbuterol, on growth, body composition and protein turnover in dwarf mice

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The responses of dwarf mice to dietary administration of clenbuterol (3 mg/kg diet), daily injections of growth hormone (15 μg/mouse per d) or both treatments combined were investigated and their actions, and any interactions, on whole-body growth, composition and protein metabolism, and muscle, liver and heart growth and protein metabolism, were studied at days 0, 4 and 8 of treatment. Growth hormone, with or without clenbuterol, induced an increase in body-weight growth and tail length growth; clenbuterol alone did not affect body-weight or tail length. Both growth hormone and clenbuterol reduced the percentage of whole-body fat and increased the protein:fat ratio. They also increased protein synthesis rates of whole body and muscle, although the magnitude of the increase was greater in response to growth hormone than to clenbuterol. Clenbuterol specifically induced growth of muscle, with a decrease in liver protein content, whereas growth hormone exhibited more general anabolic effects on tissue protein. Previous reports have suggested that effects of clenbuterol on skeletal muscle are mediated, at least in part, via decreased rates of protein degradation; we could find little evidence of any decrease in whole-body or tissue protein degradation and anabolic effects were largely due to increases in protein synthesis rates. However, small increases in muscle protein degradation rate were observed in response to growth hormone. Growth hormone induced a progressive increase in serum insulin-like growth factor-1 concentration, whereas there was no change with clenbuterol administration. Anabolic effects on whole-body and skeletal muscle protein metabolism, therefore, appear to be initially via independent mechanisms but are finally mediated by a common response (increased protein synthesis) in dwarf mice.

Body composition: Protein turnover: Growth hormone: Clenbuterol

Growth hormone (GH) and the β_2-agonists, for example clenbuterol, both induce increases in whole-body lean tissue content whilst decreasing total fat content (Baker *et al.* 1984; Ricks *et al.* 1984; Hart & Johnsson, 1986); increases in rates of daily live-weight gain can also be observed, depending on the balance of muscle gain *v.* fat loss. These changes in growth rate and body composition are of benefit to the agricultural industry in terms of production efficiency and also to the consumer because lean meat is currently desirable, excessive dietary fat being perceived as detrimental to health.

The anabolic action of GH on skeletal muscle in vivo, is mediated primarily via an increase in protein synthesis rates (Pell & Bates, 1987; Bates & Holder, 1988); protein degradation remains unchanged or even tends to increase. However, the effects of β_2-agonists on muscle protein turnover are more equivocal. Initial reports suggested that muscle protein synthesis rates (Deshaies *et al.* 1981; Emery *et al.* 1984) and muscle RNA

contents, indicative of protein synthetic capacity (Beerman *et al.* 1987*b*; Maltin *et al.* 1987; Babij & Booth, 1988), were increased in β_2-agonist-treated animals. However, Reeds *et al.* (1986) and Bohorov *et al.* (1987) found no increase in muscle protein synthesis rates in rats and sheep respectively, and concluded that β_2-agonists must induce increases in muscle mass by decreasing rates of protein degradation. In addition, Williams *et al.* (1987*b*), Hovell *et al.* (1988) and MacRae *et al.* (1988) all suggested that clenbuterol-treated animals have decreased rates of whole-body protein degradation, although their findings are difficult to interpret as responses of muscle and non-muscle components cannot be isolated. Recently, Maltin *et al.* (1989) have suggested that transient increases in normal protein synthesis rates may occur in response to β-agonists. If the major actions of GH and of β_2-agonists on skeletal muscle are, respectively, to increase rates of protein synthesis and decrease rates of protein degradation, then treatment of adequately nourished animals with both agents should induce greater increases in net muscle growth than would occur with individual treatments. This hypothesis has been suggested by Williams *et al.* (1987*a*) but, in their study, little response to exogenous GH was observed.

Snell mice carry a recessive gene which causes dwarfism in the homozygote; they grow to only 25–30% of the adult size of their normal littermates. The defect is primarily in the anterior pituitary gland which releases minimal amounts of GH, thyroid hormones and prolactin into the circulation although concentrations of other anabolic hormones are normal (van Buul-Offers, 1983). In terms of muscle protein metabolism, this defect is expressed as reduced rates of protein synthesis, protein degradation being normal (Bates & Holder, 1988). The Snell dwarf mouse is, therefore, a sensitive model animal in which to study the anabolic action of GH and its relationship with other growth promoters. The objectives of the present study were to determine the individual and combined actions of GH and clenbuterol in Snell dwarf mice so that any interactions between these two anabolic agents could be assessed.

EXPERIMENTAL

Animals and diet

Eighty-eight Snell dwarf mice were bred from a colony at the Institute of Child Health, University of London. They were weaned at 4 weeks of age, after which time they were offered a powdered diet (250 g crude protein (nitrogen × 6·25)/kg) *ad lib.*, consisting of milk powder, wheat germ, powdered Spratts diet-1 and egg powder in the proportions 100:25:70:3 by weight; water was always available. When the mice were 8 weeks old they were randomly allocated to eleven treatment groups of eight mice (see pp. 116–117), and were housed at a temperature of 25° on a 12 h light – 12 h dark cycle.

Experimental design

At 9 weeks of age, one group of eight mice (day −4) were slaughtered by decapitation. Blood was collected from the neck, kept on ice until clotted and then centrifuged at 2000 ***g*** for 15 min; serum was removed and stored at −20°. Skeletal muscle (combined gastrocnemius/plantaris), liver and heart (ventricle) were dissected rapidly, cooled on ice, weighed and stored in liquid N_2. The gastrointestinal tract was dissected, the digesta were removed and the whole carcass plus empty gastrointestinal tract, was frozen and stored in liquid N_2.

The remaining eighty mice (ten cages) were weighed daily for 4 d to establish baseline body-weights and growth rates. The treatment groups (sixteen mice per group) were as follows: time-zero control, pituitary extract control, bovine GH (bGH) (15 μg/mouse per d), clenbuterol (3 mg/kg diet) and GH plus clenbuterol. GH was administered as a subcutaneous injection (0·1 ml bGH solution (150 μg/ml) in carbonate-buffered

(25 mmol/l, pH 9·4) saline (9 g sodium chloride/l) and mice not treated with GH received equivalent injections of vehicle.

The untreated zero controls were slaughtered before treatments were administered to the other mice. At 15 min before slaughter, they were each injected intraperitoneally with 5 μCi [^{3}H]phenylalanine ([2,6-^{3}H]phe; New England Nuclear, Stevenage, Herts) and 30 μmol unlabelled phenylalanine in a volume of 0·2 ml saline for the determination of protein synthesis rates (Bates & Holder, 1988). At slaughter, tissues were dissected, weighed and stored as described previously.

The remaining four groups were injected with [^{3}H]phenylalanine and slaughtered as described previously, on days 4 and 8 of treatment; four mice were killed from each cage on day 4 and the remaining mice were regrouped into four cages. The amounts of feed offered and refused were determined daily so that the feed intake per cage could be calculated throughout the study.

Tissue processing and chemical analysis

Whole-body composition. Carcasses (whole body including empty gastrointestinal tract but excluding dissected liver, heart and gastrocnemius/plantaris muscle) were homogenized in 4 vol. water using a Polytron homogenizer (Northern Media Supplies, Hessle, North Humberside). A small portion (2 ml) was removed and used for the determination of whole-body protein synthesis rates. The remainder was evaporated to dryness at 90° and analysed for fat and protein contents. Whole-body fat was determined directly via chloroform–methanol (2:1, v/v) extraction using Soxhlet cellulose thimbles (Whatman, Maidstone, Kent). Whole-body protein content was estimated on samples of the fat-free dried carcass from the N content (protein being N × 6·25) measured on an automated Kjeldahl N analyser (Kheltec 1300; Tecator Instruments, Bristol, Avon).

Protein synthesis, protein and RNA contents. Muscle, liver and heart samples were homogenized in ice-cold trichloroacetic acid (100 g/l) and centrifuged at 2800 ***g*** for 20 min. Trichloroacetic acid-soluble phenylalanine specific radioactivity (S_I) was measured by the enzymic-fluorimetric method of Garlick *et al.* (1980). The trichloroacetic acid-precipitated pellets were treated as in Garlick *et al.* (1980) to determine protein-bound phenylalanine specific radioactivity (S_B), protein concentration, by the method of Lowry *et al.* (1951), and RNA concentration (Millward *et al.* 1974).

Insulin-like growth facor-1 (IGF-1). Serum IGF-1 concentrations were determined in acid/ethanol-extracted serum samples (Daughaday *et al.* 1982) using [^{125}I]IGF-1 and polyclonal antiserum supplied by the Institute of Child Health, University of London.

Calculations

Protein synthesis. Whole-body and tissue fractional protein synthesis rates (k_s) were calculated as a percentage of the total protein pool/d as in Bates & Holder (1988).

Fractional growth and degradation rates. Whole-body and tissue growth rates for the protein pools were calculated over 4-d periods. The protein content of the tissues was calculated as a proportion of whole-body weight for each treatment group (where appropriate) for days −4, 0 and +4 from their protein concentrations and tissue weights at slaughter. Since the body-weight of the mice had a low variability at day 0 and within groups, these proportions could be used to predict the initial tissue protein contents for animals killed on days 0, +4 and +8 respectively. The gain in tissue protein content for each mouse was calculated from its measured protein content at slaughter minus its calculated protein content 4 d previously; the fractional rate of protein deposition (k_g) could then be calculated for their slaughter time-points. As emphasized by Reeds *et al.* (1986), this approach uses body-weight as a base measurement but does not use rates of

weight gain for the calculation. Protein degradation rates (k_d) were calculated from the differences between k_s and k_g.

Statistics

Statistical differences between treatment groups were assessed using analysis of variance with treatment effects split into a factorial design. Hence, overall effects of GH and clenbuterol, as well as their interaction, could be identified. In addition, the effects of length of exposure to each treatment (4 or 8 d) and its interaction with individual and combined treatments was determined. The full analysis of the variance therefore had fifty-six residual degrees of freedom. Where appropriate, the day 0 weights of the mice were used as a covariate.

RESULTS

Whole-body growth, composition and protein metabolism and feed intake

Table 1 shows the initial and final body-weights and body-weight growth rates for control and treated mice at days 0, 4 and 8 of the study. Mice treated with clenbuterol alone had similar final body-weights to those of the controls, whereas there was a significant increase in the final body-weight of all GH-treated mice ($P < 0{\cdot}001$), whether alone or in combination with clenbuterol. GH also induced a significant ($P < 0{\cdot}01$) increase in tail length growth over the treatment period, which clenbuterol did not (mm/8 d: control 0·125, clenbuterol 0·138, GH 0·325, clenbuterol plus GH 0·286; standard error of difference (SED) 0·042, n 8 (per group)).

Average daily feed intakes could only be determined for each cage of eight mice. Feed intake (g/mouse per d) was increased ($P < 0{\cdot}05$) in all GH-treated mice (control 1·11, clenbuterol 1·07, GH 1·22, GH plus clenbuterol 1·29; SED 0·051, n 8 (mean observations per treatment)). Clenbuterol alone had no overall effect on feed intake, except on day 1 of treatment when intake appeared to be depressed to 0·99 g/mouse per d. The average dose rate of clenbuterol was, therefore, calculated to be 3·2 μg/mouse per d for mice treated with clenbuterol alone and 3·8 μg/mouse per d for those treated with GH plus clenbuterol; on a per unit body-weight basis these dose rates were not statistically different.

Whole-body fat and protein contents are presented in Table 2. Treatment of mice with clenbuterol alone induced a significant decrease in fat content ($P < 0{\cdot}05$). When both time-points (day 4 and day 8) were considered together, clenbuterol had no significant effect on whole-body protein content. However, more detailed examination of treatment and time effects demonstrated that there was an increase in absolute protein content between days 4 and 8. Clenbuterol, therefore, induced an overall increase ($P < 0{\cdot}05$) in the protein:fat ratio from 0·415 for control to 0·466 in treated mice. A further increase in overall protein:fat ratio was observed in GH-treated mice (mean 0·548, $P < 0{\cdot}001$ compared with controls) and this was reflected by the significant decrease in percentage fat ($P < 0{\cdot}001$) and increase in protein ($P < 0{\cdot}001$) contents. Carcass water contents were significantly increased in all GH-treated mice ($P < 0{\cdot}01$) but not in mice treated with clenbuterol alone (control 59·9, clenbuterol 60·9, GH 62·6, GH plus clenbuterol 63·3% final body-weights; SED 1·1, n 16 (per group)). No interactions of combined GH and clenbuterol treatments were observed for any aspect of carcass composition.

Whole-body protein turnover is also presented in Table 2. Growth rate of the whole-body protein pool (k_g) was significantly increased by GH but not by clenbuterol treatment. However, this overall GH-induced increase was dependent on treatment time, being increased over 3-fold at day 4 but actually decreased on day 8. Whole-body protein synthesis rates (k_s) were increased by clenbuterol ($P < 0{\cdot}05$) and GH ($P < 0{\cdot}001$) when both days 4 and 8 were considered together. Total RNA concentrations were increased in GH-treated but not in clenbuterol-treated mice, illustrated by the changes in RNA:protein ratios. RNA activity (k_{RNA}, g protein synthesized/g RNA per d) increased significantly in

Table 1. *Whole-body-weights and growth rates of dwarf mice killed on days 0, 4 or 8 following treatment with dietary clenbuterol (Clen) or injected growth hormone (GH), or both*†

(Day 0 values are means with their standard errors in parentheses (n 16); all other values are group means (n 8 (per group)) with the pooled standard error of difference (SED). Initial body-weights are the weights on day 0 of the mice killed on the relevant day)

	Treatment					Main effects			
	Control	Clen	GH	Clen + GH	SED	Clen	GH	Clen × GH	Day
Initial body-wt (g)									
Day 0	7·74 (0·16)	—	—	—	0·28	NS	NS	NS	NS
Day 4	7·64	7·83	7·61	7·55					
Day 8	7·93	7·84	7·80	7·99					
Final body-wt (g)									
Day 0	—	—	—	—	0·14	NS	***	NS	***
Day 4	8·22	7·98	8·88	8·67					
Day 8	8·34	8·40	9·46	9·49					
Body-wt growth (%/d)									
Day 0	2·08 (0·30)	—	—	—	0·34	*	***	NS	***
Day 4	1·81	0·40	3·63	3·21					
Day 8	0·57	0·80	1·67	1·88					

NS, not significant.
* $P < 0·05$, *** $P < 0·005$.
† For details of treatments, see pp. 116–117.

all GH-treated mice ($P < 0·01$) at both day 4 and day 8 but only tended to increase in mice given clenbuterol due to a response at day 4. Thus, GH induced increases in protein synthesis rate by increasing both protein synthetic capacity (illustrated by RNA:protein) and also the efficiency of protein synthesis (k_{RNA}) whereas clenbuterol only tended to increase RNA activity. When the entire treatment period was considered, neither GH nor clenbuterol induced any changes in protein degradation rates (k_d); however, on day 8, all mice treated with GH exhibited a significant increase.

Muscle (combined gastrocnemius plus plantaris) weights and protein metabolism

Both GH ($P < 0·001$) and clenbuterol ($P < 0·01$) induced significant increases in gastrocnemius plus plantaris weight (Table 3) and these increases were additive in mice given clenbuterol plus GH when the entire treatment period was considered. However, only mice treated with clenbuterol or clenbuterol plus GH exhibited a significant increase ($P < 0·001$) in muscle weight expressed as a percentage of final body-weight. Muscle total protein content was also significantly increased for clenbuterol- ($P < 0·001$) and GH-treated ($P < 0·01$) mice. Surprisingly, the clenbuterol-induced increase in protein concentration was restored to control values when in combination with GH (mg/g muscle: control 152·8, clenbuterol 157·1, GH 149·2, clenbuterol plus GH 149·7; SED 3·2, n 16 (per group)). Rates of protein synthesis (k_s) were significantly ($P < 0·001$) increased in both GH- and clenbuterol-treated mice; these increases tended to be greater on day 4 than on day 8 of treatment but were almost additive for the mice receiving the combined treatments at both time-points. Protein synthetic capacity, implied by RNA:protein ratio, was also significantly ($P < 0·001$) increased by GH and clenbuterol. In GH-treated mice RNA activity was elevated ($P < 0·05$) on day 4 of treatment but not on day 8, such that no overall mean increase was observed. Therefore, the increases in muscle protein synthesis rates appear to be mediated largely by increases in the capacity for protein synthesis in clenbuterol- and GH-treated mice with a transient increase in translational efficiency also occurring in GH-treated mice. Calculated rates of protein degradation were significantly

Table 2. *Fat, protein and RNA content and rates of protein synthesis* (k_s), *growth* (k_g), *degradation* (k_d) *and RNA activity* (k_{RNA}) *in the whole body (including empty gastrointestinal tract but minus liver, heart and gastrocnemius/plantaris muscle) of dwarf mice killed on days 0, 4 or 8 following treatment with clenbuterol (Clen) or growth hormone (GH), or both*†

(Day 0 values are means with their standard errors in parentheses; values for days 4 and 8 are group means with the pooled standard error of the difference (SED))

	Treatment					Main effects			
	Control	Clen	GH	Clen + GH	SED	Clen	GH	Clen × GH	Day
Fat (mg)									
Day 0	1949 (93)	—	—	—					
Day 4	2019	1828	1883	1513	145	*	NS	NS	***
Day 8	2123	1996	2111	2120					
Fat (g/kg)									
Day 0	247 (8)	—	—	—					
Day 4	246	229	209	175	16	*	***	NS	*
Day 8	254	238	223	223					
Protein (mg)									
Day 0	784 (15)	—	—	—					
Day 4	829	832	1030	1044	38	NS	***	NS	*
Day 8	868	919	1054	1060					
Protein (g/kg)									
Day 0	99·6 (1·5)	—	—	—					
Day 4	101·0	104·5	114·1	120·4	4·4	NS	***	NS	NS
Day 8	104·1	109·4	111·4	111·8					
RNA:protein ($\times 10^{-3}$)									
Day 0	33·9 (0·5)	—	—	—					
Day 4	32·2	31·9	34·7	32·6	1·3	NS	**	NS	*
Day 8	31·0	31·3	32·9	31·9					
k_s (%/d)									
Day 0	20·0 (0·4)	—	—	—					
Day 4	21·3	24·0	24·3	24·9	1·0	*	***	NS	***
Day 8	19·8	20·3	22·4	23·4					
k_g (%/d)									
Day 0	2·20 (0·41)	—	—	—					
Day 4	1·45	1·60	6·20	6·38	0·89	NS	***	NS	***
Day 8	1·24	2·23	0·48	0·17					
k_d (%/d)									
Day 0	17·8 (0·7)	—	—	—					
Day 4	19·8	22·2	18·1	18·5	1·27	NS	NS	NS	NS
Day 8	18·6	18·1	21·9	23·3					
k_{RNA} (g protein/g RNA per d)									
Day 0	6·01 (0·17)	—	—	—					
Day 4	6·72	7·49	7·04	7·71	0·46	NS	***	NS	*
Day 8	6·41	6·52	6·85	7·37					

NS, not significant.
* $P < 0·05$, ** $P < 0·01$, *** $P < 0·005$.
† For details of treatments, see pp. 116–117.

increased ($P < 0·001$) in all GH-treated mice but not in mice administered clenbuterol alone. As with whole-body protein metabolism, no evidence for any interaction between GH and clenbuterol was found in mice treated with both agents.

Liver weight and protein synthesis

Clenbuterol treatment induced a significant ($P < 0·001$) overall decrease in liver weight (Table 4) whether expressed in absolute terms or as a percentage of whole body-weight; this

Table 3. *Skeletal muscle (combined gastrocnemius and plantaris) composition and rates of protein synthesis* (k_s), *growth* (k_g), *degradation* (k_d) *and RNA activity* (k_{RNA}) *of dwarf mice killed on days 0, 4 or 8 following treatment with clenbuterol (Clen) or growth hormone (GH), or both*†

(Day 0 values are means with their standard errors in parentheses; values for days 4 and 8 are group means with the pooled standard error of the difference (SED))

	Treatment					Main effects			
	Control	Clen	GH	Clen + GH	SED	Clen	GH	Clen × GH	Day
Wt (mg)									
Day 0	60·9 (0·9)	—	—	—					
Day 4	66·7	68·6	72·2	75·8	2·3	**	***	NS	***
Day 8	72·0	77·2	79·9	83·4					
Wt (g/kg)									
Day 0	7·84 (0·31)	—	—	—					
Day 4	8·08	8·58	8·11	8·73	0·26	***	NS	NS	**
Day 8	8·52	9·15	8·47	8·79					
Protein content (mg)									
Day 0	10·10 (0·53)	—	—	—					
Day 4	10·26	10·81	10·85	11·20	0·42	***	**	NS	***
Day 8	11·01	12·13	11·87	12·76					
RNA: protein ($\times 10^{-3}$)									
Day 0	6·44 (0·22)	—	—	—					
Day 4	6·62	6·88	7·67	8·19	0·30	***	***	NS	**
Day 8	6·74	7·40	8·33	8·91					
k_s (%/d)									
Day 0	6·83 (0·34)	—	—	—					
Day 4	6·72	8·22	9·28	10·56	0·52	***	***	NS	NS
Day 8	7·02	8·02	8·85	9·54					
k_g (%/d)									
Day 0	1·09 (0·16)	—	—	—					
Day 4	0·28	1·61	1·60	2·35	0·64	***	***	NS	NS
Day 8	1·13	2·43	2·24	2·39					
k_d (%/d)									
Day 0	5·67 (0·35)	—	—	—					
Day 4	6·44	6·61	7·67	8·21	0·68	NS	***	NS	NS
Day 8	5·89	5·60	6·61	7·15					
k_{RNA} (g protein/g RNA per d)									
Day 0	10·71 (0·58)	—	—	—					
Day 4	10·23	11·97	12·17	12·51	0·75	NS	NS	NS	**
Day 8	10·42	10·75	10·65	10·83					

NS, not significant.
** $P < 0·01$, *** $P < 0·005$.
† For details of treatments, see pp. 116–117.

was due to a marked decrease on day 4 rather than on day 8. GH-treated mice had significantly heavier livers ($P < 0·001$). Liver protein content was also significantly decreased ($P < 0·01$) for clenbuterol-treated mice and increased ($P < 0·001$) for GH-treated mice. Total (fixed plus export) hepatic protein synthesis rates were significantly increased ($P < 0·001$) in all mice treated with GH. Clenbuterol appeared to have little effect on liver protein synthesis and this was supported by the unchanged RNA:protein, on days 4 and 8, and RNA activity, on day 8, in clenbuterol-treated mice. GH induced significant increases in both RNA:protein and RNA activity. Thus, from these findings, clenbuterol seems to exert minimal effects on hepatic protein metabolism whereas GH induces an overall increase in total protein synthesis rates. Export protein synthesis could not be

Table 4. *Liver composition and rates of protein synthesis* (k_s) *and RNA activity* (k_{RNA}) *of dwarf mice killed on days 0, 4 or 8 following treatment with clenbuterol (Clen) or growth hormone (GH), or both* †

(Day 0 values are means with their standard errors in parentheses; values for days 4 and 8 are group means with the pooled standard error of the difference (SED))

	Treatment					Main effects			
	Control	Clen	GH	Clen+GH	SED	Clen	GH	Clen × GH	Day
Wt (mg)									
Day 0	312 (9)	—	—	—					
Day 4	340	298	390	353	13	***	***	NS	***
Day 8	346	345	424	401					
Wt (g/kg)									
Day 0	40·4 (0·6)	—	—	—					
Day 4	41·3	37·3	43·9	40·4	1·2	***	***	NS	**
Day 8	41·4	41·1	44·8	42·3					
Protein content (mg)									
Day 0	61·4 (2·1)	—	—	—					
Day 4	63·80	57·61	70·17	63·47	2·46	**	***	NS	***
Day 8	68·12	67·47	78·85	78·01					
RNA:protein ($\times 10^{-3}$)									
Day 0	40·66 (0·52)	—	—	—					
Day 4	41·50	41·15	45·51	46·56	0·87	NS	***	NS	**
Day 8	42·35	41·69	47·11	47·61					
k_s (%/d)									
Day 0	56·0 (1·1)	—	—	—					
Day 4	57·2	63·3	76·8	79·3	3·3	NS	***	NS	NS
Day 8	57·3	57·3	75·4	75·4					
k_{RNA} (g protein/g RNA per d)									
Day 0	13·8 (0·2)	—	—	—					
Day 4	13·8	15·4	16·8	17·0	0·60	NS	***	NS	**
Day 8	13·5	13·7	16·0	15·8					

NS, not significant.
** $P < 0·01$, *** $P < 0·005$.
† For details of treatments, see pp. 116–117.

quantified separately in the present experiment and therefore net growth of the total hepatic protein pool and protein degradation rates were not calculated.

Heart (ventricle) weight and protein metabolism

Both clenbuterol and GH induced a significant increase ($P < 0·001$) in absolute heart ventricle weight but this was only increased in proportion to final body-weight for clenbuterol-treated mice (Table 5). Heart protein content was also increased by both clenbuterol ($P < 0·01$) and GH ($P < 0·001$) treatments. Over the entire treatment period, clenbuterol did not induce a statistical change in growth rate whereas GH did ($P < 0·001$). Protein synthesis rates were increased in response to both GH and clenbuterol and these increases were sustained until day 8. Calculated rates of protein degradation were consistently increased in GH-treated and clenbuterol plus GH-treated mice on both day 4 and day 8 but were only increased on day 8 in the mice treated with clenbuterol alone. The only statistically significant interaction between combined treatments of GH and clenbuterol was observed for heart ventricle k_d. This is difficult to interpret since their combined treatments were more than additive on day 4 and less than additive on day 8.

Table 5. *Heart (ventricles) composition and rates of protein synthesis* (k_s), *growth* (k_g), *degradation* (k_d) *and RNA activity* (k_{RNA}) *of dwarf mice killed on days 0, 4 or 8 following treatment with clenbuterol (Clen) or growth hormone (GH), or both* †

(Day 0 values are means with their standard errors in parentheses; values for days 4 and 8 are group means with the pooled standard error of the difference (SED))

	Treatment					Main effects			
	Control	Clen	GH	Clen + GH	SED	Clen	GH	Clen × GH	Day
Wt (mg)									
Day 0	23·6 (0·7)	—	—	—	1·1	***	***	NS	**
Day 4	24·8	26·6	27·3	28·8					
Day 8	26·0	27·9	29·4	31·8					
Wt (g/kg)									
Day 0	3·05 (0·09)	—	—	—	0·12	***	NS	NS	NS
Day 4	3·01	3·36	3·07	3·35					
Day 8	3·11	3·32	3·11	3·35					
Protein content (mg)									
Day 0	3·77 (0·10)	—	—	—	0·16	**	***	NS	***
Day 4	4·00	4·42	4·31	4·40					
Day 8	4·23	4·43	4·60	4·94					
RNA : protein ($\times 10^{-3}$)									
Day 0	28·02 (0·41)	—	—	—	0·70	NS	***	NS	***
Day 4	28·18	27·32	29·25	30·31					
Day 8	27·20	26·44	27·72	27·29					
k_s (%/d)									
Day 0	12·08 (0·57)	—	—	—	0·64	***	***	NS	NS
Day 4	11·49	12·62	14·82	16·32					
Day 8	12·38	14·58	15·08	14·88					
k_g (%/d)									
Day 0	1·19 (0·19)	—	—	—	0·62	NS	***	NS	***
Day 4	1·33	3·11	3·13	3·54					
Day 8	1·62	0·59	1·77	2·27					
k_d (%/d)									
Day 0	10·92 (0·53)	—	—	—	0·90	NS	**	**	***
Day 4	10·16	9·51	11·69	12·79					
Day 8	10·76	13·99	13·25	12·61					
k_{RNA} (g protein/g RNA per d)									
Day 0	4·39 (0·23)	—	—	—	0·25	***	***	NS	***
Day 4	4·08	4·70	5·08	5·35					
Day 8	4·58	5·51	5·45	5·45					

NS, not significant.
** $P < 0·01$, *** $P < 0·005$.
† For details of treatments, see pp. 116–117.

Serum IGF-1 concentrations

Fig. 1. shows the serum IGF-1 concentrations for days −4, 0, 4 and 8. As expected, untreated dwarf mice have very low concentrations of IGF-1 and these were unchanged in mice treated with only clenbuterol. GH-treatment, whether alone or in combination with clenbuterol, induced a significant ($P < 0·001$) time-dependent ($P < 0·001$) increase in IGF-1 concentrations. No interaction between GH and clenbuterol on IGF-1 concentrations was observed.

Effects of treatment time on responsiveness to GH and clenbuterol

The mice in the present study were slaughtered after 4 or 8 d of treatment and, therefore, some assessment could be made of temporal responsiveness to GH and clenbuterol.

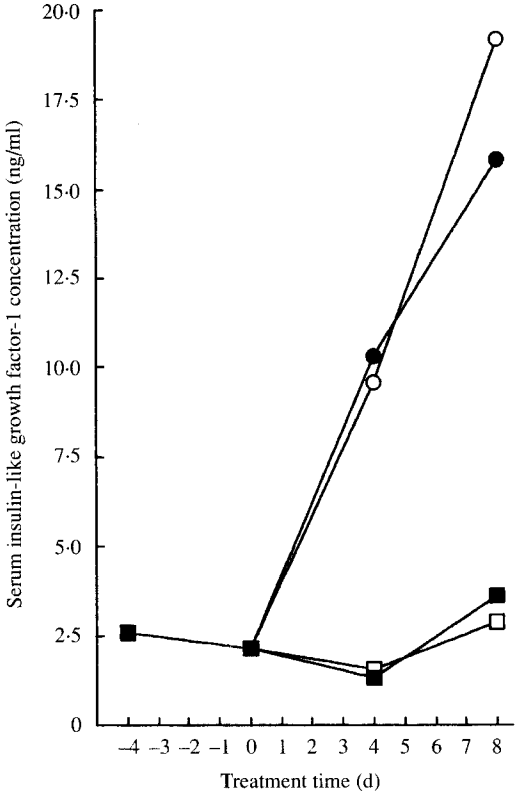

Fig. 1. Serum insulin-like growth factor-1 concentrations of dwarf mice killed on days −4, 0, 4 or 8 following treatment with saline (9 g sodium chloride/l) (□), clenbuterol (■), growth hormone (○) or both clenbuterol and growth hormone (●). Values are means from groups of eight mice and the pooled standard error of the difference was 2·22.

Generally, the magnitude of the response was greater on day 4 than on day 8 in GH-treated, but not always in clenbuterol-treated, mice. This phenomenon was especially apparent for whole-body actions of GH so that increases in whole-body growth and protein content and decreases in whole-body fat content were much greater between days 0 and 4 than between days 4 and 8. It also occurred for both clenbuterol- and GH-treated mice for whole-body protein synthesis rate and RNA activity and for various tissue responses such as muscle protein synthesis and heart protein growth rates. The change in responsiveness with treatment time was sufficiently extreme in some cases to produce mean treatment responses for the entire period of study which were different from the individual responses on days 4 or 8; for example, whole-body protein content was significantly increased on day 8 in clenbuterol-treated mice but was statistically unchanged when the entire treatment period was considered. It is, therefore, important to be aware of treatment time when overall interpretations are made concerning GH or clenbuterol action. Unfortunately, since only two time-points were investigated here, limited conclusions can be made on temporal responses to either anabolic agent.

A further necessary consideration is the methodology for the determination of protein synthesis and degradation rates. Protein synthesis was measured directly over a period of 15 min only, on days 0, 4 and 8 of treatment. However, protein degradation was calculated from mean growth rates which occurred between days 0 to 4 and 4 to 8 and the 15 min

'point' determinations of protein synthesis. It is possible that neither protein synthesis or degradation are linear over these 4-d periods and that equivalent physiological situations may not have been compared; transient changes in net protein growth, protein synthesis or protein degradation could have been missed. Therefore, even though no evidence was found for a decrease in muscle protein degradation rates in the present study this could have occurred if decreases were not great enough to influence mean net protein growth sufficiently.

DISCUSSION

Use of the dwarf mouse to investigate the control of protein metabolism

The dwarf mouse (Snell, 1929) has an autosomal recessive mutation resulting in an absence of anterior pituitary acidophil and thyrotroph cells producing low circulating concentrations of thyroid hormones, prolactin and GH and, therefore, of IGF-1. These hormonal deficiencies influence skeletal muscle development; the appearance of the adult fast-type myosin chain is retarded and there is an increase in the number of fibres containing the slow-type heavy chain (Butler-Browne *et al.* 1987). Therefore, dwarf mouse skeletal muscle will contain a slightly higher proportion of slow, oxidative fibres and a lower proportion of fast-twitch fibres than those of normal littermates. As far as protein turnover is concerned, dwarf mice exhibit reduced rates of muscle protein synthesis but normal rates of protein degradation; heart synthetic rates are normal but liver protein synthesis is decreased (Bates & Holder, 1988). The reduced rates of protein synthesis in skeletal muscle are probably a result of low circulating hormone concentrations rather than changes in fibre type because their response to exogenous hormone is rapid (Bates & Holder, 1988).

Denervation-induced muscle atrophy has been used as a model for the study of β-agonist action (Maltin *et al.* 1986*b*, 1987, 1989) the decreased muscle protein content being due to the loss of muscle stretch. Denervation makes muscle more sensitive to β-agonist action because the β_2-adrenergic receptor population in muscle fibres and their associated blood vessels will be up-regulated (Lavenstein *et al.* 1979). As far as protein turnover is concerned, denervation appears to increase protein degradation with the response of protein synthesis being dependent on muscle type (Maltin *et al.* 1989).

Responses of whole-body composition and protein metabolism to clenbuterol and GH

Both clenbuterol and GH repartitioned body composition away from fat accumulation and towards lean tissue deposition in dwarf mice, but the way in which this was achieved was strikingly different. Clenbuterol appeared to specifically change tissue proportions only, illustrated by the unchanged rate of whole-body weight gain of treated mice compared with controls. This response must be, in part, related to food intake; MacRae *et al.* (1988) have summarized current findings and have concluded that in experiments where voluntary food intake has not increased, the major effect of clenbuterol is on fat:protein ratios. However, when food intake has increased, fat deposition is not inhibited but rather protein growth is enhanced. In contrast, GH stimulated whole-body-weight gain significantly and, even though it did not inhibit absolute fat deposition, it must have stimulated growth of other tissues besides muscle, particularly bone growth. This was illustrated by the significant GH-stimulated increase in tail length, which is correlated with cartilage growth in vivo (Holder *et al.* 1982).

The effect of GH on tissue protein turnover

Most studies on the action of GH on protein metabolism have reported a stimulation of muscle protein synthesis (Flaim *et al.* 1978; Albertsson-Wikland *et al.* 1980; Pell & Bates, 1987; Bates & Holder, 1988). Bates & Holder (1988) have discussed this, as well as the GH-induced increase in liver and heart synthetic rates, in some detail for dwarf mice. The

response of skeletal muscle protein degradation to GH depends on the physiological state of the muscle but, in vivo, degradation tends to increase or is unchanged (Pell & Bates, 1987; Bates & Holder, 1988). Serum IGF-1 concentrations increased in all mice receiving GH. It is not yet known whether the stimulatory effect of GH on protein metabolism is direct or via IGF-1 or some other growth factor. However, exogenous IGF-1 can increase muscle, but not liver, protein synthesis in dwarf mice (Pell & Bates, 1989) and therefore GH action must be different in these two tissues. Little is known of the effects of GH on muscle fibre types; Beerman *et al.* (1987*a*) reported that GH did not change fibre composition in the semitendinosus muscle of lambs and also suggested that muscle growth appeared to be via satellite cell proliferation rather than the true hypertrophy which is induced by clenbuterol. Hypophysectomy reduced the proportion of slow-twitch fibres in the soleus and extensor digitorum muscles of rats (Ayling *et al.* 1989) but this could be reversed by administration of human GH. It is possible that GH has a more stimulatory effect on slow-twitch fibres, since it induced a greater increase in protein synthetic rate in the biceps femoris than in the semitendinosus muscle of lambs (Pell & Bates, 1987).

The liver has the highest concentration of GH receptors in the body (Wallis, 1980) and, therefore, it is not surprising that GH stimulates hepatic protein synthesis rates. Since both fixed and export protein synthesis were measured simultaneously it is not possible to distinguish whether a greater increase occurred in one or the other.

The effect of clenbuterol on tissue protein turnover

Even though clenbuterol consistently induces an increase in whole-body lean tissue mass, and particularly of muscle, its mechanism of action on protein turnover in normal muscle is still equivocal. In a thorough examination of muscle responses in rats, Reeds *et al.* (1986) could find no evidence for increased rates of protein synthesis and concluded that its anabolic effects must be mediated primarily by a decreased rate of protein degradation, which was supported by several other studies (Bohorov *et al.* 1987; Williams *et al.* 1987*b*; Higgins *et al.* 1988; Wang & Beerman, 1988). However, MacRae *et al.* (1988) did obtain a transient increase in whole-body synthetic rate after 11 d of dietary clenbuterol and Emery *et al.* (1984) obtained a 34% increase in muscle synthetic rate in rats injected daily with clenbuterol. Eisemann *et al.* (1988) observed an increase in α-amino-N uptake by the hindquarters of clenbuterol-treated steers and concluded that an increase in synthetic rate could occur, even though increased N retention could be due to changes in protein degradation. Additionally, even though Reeds *et al.* (1986) could not detect changes in muscle protein synthesis, they did record increases in RNA content and, in later studies by this group, transient but consistent increases occurred in denervated muscle and in some innervated muscle types (Maltin *et al.* 1987, 1989).

Further insight into the mechanism of action of clenbuterol on muscle may be obtained when different fibre types are considered. Williams *et al.* (1984) have reported that muscle β_2-agonist receptor number is correlated with oxidative capacity, the soleus having a greater binding capacity than the gastrocnemius muscle in sedentary rats. However, this finding cannot easily be correlated with responsiveness to clenbuterol in terms of muscle hypertrophy; increases in cross-sectional area have been observed for both type I slow- (Maltin *et al.* 1986*a*; Beerman *et al.* 1987*b*) and type II fast- (Maltin *et al.* 1986*a*; Beerman *et al.* 1987*b*; Zeman *et al.* 1988) twitch fibres with no consistent trends in the relative responsiveness of either fibre type. In addition, there appears to be little correlation between muscle type and protein synthetic response to clenbuterol in innervated muscle, although not all muscle types were examined in a single study (Maltin *et al.* 1987, 1989). It would appear that one of the responses of muscle to clenbuterol may be a change in fibre type characteristics; in both soleus and extensor digitorum longus in rats (Maltin *et al.*

1986*a*, 1987; Zeman *et al.* 1988) and longissimus dorsi and semitendinosus muscles in sheep (Beerman *et al.* 1987*b*; Kim *et al.* 1987) there are significant alterations towards a predominance of, and hypertrophy of, type II fibres. Fast-twitch fibres have only about 50% of the protein synthetic rate of slow-twitch fibres (Laurent *et al.* 1978; Bates & Millward, 1983) and, therefore, depending on the exact time-course of the switch in fibre type, this could explain why sustained increases in protein synthetic rate are not observed in normal clenbuterol-treated animals. It could also explain the sustained increase in synthesis observed in the present study, because dwarf mice have an impaired ability to reduce their proportion of slow-twitch fibres (Butler-Brown *et al.* 1987).

It is not yet clear whether β-agonists manipulate protein metabolism directly, or if they act via some secondary factor. It is unlikely that β_2-agonists interact with the circulating GH or IGF-1 since they are effective in the dwarf mice of the current study and in hypophysectomized rats (James & Barker, 1987; Thiel *et al.* 1987), although this does not preclude the possibility of some locally produced autocrine or paracrine factors. There is evidence to suggest that clenbuterol action may be mediated, at least in part, via an increased muscle sensitivity to insulin. Administration of β_2-agonists to diabetic mice induces decreased glucose and increased insulin concentrations (Carroll *et al.* 1985). Also, treatment of rats with adrenaline can induce increased binding of insulin to muscle sarcolemmal membrane preparations (Webster *et al.* 1986). In lambs fed on cimaterol, insulin concentrations decrease (Beerman *et al.* 1987*b*); unfortunately insufficient serum could be obtained from the mice in the current study to perform insulin and glucose assays. Since insulin has a primary role in the control of protein synthesis (Millward *et al.* 1983), clenbuterol and other hormones, for example corticosterone (Odedra *et al.* 1982), may change insulin sensitivity.

Clenbuterol action appears to be tissue specific; it exerted little effect on total hepatic protein synthesis rates even though liver weight and protein content were decreased. This decline in liver weight has also been reported by Reeds *et al.* (1986), Williams *et al.* (1987*b*) and Sainz & Wolff (1988), although the mechanism by which it occurs is unknown. Clenbuterol treatment had an apparent anabolic effect on the heart; however, to what extent this is due to its direct actions or to work-induced hypertrophy following tachycardia is difficult to assess. Williams (1987) has discussed the actions of clenbuterol in relation to tissue specificity and concluded that any cardiac muscular action is apparently of short duration.

Conclusions

This investigation has demonstrated that the anabolic effects of GH and clenbuterol on muscle and whole-body protein metabolism may be mediated primarily via increased protein synthesis rates. Since treatment was only for up to 8 d, it is difficult to assess how long the increase would have been sustained; it may be that synthetic rate decreases to control values after several weeks of treatment. In addition, no evidence could be found for the proposal that β_2-agonists induce increased muscle growth via a decrease in protein degradation.

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