Effect of the β -adrenoceptor agonist clenbuterol and phytohaemagglutinin on growth, protein synthesis and polyamine metabolism of tissues of the rat

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1 The kidney bean lectin, phytohaemagglutinin (PHA), induced a marked atrophy of skeletal muscle which was evident from the changes in tissue composition (protein, RNA, DNA and polyamine content) and from the reduction in weight and protein synthesis of hind leg muscles of rats fed on kidney bean-diets for four days. The β -adrenoceptor agonist, clenbuterol, induced skeletal muscle hypertrophy by transiently stimulating protein synthesis. As a consequence, the muscle loss caused by a short exposure to PHA was, in part, ameliorated by clenbuterol treatment.

2 Cardiac muscle was affected to a lesser extent than skeletal muscle by both clenbuterol and the lectin. However, there was evidence that protein synthesis in heart was reduced by PHA.

3 PHA had opposite effects on the gut, the lectin-induced hyperplasia of the jejunum was accompanied by a large increase in protein synthesis. Clenbuterol alone had no effect on the jejunum whereas a combination of PHA and clenbuterol appeared to exacerbate the effect of the lectin on gut.

4 Both the lectin-induced gut growth and the hypertrophy of skeletal muscle caused by clenbuterol were preceded by the accumulation of polyamines in the respective tissues. Of particular note was the observation that a significant increase in the proportion of the intraperitoneally injected ¹⁴C-labelled spermidine or putrescine taken up by the growing tissues could be detected by the second day. Therefore, the measurement of uptake of labelled polyamines may be used as a sensitive indicator of early alterations in tissue metabolism.

Keywords: Clenbuterol; phytohaemagglutinin (PHA); protein synthesis; small intestine; skeletal muscle; heart; liver; polyamines; growth; atrophy

Introduction

The lectin component of raw kidney bean, the phytohaemagglutinin (PHA) is a potent growth factor for the rat small intestine and induces extensive hyperplastic and hypertrophic growth of the tissue (Oliveira *et al.*, 1988; Pusztai *et al.*, 1988). The basis of this striking physiological activity of PHA is its resistance to proteolytic degradation and consequent binding to the small intestinal epithelium. The lectin survives in an immunologically intact form nearly completely on passage through the entire digestive system (Pusztai *et al.*, 1991a), particularly when bound to the complex carbohydrate moieties of receptors of the luminal membranes of gut epithelial cells (Pusztai *et al.*, 1991b). Binding is followed by endocytosis (King *et al.*, 1986) and partial transcytosis (Pusztai *et al.*, 1991c). Only lectins that are bound and endocytosed by gut cells act as major stimulants of gut growth (Pusztai *et al.*, 1990).

The absorbed PHA induces major shifts in the hormonal balance of the body (Pusztai *et al.*, 1989; 1991a) and interferes with the metabolism of some tissues (Oliveira *et al.*, 1988). One of the most striking effects is the loss of about 30% of skeletal muscle tissue in rats fed with kidney bean diets for a week. Although the mechanism of this atrophy is not fully clear, the reduction of the fractional rate of protein synthesis in the skeletal muscle (Palmer *et al.*, 1987) might be the result of the lowering of the blood insulin levels (Pusztai *et al.*, 1991c).

The β -adrenoceptor agonist, clenbuterol, increases the mass and protein content of skeletal muscle in many species. It has been proposed that the hypertrophy in rodents was mediated by promoting muscle protein deposition, partly by decreasing protein degradation (Reeds *et al.*, 1986) and partly by a

transient rise in protein synthesis rate (Emery *et al.*, 1984; Maltin *et al.*, 1987). The effects of clenbuterol on tissues other than muscle and fat have not been extensively studied although a small reduction in liver weight after feeding rats on clenbuterol for 3 weeks has been reported (Reeds *et al.*, 1986), and another β -agonist, ractopamine, had a similar effect in pigs (Aalhus *et al.*, 1990). Reductions in the protein content of both liver and gut have also been reported in steers (Williams *et al.*, 1987). The mechanism of action of clenbuterol is not known; its effectiveness in diabetic rats suggests that insulin may not be involved (McElligott *et al.*, 1987) but others have shown changes in insulin binding (Webster *et al.*, 1986) and insulin concentrations (Beermann *et al.*, 1986; O'Connor *et al.*, 1988) after feeding clenbuterol. It has also been suggested that clenbuterol may exert some of its effects through interaction with hormones, in the case of cardiac hypertrophy these effects may be mediated by prostaglandins (Palmer *et al.*, 1990). Since clenbuterol might have the opposite effect to PHA on hormones, it may prevent or compensate for the PHA-induced muscle atrophy by reversing some of the changes caused by the lectin.

The intimate involvement of polyamines in growth has long been established (Jänne *et al.*, 1978), although their precise role(s) and mode of action remain unknown. PHA-induced gut growth is preceded by polyamine accumulation in the tissue (Pusztai *et al.*, 1989), not as the result of increased *de novo* synthesis of polyamines *in situ*, but by stimulation of polyamine uptake from the circulation (Bardocz *et al.*, 1990a). Thus, the uptake of putrescine through the basolateral membrane increased within the first hour of lectin exposure. However, the effect wore off quickly, lasting for 24 h only (Bardocz *et al.*, 1990b). In contrast, the stimulation of spermidine uptake from the circulation was not immediate but remained elevated and was stimulated

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further for as long as PHA was present in the bowel (Bardocz *et al.*, 1990b).

The present study was undertaken to establish firstly whether accumulation of polyamines occurs during clenbuterol-induced muscle hypertrophy and whether clenbuterol affects the uptake of polyamines from the circulation by skeletal muscle. Since PHA and clenbuterol have opposite actions on skeletal muscle, a second objective was to investigate the possibility that clenbuterol could protect the muscle against the lectin-induced atrophy by preventing the fall in rates of protein synthesis. The skeletal musculature represents a pool of amino acids which may be mobilised in times of dietary insufficiency (Millward *et al.*, 1976), trauma and sepsis (Clowes *et al.*, 1983; Jepson *et al.*, 1986). Therefore the study was designed also to investigate the possibility that, if the muscle atrophy was a prerequisite for the gut hyperplasia, i.e. substrates for the latter were released by mobilising compounds stored in muscle, then clenbuterol might also reduce gut growth. The feasibility of using measurements of polyamine uptake to detect early changes in the metabolism of the gut and gastrocnemius muscle in rats given both PHA and clenbuterol was also investigated.

Methods

Male Hooded-Lister rats of the Rowett strain (about 80 g) were pre-fed *ad libitum* on a diet containing powdered egg albumin (10% protein) for 3 days, then divided into 12 groups, each containing 8 rats; 6 groups were pair-fed with egg albumin (control) diet with or without 4 mg kg⁻¹ clenbuterol (Boehringer-Ingelheim). The other 6 groups were fed on a diet in which raw kidney bean and egg albumin each provided 5% protein (Oliveira *et al.*, 1988), with or without 4 mg kg⁻¹ clenbuterol. Phytohaemagglutinin (PHA), which is responsible for most of the antinutritional effects of kidney bean diet (Oliveira *et al.*, 1988), accounted for 15% of the bean protein and therefore for 7.5% of total dietary protein. Food consumption of all groups of rats was restricted to 5.5 g per rat daily, with free access to acidified water. The animals were fed for 2, 4 or 7 days, fasted overnight and then refed with 2 g of the appropriate diet 2 h before being killed by decapitation.

Four animals of each group were injected intraperitoneally with 8.5 nmol (3.0 × 10⁶ d.p.m.) [¹⁴C]-putrescine and the other four with 9.9 nmol (3.3 × 10⁶ d.p.m.) [¹⁴C]-spermidine precisely 1 h before they were killed. Some of the rats also received 150 µmol L-phenylalanine plus 75 µCi L-[2,6-³H]-phenylalanine per 100 g body weight by injection via the lateral tail vein 10 min before death for the measurement of protein synthesis rates (Garlick *et al.*, 1980). All isotopes used were from Amersham International plc (Amersham, Bucks.). Tissues were dissected as appropriate for measuring polyamine uptake, chemical composition or protein synthesis rate.

The small intestine was removed, washed with ice-cold saline, weighed and its length measured. Six sections of 2 cm were cut out from each small intestine at 3; 9; 21; 41; 63 and 81 cm from the pyloric junction and placed into NE 265 scintillation fluid (NE Technology Limited, Edinburgh). After two days of extraction, the sections were counted for radioactivity as previously described (Bardocz *et al.*, 1990a). Extraction for this time yielded maximum counts. The uptake of polyamines by the rat small intestine was calculated *pro rata* from the amounts of radioactivity incorporated into the 6 sections during the 1 h labelling period. The remaining parts of the small intestine were freeze-dried with the exception of a 2 cm section (7–9 cm from pyloric junction) which was used for the protein synthesis measurement.

Both gastrocnemius muscles were excised. That from the right hind limb was treated with 1 ml of NCS tissue digester (Amersham International plc) at 60°C for 16 h. The solution was neutralized by the addition of 30 µl of concentrated

acetic acid and the samples were counted for radioactivity after the addition of 10 ml scintillation liquid, NE 265. The left gastrocnemius was freeze dried.

The plantaris, half of the heart, a 250 mg sample of liver and a 2 cm section of the jejunum were immediately frozen in liquid N after dissection. These samples were used for measurement of protein synthesis (Garlick *et al.*, 1980) as described previously (Reeds *et al.*, 1986).

Chemical analysis was performed on the freeze-dried small intestine and muscle tissues. An 18 cm piece of the upper jejunum and the whole left gastrocnemius muscle from each rat were homogenized in 25 and 20 ml of 2% (w/v) perchloric acid respectively and the homogenates centrifuged for 15 min at 10,000 g. The polyamine content of the supernatants was analyzed by high performance liquid chromatography (h.p.l.c.) according to Seiler & Knödgen (1980). Protein (Schacterle & Pollak, 1973), RNA (Sneider, 1957), and DNA (Lovtrup & Roos, 1961) estimations were carried out on the precipitates redissolved in a volume of 0.3 M NaOH which was the same as that of perchloric acid used for homogenization. All chemicals used were the highest grade of purity from Sigma Chemical Co. (Poole, Dorset).

Statistical analysis

All data are expressed as means for 4 rats per group. Statistical analysis was carried out by a multiple way analysis of variance 'ANOVA' from 'Minitab' (Statistical Software) and this programme was used to calculate pooled standard deviations. The residual variance (δ^2) was obtained from the analysis of variance tables. The standard errors of difference (s.e.d.) for comparing two groups of size 4 was calculated from the formula:

$$\text{s.e.d.} = \sqrt{\delta^2 (1/4 + 1/4)}$$

For the analysis of variance the following factors had been taken into consideration: time (2, 4 and 7 days), clenbuterol and kidney bean treatments. Groups statistically different from control at any time were marked with different superscripts. ^a and ^b refer to significant ($P < 0.01$) effects of clenbuterol or kidney bean treatment; ^c mark significant exchanges by time and ^d refers to interaction between clenbuterol and kidney bean treatment.

Results

The lectin from kidney bean was found to be a potent growth factor for the rat small intestine. The dry weight of jejunum increased steadily and by 7 days it was 53% above the control value (Table 1). The jejunal contents of RNA, putrescine and spermidine were also significantly increased by the second day (Table 1 and Figure 1). Similarly, 20% and 46% increases in the rate of gut protein synthesis were observed on days 2 and 4 respectively (Table 2). In contrast, the total protein, spermine and DNA contents of the jejunum were not significantly increased until the fourth day. Jejunal hypertrophy continued until day 7 with further increases of 96% in spermine and spermidine and a 120% increase in putrescine content (Figure 1). Clenbuterol had no effect on the growth of the jejunum in rats given either the control or bean-containing diet (Table 1), but it did inhibit the PHA-induced accumulation of putrescine in the jejunum at day 4 and the increments in jejunal polyamine contents in rats fed kidney beans for 7 days (Figure 1). The amounts of polyamines in the jejunum of clenbuterol-treated rats were 40, 83 and 80%, respectively of those in rats given kidney bean alone (Figure 1). In contrast, the presence of clenbuterol appeared to exacerbate the lectin-induced increase in gut protein synthesis by 20% on day 2 and by 12% on day 4 (Table 2).

Table 1 Effect of kidney beans and clenbuterol on the growth of rat jejunum

Diet:	Days treatment	(mg)	Dry weight	Protein	RNA	DNA
			(per 100 g body weight)	(mg)	(mg)	(mg)
Control	2	118	0.60	69	7.0	3.1
	4	120	0.60	71	6.0	3.0
	7	121	0.58	71	7.0	3.0
Clenbuterol	2	107 ^a	0.60	70	5.4 ^a	3.0
	4	111 ^a	0.55 ^a	72	6.7 ^{ac}	3.2
	7	109 ^a	0.55 ^a	66	7.4	3.5
Kidney bean	2	142 ^b	0.70 ^b	73 ^b	9.2 ^b	3.6 ^b
	4	155 ^{bc}	1.06 ^{bc}	97 ^{bc}	16.0 ^{bc}	4.0 ^b
	7	185 ^{bc}	1.07 ^{bc}	112 ^{bc}	17.0 ^{bc}	5.2 ^{bc}
Clenbuterol + kidney bean	2	136 ^d	0.70 ^b	77 ^b	9.3 ^b	3.8 ^b
	4	148 ^{dc}	1.02 ^d	89 ^d	16.2 ^b	4.7 ^d
	7	189 ^{bc}	1.10 ^{dc}	119 ^{dc}	18.4 ^{bc}	5.7 ^{dc}
s.e.d.		3	0.01	2	0.3	0.2

Rats (groups of 8) were fed on egg albumin (control) or kidney bean diets with or without 4 mg kg⁻¹ clenbuterol; 20 cm of the upper jejunum was used for analysis. Values are means of 4 and s.e.d. Statistical treatment was by analysis of variance taking out time, clenbuterol and kidney bean effects and their interactions. Data within a column with different superscripts are significantly different from control: $P < 0.01$. ^a and ^b represent significant effects of clenbuterol or kidney bean treatments, ^c marks the effect of time on a treatment and ^d represents interaction between clenbuterol and the kidney bean treatments.

Table 2 Fractional rates of protein synthesis (k_s; % per day) in the liver and jejunum of rats fed kidney beans (PHA) and/or clenbuterol for 2 or 4 days

	Jejunum	Liver
<i>2 days</i>		
Control	108.9	90.7
Clenbuterol	107.1	93.6
PHA	130.8 ^b	87.5
PHA + Clenbuterol	157.7 ^d	93.2
<i>4 days</i>		
Control	99.2	88.5
Clenbuterol	108.6	89.4
PHA	145.0 ^b	75.1 ^b
PHA + Clenbuterol	162.5 ^b	68.1 ^b
s.e.d.	10.0	8.6

Values are means of 4 and s.e.d. Statistical treatment was by analysis of variance taking out time, clenbuterol and kidney bean effects and their interactions. Data within a column with different superscripts are significantly different from control: $P < 0.01$. ^a and ^b represent significant effects of clenbuterol or kidney bean treatments, ^c marks the effect of time on a treatment and ^d represents interaction between clenbuterol and the kidney bean treatments.

The expected effects of clenbuterol on skeletal muscle hypertrophy were evident from the increased weight, protein and DNA contents of the hind leg gastrocnemius tissue by the 4th day of treatment (Table 3). Moreover, RNA (Table 3) and spermidine (Figure 2) contents of the gastrocnemius muscle were significantly increased by the second day. In contrast, in rats fed on kidney beans for 4 or more days, muscle atrophy was observed (Table 3). Clenbuterol partially protected the muscle against the atrophy caused by the kidney bean lectin by ameliorating the loss of weight, protein, and RNA (Table 3) and reversing the kidney bean-induced loss of spermidine and spermine (Figure 2).

Fractional rates of protein synthesis and RNA content were measured in the plantaris muscle and were affected by both clenbuterol and kidney bean lectin (Table 4). By day 2 of treatment clenbuterol had increased the RNA content by 15% and the fractional rate of protein synthesis by over 30%, whilst the lectin had significantly decreased both RNA and protein contents and the fractional rate of protein synthesis. Clenbuterol appeared partially to protect the muscle from the lectin-induced atrophy at this time. Both the RNA content of the muscle and the rate of protein synthesis were significantly higher on the combined treatment than when the

Table 3 Effect of clenbuterol and kidney bean on the growth of rat gastrocnemius

Diet:	Days treatment	(mg)	Dry weight	Protein	RNA	DNA
			(per 100 g body weight)	(mg)	(mg)	(mg)
Control	2	87	0.38	68	0.97	0.27
	4	93 ^c	0.39	70	1.07 ^c	0.29
	7	98 ^c	0.39	69	1.03 ^c	0.29
Clenbuterol	2	89	0.39	70	1.22 ^a	0.29
	4	104 ^a	0.46 ^a	76 ^{ac}	1.40 ^{ac}	0.33 ^a
	7	111 ^{ac}	0.46 ^a	79 ^{ac}	1.70 ^{ac}	0.44 ^{ac}
Kidney bean	2	80 ^b	0.41 ^b	67	0.76 ^b	0.24 ^b
	4	73 ^{bc}	0.39	55 ^b	0.67 ^{bc}	0.24 ^b
	7	71 ^{bc}	0.35 ^b	43 ^{bc}	0.43 ^{bc}	0.22 ^b
Clenbuterol + kidney bean	2	86	0.43 ^d	70	0.85 ^d	0.28
	4	78 ^d	0.42 ^d	63 ^d	0.80 ^{cd}	0.24 ^b
	7	84 ^{dc}	0.41 ^d	57 ^b	0.65 ^{bc}	0.22 ^b
s.e.d.		1	0.01	2	0.2	0.1

Rats (groups of 8) were fed on egg albumin (control) or kidney bean diets with or without 4 mg kg⁻¹ clenbuterol. Freeze dried gastrocnemius muscles from both hind limbs were analysed. Values are means of 4 and s.e.d. Statistical treatment was by analysis of variance taking out time, clenbuterol and kidney bean effects and their interactions. Data within a column with different superscripts are significantly different from control: $P < 0.01$. ^a and ^b represent significant effects of clenbuterol or kidney bean treatments, ^c marks the effect of time on a treatment and ^d represents interaction between clenbuterol and the kidney bean treatments.

Figure 1 Effect of kidney bean and clenbuterol on the polyamine content of rat jejunum. Rats (groups of 4) were fed on egg albumin (control) with (hatched column) or without (open column) 4 mg kg⁻¹ clenbuterol or kidney bean diets with (cross-hatched column) or without (solid column) 4 mg kg⁻¹ clenbuterol; 20 cm of their upper jejunum was measured and analysed. Values are means of 4 and s.e.d. (vertical bars). Statistical treatment was by analysis of variance taking out time, clenbuterol and kidney bean effects and their interactions. Data with different superscripts are significantly different from control: $P < 0.01$. ^a and ^b represent significant effects of clenbuterol or kidney bean treatments, ^c marks the effect of time on a treatment and ^d represents interaction between clenbuterol and the kidney bean treatments.

lectin was fed alone and were not significantly different from the control group. By 4 days the lectin had induced further losses of RNA and protein from the plantaris muscle and the fractional synthesis rate remained depressed. The effect of clenbuterol on protein synthesis rate appeared to have diminished and was no longer significant. Also, the ability of clenbuterol to protect against the lectin-induced atrophy appeared to have been lost as both the RNA and the fractional rate of protein synthesis were significantly below the control values by this time (Table 4).

Effects of both lectin and clenbuterol on the heart (Table 5) were similar to those on the skeletal muscle. Clenbuterol increased the heart weight and RNA content but did not reduce the rate of protein synthesis. Kidney bean lectin tended to reduce heart weight and protein synthesis rates were lower in the two lectin-fed groups (with or without clenbuterol) at both 2 and 4 days.

Liver protein synthesis rates were unaffected by any treatment on day 2 (Table 2), polyamine content of the liver was also unaffected (data not shown). The only significant effect on the liver was a reduction in the rate of protein synthesis by day 4 on the combined kidney bean plus clenbuterol diet and there were effects on polyamine content at the same time. Spermidine content of the liver was unchanged by clenbuterol (3122 ± 205 nmol; control = 3328 ± 170 nmol) but significantly decreased by the lectin (2434 ± 26 nmol, $P < 0.05$) and by the combined diet (2284 ± 52 nmol, $P < 0.05$). Spermine content was unaffected by lectin or clenbuterol alone (1905 ± 92 nmol and 1946 ± 98 nmol respectively; control 1922 ± 135 nmol) but, like spermidine, was decreased on the diet containing both kidney beans and clenbuterol (1717 ± 29 nmol, $P < 0.05$).

Early changes in metabolic activity of all the tissues studied were detected by measurement of the uptake of ¹⁴C-labelled polyamines from the peritoneum. Rats given PHA

Figure 2 Effect of clenbuterol and kidney bean on the polyamine content of rat gastrocnemius. Rats (groups of 4) were fed on egg albumin (control) with (hatched column) or without (open column) 4 mg kg⁻¹ clenbuterol or kidney bean diets with (cross-hatched column) or without (solid column) 4 mg kg⁻¹ clenbuterol. Freeze-dried gastrocnemius muscles from both hind limbs were analysed. Values are means of 4 and s.e.d. (vertical bars). Statistical treatment was by analysis of variance taking out time, clenbuterol and kidney bean effects and their interactions. Data with different superscripts are significantly different from control: $P < 0.01$. ^a and ^b represent significant effects of clenbuterol or kidney bean treatments, ^c marks the effect of time on a treatment and ^d represents interaction between clenbuterol and the kidney bean treatments.

Table 4 Effect of kidney beans (PHA) and clenbuterol on weight, RNA and protein content and fractional rate of protein synthesis of plantaris muscle

	Wt per 100 g body weight	RNA (μ g)	Protein (mg)	k_s (%per day)
<i>2 days</i>				
Control	91.9	119.4	17.5	15.2
Clenbuterol	98.8 ^a	137.6 ^a	17.7	20.3 ^a
PHA	82.9 ^b	91.9 ^b	14.0 ^b	10.3 ^b
PHA + clenbuterol	91.5	107.9 ^d	15.5 ^b	14.7
<i>4 days</i>				
Control	90.6	111.7	15.1	15.3
Clenbuterol	98.5 ^a	138.6 ^a	15.6	17.7
PHA	84.4 ^b	68.2 ^{bc}	12.1 ^{bc}	10.1 ^b
PHA + clenbuterol	91.4	78.2 ^b	13.0 ^{bc}	12.5 ^d
s.e.d.	2.8	6.0	0.9	1.3

Values are means of 4 and s.e.d. Statistical treatment was by analysis of variance taking out time, clenbuterol and kidney bean effects and their interactions. Data within a column with different superscripts are significantly different from control: $P < 0.01$. ^a and ^b represent significant effects of clenbuterol or kidney bean treatments, ^c marks the effect of time on a treatment and ^d represents interaction between clenbuterol and the kidney bean treatments.

Table 5 Effect of kidney beans (PHA) and clenbuterol on weight, RNA and protein content and fractional rate of protein synthesis of heart

	Wt per 100 g body weight	RNA (mg)	Protein (mg)	k_s (%per day)
<i>2 days</i>				
Control	389	1.42	59.3	14.3
Clenbuterol	472 ^a	2.02 ^a	59.5	15.2
PHA	392	1.32 ^b	56.6 ^b	12.4
PHA + clenbuterol	443 ^d	1.75 ^d	66.6 ^d	13.1
<i>4 days</i>				
Control	393	1.60	54.2	13.9
Clenbuterol	430 ^{ac}	1.97 ^a	55.4	13.4
PHA	350 ^b	1.21 ^b	54.2	11.4 ^b
PHA + clenbuterol	401 ^d	1.33 ^d	51.3 ^d	11.2 ^b
s.e.d.	15	0.04	1.2	1.2

Values are means of 4 and s.e.d. Statistical treatment was by analysis of variance taking out time, clenbuterol and kidney bean effects and their interactions. Data within a column with different superscripts are significantly different from control: $P < 0.01$. ^a and ^b represent significant effects of clenbuterol or kidney bean treatments, ^c marks the effect of time on a treatment and ^d represents interaction between clenbuterol and the kidney bean treatments.

showed increased jejunal uptake of both putrescine and spermidine (Figure 3). Clenbuterol did not significantly affect the uptake of either putrescine and spermidine by the gut in rats fed egg albumin but further stimulated spermidine uptake in rats fed the bean diet (Figure 3).

The negative effect of PHA on muscle growth was reflected by a significant decrease in the uptake of labelled spermidine by the gastrocnemius muscle on day 4 (Figure 4). Conversely clenbuterol-induced muscle hypertrophy was accompanied by a stimulation of putrescine uptake (+80%) and spermidine uptake (+137%) on day 2 (Figure 4). Like the effects of clenbuterol on protein synthesis (Table 4), there was a tendency for the stimulation by clenbuterol of putrescine and spermidine uptake to fall with time.

Discussion

Although the biochemical mechanism whereby clenbuterol promotes muscle protein deposition is unclear, it has been suggested that it involves mobilization of body lipids through interaction with β -adrenoceptors and occurs at the expense of the growth of other tissues of the body (Reeds *et al.*, 1986). The growth of the small intestine induced by PHA also involves mobilization of body lipids (Oliveira *et al.*, 1988; Pusztai *et al.*, 1989). The present study was designed therefore to investigate the combined effects of the two different growth factors on muscle and small intestinal metabolism.

The effects of clenbuterol on muscle mass were small in comparison with previous studies where increases in protein content in excess of 20% have been observed (Reeds *et al.*, 1986; Maltin *et al.*, 1989). The rats in earlier experiments were fed *ad libitum* whilst those in the present study were pair-fed on the amount of diet consumed by the kidney bean-treated groups, receiving approximately half their normal dietary intake. Although the clenbuterol content of the diet was doubled to ensure similar daily intakes of the drug, the dietary restriction may account for the relatively poor hypertrophy of the plantaris. In other respects however the plantaris did respond well to clenbuterol, the significant increase in RNA content and fractional rate of protein synthesis by the second day, and the tendency for synthesis rates to

Figure 3 The effects of clenbuterol and kidney bean on the uptake of [14 C]-putrescine or [14 C]-spermidine from the peritoneum by the rat small intestine. Rats (groups of 4) were pair fed with egg albumin (control) with (hatched column) or without (open column) 4 mg kg⁻¹ clenbuterol or kidney bean diets with (cross-hatched columns) or without (solid column) containing 4 mg kg⁻¹ clenbuterol (22 μ g per rat daily) for 2 or 4 days. Exactly 1 h before killing each group was injected with either [14 C]-putrescine or [14 C]-spermidine intraperitoneally, and the radioactivity accumulated to their entire small intestine measured. Values are means of 4 and s.e.d. (vertical bars). Statistical treatment was by analysis of variance taking out time, clenbuterol and kidney bean effects and their interactions. Data with different superscripts are significantly different from control: $P < 0.01$. ^a and ^b represent significant effects of clenbuterol or kidney bean treatments, ^c marks the effect of time on a treatment and ^d represents interaction between clenbuterol and the kidney bean treatments.

Figure 4 Effect of clenbuterol and kidney bean on the growth of rat gastrocnemius. Rats (groups of 4) were fed on egg albumin (control) with (hatched column) or without (open column) 4 mg kg⁻¹ clenbuterol or kidney bean diets with (cross-hatched column) or without (solid column) 4 mg kg⁻¹ clenbuterol. Freeze dried gastrocnemius muscles from both hind limbs were analysed. Values are means of 4 and s.e.d. (vertical bars). Statistical treatment was by analysis of variance taking out time, clenbuterol and kidney bean effects and their interactions. Data with different superscripts are significantly different from control: $P < 0.01$. ^a and ^b represent significant effects of clenbuterol or kidney bean treatments, ^c marks the effect of time on a treatment and ^d represents interaction between clenbuterol and the kidney bean treatments.

return towards control values by day 4 (Table 4) seem to be typical of the response of rats to the drug. It has been proposed that in rats, clenbuterol both increases synthesis and decreases degradation of muscle protein, the former of these effects being transient in nature and responsible for the early hypertrophic effects of the drug (Table 4 and Maltin *et al.*, 1989). It is interesting therefore that the protection against the lectin-induced muscle atrophy also appears to be transient and occurred only during the period when clenbuterol was acting to stimulate the rate of protein synthesis (Table 3).

As with other growth processes (Jänne *et al.*, 1978), the clenbuterol-induced muscle hypertrophy was accompanied by an increase in the polyamine content of the gastrocnemius muscle (Figure 2). In fact, the accumulation of spermidine was significantly increased within two days of exposure (before the changes in weight, protein or DNA content were observed). Therefore, like the changes in muscle RNA content and in the fractional rate of protein synthesis, which, in clenbuterol-induced hypertrophy usually precede detectable muscle growth (Table 4), changes in polyamine levels (Figure 2) may be a useful early marker for muscle hypertrophy.

In the muscle tissue of PHA-fed rats, treatment with clenbuterol initially maintained the concentration of putrescine at levels close to those in control rats whilst rats fed on the lectin alone showed reduced muscle spermidine levels on day 2 (Figure 2). By 4 days, putrescine content in the gastrocnemius of rats fed lectin plus clenbuterol had declined dramatically and was no longer different from the level in the rat fed the lectin only; by day 7 the spermidine content was also significantly below control values (Figure 2). The reduc-

tion in polyamines in response to the lectin suggests that a drop in tissue polyamine levels may provide an indication of incipient tissue loss just as increases in tissue polyamine levels can be used as indicators of gut hypertrophy (Pusztai *et al.*, 1989). Furthermore the transient partial protection by clenbuterol against the effect of the lectin seems to coincide with the partial protection against the lectin-induced muscle hypertrophy discussed above.

Cardiac hypertrophy is a well known effect of clenbuterol in the rat (Reeds *et al.*, 1986) but the data presented here suggest that this effect is not associated with an increase in the fractional rate of protein synthesis (Table 5). Although the lectin did not affect heart weight and clenbuterol did, it was the lectin treatment which was shown to have significant effects on cardiac protein synthesis. These observations suggest that in response to lectin treatment, cardiac atrophy is prevented by proportionately similar decreases in the rates of protein synthesis and degradation. In this respect the heart appears to respond similarly to the tonic soleus muscle which, unlike the phasic plantaris and gastrocnemius, did not atrophy in response to kidney bean lectin in spite of a marked reduction in the rate of protein synthesis (Palmer *et al.*, 1987).

The liver was the tissue least affected by either clenbuterol or the kidney bean lectin. The only significant effect on the rates of liver protein synthesis was a reduction by day 4 in the group of rats fed on kidney beans plus clenbuterol (Table 2). The close temporal correlation between this effect on protein synthesis and the changes in polyamine content, which were only apparent in the combined diet (lectin plus clenbuterol) provide further support for the suggestion that polyamines may be a useful early marker for changes in rates of tissue metabolism that precede growth.

The phytohaemagglutinin, PHA, is a powerful growth stimulant for the gut. Thus, raw kidney bean (Pusztai *et al.*, 1988) and particularly its lectin component (PHA) (Oliveira *et al.*, 1988) induce hypertrophic and hyperplastic growth of the rat small intestine *in vivo* (Pusztai *et al.*, 1991a,b,c). Both the binding of PHA to gut carbohydrate receptors and its endocytosis by epithelial cells appear to be necessary for the stimulation of cellular protein (Table 2) and glycoprotein synthesis (Palmer *et al.*, 1987) and the increase of RNA, DNA and polyamine content of the gut tissue (Table 1, Pusztai *et al.*, 1988).

The increased rate of uptake of ¹⁴C-labelled putrescine and spermidine by the jejunum of rats given kidney bean diet (Figure 3) and by the gastrocnemius muscle of rats treated with clenbuterol (Figure 4) indicates that polyamines may play an early role in the growth process. Previous studies have indicated that most of the polyamines required to support the growth of the gut come from external sources via the circulation (Bardocz *et al.*, 1990a,b), suggesting that polyamines may be exchanged between various organs of the body following a growth stimulus. This idea is very attractive. On exposure to PHA, the gastrocnemius (which is representative of the majority of the skeletal musculature) loses more than 20% of its mass and polyamine content in a week (Table 3). Therefore, from the atrophy of the skeletal muscles, which represent about 40% of the total body mass and protein content, relatively large amounts of polyamines might be available for the replacement of those which had been taken up by organs such as the gut, whose growth is stimulated by PHA.

As both the increase in gut polyamine levels and the outflow of polyamines from the muscle occurs very early on PHA stimulation, it seems probable that the inter-organ communication link is not passive and that there is some hormonal signal. The finding that PHA in the gut lumen can immediately reduce circulating insulin levels (Pusztai *et al.*, 1991c) appears to support a direct signalling mechanism. On the basis of the present experimental evidence it appears that there is a parallel between polyamine uptake and protein synthesis rate in the tissues examined. However, it is difficult

to suggest a precise role for polyamines in this signalling process, i.e. whether they act as second messengers or simply follow the changes occurring in muscle. The latter view appears to be more realistic. As the polyamine pool of the body is limited in size, there is competition between the organs for the polyamines available to support the growth induced by a combination of growth factors.

In this paper three different effects of clenbuterol and the kidney bean lectin PHA on tissue growth have been identified. In phasic skeletal muscle clenbuterol imparts a partial and temporary protection against the severe atrophy caused by the lectin. In the heart a previously unidentified action of the lectin appears to be a reduction in protein synthesis; this however does not result in a marked cardiac

atrophy, nor is it affected by clenbuterol. Finally, the PHA-induced hyperplasia of the jejunum leading to rapid increase in protein synthesis, is not reversed but accentuated by clenbuterol. It has been shown for the first time that clenbuterol-induced skeletal muscle hypertrophy is accompanied by polyamine accumulation in that tissue. Indeed, this increase is clear before growth and protein accretion could be detected. More importantly, measurements of the uptake of labelled putrescine and spermidine from the circulation by specific organs induced to grow by different stimuli can predict metabolic changes in these tissues at an even earlier stage and can reflect changes in growth and metabolism even when they result from the combined effects of more than one stimulant.

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