Muscle Protein Waste in Tumor-bearing Rats Is Effectively Antagonized by a β_2-Adrenergic Agonist (Clenbuterol)

Role of the ATP-Ubiquitin-dependent Proteolytic Pathway

Paola Costelli,* Célia García-Martínez,‡ Marta Llovera,‡ Neus Carbó,‡ Francisco J. López-Soriano,‡ Neus Agell,§ Luciana Tessitore,* Francesco M. Baccino,*‖ and Josep M. Argilés‡

*‡Departament de Bioquímica i Fisiologia, Facultat de Biologia, and §Departament de Biologia Cel.lular, Facultat de Medicina, Universitat de Barcelona, 08071 Barcelona, Spain; *Dipartimento di Medicina ed Oncologia Sperimentale, Sezione di Patologia Generale, Università di Torino, 10125 Torino, Italy; and ‖Centro CNR di Immunogenetica ed Oncologia Sperimentale, 10125 Torino, Italy*

Abstract

Tissue protein hypercatabolism (TPH) is a most important feature in cancer cachexia, particularly with regard to the skeletal muscle. The rat ascites hepatoma Yoshida AH-130 is a very suitable model system for studying the mechanisms involved in the processes that lead to tissue depletion, since it induces in the host a rapid and progressive muscle waste mainly due to TPH (Tessitore, L., G. Bonelli, and F. M. Baccino. 1987. *Biochem. J.* 241:153–159). Detectable plasma levels of tumor necrosis factor-α associated with marked perturbations in the hormonal homeostasis have been shown to concur in forcing metabolism into a catabolic setting (Tessitore, L., P. Costelli, and F. M. Baccino. 1993. *Br. J. Cancer*. 67:15–23). The present study was directed to investigate if β_2-adrenergic agonists, which are known to favor skeletal muscle hypertrophy, could effectively antagonize the enhanced muscle protein breakdown in this cancer cachexia model. One such agent, i.e., clenbuterol, indeed largely prevented skeletal muscle waste in AH-130–bearing rats by restoring protein degradative rates close to control values. This normalization of protein breakdown rates was achieved through a decrease of the hyperactivation of the ATP-ubiquitin-dependent proteolytic pathway, as previously demonstrated in our laboratory (Llovera, M., C. García-Martínez, N. Agell, M. Marzábal, F. J. López-Soriano, and J. M. Argilés. 1994. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 338:311–318). By contrast, the drug did not exert any measurable effect on various parenchymal organs, nor did it modify the plasma level of corticosterone and insulin, which were increased and decreased, respectively, in the tumor hosts.

The present data give new insights into the mechanisms by which clenbuterol exerts its preventive effect on muscle protein waste and seem to warrant the implementation of experimental protocols involving the use of clenbuterol or alike drugs in the treatment of pathological states involving TPH, particularly in skeletal muscle and heart, such as in the present model of cancer cachexia. (*J. Clin. Invest.* 1995. 95:2367–2372.) Key words: clenbuterol • tumor • protein turnover • cachexia • ubiquitin

Introduction

Malignant neoplasms frequently induce a progressive loss of lean body mass in the host associated with marked alterations in the endocrine and metabolic homeostasis. The skeletal muscle, which accounts for almost half of the whole body protein mass, is severely affected in cancer cachexia (1–3) and evidence has been provided for muscle protein waste as being associated with enhanced turnover rates (4–9). Since cachexia tends to develop at a rather advanced stage of the neoplastic growth, preventing muscle waste in cancer patients is of a great potential clinical interest. Whether the negative protein balance results from altered rates of synthesis or breakdown or from changes on both sides of muscle protein turnover is still debated, however (6, 10–12).

The rat ascites hepatoma Yoshida AH-130 is a suitable model system to study the mechanisms involved in the establishment of cachexia. Its growth causes in the host rapid and progressive loss of body weight and tissue waste, particularly in skeletal muscle. Acceleration of tissue protein breakdown accounts for most of the waste in the AH-130 bearers (6, 13–15). In particular, skeletal muscle hypercatabolism is mainly due to hyperactivation of the ATP-ubiquitin-dependent proteolytic system (16). Detectable plasma levels of tumor necrosis factor-α (TNF) and perturbations in the hormonal homeostasis (17) likely play an important role in forcing the metabolic balance towards the catabolic side.

β_2-adrenergic agonists are potent muscle growth promoters in many animal species (18), resulting in skeletal muscle hypertrophy, while they cause a reduction of the body fat content (19). These compositional alterations are associated with a redistribution of energy substrates, which are mobilized from storage sites for utilization by tissues such as muscle and brown adipose tissue (19). The intimate mechanisms by which these

Address correspondence to Dr. Josep M. Argilés, Unitat de Bioquímica i Biologia Molecular B, Departament de Bioquímica i Fisiologia, Facultat de Biologia, Universitat de Barcelona, Diagonal 645, 08071-Barcelona, Spain. Phone: 3-4021519; FAX: 3-4021559.

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compounds exert such effects at the cellular level are still uncertain (19), although changes in protein turnover are clearly involved. Little is known, however, as to whether β_2-agonists act by potentiating the anabolic processes or depressing the catabolic ones.

Aim of the present investigation has been to study the effects of a β_2-adrenergic agonist (clenbuterol), well known for its selective anabolic action on the skeletal muscle, on tissue protein turnover in rats bearing a cachexia-generating fast-growing tumor.

Methods

Animals, tumor inoculation, and treatment. Male Wistar rats (Interfauna, Barcelona, Spain) weighing ~ 100 g were used. The animals were maintained on a regular light–dark cycle (light on from 08:00 a.m. to 08:00 p.m.) and had free access to food and water. The diet (Panlab, Barcelona, Spain) consisted of 54% carbohydrate, 17% protein, and 5% fat (the residue was nondigestible material); the food intake was measured daily. Rats were divided into two groups, namely controls and tumor hosts. The latter received an intraperitoneal inoculum of 10^8 AH-130 Yoshida ascites hepatoma cells obtained from exponential tumors (for details see reference 6). Both groups were further divided into treated and untreated, the former being administered a daily s.c. dose of clenbuterol (1 mg/kg b.w., dissolved in physiological solution), the latter a corresponding volume of solvent.

On days 0,4, and 8 after tumor transplantation animals were weighed and anesthetized with diethyl ether. The tumor was harvested from the peritoneal cavity, its volume and cellularity evaluated, and cells separated from the ascitic fluid by centrifugation at 100 *g* for 10 min. Blood was collected from the abdominal aorta into heparinized tubes and centrifuged (3500 *g*, 10 min, 4°C) to obtain plasma. Tissues were rapidly excised, weighed, and frozen in liquid nitrogen (see reference 6; for the adipose tissues see reference 20).

Protein turnover. Protein turnover rates were determined by a method that, as previously discussed (6, 15), is very suitable for the liver (21), yet suffers from considerable label recycling in skeletal muscle (22); however, it offers the best compromise to monitor simultaneously protein synthesis and degradation in the same animal (see reference 23). Briefly, apparent rates of synthesis and degradation for proteins of the slow turnover pool were evaluated by measuring the decay in specific and total protein radioactivity after labeling in vivo, 24 h before tumor transplantation, with a single intraperitoneal dose of sodium [^{14}C]bicarbonate (250 μCi/kg b.w.). Fractional rates of protein degradation (k_d), synthesis (k_s), and accumulation (k_a) were calculated as follows:

k_d = ln (total protein radioactivity)/t

k_s = ln (specific protein radioactivity)/t

k_a = ln (total protein)/t

and expressed as percent per day.

Tissue protein was determined by the method of Lowry et al. (24), using bovine serum albumin as working standard.

RNA isolation and Northern blot analysis. Total RNA from gastrocnemius muscle was extracted using the acid guanidinium isothiocyanate/phenol/chloroform method as described by Chomczynski and Sacchi (25). RNA samples (40 μg/ml) were denatured, subject to 1.2% agarose gel electrophoresis and transferred to Hybond H membrane (Amersham International, Buckinghamshire, UK). RNA was fixed to membrane by illuminating with UV for 4 min.

Prehybridization was done in 50% formamide/5× SSC (0.3 M NaCl, 65 mM sodium citrate)/5× Denhart's solution (1× Denhart's solution is 0.1% polyvinylpyrolidone, 0.1% Ficoll, 0.1% BSA)/20 mM sodium phosphate pH 6.8/0.1% SDS/100 μg/ml denatured salmon sperm DNA overnight at 42°C. Membranes were hybridized with appropriate probes (10^6–10^7 cpm/ml) at 42°C for 18 h. Nonspecifically bound probe was removed by successive washes in 2× SSC (15 min at 55°C, twice), 2× SSC + 0.1% SDS (30 min at 55°C) and 0.1× SSC + 0.1% SDS (15 min at 55°C, twice). Specific hybridization was then detected by autoradiography (for more details see 16).

Radiolabeled probes were prepared by the random priming method (Boehringer-Mannheim, Barcelona, Spain). The ubiquitin probe used was a cDNA clone containing 12 bp of the second ubiquitin coding sequence plus a complete third and fourth ubiquitin coding sequence and 120 bp of the 3′-untranslated region of the chicken polyubiquitin gene UB1 (26). An actin probe was used as a control of loading. Filters were exposed to X Omat AR-5 films (Eastman Kodak Co., Rochester, NY) at −70°C for 2–4 d.

Plasma hormones. Circulating corticosterone was evaluated by a rat radioimmunoassay (IDS, Boldon, England). Insulin was measured by radioimmunoassay by the method of Albano et al. (27), using rat insulin as working standard.

Data presentation. Data are given as means±SD. Two-way factorial analysis of variance was used to calculate the significance of differences. As for fractional rates of protein turnover, significance of differences was calculated by analysis of variance on linear regressions (28).

Chemicals. All enzymes and coenzymes were obtained from Boehringer-Mannheim or Sigma Chemical Co, (St. Louis, MO), sodium ^{14}C-bicarbonate (53 mCi/mmol) from New England Nuclear (Boston, MA). Clenbuterol was kindly provided by Smithkline Beechman Pharmaceuticals (Betchworth, Surrey, UK).

Results

The rat ascites hepatoma Yoshida AH-130 grew exponentially for 4–5 d then shifted into a stationary phase approximately by day 8 after transplantation, as previously shown (29). This growth pattern was not significantly affected by clenbuterol (Table I).

Clenbuterol did not alter the food intake either in controls or in tumor bearers. The hypophagia occurring during tumor growth (15, 17) persisted unchanged in treated animals: the average daily food intake was 25 and 23 g on day 0, and 14, and 15 g on day 8 after tumor inoculation, respectively, in untreated and treated tumor hosts; in treated and untreated non-tumor bearing animals it amounted to 24 and 23 g, respectively, over the whole experimental period.

As shown in Table I, the loss of body weight in tumor bearers became prominent by day 8 and was not affected by clenbuterol. A marked reduction of white adipose tissue mass was already detectable at day 4 in clenbuterol-treated controls; this tissue was also diminished in day 8 tumor bearers, yet not further affected to a significant degree by the drug treatment. Both liver and brown adipose tissue were hypotrophic 4 d after tumor inoculation and even more so by day 8; no significant protective effect was exerted by clenbuterol on either tissue.

Quite different was the pattern with regard to skeletal and heart muscles. An early and progressive decrease in wet weight (data not shown) and protein content was elicited by tumor growth (Table II), with gastrocnemius and heart being affected earlier and more severely than soleus, as previously reported (6). Treatment with clenbuterol substantially prevented the protein waste in each tissue, since day 4 for gastrocnemius and heart, by day 8 for the soleus. This protective effect was also noticeable in terms of wet tissue weight for the skeletal muscles, but not for the heart (data not shown); a similar discrepancy between heart wet weight and protein content had already been observed in AH-130 tumor bearers treated with anti-TNF antibodies (30). In agreement with previous observations, clenbu-

Table I. Body and Tissue Weight and Tumor Growth in AH-130 Hosts after Clenbuterol Administration

Time	Tumor	Treatment	Body weight (g)		Liver	WAT	IBAT	Total tumor cell number ($\times 10^{-6}$)	Tumor volume
			Initial	Final					
					mg	*mg*	*mg*		*ml*
Day 4	No	None	183±9	220±13	5725±164	397±41	163±21		
		Clenbuterol	189±6	217±8	5605±267	318±36	167±32		
	Yes	None	181±9	206±25	5168±320*	370±47	110±15*	2976±220	19±2
		Clenbuterol	185±8	205±13	5051±275*	359±56	110±24*	2631±799	17±3
Day 8	No	None	185±12	253±17	6437±267	564±31	162±23		
		Clenbuterol	182±12	216±23	6378±718	459±22	135±20		
	Yes	None	188±11	199±15‡	4829±740‡	421±23*	83±9‡	8133±937	56±14
		Clenbuterol	183±9	198±17‡	4890±428‡	381±68*	103±17‡	7406±1206	51±15

Data are expressed as means±SD. Tissue weights are expressed as percentages of initial body weight. Significance of the differences (two-way factorial analysis of variance): * $P < 0.05$; ‡ $P < 0.01$ (tumor burden) $n = 4$ and 6 for nontumor and tumor bearers, respectively.

terol afforded a significant trophic action (protein increase) on muscles in nontumor bearers as well, particularly on the gastrocnemius and less on the heart, while the soleus was not significantly affected (Table II).

Protein turnover in gastrocnemius and heart was evaluated to assess by which regulation, whether on synthesis or on degradation or both, the protein-sparing action of clenbuterol was exerted. As previously reported (6, 15), rates of protein degradation were enhanced in both muscles examined in consequence of tumor growth, while synthesis remained virtually unchanged, resulting in protein accumulation rates lower than in controls (Table III). On treatment with clenbuterol, the elevation of protein breakdown rates was suppressed and protein accumulation rates returned similar to those in controls (Table III). No detectable effect was observed with regard to protein synthesis rates. Likewise in nontumor bearers, the gastrocnemius and heart hypertrophy observed on drug treatment appeared to result from a marked decrease in protein catabolic rates, while synthesis rates were not significantly affected (Table III).

As previously shown (16), the accelerated muscle protein breakdown in the AH-130 hosts may be achieved through activation of the ATP-ubiquitin–dependent proteolytic system. As shown in Fig. 1, two polyubiquitin mRNA species (2.4 and 1.2 kb) were found in gastrocnemius muscle. Tumor-bearing animals showed an increased expression of the polyubiquitin genes in relation with the corresponding control animals: over severalfold (2.4 kb) and 14-fold (1.2 kb). When the tumor-bearing animals received clenbuterol, this activation was suppressed, the values obtained being over twice than those obtained in the untreated controls for both ubiquitin mRNA (Fig. 1).

Corticosterone was elevated and insulin decreased in the blood plasma of AH-130 hosts, as previously observed (17). When these animals were administered clenbuterol, neither insulin nor corticosterone changes were affected, suggesting that the drug effects were not mediated through these hormones. In nontumor bearers the treatment did not modify corticosterone, while insulin concentrations decreased below the control values (Table IV).

Table II. Muscle Protein Content

Time	Tumor	Treatment	Gastrocnemius	Soleus	Heart
Day 0	No	None	45.8±3.5	2.29±0.11	45.5±3.4
Day 4	No	None	70.6±1.4	2.70±0.08	62.2±2.3
	No	Clenbuterol	73.2±1.3	2.84±0.16	59.1±10.1
	Yes	None	60.4±3.9**	2.62±0.20	54.2±4.2*
	Yes	Clenbuterol	64.8±2.2‡	2.89±0.26	67.3±9.1‡
Day 8	No	None	87.8±2.7	3.51±0.24	67.2±4.3
	No	Clenbuterol	99.1±3.6*	3.44±0.31	74.1±3.2*
	Yes	None	55.7±4.1**	2.70±0.26**	55.8±6.0*
	Yes	Clenbuterol	78.1±3.8‡‡	3.16±0.18‡‡	87.6±9.4**‡‡

Data (means±SD) are expressed as mg of protein per whole organ. Statistical comparison of the data (two-way factorial analysis of variance) showed significant effects for both tumor burden and treatment. Interactions: * $P < 0.05$ and ** $P < 0.01$ (tumor bearing vs. nontumor bearing); ‡ $P < 0.05$ and ‡‡ $P < 0.01$ (treatment vs. placebo). $n = 4$ and 6 for nontumor and tumor bearers respectively.

Discussion

Muscle protein waste is a main feature in cancer cachexia and, generally, is mostly ascribed to enhanced tissue protein catabolism (4–9, 15). So far, most therapeutic approaches to cancer cachexia have been designed on the assumption that tissue waste merely results from undernutrition or tumor-host competition, yet parenteral nutrition or overfeeding have proven to be only marginally or, at best, temporarily effective (31–33). Moreover, in some cases the tumor itself apparently took advantage of such regimen more than the patient (32, 34).

The rat tumor model used in the present study quickly causes progressive body weight loss and tissue protein waste, the latter associated with TPH (6, 15) likely mediated by production of cytokines such as TNF and alterations in the hormonal homeostasis (17). The beneficial effects of treatments with anti-TNF antibodies (30) or with drugs interfering with the development of TPH (35) have been previously reported. The present observations show that treatment of the AH-130 hosts with clenbuterol largely abolished the protein waste in the gastrocnemius and heart by restoring normal rates of protein degradation. This is in agreement with previous reports that β_2-adrenergic agonists may antagonize the skeletal muscle depletion in different situa-

Table III. Muscle Protein Turnover

	k_s	k_d	k_a
Gastrocnemius			
Controls	10.30	2.36	5.65
r	−0.992	−0.989	0.996
slope	−1.92±0.29	−32±1.16	3.98±0.19
Controls + clenbuterol	10.28	1.05**	7.18**
r	−0.986	−0.995	0.990
slope	−1.95±0.25	−15±2.41	5.38±0.86
F	5.51	396	21.32
AH-130	9.06	7.02**	−1.01**
r	−0.995	−0.991	−0.858
slope	−1.77±0.27	−79±7.53	−0.63±0.26
F	4.68	498	248
AH-130 + clenbuterol	8.86	2.43††	4.21††
r	−0.978	−0.994	0.966
slope	−1.75±0.17	−33±1.49	2.79±0.35
F	4.97	500	140
Heart			
Controls	10.81	6.54	4.46
r	−0.991	−0.994	0.993
slope	−2.15±0.44	−481±24	2.39±0.17
Controls + clenbuterol	10.45	3.25**	6.65**
r	−0.994	−0.998	0.996
slope	−2.08±0.50	−257±23	4.00±0.15
F	3.28	409	512
AH-130	12.94	9.87**	1.81**
r	−0.988	−0.990	−0.991
slope	−2.19±0.57	−639±99	−0.89±0.21
F	4.12	48.24	197
AH-130 + clenbuterol	10.63	6.96‡	8.20‡‡
r	−0.992	−0.987	0.990
slope	−2.12±0.46	−506±61	5.32±0.22
F	5.03	24.07	1560

For further details see Methods section. Fractional rates of protein synthesis (k_s), degradation (k_d) and accumulation (k_a) are expressed as percent per day ($n = 5$ for each time point) and were calculated over the time interval 0–8 d, since linearity of radioactivity decay during the experimental period (days 0-2-4-6-8) was tested. Variance between groups is indicated by SE of the slope (t × SE of the slope, t being the 95% value of student's t test for n-2 (see Lee and Lee) (28). Significance of the differences: ** $P < 0.01$ vs. controls; ‡ $P < 0.05$ and ‡‡ $P < 0.01$ vs. AH-130.

tions. Thus clenbuterol markedly attenuates the muscle atrophy by denervation (36, 37) or by hindlimb suspension (38). Moreover, this drug can increase the skeletal muscle mass in mice with genetic muscle dystrophy (39). More recently, clenbuterol has been reported to accelerate the recovery of the skeletal muscle mass after challenge with bacterial lipopolysaccharides or surgical stress (40, 41).

It is not clear how these agents modulate tissue protein turnover, though some indirect evidence for an effect on protein catabolism has been provided. Reeds et al. (42) did not observe any change in the gastrocnemius protein synthesis rates after dietary administration of clenbuterol to rats, and concluded that decreased breakdown had to be involved. An indirect confirmation that β_2-agonists may act by reducing protein catabolism came from a recent study in which the intracellular amino acid content of rat gastrocnemius was shown to increase after treatment with metaproterenol (43). In addition, glutamine, and alanine were found to be released at reduced rates from isolated muscles in the presence of the β-adrenergic agonist isoprenaline (44). The present study is totally consistent with these previous reports. Both the prevention of muscle protein depletion in AH-130 tumor bearers and the increase of muscle protein mass in nontumor bearers afforded by clenbuterol appeared to result from regulations on the catabolic side, respectively, by restoring protein breakdown to normal rates or by reducing them to less than normal levels. Moreover, in agreement with previous reports (45, 46), the effects of the drug appeared more marked on a fast-twitch (gastrocnemius) than on a slow-twitch muscle (soleus). The trophic action of β_2-adrenergic agonists is generally regarded as quite selective for the skeletal muscle (19, 38, 42). After treatment with such drugs, however, cardiac hypertrophy has been reported in some studies (42, 47), yet not in others (38). In the present study, clenbuterol administration exerted quite comparable effects on gastrocnemius and heart in both control and tumor-bearing animals.

The precise mechanisms by which intracellular proteins are degraded is largely unknown, although it is accepted that proteolysis may occur inside and outside the lysosomes. Lysosomic proteases, in particular cathepsins, do not seem to be largely involved in the degradation of myofibrillar proteins in rat skeletal muscle (35, 48). The ATP-ubiquitin–dependent proteolytic system is postulated to account for the turnover of short-lived proteins (49) or for abnormal proteins formed during stress such as heat-shock (50). However, it has been suggested that the activity of this system can also be related to the turnover of long-lived proteins such as those found in skeletal muscle (51). Recently, this proteolytic system has been involved in the perturbations of protein metabolism consequent to fasting and denervation atrophy (52). Previous studies from our laboratory have shown that the lysosomal pathway is only marginally involved in the development of muscle protein hypercatabolism in the AH-130 hosts (16, 35), while an important activation of the ATP-dependent proteolysis seems to be the leading mechanism (16). It is very interesting to observe that the preventive effect exerted by clenbuterol on the acceleration of muscle protein breakdown is due to complete repression of the hyperexpression of the ubiquitin mRNAs. The association between the increased ubiquitin gene expression and the proteolytic activity in this tumor model has been recently studied by our laboratory (53). Moreover, it is important to underline that in control rats receiving clenbuterol, the ubiquitin mRNAs expression is below the basal value. To the best of our knowledge, this is the first report showing that the effect of clenbuterol on muscle protein turnover is due to down-regulation of the ATP-ubiquitin–dependent proteolytic system.

β_2-adrenergic agonists are also known to display their anabolic effects in different situations of perturbed endocrine status such as diabetes, adrenalectomy, or castration (47, 54), suggesting that their action might involve hormonal modulations. However, as far as insulin and corticosterone are concerned, the present study suggests that this is not the case for the AH-130 hosts, since the altered plasma level of neither hormone was corrected by the treatment. However, the hormonal involvement

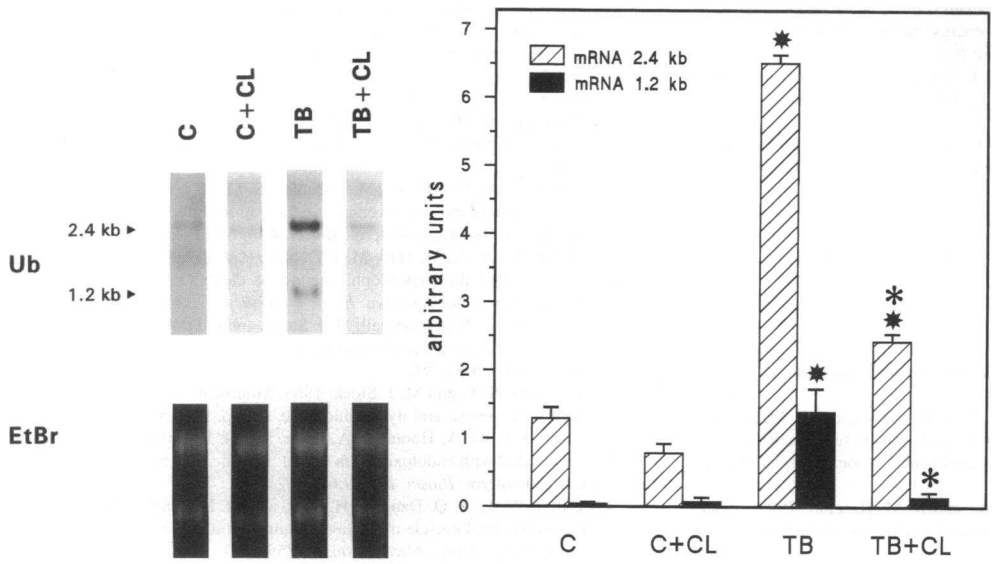

Figure 1. Northern blots of gastrocnemius muscle extracts from tumor-bearing rats. Expression of ubiquitin mRNA in skeletal gastrocnemius muscles from control (*C*), control treated with clenbuterol (*C+CL*), 7-d tumor-bearing (*TB*) and 7-d tumor-bearing treated with clenbuterol (*TB+CL*). It was detected after hybridization with a cDNA probe containing a region of the chicken polyubiquitin gene UB1. Autoradiographs were subjected to scanning densitometry. Ethidium bromide (*EtBr*) was used for total RNA quantitation. The results of 3 different animals are shown and expressed as arbitrary units. Significance of the differences (two-way factorial analysis of variance): $P < 0.01$ (tumor burden); $P < 0.01$ (treatment).

cannot be ruled out since the β_2-agonist could have altered hormone sensitivity (55).

Another important action of β_2-adrenergic agonists is to reduce the body fat content by directly stimulating lipolysis (19); tissue-specific modulations in lipoprotein lipase activity could constitute an additional mechanism for promoting a decreased lipid accretion in adipose tissue (56). In addition, these drugs enhance the overall energy expenditure and increase the thermogenesis, thereby dissipating the excess energy derived from the lipids mobilized (19). The present cachexia model in itself is characterized by extensive lipid mobilization (20, 57), associated with decreased activity of tissue lipoprotein lipase (20). The lack of effect of clenbuterol on adipose tissue in AH-130 hosts thus probably reflects the fact that lipid mobilization was already occurring in these animals and could not be easily stimulated further. When the drug treatment was applied to control rats, however, the adipose tissue mass decreased with respect to untreated controls (by 20%), in agreement with previous reports (38, 58).

In conclusion, the present results indicate that clenbuterol exerted a selective, powerful protective action on heart and skeletal muscle by antagonizing the enhanced protein degradation and the resulting protein loss that characterize cachexia in AH-130 tumor-bearing rats. These observations suggest that, in spite of its numerous side effects, clenbuterol could reveal as a convenient therapeutic tool in pathological states wherein muscle protein hypercatabolism is a critical feature such as cancer cachexia or other wasting diseases.

Table IV. Plasma Levels of Insulin and Corticosterone

Time	Tumor	Treatment	Corticosterone	Insulin
			mg/dl	*μU/ml*
Days 4–8	No	None	156±37	200±26
Day 4	No	Clenbuterol	177±47	115±20
	Yes	None	463±156*	86±9*
	Yes	Clenbuterol	434±186*	70±10*
Day 8	No	Clenbuterol	167±42	151±13
	Yes	None	463±145*	98±11*
	Yes	Clenbuterol	580±171*	79±10*

Data are means±SD. Significance of the differences (two-way factorial analysis of variance): * $P < 0.05$ (tumor burden). $n = 4$ and 6 for nontumor and tumor bearers, respectively.

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