

Anticachectic Effects of Formoterol: A Drug for Potential Treatment of Muscle Wasting

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ABSTRACT

In cancer cachexia both cardiac and skeletal muscle suffer an important protein mobilization as a result of increased proteolysis. Administration of the β_2 -agonist formoterol to both rats and mice bearing highly cachectic tumors resulted in an important reversal of the muscle-wasting process. The anti-wasting effects of the drug were based on both an activation of the rate of protein synthesis and an inhibition of the rate of muscle proteolysis. Northern blot analysis revealed that formoterol treatment resulted in a decrease in the mRNA content of ubiquitin and proteasome subunits in gastrocnemius muscles; this, together with the decreased proteasome activity observed, suggest that the main anti-proteolytic action of the drug may be based on an inhibition of the ATP-ubiquitin-dependent proteolytic system. Interestingly, the β_2 -agonist was also able to diminish the increased rate of muscle apoptosis (measured as DNA laddering as well as caspase-3 activity) present in tumor-bearing animals. The present results indicate that formoterol exerted a selective, powerful protective action on heart and skeletal muscle by antagonizing the enhanced protein degradation that characterizes cancer cachexia, and it could be revealed as a potential therapeutic tool in pathologic states wherein muscle protein hypercatabolism is a critical feature such as cancer cachexia or other wasting diseases.

INTRODUCTION

The development of cancer cachexia is the most common manifestation of advanced malignant disease. Indeed, cachexia occurs in the majority of cancer patients before death, and it is responsible for the death of 22% of cancer patients (1). The abnormalities associated with cancer cachexia include anorexia, weight loss, muscle loss and atrophy as well as anemia and alterations in carbohydrate, lipid, and protein metabolism (2). The degree of cachexia is inversely correlated with the survival time of the patient, and it always implies a poor prognosis (3–5). Perhaps one of the most relevant characteristics of cachexia is that of asthenia, which reflects the important muscle waste that takes place in the cachectic cancer patient (6). Lean body mass depletion is one of the main trends of cachexia, and it involves not only skeletal muscle, it also affects cardiac proteins resulting in important alterations in heart performance. In addition to the increased muscle protein degradation found during cancer growth, the presence of the tumor also induces an increased rate of DNA fragmentation in skeletal muscle in both rats and mice (7).

β_2 -adrenergic agonists are potent muscle growth promoters in many animal species (8, 9), resulting in skeletal muscle hypertrophy

(10–13), whereas they cause a reduction of the body fat content (14, 15). 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 (14). The intimate mechanisms by which these compounds exert such effects at the cellular level are still uncertain (9, 14, 15), although changes in protein turnover are clearly involved (16). The many physiologic functions controlled by β -adrenergic receptors suggests that the mechanism(s) for the observed changes in carcass composition may be extremely complex. Any proposed mechanism must begin with the possibility of direct effects of the agonist on skeletal muscle and adipocyte β -adrenergic receptors. Clenbuterol is one of these compounds with important anti-cachectic effects in animal models. The mode of action of this drug is based on its ability to prevent muscle wasting by inhibiting proteolysis in skeletal muscle (17). However, the toxicity of the drug in humans (18, 19) has not favored the undertaking of clinical trials.

Formoterol is a highly potent, β_2 -adrenoceptor-selective agonist combining the clinical advantages of rapid onset of action with duration of action. This compound is already in use in humans for the treatment of bronchospasm associated with asthma. *In vitro*, formoterol is a potent airway smooth muscle relaxant with high efficacy and very high affinity and selectivity for the β_2 -adrenoceptor (20). Moreover, formoterol relaxes bronchial smooth muscle and also provides important clinical benefits in symptomatic patients with chronic obstructive pulmonary disease (21). Formoterol, as other long-acting β_2 -adrenoceptor agonists, attenuates the allergen-induced late asthmatic reaction (22) and, under certain conditions, has more effect than other β_2 -adrenoceptor agonists (salbutamol and salmeterol), as has been described previously (23).

Bearing all this in mind, the aim of the present investigation has been to test, in two different animal models of cancer cachexia, the anti-cachectic efficiency of the relatively non-toxic β_2 -adrenergic formoterol.

MATERIALS AND METHODS

Animals. Male Wistar rats (Interfauna, Barcelona, Spain), of 5 weeks of age, and C57Bl/6 mice (Criffa, Barcelona, Spain), of about 12 weeks of age, were used in the different experiments. The animals were maintained at $22 \pm 2^\circ\text{C}$ with a regular light-dark cycle (light on from 8:00 a.m. to 8:00 p.m.) and had free access to food and water. The food intake was measured daily. All animal manipulations were made in accordance with the European Community guidelines for the use of laboratory animals.

Tumor Inoculation and Treatment

Yoshida AH-130 Ascites Hepatoma. 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 (24). Both groups were further divided into treated and untreated, the former being administered a daily intraperitoneal dose of formoterol (2 mg/kg body weight, dissolved in physiologic solution) and the latter a corresponding volume of solvent. On day 7 after tumor transplantation, the animals were weighed and anesthetized with an intraperitoneal injection of ketamine/xylazine mixture (3:1, Imalgene and Rompun, respectively). The tumor was

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harvested from the peritoneal cavity and its volume and cellularity evaluated. Tissues were rapidly excised, weighed, and frozen in liquid nitrogen.

Lewis Lung Carcinoma. Mice were divided into two groups, namely controls and tumor hosts. The tumor hosts received an inoculum of 5×10^5 Lewis lung carcinoma cells obtained from exponential tumors, given intramuscularly (left thigh). Both groups were further divided into treated and untreated, the former being administered, during the last 9 days before sacrifice, a daily intraperitoneal dose of formoterol (2 mg/kg body weight, dissolved in physiologic solution) and the latter a corresponding volume of solvent. On day 15 after tumor transplantation, the animals were weighed and anesthetized with ketamine/xylazine mixture (intraperitoneal, Imalgene and Rompun, respectively). The tumor was excised from the hind leg and its mass determined. Samples of tissues were rapidly excised, weighed, and frozen in liquid nitrogen.

Biochemicals

They were all reagent grade and obtained either from Roche S.A. (Barcelona, Spain) or from Sigma Chemical Co. (St. Louis, MO). Radiochemicals were purchased from Amersham (Bucks, United Kingdom). Formoterol fumarate micronized was kindly provided by Industriale Chimica s.r.l. (Saronno, Italy).

Lipid and Water Content

Lipid content was measured by the method of Folch *et al.* (25). Eighty milligrams of gastrocnemius muscle were used for tissue water content analysis and placed in weighed dry glass. Tissue samples were dried to a constant weight at 90°C for 72 hours to determine water content.

Muscle Preparations and Incubations

The dissection, isolation, and incubation of the extensor digitorum longus (EDL) muscles were carried out in rats under ketamine/xylazine mixture anesthesia as described previously (26, 27). The muscles were preincubated for 60 minutes: 30 minutes in Krebs-Henseleit buffer and 30 minutes in supplemented medium containing 10^{-4} mol/L formoterol, 10^{-4} mol/L clenbuterol, or none and then incubated for 120 minutes in fresh supplemented medium. Such muscles are able to maintain normal ATP concentrations during a 3-hour incubation period [3.6 ± 0.8 (5) control; 3.7 ± 0.6 (6) formoterol; 3.4 ± 0.9 (7) clenbuterol nmol ATP/mg EDL; ref. 28]. Total protein degradation by the isolated muscles was calculated as the rate of tyrosine released in the last 2 hours of incubation into the medium in the presence of 0.5 mmol/L cycloheximide to block the reincorporation of tyrosine into tissue protein. Tyrosine was measured fluorimetrically as described previously (29). Total protein synthesis rate in isolated muscles was performed as described previously (30).

Determination of Proteasome Activity

The chymotrypsin-like activity of the muscle proteasome was determined by evaluating the cleavage of the specific fluorogenic substrate succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin. The gastrocnemius muscle was homogenized in 20 mmol/L TRIS-HCl (pH 7.2) containing 0.1 mmol/L EDTA, 1 mmol/L 2-mercaptoethanol, 5 mmol/L ATP, 20% glycerol, and 0.04% (v/v) Triton X-100. Muscle homogenates were then centrifuged at $13,000 \times g$ for 15 minutes at 4°C. The supernatant was collected, and protein concentration was determined with the bicinchoninic acid assay (Pierce Chemical Co., Rockford, IL). Aliquots of 40- μ g protein were then incubated for 60 minutes at 37°C in the presence of succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (40 μ mol/L). The incubation buffer for the evaluation of proteasome activity was 50 mmol/L HEPES (pH 8.0) containing 5 mmol/L EGTA. Fluorescence was read with a spectrofluorometer (380 nm excitation and 460 nm emission; RF-5001 PC spectrofluorophotometer, Shimadzu, Tokyo, Japan). The activity, expressed as nkat/mg protein referred to the total protein pool, was calculated by using free amidocoumarin as working standard. The final result is the difference between the nkatals obtained with and without the presence of 25 μ mol/L lactacystin, a specific proteasome inhibitor.

RNA Isolation and Northern Blot Analysis

Total RNA from gastrocnemius muscle was extracted with the acid guanidinium isothiocyanate/phenol/chloroform method (31). Northern blot analyses were performed as described previously (26).

Apoptosis Assays

DNA fragmentation assay in tibialis muscle was performed as described previously (7). Caspase-3 activity in tibialis muscle was determined with the BD ApoAlert Caspase-3 Colorimetric assay kit (BD Biosciences Clontech, Palo Alto, CA).

Electrophoretic Mobility Shift Analysis

Nuclear protein extracts from tibialis muscle were isolated as reported previously (32), and protein concentration was determined by BCA Protein Assay kit (Pierce). Oligonucleotide corresponding to the consensus sequence to nuclear factor- κ B (NF- κ B), activating protein-1 (AP-1), and CCAAT/enhancer-binding protein (C/EBP) were end-labeled with [α^{32} P]dCTP and Klenow enzyme. The double-stranded oligonucleotides end-labeled (30,000 cpm) were incubated for 10 minutes on ice with 150 μ g of nuclear protein extract. Reactions were carried out in a final volume of 20 μ L containing 12% glycerol, 12 mmol/L HEPES (pH 7.9), 4 mmol/L Tris-HCl (pH 7.9), 1 mmol/L EDTA (pH 8), 1 mmol/L DTT, 25 mmol/L KCl, 5 mmol/L MgCl₂, 40 μ g/ml polydeoxyinosinic-deoxycytidylic acid and protease inhibitors (aprotinin and leupeptin). At the end of the incubation, mix samples were electrophoresed at 4°C at 325 V for 60 to 80 minutes on a 7% nondenaturing polyacrylamide gel in 0.5 \times Tris-borate-EDTA. After electrophoresis, the gel was dried for 120 minutes in a Bio-Rad Gel Dryer (Bio-Rad, Hercules, CA) and exposed overnight to an X-ray-sensitive film (Hyperfilm-MP, Amersham Biosciences) at -80°C with intensifying screens. The specificity of the NF- κ B, AP-1, and C/EBP bands has been confirmed by reactions with mutant oligonucleotides end-labeled.

Statistical Analysis

Statistical analysis of the data were performed by one-way and two-way ANOVA.

RESULTS

With the aim of investigating the potential anticachectic effects of the β_2 -agonist formoterol, we choose two different tumor models that share the fact that they have a very great cachectic response: the rat Yoshida AH-130 ascites hepatoma and the mouse Lewis lung carcinoma. The growth of these tumors causes in the host rapid and progressive loss of body weight and tissue waste, particularly in skeletal muscle (33, 34). Acceleration of tissue protein breakdown accounts for most of the waste in the AH-130 bearers (24, 35).

Although formoterol treatment resulted in substantial increases in food intake in both rats and mice (Table 1) from the nontumor-bearing groups, the β_2 -agonist treatment did not result in either changes in food intake or tumor growth in the rat or mouse tumor-bearing animals (Table 1). Therefore, the hypophagia occurring during tumor growth persisted unchanged in the treated rats (Table 1A).

Quite different was the pattern with regard to skeletal and heart muscles. Thus, as shown in Table 1A, the implantation of the Yoshida AH-130 ascites hepatoma resulted in a decrease in muscle weights (13% for gastrocnemius, 16% for tibialis, 11% for soleus, 19% for EDL, and 11% for heart) as we reported previously (33). Formoterol treatment resulted in an increase in most muscle types including gastrocnemius (28%), tibialis (25%), EDL (32%), and cardiac muscle (21%) in the control group. Interestingly, the drug did not alter either the lipid or water content [lipid content (%) control 2.7 ± 0.2 (4), formoterol 2.7 ± 0.1 (5); water content (%) control 76 ± 0.4 (4), formoterol 78 ± 0.9 (4)]. In tumor-bearing animals, the β_2 -agonist

Table 1 Food intake, tissue weights, and tumor growth

	C	C+F	TB	TB+F	ANOVA	
					Tumor	Treatment
A. Rats bearing the Yoshida AH-130 ascites hepatoma						
No. of Animals	(4)	(5)	(12)	(12)		
Food intake	121 ± 1	135 ± 2	105 ± 4	109 ± 5	<0.001	<0.05
Muscle weights						
Gastrocnemius	673 ± 12	859 ± 38	585 ± 10	639 ± 16	<0.001	<0.001
Tibialis	219 ± 7	275 ± 12	184 ± 4	204 ± 4	<0.001	<0.001
Soleus	46 ± 1	55 ± 4	41 ± 1	40 ± 1	<0.001	NS
EDL	53 ± 2	70 ± 3	43 ± 1	50 ± 1	<0.001	<0.001
Heart	430 ± 17	520 ± 15	382 ± 7	426 ± 17	<0.001	<0.001
Adipose tissues weights						
White (dorsal)	963 ± 103	759 ± 126	516 ± 90	461 ± 49	<0.001	NS
Brown (interscapular)	260 ± 13	278 ± 21	123 ± 11	177 ± 11	<0.001	<0.05
Carcass weight	92 ± 1	113 ± 11	80 ± 1	81 ± 1	<0.001	NS
Tumor cell content	—	—	3886 ± 444 (5)	3403 ± 88 (5)		NS
B. Mice bearing the Lewis lung carcinoma						
No. of animals	(7)	(6)	(8)	(7)		
Food intake	46 ± 1	53 ± 3	51 ± 2	53 ± 2	NS	<i>P</i> < 0.05
Muscle weights						
Gastrocnemius	563 ± 4	669 ± 13	492 ± 9	548 ± 13	<0.001	<0.001
Tibialis	192 ± 2	217 ± 4	152 ± 3	169 ± 3	<0.001	<0.001
Soleus	28 ± 1	36 ± 1	23 ± 2	36 ± 2	<i>P</i> = 0.07	<0.001
EDL	40 ± 1	42 ± 2	35 ± 2	40 ± 2	<i>P</i> < 0.05	<i>P</i> = 0.06
Heart	432 ± 15	462 ± 31	401 ± 17	496 ± 40	NS	<0.05
Adipose tissues weights						
White (dorsal)	238 ± 18	217 ± 25	58 ± 12	68 ± 9	<0.001	NS
Brown (interscapular)	208 ± 12	249 ± 6	127 ± 5	153 ± 13	<0.001	<0.01
Carcass weight	80 ± 1	87 ± 1	69 ± 1	73 ± 2	<0.001	<0.001
Tumor weight	—	—	4.26 ± 0.3	4.58 ± 0.3		NS

NOTE. For more details see Materials and Methods. Results are mean ± SEM for the number of animals indicated in parentheses. Food intake is expressed in grams and refers to the ingestion during the period of the experiment prior to sacrifice which took place 7 days after tumor inoculation (A) and 15 days after tumor inoculation (B). Tissue weights are expressed as milligrams per 100 grams of initial body weight. Carcass (body without organs or tumor) is expressed as grams per 100 grams of initial body weight. Tumor cell content is expressed in millions of cells (A) and in grams (B). Statistical significance of the results by two-way ANOVA.

Abbreviations: C, control; F, formoterol-treated; TB, tumor-bearing; E2, E2-conjugating enzyme; NS, nonsignificant differences; —, No tumor present.

induced an increase in gastrocnemius (9%), tibialis (11%), EDL (16%), and heart (11%) weight, thus preventing muscle wasting (Table 1A). Treatment with formoterol had therefore a clear protective effect, in terms of wet tissue weight for the skeletal muscles and for the heart. Then, formoterol afforded a substantial trophic action on muscles in nontumor bearers as well, particularly on the gastrocnemius, tibialis, EDL, and cardiac muscle, whereas the soleus was not significantly affected (Table 1A), in agreement with our previous observations with other β_2 -agonists (16).

In the Lewis lung model, similar findings were observed because tumor burden induced a great decrease in muscle weights (13% for gastrocnemius, 21% for tibialis, and 18% for soleus; Table 1B) as we reported previously (34). Formoterol treatment increased the muscle weights of gastrocnemius (19%), tibialis (13%), and soleus (28%) significantly in the nontumor-bearing animals (Table 1B). The drug did not alter either the lipid or water content in mice [lipid content (%) 2.9 ± 0.3 (4) control, 2.6 ± 0.2 (4) formoterol; water content (%) 75 ± 0.2 (4) control, 76 ± 0.1 (3) formoterol]. Interestingly, treatment with the β_2 -agonist had a substantial protective effect on skeletal muscle weights [gastrocnemius (11%), tibialis (11%) and soleus (56%)] and in cardiac muscle (24%) in tumor-bearing animals (Table 1B).

Regarding adipose tissues, tumor growth inflicted large reductions in white adipose tissue mass in the Yoshida model (46%; Table 1A) and also in the Lewis lung one (76%; Table 1B). A similar situation was observed for brown adipose tissue [53% decrease in the Yoshida model (Table 1A) and 39% in the Lewis lung one (Table 1B)]. In the rat tumor model, formoterol treatment was associated with a tendency toward a decrease in white adipose tissue in both the control and tumor-bearing animals, although the results did not reach statistical significance, whereas it induced an increase in brown adipose tissue weight (Table 1A). In the Lewis lung model, the β_2 -agonist did not alter the white adipose tissue mass in either control or tumor-bearing

animals, but it induced an increase in brown adipose tissue in both control and tumor bearers (Table 1B).

We therefore decided to investigate if the effects of formoterol were based on alterations in the rate of protein degradation of skeletal muscle. We performed incubations of isolated incubated muscles in the presence of formoterol. As can be seen in Fig. 1B, formoterol decreased the rate of protein degradation by 20%, a similar value to that obtained for clenbuterol (36), thus confirming that the action of the molecule is based on a decrease in the proteolytic rate. In addition, the proteolytic rate was also significantly decreased (12%) in rats that had been pretreated previously for 6 consecutive days with the β_2 -agonist (Fig. 1A).

As our group has shown previously (33, 34, 37), the accelerated muscle protein breakdown in both the AH-130 and Lewis lung hosts is achieved basically through activation of the ATP-ubiquitin-dependent proteolytic system. We therefore decided to investigate the effects of formoterol on the gene expression of different proteolytic systems present in skeletal muscle. We studied the gene expression in gastrocnemius muscle of two *polyubiquitin* mRNA species (2.4 and 1.2 kb), *C8* and *C9 proteasome* subunits, as well as *E2 ubiquitin-conjugating* enzyme (all of them involved in the ATP-ubiquitin-dependent proteolytic pathway), *m-calpain* (calcium-dependent system), and *cathepsin B* (lysosomal system). As shown in Table 2A and B, tumor-bearing animals showed an increased expression of the *polyubiquitin* genes in relation with the corresponding control animals: over 2-fold (2.4 kb) and 6-fold (1.2 kb) in Yoshida model, and over 2-fold (2.4 kb) and 3-fold (1.2 kb) in Lewis lung carcinoma model. When the tumor-bearing animals received formoterol, this activation was suppressed. Interestingly, similar findings were found for the *C8* and *C9 proteasome* subunits, the expression of which was increased as a result of tumor growth, and formoterol treatment suppressed this activation (Table 2A and B). Similar findings were observed with *E2 ubiquitin-conjugating* enzyme in the Yoshida rat

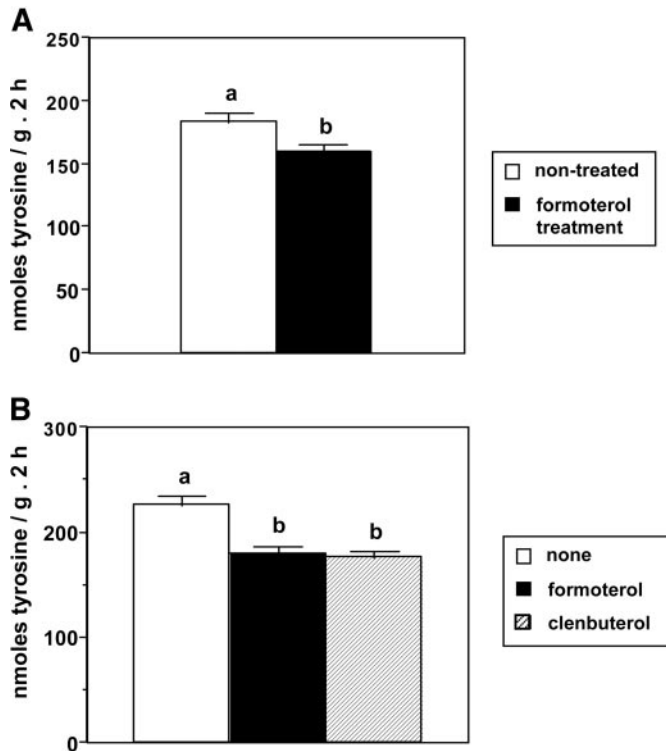


Fig. 1. Proteolytic rates in isolated EDL rat muscles. For more details, see Materials and Methods. Proteolytic rates were measured in the presence of cycloheximide (0.5 mmol/L) and are expressed as nanomoles tyrosine per gram and 2 hours of incubation. In A, the results correspond to rats given formoterol (2 mg/kg body weight) treatment for 6 consecutive days before sacrifice. Statistical significance of the results (by one-way ANOVA); non-treated *versus* treated, $P < 0.05$. In B, the effect of the β_2 -agonists (formoterol and clenbuterol) on incubated muscles of non-treated animals. Statistical significance of the results by one-way ANOVA, between groups $P < 0.001$; statistically significant difference by *post hoc* Duncan test. Different superscripts indicate significant differences between groups.

model (Table 2A). Taking into account the increase in proteasome activity in skeletal muscle of tumor-bearing mice (38), we decided to test if formoterol was also able to revert this activity in skeletal muscle

of tumor-bearing rats. We measured the most dominant catalytic activity (chymotrypsin-like activity) in gastrocnemius muscle of rats bearing the Yoshida AH-130 tumor. The results obtained show a decrease of proteasome activity in both nontumor- (64%) and tumor-bearing rats (47%) treated with formoterol (Table 3).

Concerning other proteolytic systems, tumor burden also resulted in an increase in the expression of the calcium-dependent system *m-calpain* (3-fold in Yoshida model and 2-fold in Lewis lung model), as reported previously (39) but formoterol treatment did not abolish this activation in any of the tumor models tested (Table 2). Concerning the action of formoterol on the calcium-dependent proteolytic system, the increase of mRNA content observed after formoterol treatment does not seem to support the observation made by other authors (40); however, other reports (41) agree with the results observed here. Finally, formoterol treatment decreased lysosomal *cathepsin B* expression in tumor-bearing rats (Table 2A) and, conversely, the slight increase in mRNA content observed in the mice bearing Lewis lung carcinoma in the lysosomal *cathepsin B* was not reverted by formoterol treatment (Table 2B).

Both formoterol and clenbuterol influenced the rate of protein synthesis that was increased by 20%, both for formoterol and clenbuterol (Table 4). These results agree with similar observations made in previous studies involving β_2 -agonists in incubated rat muscles (42).

Table 3 Proteasome activity in skeletal muscle homogenates from tumor-bearing rats

Experimental group	Treatment	Proteasome activity
Control	None	13.3 ± 2.5 (3)
	Formoterol	4.8 ± 1.4 (3)†
Tumor	None	20.3 ± 4.7 (3)*
	Formoterol	10.7 ± 2.4 (5)*,†

NOTE. For more details see Materials and Methods. The activities, expressed as nkatals/mg protein referred to the total protein pool, was calculated by using free amidocoumarin as working standard, and are mean ± SEM for the number of animals indicated in parentheses. The final result is the difference between the nkatals obtained with and without the presence of 25 $\mu\text{mol/L}$ lactacystin, a specific proteasome inhibitor. Statistical significance of the results by two-way ANOVA.

* Nontumor *versus* tumor $P < 0.05$.

† Nontreatment *versus* treatment $P < 0.01$.

Table 2 Gene expression of the different proteolytic systems in gastrocnemius muscles

Proteolytic system	C	C+F	TB	TB+F	ANOVA	
					Tumour	Treatment
A. Rats bearing the Yoshida AH-130 ascites hepatoma						
Ubiquitin-dependent						
Ubiquitin 2.4 kb	100 ± 12 (4)	92 ± 12 (5)	233 ± 23 (3)	128 ± 19 (5)	<0.01	<0.05
Ubiquitin 1.2 kb	52 ± 10 (3)	57 ± 12 (5)	311 ± 71 (3)	73 ± 15 (4)	<0.05	<0.05
Proteasome subunit C8	100 ± 9 (4)	62 ± 7 (5)	155 ± 7 (5)	78 ± 9 (5)	<0.01	<0.001
Proteasome subunit C9	100 ± 5 (4)	104 ± 7 (5)	126 ± 6 (5)	80 ± 4 (5)	NS	<0.01
E2 1.8 kb	100 ± 4 (4)	91 ± 3 (5)	163 ± 4 (5)	136 ± 5 (5)	<0.001	<0.01
E2 1.2 kb	100 ± 7 (4)	88 ± 6 (5)	157 ± 8 (5)	128 ± 5 (5)	<0.001	<0.01
Calcium-dependent						
m-calpain	100 ± 13 (4)	187 ± 14 (5)	282 ± 33 (5)	212 ± 9 (4)	<0.01	NS
Lysosomal						
Cathepsin B	100 ± 8 (4)	106 ± 6 (5)	113 ± 3 (5)	65 ± 6 (5)	$P = 0.09$	<0.05
B. Mice bearing Lewis lung carcinoma						
Ubiquitin-dependent						
Ubiquitin 2.4 kb	100 ± 9 (5)	50 ± 9 (5)	227 ± 59 (4)	90 ± 21 (6)	<0.01	<0.01
Ubiquitin 1.2 kb	36 ± 3 (5)	25 ± 5 (5)	113 ± 14 (4)	27 ± 5 (6)	<0.01	<0.001
Proteasome subunit C8	100 ± 8 (5)	108 ± 7 (4)	177 ± 11 (5)	129 ± 12 (6)	<0.001	$P = 0.08$
Proteasome subunit C9	100 ± 10 (5)	114 ± 7 (4)	145 ± 3 (5)	101 ± 12 (5)	NS	$P = 0.08$
Calcium-dependent						
m-calpain	100 ± 21 (5)	136 ± 20 (5)	222 ± 27 (5)	254 ± 33 (7)	<0.001	NS
Lysosomal						
Cathepsin B	100 ± 14 (5)	134 ± 5 (3)	157 ± 11 (5)	161 ± 18 (7)	<0.05	NS

NOTE. For more details see Materials and Methods. Expression of the different proteolytic systems components mRNAs was detected after hybridization with cDNA probes. Loading correction was carried out by performing blots with the rat 18S ribosomal subunit probe. Autoradiographs were subjected to scanning densitometry. Results are mean ± SEM for the number of animals indicated in parentheses. Statistical significance of the results by two-way ANOVA.

Abbreviations: C, control; F, formoterol-treated; TB, tumor-bearing; E2, E2-conjugating enzyme; NS, nonsignificant differences.

Another aspect that was included in the present study was the evaluation of muscle apoptosis during cancer. The results presented in Fig. 2 show that tumor growth is associated with an increased rate of apoptosis both determined as DNA fragmentation (68%; panels A and B) and as activation of caspase-3 (59%; panel C). Very interestingly, formoterol treatment completely abolished the increased rate of muscle apoptosis (Fig. 2 A-C).

Finally, the results shown in Table 5 show that none of the transcription factors studied (neither NF- κ B, AP-1, nor C/EBP) were involved in the development of muscle wasting during tumor burden.

DISCUSSION

Muscle protein waste is a main feature in cancer cachexia and is mostly ascribed to enhanced tissue protein catabolism (33, 43–45). The present observations show that treatment of the AH-130 hosts with formoterol largely abolished the wasting in gastrocnemius, tibialis, EDL, and heart. This is in agreement with previous reports that β_2 -adrenergic agonists may antagonize the skeletal muscle depletion in different situations. Thus, clenbuterol markedly attenuates muscle wasting during cancer cachexia (16, 17) and the muscle atrophy by denervation (46–48) or by hindlimb suspension (13, 49, 50). Moreover, this drug can increase the skeletal muscle mass in mice with genetic muscle dystrophy (51). A disadvantage associated with clenbuterol treatment is that it also has negative effects on heart performance on both experimental animals (52, 53) and humans (54). The pronounced anabolic effect of clenbuterol in animals has led to an excessive use of this drug in animal breeding, and cases of intoxication in humans after ingestion of bovine liver from cattle treated in this way have been described previously (54). Another β_2 -adrenergic agonist, fenoterol, a synthetic compound with bronchodilatory properties, has also anabolic properties in skeletal muscle (49, 55). Ryall *et al.* (55) demonstrated that fast-twitch EDL and slow-twitch soleus muscles of rats treated with fenoterol for 4 weeks had a greater force-producing capacity than muscles from rats that received an equimolar dose of clenbuterol. It is not clear how these agents modulate tissue protein turnover, although some indirect evidence for an effect on protein catabolism has been provided. Reeds *et al.* (56) 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. Other studies suggest that catecholamines may increase the rate of protein synthesis in oxidative muscles, leading to increased protein accretion (40).

Both the prevention of muscle wasting in AH-130 and Lewis lung carcinoma tumor bearers and the increase of muscle protein mass in nontumor bearers afforded by formoterol appeared to result from regulations on the catabolic side, respectively, by restoring decreased protein breakdown to normal rates or by reducing them to less than normal levels. Moreover, in agreement with previous reports (57, 58), the effects of the drug appeared more marked on a fast-twitch (gastrocnemius) than on a slow-twitch muscle (soleus), as we observed in

Table 4 Protein synthesis rate in isolated EDL muscles from control rats

Additions to incubation medium	Rate of protein synthesis
None	7.82 \pm 0.5 (6)*
Formoterol	9.36 \pm 0.3 (7)†
Clenbuterol	9.36 \pm 0.5 (9)†

NOTE. For full details see Materials and Methods section. Synthetic rates are expressed as nanomoles of [¹⁴C]phenylalanine incorporated per gram and 30 minutes of incubation. The results are mean values \pm SEM for the number of animals indicated in parentheses. Statistical significance of the results by one-way ANOVA, between groups, *P* < 0.05; † statistically significant difference by *post hoc* Duncan test. Different superscripts indicate significant differences between groups.

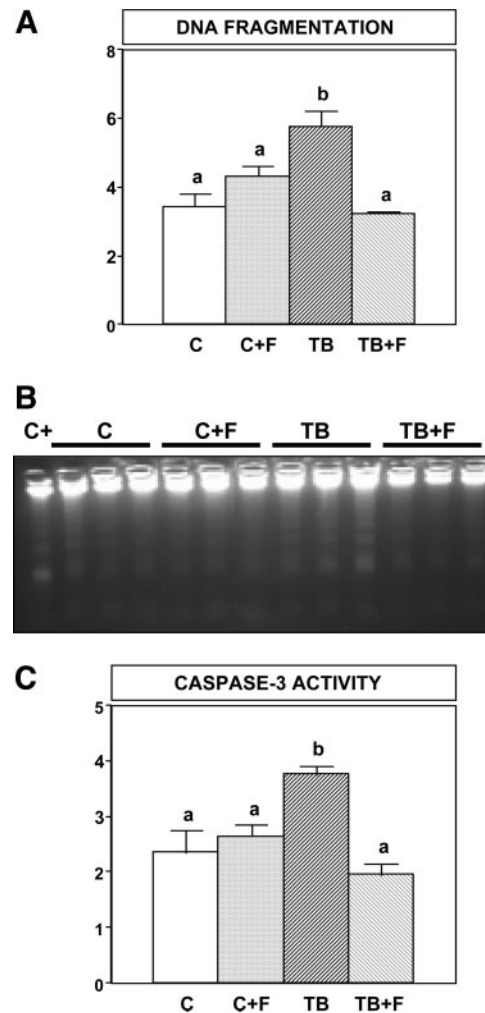


Fig. 2. Apoptosis in tibialis muscles from tumor-bearing rats. For more details, see Materials and Methods. Results are mean \pm SEM for five animals in each group. In A and B, DNA fragmentation was assessed by monitoring the laddering in an agarose gel. The percentage of DNA fragmentation was quantified by scanning densitometry. Liver from anti-Fas antibody-treated mice (C+) was used as positive control. In C, caspase-3 activity was determined by a colorimetric assay, and the results are expressed as nanomoles per hour. C, control; F, formoterol-treated; TB, tumor-bearing. Statistical significance of the results by one-way ANOVA, between groups, *P* < 0.001; † statistically significant difference by *post hoc* Duncan test. Different superscripts indicate significant differences between groups.

Yoshida model in the present study. The trophic action of β_2 -adrenergic agonists is generally regarded as quite selective for the skeletal muscle (14, 49, 56). After treatment with such drugs, however, cardiac hypertrophy has been reported in some studies (56, 59) but not in others (49). In the present study, formoterol administration exerted quite comparable effects on gastrocnemius, tibialis, EDL (in Yoshida model), soleus (in Lewis lung model), and heart in both control and tumor-bearing animals.

The precise mechanisms by which intracellular proteins are degraded are largely unknown, although it is accepted that proteolysis may occur inside and outside the lysosomes. 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 (60), whereas an important activation of the ATP-dependent proteolysis seems to be the leading mechanism (33, 60). Formoterol, as we report in the present study, plays a role in regulating gene expression of *ubiquitin* and *proteasome subunits* (all of them genes of the ATP-ubiquitin-dependent proteolytic system). Northern blot analysis revealed that formoterol treatment resulted in a

Table 5 NF- κ B, AP-1, and C/EBP DNA-binding activity in the tibialis muscles from rats bearing the Yoshida AH-130 ascites hepatoma

Transcription factor	ANOVA					
	C	C+F	TB	TB+F	Tumor	Treatment
NF- κ B	100 \pm 7 (4)	118 \pm 7 (5)	100 \pm 16 (4)	93 \pm 13 (5)	NS	NS
AP-1	100 \pm 14 (4)	85 \pm 8 (5)	112 \pm 10 (5)	105 \pm 12 (4)	NS	NS
C/EBP	100 \pm 20 (4)	128 \pm 13 (4)	122 \pm 20 (4)	107 \pm 13 (5)	NS	NS

NOTE. For more details see Materials and Methods. NF- κ B, AP-1, and C/EBP DNA-binding were determined in nuclear extracts from tibialis muscle. Autoradiographs were subjected to scanning densitometry. Results are mean \pm SEM for the number of animals indicated in parentheses. Statistical significance of the results by two-way ANOVA. Abbreviations: C, control; F, formoterol-treated; TB, tumor-bearing; E2, E2-conjugating enzyme; NS, nonsignificant differences.

decrease in mRNA content in gastrocnemius muscles in *ubiquitin* and *proteasome subunits* and in a decrease in proteasome activity in all experimental groups, therefore suggesting that the main anti-proteolytic action of the drug may be based on an inhibition of the ATP-ubiquitin-dependent proteolytic system. Moreover, we demonstrate that formoterol decreased the rate of protein degradation by 20% in incubated EDL in non-treated rats, and by 12% in pretreated rats. This result has a similar value to the study obtained for clenbuterol (36), thus confirming that the action of the molecule is based on a decrease in the proteolytic rate. Interestingly, we also observed an increment in the rate of protein synthesis in incubated EDL muscles in the presence of the β_2 -agonist, therefore suggesting a role of formoterol in both catabolic and anabolic pathways of protein metabolism in skeletal muscle.

Another distinctive feature associated with muscle wasting during tumor growth is muscle apoptosis. Indeed, our research group reported that an enhanced DNA fragmentation rate takes place in the skeletal muscle of tumor-bearing animals (7). It is therefore interesting to point out that the formoterol clearly acted as an antiapoptotic agent, completely abolishing the increased apoptotic rate, both determined as caspase-3 activity and DNA fragmentation. These approximations include both initial apoptotic events (activation of caspase-3) and the final stage of the apoptosis as measured by the DNA laddering. Previous reports have already described the anti-apoptotic effects of clenbuterol in liver (61).

Finally, to explain the mechanism(s) associated with the action of formoterol in muscle, we decided to evaluate the possible role of several transcription factors. Initially we studied the role of NF- κ B in muscle wasting in both tumor models. We chose this transcription factor because previous *in vitro* studies showed its possible involvement in cachexia (62, 63). The results found (Table 5) were in disagreement with the mentioned reports, because no activation associated with tumor growth was present in our tumor models. We then decided to examine other transcription factors (AP-1 and C/EBP) that might have been involved in muscle wasting during sepsis (64, 65). The results presented in Table 5 show that neither of these transcription factors were affected by tumor growth nor by the β_2 -agonist treatment.

In conclusion, the present results indicate that formoterol exerted a selective, powerful protective action on heart and skeletal muscle by antagonizing the enhanced protein degradation that characterizes cancer cachexia; in addition, the β_2 -agonist also had a protective action against the apoptotic events on skeletal muscle. These observations suggest that, conversely to what it is found with other β_2 -agonists that have numerous side effects and considerable toxicity in humans, formoterol could be revealed as a potential therapeutic tool in pathologic states wherein muscle protein hypercatabolism is a critical feature such as cancer cachexia or other wasting diseases. However, and although in experimental animals the dose used in this and other studies (66) showed no toxicity, safety studies on formoterol in humans are required.

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