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ORIGINAL RESEARCH

Lack of Synergy Between β-Agonist Treatment and a Blockage of Sarcoplasmic Calcium Flow in a Rat Cancer Cachexia Model

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Background: During cancer cachexia, both skeletal muscle and adipose tissue losses take place. The use of β2-agonists, formoterol in particular, has proven to be very successful in the treatment of the syndrome in pre-clinical models. The object of the present research was to study the effects of a combination of formoterol and dantrolene, an inhibitor of the ryanodine receptor 1 (RyR1), on body weight loss and cachexia in tumour-bearing animals.
Methods: Rats were separated into two groups: controls (C) and tumour bearing (TB). TB group was further subdivided into four groups: untreated (saline as a vehicle), treated with Formoterol (TF) (0,3 mg/kg body weight in saline, subcutaneous (s.c.), daily), treated with Dantrolene (TD) (5 mg/kg body weight in saline, subcutaneous (s.c.), daily), and double-treated treated (TFD) with Formoterol (0,3 mg/kg body weight, subcutaneous (s.c.), daily) and Dantrolene (5 mg/kg body weight, subcutaneous (s.c.), daily). 7 days after tumour transplantation, muscle weight, grip force, and total physical activity were specified in all experimental groups.
Results: While formoterol had, as in previous studies, a very positive effect in reducing muscle weight loss, dantrolene had no effects, neither on skeletal muscle nor on any of the parameters studied. Finally, the combined treatment (formoterol and dantrolene) did not result in any significant benefit on the action of the β2-agonist.
Conclusion: It is concluded that, in the preclinical cachectic model used, no synergy exists between β2-agonist treatment and the blockade of sarcoplasmic-calcium flow.
Keywords: cancer cachexia, skeletal muscle, dantrolene, formoterol, calcium, ryanodine receptor 1

Background

The percentage of cancer patients affected by cachexia varies from 50 to 80% constituting a useful survival estimate. Cachexia is responsible for more than 20% of cancer deaths in humans.[1] Moreover, cachexia is linked with a decrease in physical activity[2] and results in a poor quality of life and a decreased efficacy and outcome of antitumoural treatment.[3,4] In 2008 a consensus,[5] defined cachexia as a

> complex metabolic syndrome associated with underlying illness and characterised by loss of muscle with or without loss of fat mass. The prominent clinical feature of cachexia is weight loss. Anorexia, inflammation, insulin resistance and increased muscle protein breakdown are frequently associated with cachexia.[5]

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Loss of both adipose tissue and skeletal muscle are important but the loss of skeletal muscle is the most important factor in the outcome of cachexia since it influences survival together with muscle force and functioning, essential in the recovery of the patient.[6]

The treatment of cancer cachexia includes many approaches and strategies but, unfortunately, they cannot completely abolish weight loss. Those approaches concentrate, basically, in either fighting anorexia and normalizing metabolic alterations or both.[7,8] Concerning the latter, β2-agonists, formoterol, in particular, have important anti-cachectic effects.[9] The action of formoterol is based on the prevention of muscle wasting by inhibiting proteolysis (decreasing the activation of the ubiquitin-dependent proteolytic system, main mechanism activated in muscle wasting conditions) and apoptosis in muscle tissue.[9,10] The anti-cachectic effects of the drug affect both physical activity and grip force, thus improving physical performance in cachectic tumour-bearing animals.[11] In humans, the combination of formoterol and the orexigenic drug megestrol acetate also resulted in a promising therapy in cancer cachexia.[12]

It is well known that mitochondria and sarcoplasmic reticulum (SR) play a key role in muscular function. Calcium released from SR, stimulates mitochondrial ATP production helping to meet increased energy demand during muscle contraction (process called excitation-contraction coupling).[13] Moreover, functionally intact mitochondria inhibit undesired localized SR calcium release by controlling the local redox environment of the calcium release units in normal cases.[13] Thus, bidirectional SR-mitochondrial communication provides a powerful local control mechanism for integrating calcium release/reuptake and ATP utilization during muscle contraction with ATP production and skeletal muscle bioenergetics.[14] In the case of cachexia, an uncontrolled release of calcium ions from the SR, due to the oxidation of ryanodine receptor 1 (RyR1), contributes to muscle weakness.[15] In addition, the utilization of S107, which prevent RyR1 dysfunction by increasing the link with it endogenous regulator calpastatin, protects muscle weakness acquired during cancer cachexia (breast cancer cells DA-MB-231), with recovery of the contraction force, but has no beneficial effect on muscle wasting.[15] On the other hand, dantrolene, an inhibitor of the RyR1, is able to suppress the calcium overload release from the SR,[16] but its use in animals bearing the AH-130 Yoshida ascites hepatoma –a highly cachectic tumour– failed to prevent muscle wasting and had no effects on the skeletal muscle protein degradation rate.[17] The aim of the present investigation was to explore if the combination of formoterol and dantrolene had any synergistic effect on cancer-related cachexia.

Methods

Animals

Male Wistar rats (6 weeks-old) (Harlan, Barcelona, Spain) were kept in individual cages at a constant temperature of 22 ± 2 °C with a regular light-dark cycle (light from 08:00 a.m. to 08:00 p.m.) and access to food and water (free). Intraperitoneal injection of 10^8 AH-130 Yoshida ascites hepatoma cells obtained from exponential tumours resulted in experimental cachexia as previously described[18] Food intake was measured every day. The protocol was approved by the Bioethical Committee of the University of Barcelona In addition, all animal handling was made following the European Community guidelines for the use of experimental animals.[19]

Experimental Design

Animals were randomized and divided into two groups, the so-called controls (C) and tumour bearing (TB). TB group was further subdivided into four subgroups: untreated (saline as a vehicle), treated with Formoterol (TF) (0,3 mg/kg body weight in saline, subcutaneous (s.c.), daily), treated with Dantrolene (TD) (5 mg/kg body weight in saline, subcutaneous (s.c.), daily), and double-treated treated (TFD) with Formoterol (0,3 mg/kg body weight, subcutaneous (s.c.), daily) and Dantrolene (5 mg/kg body weight, subcutaneous (s.c.), daily). 7 days following tumour inoculation, animals were weighed and anesthetized with an intraperitoneal (i.p.) injection of ketamine/xylazine mixture (3:1) (Imalgene® and Rompun® respectively). Animals were euthanized by performing an incision in the aorta. Tumour volume was determined on the day of sacrifice. Tissues samples were rapidly extracted, weighed, and frozen using liquid nitrogen.

Biochemicals

Formoterol was kindly given by Industriale Chimica s.r.l. (Saronno, Italy), Dantrolene was purchased from Abcam Co. (Cambridge, England).

Total Physical Activity

During the last 24 hours prior to the sacrifice, total physical activity (TPA) (IR ACTIMETER System and ACTITRAK software from Panlab, Barcelona) was measured in the different experimental groups, using activity sensors that convert individual changes in the infrared pattern –caused by movements of the animals– into arbitrary activity counts.[20] The animals remained in their individual cages while performing the measurements. In order to minimize stress to the animals, a frame containing an infrared beam system was placed outside the cage.

Grip Force Assessment

The grip-strength test[20] was used to determine rat skeletal muscle strength (GS). The grip-strength apparatus (Panlab-Harvard Apparatus, Spain) includes a pull bar attached to a dynamometer that acts as an isometric force transducer. The grip strength meter apparatus was placed horizontally, the animals being held by the tail and lowered towards the apparatus. Rats were permitted to grasp the bar being then pulled backward in the horizontal plane. Just before it lost grip, the force applied to the bar was measured as the peak tension. A minimum of three measurements per animal were taken, the results being averaged for analysis. The data are given as g/g initial body weight.

Statistical Analysis

The means and the standard error of the mean (SEM) were calculated for each studied parameter. One-way ANOVA was used for statistical analysis of the results. Post-hoc pairwise comparisons Dunnett's test were used. (Tables 1 and 2). Statistical analysis of the results was done using the Student's *t*-test (Activity data in Table 3).

Results

As can be seen in Table 1, the presence of the tumour resulted in an important loss of body weight, –shown as decrement in weight increase– which is associated with anorexia. Indeed, the animals ate at least 30% less than the control non-tumour-inoculated animals. This is linked with a significant decrease in the weight of the gastrointestinal tract.

Table 2 depicts the skeletal muscles and adipose tissue weights in both control and tumour-bearing rats. As can be seen, the tumour resulted in important decreases in the mass of all muscles studied including the heart. The significant decreases affected gastrocnemius (GSN) (15%) (Figure 1), soleus (8%), tibialis (10%) and extensor digitorum longus (EDL) (14%). These results reinforce the idea that muscle wasting is one of the main characteristics of cancer cachexia[5,21] and leads to decreased muscle performance and consequently quality of life.[20] In the case of the heart, the decrease was 11% (Table 2); this decrease in heart weight also affects cardiac function, as we have previously described.[22,23] Concerning adipose tissue, tumour burden also resulted in significant decreases that affected both white adipose (38%) and brown adipose tissues (33%). These results agree with previous studies performed using the same tumour model.[23–25]

β2-agonist treatment (formoterol) resulted in a weight gain in GSN and tibialis muscles (Table 2). In spite of these positive effects, formoterol treatment did not affect tumour volume (Table 1). The effects of formoterol on muscle weight can also be seen in function. Indeed,

Table 1 Effects of Formoterol and Dantrolene Treatments on Food Intake, Body Weight and Tumour Content in Tumour-Bearing Rats

Parameters	C	T	Experimental Groups		
			TF	TD	TFD
IBW	206 ± 5	204 ± 3	206 ± 3	203 ± 5	206 ± 3
FBW	249 ± 6	206 ± 8 ***	211 ± 4	193 ± 8	213 ± 4
ΔBW	43 ± 2	2 ± 6 ****	5 ± 1	−10 ± 4	7 ± 3
Carcass	82 ± 5	77 ± 3	79 ± 2	72 ± 2	77 ± 2
Food intake	62 ± 0.3	43 ± 4 **	45 ± 4	41 ± 4	42 ± 3
GIT	10.2 ± 0.3	6.0 ± 0.5 ****	5.8 ± 0.4	5.5 ± 0.3	5.9 ± 0.2
Tumour volume (mL)	–	58.4 ± 6	67 ± 2.7	69.5 ± 4	65 ± 3.1

Notes: Results are mean ± SEM for the number of animals: C: rats without tumour (6); T: tumour-bearing rats (6); TF: treated with formoterol (8); TD: treated with dantrolene (6); TFD: treated with both formoterol and dantrolene (8). IBW: initial body weight, FBW: final body weight (without tumour) and ΔBW (difference between IBW and FBW) are expressed as g. Food intake is expressed as g/100g IBW and refers to the cumulative intake (7 days). GIT: Gastrointestinal tract and Carcass (body without organs) are expressed in g/100g IBW. Statistical significance of the results by one-way analysis of variance (ANOVA). Post hoc pairwise comparisons (Dunnett's test) were performed. Values that are significantly different by the ANOVA test from the control group are indicated by **p < 0.01, ***p < 0.001, ****p< 0.0001; no differences are observed between the tumour non-treated and tumour treated animal groups.

Table 2 Effects of Formoterol and Dantrolene Treatments on Muscle and Adipose Tissue Weight in Tumour-Bearing Rats

Parameters	C	T	Experimental Groups		
			TF	TD	TFD
Muscle Weights					
Soleus	50 ± 1	46 ± 2	48 ± 2	42 ± 2	46 ± 1
Tibialis	213 ± 4	191 ± 9	219 ± 8 ##	180 ± 7	210 ± 6
EDL	51 ± 1	44 ± 2	50 ± 2	40 ± 1	48 ± 1
Heart	367 ± 9	325 ± 11	315 ± 9	315 ± 9	329 ± 8
Adipose Weights					
dWAT	1050± 151	646 ± 63 *	442 ± 55	535 ± 99 (5)	554 ± 64
BAT	180 ± 13	101 ± 6 **	103 ± 5	97 ± 9	119 ± 6

Notes: Results are mean ± SEM for the number of animals: C: rats without tumour (6); T: tumour-bearing rats (6); TF: treated with formoterol (8); TD: treated with dantrolene (6); TFD: treated with both formoterol and dantrolene (8). All weights are normalized by initial body weight (IBW) and are expressed in mg/100g IBW. EDL: extensor digitorum longus. dWAT: dorsal white adipose tissue; BAT: brown adipose tissue. Statistical significance of the results by one-way analysis of variance (ANOVA). Post hoc pairwise comparisons (Dunnett's test) were performed, except for BAT which post hoc Kruskal–Wallis test is performed. Values that are significantly different by the ANOVA test from the control group are indicated by *p <0.05, **p < 0.01; from the tumour non-treated animal group are indicated by ##p < 0.01.

Table 3 Last 24 Hours of Physical Activity in Rats Bearing the Yoshida AH-130 Ascites Hepatoma Treated with Formoterol and Dantrolene

Parameters	C	T	Experimental Groups		
			TF	TD	TFD
Total physical activity	39468 ± 5294	18886± 5182 *	31134 ± 1337 #	23654 ± 1871	34074 ± 435
Stereotyped movements	5074 ± 766	2216± 477 *	5678 ± 676 ##	3912 ± 653	3315 ± 256
Locomotor movements	34394 ± 4620	16671± 4819 *	25455 ± 1128 #	19742 ± 2485	30759 ± 179
Maximum speed	37 ± 6	26 ± 6	50 ± 4 ##	52 ± 4 #	57 ± 6 #
Total travelled distance	30711 ± 6181	16729± 4374	41276 ± 6448 #	32592 ± 2741 #	37793 ± 357 #

Notes: Results are mean ± S.E.M. for the number of animals: C: rats without tumour (4); T: tumour-bearing rats (4); TF: treated with formoterol (8); TD: treated with dantrolene (4); TFD: treated with both formoterol and dantrolene (2). Physical activity is expressed in activity units. Stereotyped movements include movements without shifting (eating and cleaning movements); conversely, locomotor movements include movements with shifting. Maximum speed is expressed in cm/s. Travelled distance is expressed in cm. Values that are significantly different by the Student's *t*-test from the control group are indicated by *p <0.05 and from the tumour non-treated animal group are indicated by #p <0.05, ##p < 0.01.

formoterol positively counteracts the decreased grip force induced by the tumour (Figure 2). Interestingly, it also had a beneficial effect on physical activity (Table 3).

The results clearly showed that the use of dantrolene alone had no beneficial effects on food intake, body weight (Table 1), or any of the muscle and adipose tissue weights studied (Table 2). Performance parameters (grip force and physical activity) were also not affected by dantrolene treatment (Figure 2 and Table 3) with the only exception of an increase in the maximum speed and total travelled distance. In fact, a higher dose of dantrolene was not able to decrease protein degradation rates in rats bearing the AH-130 hepatoma.[17]

Discussion

The experimental model used in this study, the Yoshida AH130 ascites hepatoma, represents a very fast growing tumour which leads to very marked cachexia involving muscle and fat wasting.[26] An exhaustive characterization of the model has been undertaken by our research team.[20,27,28]

Dantrolene is an inhibitor of the RyR1 which inhibits intracellular calcium release from the SR.[29] Ryanodine receptor is a protein that allows for calcium ions to be exported from the sarcoplasmic reticulum into the cytoplasm leading to contraction. In normal conditions, calcium is returned into the SR against a concentration gradient through the action of the sarcoendoplasmic reticulum Ca^{2+}-ATPase (SERCA) pumps.[30] However, modification of RyR1 protein, such as oxidation, can occur during cancer cachexia.[15] This results in calcium leakage associated with defective mitochondrial function and an increase in calcium-dependent proteolysis, which is mediated by the calcium-dependent proteases, also known as calpains.[31] Bearing this in mind, the objective

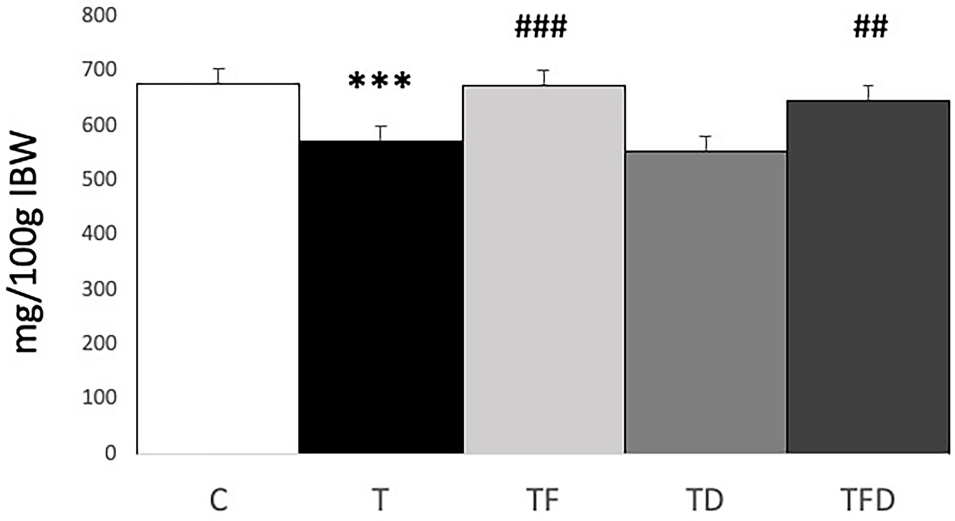

Figure 1 Gastrocnemius weight in tumour-bearing rats. Results are mean ± SEM for the number of animals: C: rats without tumour (6); T: tumour-bearing rats (6); TF: treated with formoterol (8); TD: treated with dantrolene (6); TFD: treated with both formoterol and dantrolene (8). All weights are normalized by initial body weight (IBW) and are expressed in mg/100g IBW. GSN: gastrocnemius muscle. Statistical significance of the results by one-way analysis of variance (ANOVA). Post hoc pairwise comparisons (Dunnett's test) were performed. Values that are significantly different by the ANOVA test from the control group are indicated by ***$p < 0.001$; from the tumour non-treated animal group are indicated by $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$.

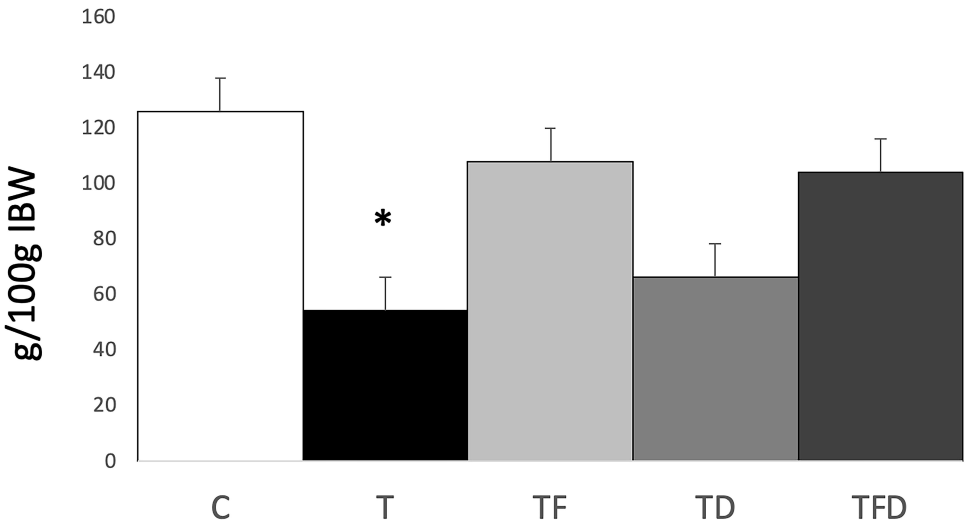

Figure 2 Grip force increase in tumour-bearing rats. Grip force increase is the difference between final versus initial force expressed in g/100g IBW. Statistical significance of the results by one-way analysis of variance (ANOVA). Post hoc pairwise comparisons (Dunnett's test) were performed. Values that are significantly different by the ANOVA test from the control group are indicated by *$p < 0.05$; no differences are observed between the tumour non-treated and tumour treated animal groups.

of the present investigation was to block the outflow of calcium by the use of dantrolene, either alone or in a combination with formoterol. In a previous investigation, Pin et al[17] used dantrolene in tumour-bearing rats as a an anti-proteolytic strategy to inhibit the calpain system which is activated in skeletal muscle during cancer cachexia.[31] Their observations suggest that the inhibition of just an individual proteolytic system is not sufficient to counteract tumour-caused muscle wasting. Bearing this in mind, we tested the combination of formoterol and dantrolene in order to test a possible additive effect on muscle wasting. No signs of toxicity were detected at the dose used (5 mg/kg). We used this dose because of previous results obtained with the drug in experiments related to inflammatory conditions (unpublished data). The rationale was that, since formoterol decreases protein degradation in skeletal muscle in cancer and dantrolene acts on a completely different mechanism, by decreasing calcium overload at the level of the sarcoplasmic reticulum, there could be could additive effects of the combination. Indeed,

sarcoplasmic alterations involving calcium overload have been described in cancer cachexia.[32] Unfortunately, dantrolene did not have any additive effects on the beneficial effect of formoterol on muscle wasting. However, dantrolene treatment alone resulted in an improvement in physical activity– speed and total travelled distance. Further investigations are needed to clarify this effect.

Conclusions

In view of all the results, it is, therefore, concluded that: a) calcium blockage does not seem to be a valid approach for treating muscle wasting, and b) that no synergy seems to exist between β2-agonist treatment and the use of the calcium outflow blocker.

Abbreviations

EDL, extensor digitorum longus; GSN, gastrocnemius muscle; RyR1, ryanodine receptor 1; s.c., subcutaneous administration; SERCA, sarcoendoplasmic reticulum Ca^{2+}-ATPase; SR, sarcoplasmic reticulum; TB, tumour bearers; TD, animals treated with dantrolene; TF, animals treated with Formoterol; TFD, double-treated animals; TPA, total physical activity.

Data Sharing Statement

All data generated or analysed during this study are included in this published article.

Ethics Approval and Consent to Participate

All animal manipulations were made in accordance with the European Community guidelines for the use of laboratory animals. They were cared for in compliance with the Policy on Humane Care and Use of Laboratory Animals (ILAR 2011) and in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. In addition, Ethics approval was granted by the Ethics Committee of the University of Barcelona and the Generalitat de Catalunya.

Consent to publish

All authors involved in the present investigation have read, approved the final form of the manuscript and have given consent to publish.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest related to employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding.

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