

# Sirtuin 1 in skeletal muscle of cachectic tumour-bearing rats: a role in impaired regeneration?

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## Abstract

**Background:** In advanced malignant disease, cachexia and muscle wasting appear to be among the most common manifestations. This phenomenon is partially related with a decreased muscle regeneration capacity, as previously described in our laboratory.

**Methods and results:** Rats bearing the Yoshida AH-130 ascites hepatoma were used in the experiments. The animals experienced a marked weight loss with decreases in skeletal muscle weights (13% gastrocnemius, 18% extensor digitorum longus, and 12% tibialis muscles). Muscle gene expression was measured using real-time polymerase chain reaction. Skeletal muscle from cachectic tumour-bearing rats is associated with a decreased expression of genes involved in regeneration such as Pax-7 (39%), myogenin (24%), and MyoD (17%). mRNA levels of Sirt1 increased (91%) in cachectic skeletal muscle. The Sirt1 gene has been shown to be associated with changes in muscle myoblast differentiation. Treatment of the tumour-bearing animals with formoterol—a beta2-agonist—normalizes the expression of genes involved in regeneration (i.e., increase of Pax7 (139%)), at the same time as it does with that of Sirt1 (42% decrease).

**Conclusions:** It is suggested that the lack of muscle regeneration observed during muscle wasting in tumour-

bearing animals is linked to the action of Sirt-1, possibly via PGC-1 $\alpha$ . These factors may constitute possible targets of pharmacological treatment against muscle loss, thus potentially contributing to the understanding and mitigation of muscle atrophy associated with disease.

**Keywords** Cachexia · Sirt1 · Muscle wasting · PGC1-alpha

## 1 Introduction

In advanced malignant diseases, cachexia appears to be one of the most common systemic manifestations. The presence of cachexia always implies a poor prognosis, having a great impact on the patients' quality of life and survival [1]. Several important molecular mechanisms have been shown to be involved in the increased muscle catabolism observed in cancer-induced cachexia, such as greater ubiquitin–proteasome-dependent proteolysis, apoptosis, and activation of uncoupling proteins [2–5]. Interaction of these mechanisms leads to muscle mass loss by promoting protein and DNA breakdown and energy inefficiency.

The sirtuin family of proteins possesses NAD<sup>+</sup>-dependent deacetylase activity and/or ADP ribosyltransferase activity. The seven mammalian sirtuins (Sirt1–7) are localized differentially within the cell and have a variety of functions [6]. Sirt1 is the most extensively studied member of the family and regulates diverse biological processes ranging from cell proliferation, differentiation, apoptosis, and metabolism [7]. Sirt3—a sirtuin present in the mitochondria [8] which seems to be able to control thermogenesis—has been linked to longevity in men, and aberrant expression of this sirtuin correlates with node-positive breast cancer in clinical biopsies from women [9], suggesting that Sirt3 serves as an important diagnostic and

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therapeutic target in human health/aging and disease, affecting men and women in unique ways. Both Sirt1 and 3 seem to be regulated both by diet [10, 11] and exercise [10].

$\beta_2$ -Adrenergic agonists are potent muscle growth promoters in many animal species [12, 13], resulting in skeletal muscle hypertrophy [14–17]. Formoterol is one of these compounds with important anti-cachectic effects in animal models. The mechanism of action of this drug is based on its ability to prevent muscle wasting by inhibiting proteolysis in skeletal muscle. Thus, this  $\beta_2$ -agonist is able to decrease the activation of the ubiquitin-dependent proteolytic system, the main mechanism activated in muscle-wasting conditions [18]. Interestingly, in addition to its anti-proteolytic effects, formoterol also decreases muscle apoptosis in muscle-wasting animals [18].

After an injury within the muscle, there is an effective restoration of its structure and function [19]. This is possible due to existence of a population of mononuclear-acquired myogenic precursors, known as satellite cells [20]. These cells in adult muscle are in quiescent phase [21]. With the appropriate environmental signals, there is an activation of them and they become precursors for the formation of new muscles during growth or to repair muscle after an injury [22].  $\beta_2$ -Adrenergic agonists increase the ability of skeletal muscle repair after injury [23–29].

Bearing all this in mind, the objective of the present investigation was to measure the levels of Sirt1 and 3 gene expression in skeletal muscle of cachectic tumour-

bearing animals and to relate them with the alterations found in skeletal muscle during cachexia. In addition, the effects of formoterol treatment (a highly effective anabolic treatment for cancer cachexia [18]) on sirtuin content were studied.

## 2 Material and methods

### 2.1 Animals

Male Wistar rats (Interfauna, Barcelona, Spain) of 5 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 08:00 A.M. to 08:00 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.

### 2.2 Tumour inoculation and formoterol treatment

Rats were divided into two groups, namely controls ( $n=14$ ) and tumour hosts ( $n=14$ ). The latter received an intraperitoneal (i.p.) inoculum of  $10^8$  AH-130 Yoshida ascites hepatoma cells obtained from exponential tumours [30]. Both groups were further divided into treated ( $n=7$ ) and untreated ( $n=7$ ), the former one being administered a daily subcutaneous dose of formoterol (0.3 mg/kg body weight,

**Table 1** Muscle weights and protein content in rats bearing the Yoshida AH-130 ascites hepatoma

|                 | C            | C+F          | TB              | TB+F            | ANOVA  |        |
|-----------------|--------------|--------------|-----------------|-----------------|--------|--------|
|                 |              |              |                 |                 | A      | B      |
| Food intake     | 106 $\pm$ 2  | 112 $\pm$ 3  | 78 $\pm$ 4      | 83 $\pm$ 2      | 0.0000 | NS     |
| Tumour content  | –            | –            | 3,648 $\pm$ 150 | 3,750 $\pm$ 180 |        | NS     |
| Muscle weights  |              |              |                 |                 |        |        |
| Gastrocnemius   | 637 $\pm$ 20 | 710 $\pm$ 23 | 553 $\pm$ 11    | 627 $\pm$ 10    | 0.0000 | 0.0001 |
| Tibialis        | 214 $\pm$ 6  | 238 $\pm$ 4  | 188 $\pm$ 3     | 206 $\pm$ 5     | 0.0000 | 0.0001 |
| Soleus          | 44 $\pm$ 1   | 48 $\pm$ 1   | 42 $\pm$ 1      | 45 $\pm$ 2      | NS     | 0.0291 |
| EDL             | 51 $\pm$ 2   | 58 $\pm$ 1   | 42 $\pm$ 2      | 47 $\pm$ 1      | 0.0000 | 0.0007 |
| GSN             |              |              |                 |                 |        |        |
| Protein content | 212 $\pm$ 10 | 290 $\pm$ 20 | 173 $\pm$ 20    | 271 $\pm$ 20    | 0.1570 | 0.0004 |

Results are mean  $\pm$  standard error of the mean for seven animals

Food intake is expressed in grams per 100 g initial body weight and refers to the ingestion during the period of the experiment prior to sacrifice which took place 7 days after tumour inoculation

Tissue weights are expressed as milligrams per 100 g of initial body weight

Tumour cell content is expressed in millions of cells. The protein content is expressed in milligrams of protein per gram GSN

Statistical significance of the results by two-way ANOVA

EDL extensor digitorum longus, C control animals, T tumour-bearing rats, F formoterol-treated animals, GSN gastrocnemius, ANOVA analysis of variance, NS non-significant differences, A tumour effect, B treatment effect

dissolved in physiological solution) and the latter one the corresponding volume of solvent. On day 7 after tumour transplantation, the animals were weighed and anesthetized with an i.p. injection of ketamine/xylazine mixture (3:1; Imalgene<sup>®</sup> and Rompun<sup>®</sup>, respectively). The tumour was harvested from the peritoneal cavity and its volume and cell content evaluated. Tissues were rapidly excised, weighed, and frozen in liquid nitrogen.

### 2.3 RNA isolation

Total RNA from gastrocnemius muscle was extracted by TriPure<sup>™</sup> kit (Roche, Barcelona, Spain), a commercial modification of the acid guanidinium isothiocyanate/phenol/chloroform method [31].

### 2.4 Real-time polymerase chain reaction

First-strand cDNA was synthesized from total RNA with oligo dT15 primers and random primers pdN6 by using a cDNA synthesis kit (Transcriptor Reverse Transcriptase, Roche, Barcelona, Spain). Analysis of mRNA levels for Sirt1, Sirt3, Pax7, MyoD, myogenin, and 18S was performed with primers designed to detect these products: Sirt1: UP—AGCTGGGGTTTCTGTTTCCTGTGG, DO—TCGAACATGGCTTGAGGATCTGGGA; Sirt3: UP—CGGCTTTGGATGTGGAGGACAC, DO—CCTGGGGATCTGAAGTCTGGGATAC; Pax7: UP—GGAAAACCAAGTGTGCCATCT, DO—CC TTGTCTTTGGCACCATT; MyoD: UP—CGACTGCTTTCTTACCACA, DO—CTCAACCCAAGCCTGAAGAG; myogenin: UP—GTCTTTTCCGACCTGATGGA, DO—GTCCCCAGTCCCTTCTCTTC, and 18S: UP—CGCAGAATTCCCACTCCCGACCC, DO—CCCAAGCTCCAACACTACGAGC. To avoid the detection of possible contamination by genomic DNA, primers were designed in different exons. The real-time polymerase chain reaction was performed using a commercial kit (Light-Cycler<sup>™</sup> 480 SYBR Green I Master, Roche, Barcelona, Spain). The relative amount of all mRNA was calculated using comparative  $C_T$  method. 18S mRNA was used as the invariant control for all studies.

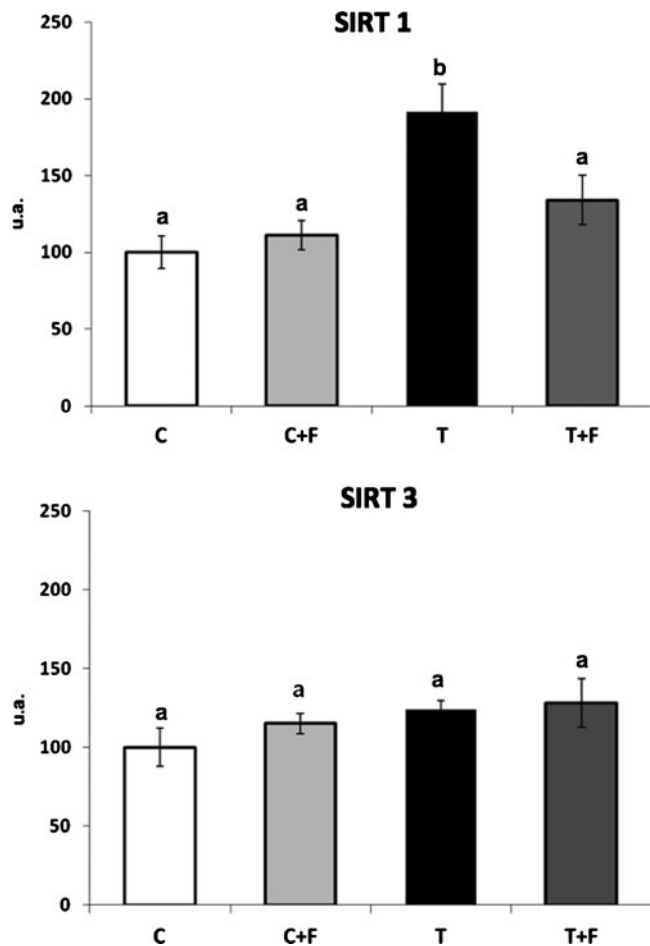
### 2.5 Protein content

Protein concentration was determined according to the method of bicinchoninic acid (Pierce, Spain) and was expressed in milligrams of protein per gram of gastrocnemius muscle.

### 2.6 Western blot

Gastrocnemius muscle was homogenized in 10 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (pH 7.5),

10 mmol/L MgCl<sub>2</sub>, 5 mmol/L KCl, 0.1 mmol/L EDTA, 0.1% Triton X-100, 1 mmol/L dithiothreitol (DTT) and 5 μL/mL of buffer of a protease inhibitor cocktail (Sigma, Spain). Tissue homogenates were then centrifuged at 7,000 rpm for 5 min at 4°C, and the supernatants were collected. Protein concentrations were determined according to the method of bicinchoninic acid (Pierce, Spain). Equal amounts of protein (50 or 100 μg) were heat-denatured in sample loading buffer (50 mmol/L Tris-HCl (pH 6.8), 100 mmol/L DTT, 2% sodium dodecyl sulfate (SDS), 0.1% bromophenol blue, 10% glycerol), resolved by SDS-polyacrylamide gel electrophoresis (10% polyacrylamide, 0.1% SDS) and transferred to Immobilon membranes (Immobilon polyvinylidene difluoride, Millipore). The filters were blocked with 5% phosphate-buffered saline with non-fat dry milk and then incubated with polyclonal antibody anti-cyclin D1 (Santa Cruz Biotechnology). Polyclonal antibody anti-



**Fig. 1** Sirtuin 1 and 3 mRNA content in gastrocnemius muscles from tumour-bearing rats. Results are mean  $\pm$  standard error of the mean for seven animals. The results of gene expression are expressed as a percentage of controls (arbitrary units). C control animals, T tumour-bearing rats, F formoterol-treated animals. Statistical significance of the results by one-way ANOVA, between groups ( $p < 0.001$ ), and statistically significant difference by post hoc Duncan test. Different superscripts indicate significant differences between groups

**Table 2** Pax7, myogenin, and MyoD mRNA content in skeletal muscle of rats bearing the Yoshida AH-130 ascites hepatoma

|          | C      | C+F    | TB    | TB+F   | ANOVA  |        |
|----------|--------|--------|-------|--------|--------|--------|
|          |        |        |       |        | A      | B      |
| mRNA     |        |        |       |        |        |        |
| Pax7     | 100±10 | 137±16 | 62±11 | 143±19 | NS     | 0.0252 |
| MyoD     | 100±5  | 97±12  | 82±5  | 79±8   | 0.0456 | NS     |
| Myogenin | 100±6  | 89±14  | 75±9  | 48±5   | 0.0322 | 0.0432 |
| Protein  |        |        |       |        |        |        |
| Cyclin D | 100±7  | 58±13  | 76±6  | 50±5   | 0.0467 | 0.0187 |

For more details, see the “Material and methods” section

Results are mean ± standard error of the mean for seven animals

The results of gene expression are expressed as a percentage of controls. Statistical significance of the results by two-way ANOVA

C control animals, T tumour-bearing rats, F formoterol-treated animals, NS non-significant differences, A tumour effect, B treatment effect

GAPDH (Sigma, Spain) was used as the invariant control. Goat anti-rabbit horseradish peroxidase conjugate (Bio-Rad) was used as secondary antibody. The membrane-bound immune complexes were detected by an enhanced chemiluminescence system (EZ-ECL, Amersham Biosciences).

## 2.7 Statistical analysis

Statistical analysis of the data was performed by means of one- and two-way analysis of variance (ANOVA).

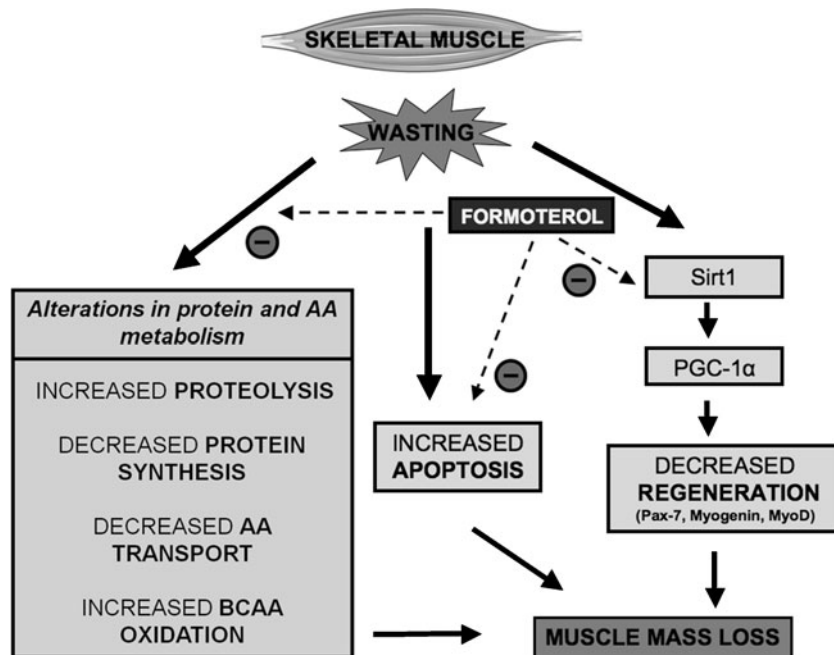
## 3 Results

As can be seen in Table 1, the implantation of the Yoshida AH-130 ascites hepatoma caused important decreases in the

weight of gastrocnemius (13%), extensor digitorum longus (18%), and tibialis muscles (12%). These changes were accompanied by significant decrease in protein content (Table 1). Formoterol treatment was able to significantly increase muscle weight and protein content (Table 1). These data agree with previous results from our own laboratory [18].

Figure 1 shows the Sirt1 and 3 mRNA content in skeletal muscle. As can be seen, tumour burden resulted in a significant increase in Sirt1 gene expression (91%) in gastrocnemius muscles, in relation with the non-tumour-bearing control animals. Interestingly, administration of formoterol results in a decrease in the levels of Sirt1 mRNA in the tumour-bearing animals without affecting the levels found in the control rats (Fig. 1). Conversely, no changes were observed in the mRNA levels of Sirt3 in any of the experimental groups studied (Fig. 1).

**Fig. 2** Hypothetical involvement of Sirt1 in muscle wasting. Formoterol treatment is able to partially block not only the changes in protein and amino acid (AA) metabolism but also in apoptosis and muscle regeneration, possibly via Sirt1. BCAA branched chain amino acids



Since Sirt1 has been associated with changes in muscle myoblast differentiation [32, 33], we decided to investigate the levels of differentiation markers in skeletal muscle from tumour-bearing rats. As can be seen in Table 2, tumour-bearing rats showed decreased mRNA content for Pax7 (39%), myogenin (24%), and MyoD (17%). Formoterol treatment increased Pax7 gene expression (139%) while reducing that of myogenin (53%) and decreased the protein levels of cyclin D (38%).

#### 4 Discussion

This is the first report on Sirt1 gene expression in skeletal muscle in catabolic conditions. Caloric restriction has been reported to increase Sirt1 content in skeletal muscle [11]; this could be relevant since tumour-bearing rats suffer from anorexia (Table 1). Formoterol does not affect food intake (Table 1); therefore, the increase in Sirt1 in muscle is not likely to have been triggered by caloric restriction in this experimental model. Other factors of inflammatory origin may be involved. From this point of view, TNF- $\alpha$ —a cytokine which is elevated in the tumour model chosen and that has been involucrated in muscle wasting [34–36]—has been shown to be responsible for increasing Sirt1 in vascular smooth muscle cells [37]. However, during sarcopenia, an increase in Sirt1 satellite cell content has been described [38]. Conversely, no changes were observed in the mRNA levels of Sirt3 in any of the experimental groups studied. This observation is in contrast with that of Alamdari et al. [39], showing an upregulation of Sirt3 in skeletal muscle during sepsis, a catabolic condition also characterized by intense muscle wasting.

Since Sirt1 has been associated with changes in muscle myoblast differentiation [32, 33], we decided to investigate the levels of differentiation markers in skeletal muscle from tumour-bearing rats. Different molecules involved in the process of muscle regeneration and in the regulation of satellite cells, such as Pax7, MyoD, and myogenin [20], are known (Fig. 2). In adult skeletal muscle, Pax7 is expressed in the majority of quiescent satellite cells. These cells, when activated, co-express Pax7 and MyoD [25, 40, 41]. When they proliferate, the levels of Pax7 decrease and other molecules involved in differentiation (such as myogenin) increase their expression. There is a part of the proliferating population that maintains the levels of Pax7, but not of MyoD, which returns to their state of quiescence [40–42]. The mRNA content decrease in tumour-bearing animals for Pax7, myogenin, and MyoD suggests a decrease in the rate of myoblast differentiation. Indeed Pax7 is a marker of satellite cell differentiation [29]. Interestingly, formoterol treatment increased Pax7 gene expression and increased myogenin gene expression, this clearly indicating that the

beta agonist is able to activate satellite cell differentiation (Fig. 2). This is supported by the decrease in the protein levels of cyclin D—a marker of cell proliferation.

Sirt1 controls the transcription of the peroxisome proliferator-activated receptor (PPAR)-gamma co-activator 1alpha (PGC-1 $\alpha$ ) in skeletal muscle [43] (Fig. 2). In fact, tumour burden resulted in significant increases in PGC-1 $\alpha$  in cachectic muscles [44]. Interestingly, formoterol treatment significantly decreased the levels of the co-activator both in control and tumour-bearing animals [44]. The results presented here indicate that the lack of muscle regeneration associated with muscle wasting during cancer [29] is likely to be associated with changes in Sirt1 content in skeletal muscle. This opens a new perspective in understanding muscle wasting associated with cancer cachexia and may lead to new approaches in the design of therapeutic strategies. In addition, Sirt1 may play a key modulatory role in animal fat deposition—it promotes fat mobilization by repressing PPAR-gamma [32] and muscle development, therefore contributing to the inter-organic metabolic cross-talk, as previously reported by our group [45].

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