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Skeletal muscle UCP2 and UCP3 gene expression in a rat cancer cachexia model

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Abstract Rats bearing the Yoshida AH-130 ascites hepatoma showed an increased expression of both uncoupling protein-2 (UCP2) (194%) and UCP3 (189%) mRNA levels in skeletal muscle 7 days after tumour inoculation. Interestingly, an even greater increase was observed in mRNA for both UCP2 (278%) and UCP3 (797%) in the pair-fed animals, suggesting that the increase in gene expression was the result of the anorexia associated with tumour burden. The results constitute the first report of UCP2 and UCP3 gene expression during cancer cachexia and agree to their possible role in the increase of energy expenditure associated with tumour growth.

Key words: Thermogenesis; Tumour growth; Skeletal muscle; Uncoupling protein-2; Uncoupling protein-3; Gene expression

1. Introduction

Until very recently, the uncoupling protein-1 (UCP1), present only in brown adipose tissue, was considered to be the only mitochondrial protein carrier that stimulated heat production by dissipating the proton gradient generated during respiration across the inner mitochondrial membrane and therefore uncoupling respiration from ATP synthesis. Recently, two new proteins sharing a similar fuction, UCP2 and UCP3, have been described. While UCP2 is expressed ubiquitously [1,2], UCP3 is expressed abundantly and dominantly in skeletal muscle in humans [3–5] and also in brown (BAT) and white (WAT) adipose tissues and heart of rodents [6]. Concerning the regulation of their expression, UCP2 mRNA levels are upregulated in WAT and skeletal muscle in obesity-resistant mice but not in an obesity-prone strain [7], therefore suggesting a possible role as a defense mechanism against high-fat induced obesity. Fasting increases UCP2 mRNA in skeletal muscle [8] while it does not seem to affect the expression of the gene in BAT. In skeletal muscle UCP3 gene expression is also modulated by food intake: its expression is increased by fasting [9].

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 [10–12] and evidence has been provided for muscle protein waste as being associated with enhanced turnover rates [13,14]. 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. Previous studies (See [15] for review) have shown that during cachectic states there is an increase in BAT thermogenesis both in humans [16] and experimental animals [17]. In addition, Roe et al. [18] have shown that there is an increased oxygen consumption in leukaemic rats. It is therefore interesting to study if tumour burden results in an enhanced UCP2 and UCP3 activation.

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 [19–21]. Detectable plasma levels of tumour necrosis factor-α (TNF) and perturbations in the hormonal homeostasis [20] likely play an important role in forcing the metabolic balance towards the catabolic side.

We decided to investigate if pathophysiological states where the levels of TNF are increased (such as tumour growth) resulted in an increased expression of both UCP2 and UCP3. Bearing this in mind, and taking into account that skeletal muscle wasting is one of the main factors contributing to cancer cachexia, the aim of the present investigation was to see if tumour burden resulted in the induction of the UCP2 or UCP3 genes in this tissue.

2. Materials and methods

2.1. Animals, tumour inoculation and treatment

Male Wistar rats (Interfauna, Barcelona, Spain) weighing about 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 non-digestible material); the food intake was measured daily. Rats were divided into three groups, namely controls, pair-fed and tumour hosts. Pair-fed were non-tumour bearing animals which were offered the same amount of food as that eaten by the tumour bearers the previous day. The latter received an intraperitoneal inoculum of 10^8 AH-130 Yoshida ascites hepatoma cells obtained from exponential tumours (for details see [19]).

On days 0, 2, 4, and 7 after tumour transplantation animals were weighed and anesthetised with diethyl ether. The tumour was harvested from the peritoneal cavity, its volume and cellularity evaluated, and cells separated from the ascitic fluid by centrifugation at $100 \times g$ for 10 min. Tissues were rapidly excised, weighed, and frozen in liquid nitrogen.

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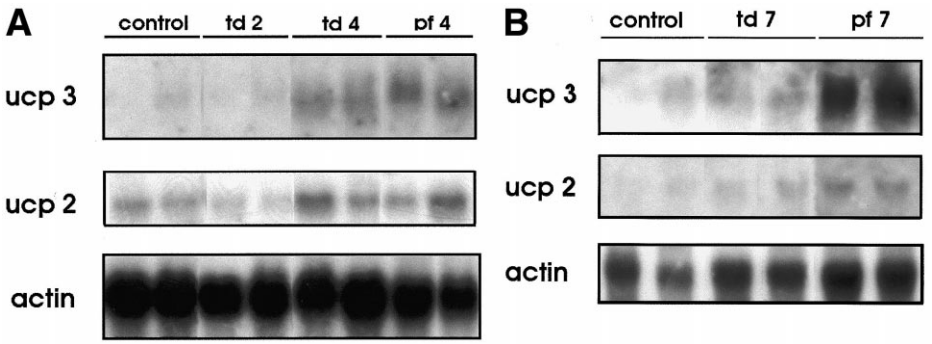

Fig. 1. Northern blots of gastrocnemius muscle extracts from control, pair-fed and tumour-bearing rats. Expression of both UCP2 and UCP3 mRNAs in skeletal gastrocnemius muscles was detected after hybridisation with cDNA probes (see Section 2). Autoradiographs were subjected to scanning densitometry. td: tumour-bearing rats (days 2, 4 and 7 after inoculation); pf: pair-fed rats.

2.2. Chemicals

All chemicals were either obtained from Boehringer-Mannheim (Barcelona, Spain) or from Sigma (St. Louis, MO, USA).

2.3. UCP2 and UCP3 gene expression

Total RNA from gastrocnemius muscle was extracted using the acid guanidinium isothiocyanate/phenol/chloroform method [22]. RNA samples (20 μg) were denaturated, subject to 1.2% agarose gel electophoresis and transferred to Hybond H membranes (Amersham). RNA was fixed to membranes by illuminating with UV for 4 min.

Prehybridisation was done in a phosphate buffer (250 mM, pH 6.8) containing 7% SDS, 1 mM EDTA and 1% BSA, during 1 h at 65°C. Membranes were hybridised in the same buffer with appropiate probes (approx. 28 Bq/ng) at 65°C for 18 h. Non-specifically bound probe was removed by successive washes in 2×SSC+0.1% SDS (10 min at 65°C) and 1×SSC+0.1% SDS (10 min at 65°C) and 0.1×SSC+0.1% SDS (15 min at 65°C, twice). Specific hybridisation was then detected by autoradiography in BioMax film (Kodak) using intensifier screens during 18–48 h and quantified by densitometry.

Radiolabeled probes were prepared by the random priming method (Boehringer-Mannheim). The probes used were the entire coding frame for mouse UCP2 [1], a cDNA clone containing the entire coding frame for mouse UCP3 (D.S. Fleury, C.F. Bouillard, GenBank accession number AF032902), and an actine probe used as hybridisation/quantification standard.

2.4. Statistical analysis

Statistical analysis of the data was performed by means of the Student's *t*-test.

3. Results and discussion

At day 2 after tumour inoculation, the food intake of the tumour-bearing animals was not decreased (in relation with the non-tumour-bearing controls) but it was significantly lower at days 4 (11%) and 7 (48%) (Table 1). Since fasting has been demonstrated to increase both UCP2 and UCP3 mRNAs in different tissues [8], we decided to include pair-fed animals which were given the same amount of food as the tumour-bearing ones.

At day 7, both pair-fed and tumour-bearing animals showed a considerable degree of body weight loss; while the control animals increased their body weight by 33.5 ± 2.43 g, the pair-fed group showed a significant decrease (9.72 ± 1.69 g, $P<0.01$) and the tumour-bearing group showed an even greater loss (28.9 ± 3.26 g, $P<0.01$). This decrease in body weight was accompanied by a decrease in adipose weight loss affecting both WAT and BAT as we have previously described [23,24]. Tumour burden resulted in an important decrease in gastrocnemius weight (21%) (Table 1). EDL, soleus and tibialis weights were decreased to a similar extent in the tumour-bearing animals but also in the pair-fed group, suggesting that the main factor involved in the muscle waste is the anorexia induced by tumour growth.

At day 2 following tumour inoculation there were no

Table 1
Food intake and body and muscle weights

	Control	Pair-fed	Tumour-bearing
Food intake (g)	117 ± 6	61.3 ± 3	60.6 ± 2
Initial body weight (g)	143 ± 3	143 ± 3	153 ± 3
Final body weight (g)	176 ± 4	169 ± 4	114 ± 4
Muscle weights (mg)			
Gastrocnemius	633 ± 24.6	566 ± 16.3	499 ± 23.2**
EDL	53.3 ± 0.55	46.3 ± 1.24**	39.6 ± 1.69***
Soleus	53.2 ± 1.44	43.3 ± 1.93***	38.9 ± 2.23**
Tibialis	200 ± 3.99	176 ± 3.55**	151 ± 6.62**
Adipose tissue weights (mg)			
White fat lumbar pads	705 ± 17.4	296 ± 37.4**	269 ± 44.9**
Brown interscapular	151 ± 18.4	72.2 ± 1.88**	75.8 ± 6.62**
Tumour cell content (cells×10^6)	–	–	68.8 ± 3.74

Data are expressed as means ± S.E.M. for five animals in each group. Data correspond to day 7 after tumour inoculation. Tissue weights are expressed as percentages of initial body weight. Statistical significance of the differences (Student's *t*-test): *$P<0.05$, **$P<0.01$, ***$P<0.001$.

Table 2
UCP2 and UCP3 gene expression in gastrocnemius muscles

	UCP2	UCP3
Control	100 ± 21	100 ± 13
Tumour, day 2	72 ± 16	148 ± 17
Tumour, day 4	169 ± 45	468 ± 17**
Tumour, day 7	194 ± 32*	189 ± 15*
Pair-fed, day 4	142 ± 23	617 ± 94**
Pair-fed, day 7	278 ± 15***	797 ± 95***,†

Data are expressed as percentage of the control values and are means ± S.E.M. for five different animals. Statistical significance of the differences (Student's *t*-test): vs. control: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; vs. tumour-bearing: $^{\dagger}P < 0.001$.

changes in either UCP2 or UCP3 gene expression in skeletal muscle (Table 2 and Fig. 1). At day 4, both the tumour-bearing animals and the pair-fed ones showed a significant increase (4.7- and 6.7-fold, respectively) in UCP3 gene expression while no changes were observed in UCP2. At day 7, both UCP2 and UCP3 mRNAs were increased in the tumour-bearing group (1.9-fold) while the increase was considerably higher in the pair-fed group (2.8-fold for UCP2 and 8-fold for UCP3). From these results it can be concluded that the activation of UCP2 and UCP3 gene expression at late stages of tumour growth is basically a consequence of the anorectic state. However, the significantly higher UCP3 mRNA levels found in the pair-fed animals at day 7 (in relation with the tumour-bearing animals) suggests the possibility that a tumour-induced circulating factor may influence skeletal muscle UCP3 mRNA levels. This gene is abundantly expressed in skeletal muscle [5], its presence having been detected only in BAT of rodents [3]. The regulation of its expression is still poorly known, it is not affected by cold acclimation [5] although it is increased following thyroid hormone treatment [25]; starvation increases UCP3 mRNA levels in skeletal muscle while it decreases them in BAT [26]. UCP3 expression is also decreased in the soleus muscle of obese Zucker rats [9] suggesting that it could possibly have a role in obesity.

Heat production in BAT, an important site of non-shivering thermogenesis in rodents [27], has been for a long time associated with the UCP1, a mitochondrial protein carrying the stimulation of heat production resulting from uncoupling respiration from ATP synthesis. The recent discovery of new members (UCP2 and UCP3) of the mammalian mitochondrial uncoupling proteins has resulted in a considerable amount of information to elucidate the role of these proteins in pathophysiological conditions. Information concerning the regulation of their mRNA levels is still in an early stage. Starvation results in an important increase in UCP2 gene expression in skeletal muscle while it does not influence in BAT [8]. Cold exposure also results in an increase in UCP2 gene expression in skeletal muscle and BAT [8]. Interestingly, UCP2 levels are upregulated in WAT in response to fat feeding, suggesting regulation by diet [1]. We have recently observed that TNF administration results in an increased skeletal muscle gene expression of UCP2 without affecting UCP3 levels in rats (S. Busquets et al., unpublished results).

Cancer cachexia is one of the worst effects of malignancy, accounting for nearly a third of cancer deaths. It is a pathological state characterised by weight loss together with anorexia, weakness, anemia and asthenia. The complications associated with the appearance of the cachectic syndrome affect both the physiological and biochemical balance of the patient and have effects on the efficiency of the anticancer treatment, resulting in a considerably decreased survival time. At the metabolic level, cachexia is associated with loss of skeletal muscle protein together with a depletion of body lipid stores. The cachectic patient, in addition to having practically no adipose tissue, is basically subject to an important muscle wastage manifested as an excessive nitrogen loss. The metabolic changes are partially mediated by alterations in circulating hormone concentrations (insulin, glucagon and glucocorticoids in particular) and cytokines, as the molecules responsible for the above referred metabolic derangements. In addition, during cachexia there is an important energetic imbalance. An increase in BAT thermogenesis has been described both in humans [16] and experimental animals [17]. Therefore, non-shivering thermogenesis due to either BAT activity or muscle increased thermogenesis (as suggested here) may be very important factors contributing to the decreased energy efficiency found in the cachectic state.

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