Hyperlipemia: a role in regulating UCP3 gene expression in skeletal muscle during cancer cachexia?

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Abstract Rats bearing the Yoshida AH-130 ascites hepatoma showed an increased expression of both uncoupling protein-2 (UCP2) (two-fold) and UCP3 (three- to four-fold) in skeletal muscle (both soleus and gastrocnemius). The increase in mRNA content was associated with increased circulating concentrations of fatty acids (two-fold), triglyceride (two-fold) and cholesterol (1.9-fold). Administration of nicotinic acid to tumor-bearing rats abolishes the hyperlipidemic increase associated with tumor burden. The vitamin treatment also resulted in a decreased UCP3 gene expression in soleus muscle but not in gastrocnemius. It is concluded that circulating fatty acids may be involved in the regulation of UCP3 gene expression in aerobic muscles during experimental cancer cachexia. Since the UCP3 protein could have a role in energy expenditure, it may be suggested that hypolipidemic agents may have a beneficial role in the treatment of the cachectic syndrome. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Energy expenditure; Uncoupling protein-2; Free fatty acid; Tumor; Nicotinic acid

1. Introduction

Until very recently, the uncoupling protein-1 (UCP1), present in brown adipose tissue (BAT), 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, therefore uncoupling respiration from ATP synthesis. Recently, new UCPs – UCP2, UCP3, UCP4 – and brain mitochondrial carrier protein-1 (BMCP-1) – in mammal tissues – have been described. While UCP2 is expressed ubiquitously [1,2], UCP3 is expressed abundantly and specifically in skeletal muscle in humans [3–6] and also in BAT of rodents [3,5,7]. BMCP-1 is a recently described mitochondrial carrier with uncoupling activity in yeast and its mRNA is highly expressed in the central nervous system of humans and rodents [8]. Finally UCP4 [9] is another brain-specific mitochondrial protein that has been seen to have an uncoupling activity in vitro. The role of these new UCPs in thermogenesis and energy balance of intact animals remains to be established. Several factors, such as T3 (3,3′,5-tri-iodo-thyronine), leptin, lipopolysaccharide and cytokines, increase the UCP2 and UCP3 mRNA levels in animals, suggesting that they may contribute to thermogenesis (see [10] for review). Different results suggest that these proteins could have different functions depending on the metabolic conditions [11–19]. Thus, it is well known that in the course of different catabolic states (infection, trauma, tumor growth) there is an increased energy expenditure (see [20] for review). It is therefore interesting to relate this increased energy expenditure to changes in the level of expression of the different UCPs, which could have a thermoregulatory role. Some studies of markers encompassing the human UCP2/UCP3 locus have revealed a linkage with resting metabolic rate [21], resting energy expenditure [22] and anorexia nervosa [23]. Other studies suggest that UCP2 and UCP3 are determinants of basal energy expenditure in humans [24,25], whereas no significant association between UCP2 and resting energy expenditure was detected in obese or diabetic humans [26,27].

Malignant neoplasms frequently induce a progressive loss of lean body mass in the host, associated with marked alterations in endocrine and metabolic homeostasis, a situation known as cachexia. Skeletal muscle tissue, which accounts for almost half of the whole body protein mass, is severely affected in cancer cachexia [28–30]. Muscle wasting in cachexia is associated with enhanced protein turnover rates [31–35]. In addition, cachexia tends to develop at rather late stages of neoplastic disease. Thus, preventing muscle wasting in cancer patients is of potential clinical interest.

We have previously shown that muscle wasting is associated with an increased expression of UCP2 and UCP3 in skeletal muscle [36]. Interestingly, the UCP mRNA content in skeletal muscle is also increased by tumor necrosis factor-α (TNF-α) administration [37], therefore suggesting that the cytokine could be responsible for the increase in expression associated with tumor growth since TNF-α plasma levels are increased during tumor growth in experimental animals [38].

Interestingly, UCP3 gene expression in skeletal muscle is increased in those pathophysiological conditions where a concomitant increase in circulating lipids – in particular free fatty acids (FFA) – is observed, such as starvation [39], high-fat diets [40] and experimentally induced diabetes [41]. Interestingly Simonyan et al. [42,43] have described not only an increase in UCP3 protein levels in rodent skeletal muscle during cold-exposure, but also a sensitizing of the membrane poten-

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tial to the uncoupling action of fatty acids, suggesting that these compounds could also have a role during cold-induced thermogenesis. In fact, it has been speculated that UCP3 could have a role in lipid metabolism, possibly as a fatty acid carrier into mitochondria [44], although some controversy still exists [45]. Different experiments have shown that when the circulating FFA concentration is decreased, by means of hypolipidemic agents, the induction in UCP2 and UCP3 gene expression is abolished [39,46]. In addition, the presence of fatty acids in C2C12 myotube cultures results in an increase in UCP3 gene expression [47].

Bearing all this in mind, we decided to investigate whether the increase in UCP2 and UCP3 gene expression in skeletal muscle of tumor-bearing rats was also associated with an increase in circulating FFA and, if by decreasing the concentration of these compounds, we could abolish the increase in UCP gene expression associated with tumor burden.

2. Materials and methods

2.1. Animals, tumor inoculation and treatment

Male Wistar rats (Interfauna, Barcelona, Spain) weighing about 130 g were used. The animals were maintained on a regular light–dark cycle (light on from 08:00) at an ambient temperature of 22 ± 2°C and had free access to food and water. The diet consisted of 45.5–48.5% carbohydrate, 18.5% protein and 3.1% fat (the residue was non-digestable material; B.K. Universal, Sant Vicent dels Horts, Barcelona, Spain).

The rats were divided into four groups, namely control non-treated, control nicotinic acid-treated, tumor-bearing non-treated and tumor-bearing nicotinic acid-treated. The two groups of tumor-bearing animals received an intraperitoneal inoculum of 10^8 AH-130 Yoshida ascites hepatoma cells obtained from exponential tumors (for details, see [48]). On day 7 after tumor transplantation, the animals were weighed and anesthetized with an i.p. injection of ketamine/xylazine mixture (Imalgene® and Rompun®, respectively). Nicotinic acid treatment was performed the day of sacrifice and was carried out intraperitoneally (100 mg/kg in three shots spaced in intervals of 2 h). Tissues were rapidly excised, weighed, and frozen in liquid nitrogen. All animal manipulations were made in accordance with the European Community guidelines for the use of laboratory animals.

2.2. RNA isolation and Northern blot analysis

Total RNA from soleus and gastrocnemius muscles was extracted using the acid guanidinium isothiocyanate/phenol/chloroform method as described by Chomczynski and Sacchi [49]. RNA samples (20 μg) were denaturated and subjected to 1.2% agarose gel electrophoresis containing 6.3% formaldehyde. The RNA in gels and in filters was visualized with ethidium bromide and photographed by UV transillumination to ensure the integrity of RNA, to check the loading of equivalent amounts of RNA and to confirm proper transfer. RNA was transferred to Hybond N membrane (Amersham) in 20× standard saline citrate (SSC; 0.15 M NaCl and 15 mM sodium citrate, pH 7.0) and fixed to membrane by Gene Linker (Bio-Rad). Prehybridization was done in a phosphate buffer (250 mM, pH 6.8) containing 7% SDS, 1 mM EDTA and 1% BSA, for 1 h at 65 °C. Membranes were hybridized in the same buffer with appropriate 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 hybridization was then detected by autoradiography in Hyperfilm-MP film (Amersham) using intensifier screens for 1–4 days at −80°C. Radiolabeled probes were prepared by the random priming method (Boehringer-Mannheim). The probes used were the entire coding frame for mouse UCP2 [11], a cDNA clone containing the entire coding frame for mouse UCP3 (D.S. Fleury, C.F. Bouillard, GenBank accession number AF032902) and a 18S rat ribosomal probe used as hybridization/quantification standard. Blots were quantified on a phosphoimager using the Phoretix 1 D gel analysis (Phoretix Int., Ltd., UK).

Table 1
Effect of nicotinic acid on UCP2 and UCP3 gene expression in tumor-bearing rats

	UCP2	UCP3
mRNA content in gastrocnemius		
Control	100 ± 12 (4)	100 ± 5 (5)
Control+NA	99 ± 7 (7)	134 ± 18 (4)
AH-130	205 ± 45 (3)*	306 ± 31 (4)***
AH-130+NA	170 ± 14 (6)***	338 ± 18 (7)***
mRNA content in soleus		
Control	100 ± 15 (4)	100 ± 26 (5)
Control+NA	239 ± 13 (4)†††	106 ± 12 (7)
AH-130	202 ± 27 (4)*	400 ± 103 (4)*
AH-130+NA	208 ± 10 (6)	71 ± 15 (7)††

The results are expressed as arbitrary units and are mean ± S.E.M. for the number of animals indicated in parentheses. Significance of the differences: none vs. tumor: $*P<0.05$, $***P<0.001$; none vs. nicotinic acid: $^{\dagger\dagger}P<0.01$, $^{\dagger\dagger\dagger}P<0.001$. NA: nicotinic acid.

2.3. Circulating metabolite analysis

Circulating FFAs were determined using the NEFA C kit (Wako Chemicals, Dallas, TX, USA). The other circulating metabolites were determined using the Spotchem II (Panel 1 and Panel 2) Reagent Strip (A. Menarini Diagnostics).

2.4. Biochemicals

All enzymes, coenzymes and other chemicals were either obtained from Roche (Barcelona, Spain) or from Sigma Chemical Co. (St. Louis, MO, USA). Radiochemicals were obtained from Amersham (Amersham, Bucks, UK).

2.5. Statistical analysis

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

3. Results and discussion

The Yoshida AH-130 rat ascites hepatoma is a suitable model system for studying the mechanisms involved in the establishment of cachexia. Its growth in the host causes rapid and progressive loss of body weight and tissue wasting, particularly in skeletal muscle. Acceleration of tissue protein breakdown accounts for most of the wasting in AH-130 bearers [48,50–52]. In particular, skeletal muscle hypercatabolism involves hyperactivation of the ATP-ubiquitin-dependent proteolytic system [53]. In addition to protein wasting [20,54], we have recently demonstrated that cachexia is associated with increased muscle DNA fragmentation (and therefore apoptosis), at least in experimental animals [55]. The implantation of the Yoshida AH-130 ascites hepatoma resulted in a considerable decrease in heart and muscle mass (data not shown). Indeed, the decrease affected gastrocnemius (22%), tibialis (20%), soleus (12%) and extensor digitorum longus (22%). The cardiac mass was reduced by 15%. This

Table 2
Circulating fatty acid levels 7 days after tumor transplantation

	FFA (mEq/l)
Control	0.214 ± 0.02 (5)
Control+NA	0.201 ± 0.04 (7)
AH-130	0.419 ± 0.02 (6)***
AH-130+NA	0.203 ± 0.03 (8)†††

For more details see Section 2. Results are expressed as mEq/l and are mean ± S.E.M. for the number of animals indicated in parentheses. Statistical significance of the results (by Student's *t*-test): AH-130 vs. control: $***P<0.001$; none vs. NA: $^{\dagger\dagger\dagger}P<0.001$. NA: nicotinic acid.

Table 3
Circulating metabolite concentrations

	Control	Control+NA	AH-130	AH-130+NA
T-Cho	58 ± 5 (5)	64 ± 6 (5)	112 ± 7 (5)***	84 ± 6 (6)†*
TG	75 ± 9 (4)	28 ± 3 (5)†††	152 ± 6 (5)***	47 ± 6 (6)†††*
Alb	3.3 ± 0.1 (5)	3.3 ± 0.07 (5)	2.4 ± 0.02 (5)***	2.6 ± 0.1 (6)***
Glu	218 ± 5 (4)	157 ± 16 (5)†	191 ± 12 (4)	127 ± 4 (5)†††
BUN	14 ± 1 (5)	16 ± 1 (4)	17 ± 2 (4)	35 ± 3 (6)††***
T-Bil	0.26 ± 0.02 (5)	0.3 ± 0.03 (5)	0.42 ± 0.02 (5)**	0.38 ± 0.02 (6)*
T-Pro	5.4 ± 0.1 (5)	5.3 ± 0.1 (5)	5.1 ± 0.1 (5)	4.9 ± 0.1 (6)*
Ca^{2+}	11.6 ± 0.2 (5)	11.6 ± 0.3 (5)	11.3 ± 0.3 (5)	11.2 ± 0.1 (6)
UA	1 ± 0.01 (5)	1.3 ± 0.1 (4)†	1.3 ± 0.1 (5)*	1.6 ± 0.1 (6)

For more details see Section 2. Results expressed as: T-Cho: total cholesterol (mg/dl), TG: triglycerides (mg/dl), Alb: albumin (g/dl), Glu: glucose (mg/dl), BUN: blood urea nitrogen (mg/dl), T-Bil: total bilirubin (mg/dl), T-Pro: total protein (g/dl), Ca^{2+}: Calcium (mg/dl) and UA: uric acid (IU/l) and are mean ± S.E.M. for the number of animals indicated in parentheses. Statistical significance of the results (by Student's *t*-test): AH-130 vs. control: $*P<0.05$, $**P<0.01$ and $***P<0.001$. NA vs. non-treatment: $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$, $^{\dagger\dagger\dagger}P<0.001$. NA: nicotinic acid.

muscle wasting was related with a decrease in carcass weight (21%) (data not shown). As previously described [36], both UCP2 and UCP3 gene expression were increased in skeletal muscle as a consequence of tumor growth. Indeed, in gastrocnemius muscle, UCP2 mRNA content was elevated two-fold, while that of UCP3 was increased three-fold (Table 1). Similarly, in soleus muscle, tumor growth induced a two-fold increase in UCP2 mRNA content and a four-fold increase in UCP3 gene expression (Table 1). The presence of the tumor was associated with a great increase in the circulating concentrations of fatty acids (two-fold) (Table 2). This increase was also associated with a generalized hyperlipidemia with parallel increases in the concentrations of cholesterol (1.9-fold) and triglycerides (two-fold) (Table 3); in addition, tumor burden resulted in a marked hypoalbuminemia (Table 3). Bearing these results in mind, we decided to investigate whether the increase in circulating fatty acids was directly responsible for the activation of the UCPs genes, as had been previously shown in starvation [39]. Therefore, in order to decrease circulating fatty acids, we administered nicotinic acid to both control and tumor-bearing groups. The vitamin has been proved to act as a hypolipidemic agent in many pathophysiological conditions [39,46,56]. Indeed, treatment of tumor-bearing animals with nicotinic acid resulted in a 52% decrease in circulating fatty acids (Table 2). Interestingly, the vitamin did not affect the levels of fatty acids in the control group (Table 2), but it decreased plasma triglycerides (63%) (Table 3). Similarly, the treatment caused a 69% decrease in plasma triglycerides and a 25% decrease in circulating cholesterol in the tumor-bearing group (Table 3). As expected, the decrease in circulating fatty acids induced by the nicotinic acid was associated with a marked decrease (82%) in UCP3 gene expression in soleus muscle, while the expression of the UCP2 gene was unchanged by the treatment (Table 1). In gastrocnemius muscle, no changes in either UCP2 or UCP3 mRNA content were observed following nicotinic acid treatment (Table 1). These results agree with those of Samec [39,46] showing that the vitamin treatment reduced the expression levels of the UCP2 and UCP3 genes in soleus, but not in gastrocnemius, during starvation. Similar results were obtained by the same group in starved animals using etomoxir – an inhibitor of the β-oxidation pathway; they observed an increase in the circulating fatty acid levels linked with increased UCP2 and UCP3 gene expression in soleus, but not in gastrocnemius [57]. The authors suggest that a positive feedback regulation mechanism could exist only in oxidative muscles, possibly as an adaptive mechanism for lipid oxidation during starvation or other pathophysiological states where glucolysis by muscle is partially substituted by fatty acid oxidation [39,46]. In addition, others have shown an increase in UCP3 gene expression in skeletal muscle (gastrocnemius, but not soleus) together with similar changes in mRNA content of different enzymes related to lipid catabolism [58]. Moreover, transgenic animals overexpressing the UCP3 gene in skeletal muscle eat 50% less food than their control littermates, but have similar circulating levels of both triglycerides and fatty acids, suggesting that fatty acid oxidation is higher in the transgenic animals [59].

The results found here suggest that during cancer cachexia, there is a differential regulation of the UCP3 gene vs. the UCP2 one, since only the former seems to respond to variation in circulating fatty acid levels. UCP2 could be regulated by other unknown factors. Since the UCP3 protein may be associated with an increase in energy expenditure [60–62] which may contribute further to the cachectic state, hypolipidemic agents may be prove to be a useful therapeutic strategy for the treatment of cancer cachexia.

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