FEBS Letters 580 (2006) 5172–5176

UCP3 in muscle wasting, a role in modulating lipotoxicity?

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Received 1 June 2006; revised 17 July 2006; accepted 18 August 2006

Available online 5 September 2006

Edited by Robert Barouki

Abstract UCP3 has been postulated to function in the defense against lipid-induced oxidative muscle damage (lipotoxicity). We explored this hypothesis during cachexia in rats (zymosan-induced sepsis), a condition characterized by increased oxidative stress and supply of fatty acids to the muscle. Muscle UCP3 protein content was increased 2, 6 and 11 days after zymosan injection. Plasma FFA levels were increased at day 2, but dropped below control levels on days 6 and 11. Muscular levels of the lipid peroxidation byproduct 4-hydroxy-2-nonenal (4-HNE) were increased at days 6 and 11 in zymosan-treated rats, supporting a role for UCP3 in modulating lipotoxicity during cachexia.

Keywords: UCP3; Lipid peroxidation; Sepsis; Lipotoxicity; Muscle; Cachexia

1. Introduction

The catabolic state present in multiple models of cachexia results in a profound and massive loss of muscle mass, and reduced cellular energy charge (for review see, e.g. [1]). Upon its discovery in 1997 and based on its homology with the *bona fide* uncoupling protein UCP1, UCP3 has been implicated in the regulation of energy expenditure and has thus been studied as a putative contributor to muscle wasting under cachectic conditions. Increased gene and protein expression of UCP3 has been reported in several cachectic diseases, including cancer [2–4], sepsis [5], burn injury [6], and rheumatoid arthritis [7,8], which would be consistent with a role of UCP3 in facilitating, or contributing to, the muscle wasting observed. In contrast, studies in pancreatic cancer [9] and COPD patients [10,11] reported unchanged or even decreased UCP3 levels. Busquets et al. [12] showed that the upregulation of UCP3 upon cachexia was only partly explained by increased plasma FFA levels, as treatment of cachectic rats with the lipolysis blocker nicotinic acid blunted the upregulation of UCP3 in soleus muscle but not in gastrocnemius. Recently, the same group showed that the UCP3 response upon cachexia may be considered an antioxidative response [13]. These findings are consistent with more recent hypotheses regarding the physiological function of UCP3, implicating a role for UCP3 in modulating lipotoxicity [14,15] through the efflux of mitochondrial matrix bound fatty acids and derived lipid peroxidation products [15–17]. The matrix is the major site of mitochondrial reactive oxygen species (ROS) production, and matrix bound fatty acids are especially prone to lipid peroxidation. Thus, highly reactive lipid peroxides can be formed, which in turn may damage mtDNA and important electron transport protein complexes in the matrix in a process termed lipotoxicity. As mitochondrial DNA repair mechanisms are limited [18,19] and electron transport complexes are vital to mitochondrial life, it is important to have a defense mechanism against lipid-induced oxidative damage. In this respect, increased UCP3 content under conditions of elevated fatty acids, and activation of UCP3 by the lipid peroxidation byproduct 4-HNE [20] could be considered such defence mechanisms. Increased oxidative stress in combination with increased UCP3 protein levels have indeed been found in experimental cancer cachexia [13,21].

Interestingly, the cachectic state is often accompanied by increased rates of adipose tissue lipolysis [22], reduced mitochondrial volume and aberrations in mitochondrial protein synthesis rate [23], a combination typically requiring increased levels of UCP3 [17].

We hypothesize that a cachexia-related increased UCP3 protein content serves to modulate lipotoxicity. To investigate this, we examined UCP3 protein content in a cachectic rat model (zymosan-induced sepsis), known to induce hypophagia [24], decrease fat oxidative capacity [25] and compromise mitochondrial protein synthesis rates [23]. Pair-fed controls were included to differentiate between the effects of (semi)starvation and the effect of zymosan-induced cachexia.

2. Methods

2.1. Animals and experimental design

Experiments were approved by the institutional animal experimental committee. Rats were individually housed (12 h dark–light cycle, 21–22 °C and 50–60% humidity). The zymosan model was applied to induce a transient septic shock, as described previously [24]. Ten-week-old, male Wistar rats with an average body mass of 300 g were given an aseptic intraperitoneal injection of zymosan (30 mg/100 g body mass) suspended in liquid paraffin (25 mg/ml). A homogeneous zymosan suspension was sterilized by incubation at 100 °C for 90 min. Four groups of rats ($n = 10$) were injected i.p. with the zymosan suspension.

Food intake and body mass were recorded daily. Rats were killed at 2 d, 6 d, and 11 d after zymosan injection. Since zymosan-induced sepsis is associated with profound decreases in food intake, age-matched control rats were pair-fed to the 2 d, 6 d, and 11 d zymosan rats. An age-matched ad libitum fed control group, which was killed at day 11 served to provide control levels of all parameters assessed.

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0014-5793/$32.00
doi:10.1016/j.febslet.2006.08.066

Rats were anaesthetized using a subcutaneous injection of ketamine (100 mg/kg body mass) and xylazine (10 mg/kg) and blood was sampled by cardiac punction after 2 h of food withdrawal, prior to dissecting the tibialis anterior muscle (TA) which was promptly frozen in melting 2-methylbutane, after which the rats were killed by cervical dislocation.

2.2. Muscle UCP3 protein content and lipid peroxidation

Skeletal muscle UCP3 protein content was determined as described previously [26]. Briefly, from each sample an equal amount of protein was loaded on a polyacrylamide gel and western blotting was performed against rat UCP3 using a rabbit polyclonal UCP3 antibody (code 1338; kindly provided by LJ Slieker, Eli Lilly), as previously described [27,28]. Blotting was also performed for cytochrome *c* (Cyt*c*; polyclonal antibody, Santa Cruz Biotechnology, CA, USA) as a marker of mitochondrial content. For valid inter gel comparison a standard sample was loaded on each gel and UCP3 levels were expressed relative to this standard. Values were expressed as UCP3/Cyt*c* ratios and as percentage of control values.

As a marker of lipid peroxidation, protein adducts of the lipid peroxidation byproduct 4-hydroxy-2-nonenal (4-HNE) were examined on frozen sections by immunofluorescence using a rabbit polyclonal antibody against 4-HNE-protein adducts (Calbiochem, San Diego, CA, USA). In the present paper, protein adducts of 4-HNE are referred to as 4-HNE content or levels. Only images in which the entire field of view comprised muscle fibers were processed for quantification. Upon conversion to 8 bits grayscale, the 4-HNE derived staining was measured as integral optical density and expressed as percentage of control values. Using this approach we ensured the examination of 4-HNE in muscle cells and avoided contamination of other cell-types, as may occur in muscle homogenates.

2.3. Plasma analyses

Blood samples were collected in tubes containing EDTA and immediately centrifuged at 4000 rpm for 10 min at 4 °C. Plasma was frozen in liquid nitrogen and stored at −80 °C until further analysis of free fatty acids (FFA) (Wako NEFA C test kit; Wako chemicals, Neuss, Germany) and triglycerides (glycerol kinase–lipase method Boehringer, Mannheim, Germany) was undertaken. All analyses were performed on an automated centrifugal spectrophotometer (Cobas Fara, La Roche, Basel, Switzerland).

2.4. Statistics

Results are presented as means ± S.E.M. For each time-point, the zymosan, pair-fed, and control group data were compared using one-way ANOVA analysis. Differences were located using the Scheffé post hoc test. Significance was set at $P < 0.05$. For UCP3 protein analyses, three muscle pools were made per zymosan group and compared to the corresponding pair-fed and control muscle pools. The effect of zymosan on UCP3 protein was compared to the effect of pair-feeding using mixed model analysis with zymosan and time as factors.

3. Results

3.1. Food intake, body mass and muscle mass

Acute peritonitis was observed during the first 2 days after zymosan injection, along with symptoms of severe illness including lethargy, hypophagia, hyperventilation, tachycardia, fever, diarrhea and loss of hemorrhagic fluid from the nose. There was a mortality of 20% in this acute phase. Food intake reduced significantly from ~20 g/day to an average of 1 g/day on days 1 and 2, after which it gradually increased to 75% of normal intake on day 8 (Fig. 1A), and remained constant from then onward. Both the zymosan and pair-fed groups showed a large loss of body mass (Fig. 1B); rats started to regain body mass between days 5 and 11. There were no significant differences in body mass between the zymosan and the pair-fed group. No catch-up growth was observed in either group.

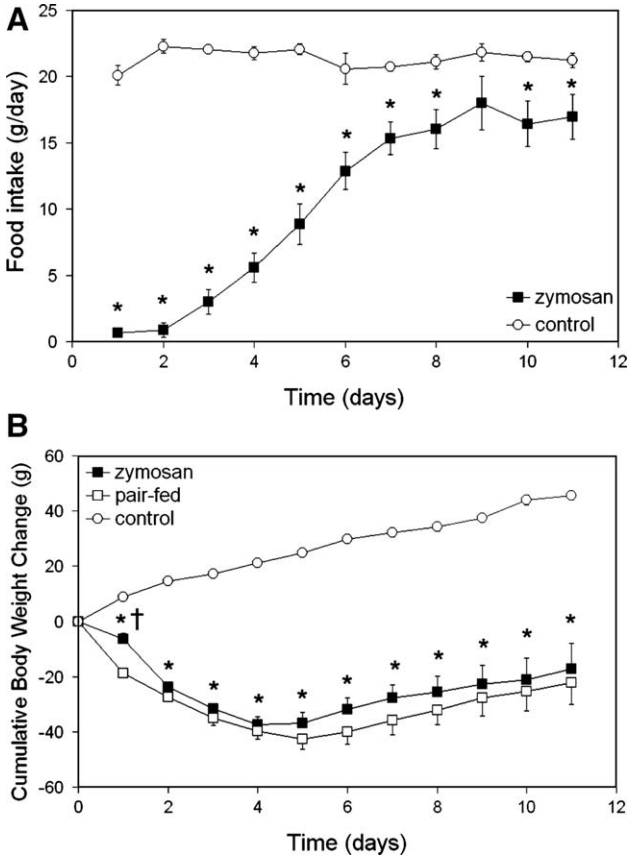

Fig. 1. (A) Food intake of zymosan-treated and ad libitum fed control rats. (B) Cumulative body weight change in zymosan-treated, pair-fed and ad libitum fed rats. $^{*}P < 0.05$ vs. ad libitum fed rats. $^{\dagger}P < 0.05$ vs. pair-fed rats.

Table 1
Tibialis anterior (TA) muscle mass in the experimental groups

Group	TA muscle mass (mg)
Control	610 ± 9
2-day zymosan	518 ± 22*†
2-day pair-fed	604 ± 14
6-day zymosan	444 ± 15*†
6-day pair-fed	545 ± 11*
11-day zymosan	522 ± 23*†
11-day pair-fed	611 ± 12

$^{*}P < 0.05$ vs. ad libitum fed rats.
$^{\dagger}P < 0.05$ vs. pair-fed rats.

Muscle mass of the main dorsiflexor (TA) muscle is shown in Table 1. TA muscle mass declined significantly (compared to pair-fed and ad libitum fed controls) as soon as 2 days after zymosan injection and up to 66 ± 2.6% of control values at day 6. The pair-fed group showed a similar but less pronounced response. TA muscle mass had started to regain in both groups by day 11.

3.2. Plasma analyses

In control rats, plasma triglycerides levels equaled 1142 ± 94 μmol/l (Fig. 2A). In line with the declined food intake the first 2 days after zymosan injection, plasma triglyceride levels dropped profoundly in both zymosan-treated

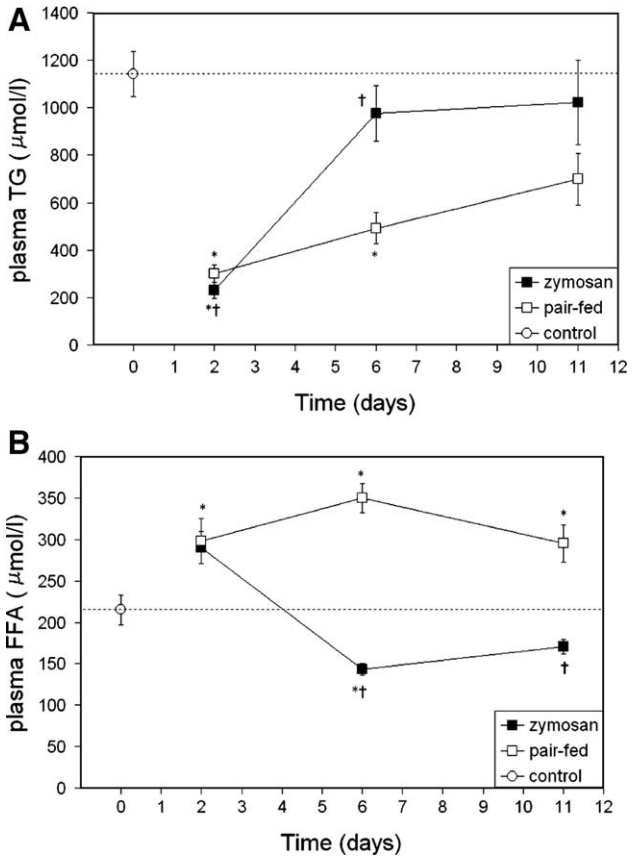

Fig. 2. Plasma levels of: (A) triglycerides and (B) free fatty acids in zymosan-treated, pair-fed and ad libitum fed control rats ($t = 0$ days). $^{*}P < 0.05$ vs. ad libitum fed rats. $^{\dagger}P < 0.05$ vs. pair-fed rats.

(230 ± 34 μmol/l, $P < 0.01$) and pair-fed control (301 ± 36 μmol/l, $P < 0.01$) rats. Six days after zymosan injection plasma triglyceride levels (976 ± 118 μmol/l) had restored to levels insignificantly different from control values (1142 ± 94 μmol/l), while in pair-fed rats (492 ± 67 μmol/l) triglyceride content remained decreased compared to zymosan treated (976 ± 118 μmol/l, $P < 0.01$) and ad libitum fed control rats ($P < 0.001$). At day 11, when food intake had partly normalized and body mass had started to regain, no significant differences in plasma TG levels were detected between groups (Fig. 2A).

At day 2 both zymosan-treated (Fig. 2B; 290 ± 19 μmol/l) and pair-fed rats (298 ± 27 μmol/l) showed significantly increased plasma free fatty acid (FFA) levels compared to ad libitum fed rats (215 ± 189 μmol/l). At six days after zymosan injection a remarkable drop in plasma FFA was observed in zymosan-treated rats (143 ± 7 μmol/l), whereas in pair-fed rats (350 ± 17 μmol/l) FFA levels were significantly ($P < 0.01$) higher compared to both zymosan-treated and control rats. At day 11, plasma FFA levels in zymosan-treated rats (171 ± 9 μmol/l) were not different from control values, while in pair-fed rats (295 ± 23 μmol/l) plasma FFA levels were still significantly higher than both zymosan-treated and control levels.

3.3. Muscle UCP3 protein content and lipid peroxidation

Compared to control values (100 ± 14%) UCP3 protein content relative to mitochondrial density, as measured by cytochrome *c* content, was increased in zymosan-injected rats (224 ± 54%) as in pair-fed controls (197 ± 32%; Fig. 3). At all time points measured UCP3/Cyt*c* content was higher in zymosan-injected rats than in pair-fed controls. After an initial rise UCP3/Cyt*c* content levels seemed to stabilize at approximately 200% at day 6 and day 11 in pair-feds, whereas in the zymosan condition UCP3/Cyt*c* content increased towards 262 ± 25% at day 6 and 256 ± 26% at day 11.

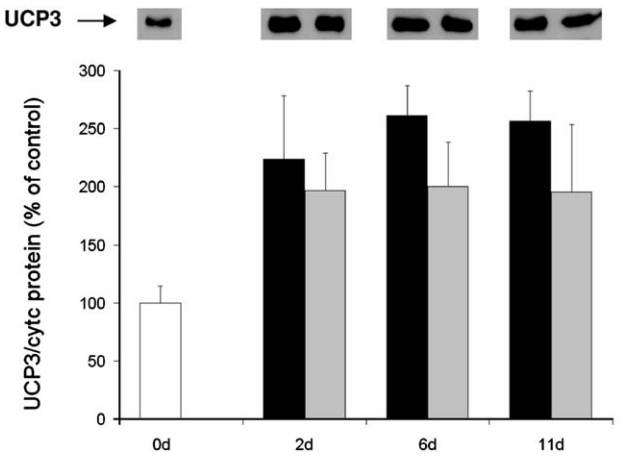

Fig. 3. UCP3/Cyt*c* protein ratios in tibialis anterior muscles from zymosan-treated (black bars), pair-fed (grey bars) and ad libitum fed control (white bar) rats. Ratios are expressed as percentages of control levels. When compared to pair-fed rats, mixed model analysis showed a significant zymosan effect on UCP3/Cyt*c* ratios ($P = 0.026$) and no time ($P = 0.927$) or time*zymosan effect ($P = 0.603$).

Lipid peroxidation was measured by immunofluorescence (representative 4-HNE stainings for control and zymosan-treated rats at day 11 are shown in Fig. 4A and B, respectively). In

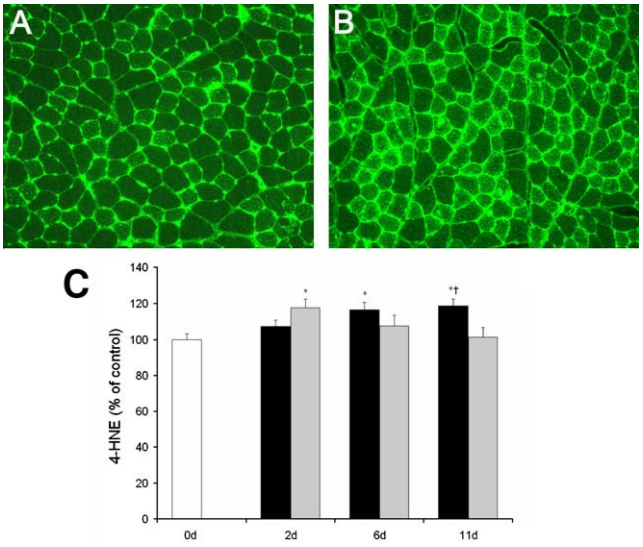

Fig. 4. 4-Hydroxy-2-nonenal (4-HNE) protein adduct levels in tibialis anterior muscles. (A–B) Representative examples of 4-HNE immunofluorescence stainings performed on tibialis anterior muscle sections of a control (panel A) and 11-day zymosan-treated (panel B) rat. (C) Quantification of 4-HNE protein adducts in muscles of zymosan-treated (black bars), pair-fed (grey bars) and ad libitum fed control (white bar) rats. $^{*}P < 0.05$ vs. ad libitum fed rats. †$P < 0.05$ vs. pair-fed rats.

control rats, 4-HNE content was set at 100%. In zymosan-treated rats, 4-HNE was not increased at day 2, but 4-HNE content was significantly elevated at day 6 (117 ± 4%) and remained significantly elevated at 11 days after zymosan injection (119 ± 4%). Interestingly, in pair-fed rats 4-HNE was increased at day 2 (118 ± 5%; $P < 0.05$), but the initial rise in 4-HNE returned to control values at day 6 and day 11.

4. Discussion

Here, we examined the hypothesis that cachexia-related increased UCP3 protein content serves to modulate lipotoxicity. Compared to controls, rats rendered cachectic by injection of zymosan possessed increased muscular UCP3 protein content lasting for at least 11 days. Pair-fed control rats showed a more modest increase in UCP3 levels than cachectic rats. In zymosan-treated rats, we observed an increase of the lipid peroxidation byproduct 4-hydroxy-2-nonenal (4-HNE) in TA muscle both 6 and 11 days after injection. Interestingly, after 2 days of pair-feeding increased 4-HNE content was observed which returned to control values at days 6 and 11. This may suggest that in food restricted but otherwise healthy animals, the increase in UCP3 protein content suffices to modulate the increased lipid peroxidation observed after 2 days of pair-feeding. In contrast to pair-fed rats, 4-HNE content was elevated in rats rendered cachectic by zymosan injection from day 6 onward, despite an increased UCP3 content. If UCP3 indeed serves to modulate lipotoxicity during cachexia, this indicates that in critically ill rats increased UCP3 protein content or its state of activation is inadequate to deal with the increased oxidative stress and concomitant lipid peroxidation products present during cachexia.

During the first 2 days after zymosan injection or pair-feeding, when food intake had reduced to virtually zero, we observed a sharp decline in plasma triglyceride levels paralleled by increased plasma FFA levels in both groups; the classical response to acute food restriction. Increased plasma FFA levels are potent activators of UCP3 gene expression [29]. Conversely, the use of anti-lipolytic agents resulted in a blunted increase in UCP3 [12], even under conditions of β-adrenergic stimulated energy expenditure [30]. Increased FFA levels in both zymosan-treated and pair-fed rats may trigger the initial rise in UCP3 protein content observed at day 2. In pair-fed rats, plasma FFA levels remained elevated at days 6 and 11, potentially explaining why UCP3 protein levels remained relatively high in this condition (albeit lower than in zymosan-injected rats). In contrast to pair-fed rats, plasma FFA levels at days 6 and 11 of zymosan-injected rats were lower than control and pair-fed levels. The decreased FFA levels in zymosan-treated rats are most likely accounted for by reduced tissue lipoprotein lipase (LPL) activity [31,32]. This notion is substantiated by the normalization of plasma triglyceride levels observed in the zymosan-treated animals at days 6 and 11. Strikingly, in zymosan-treated rats UCP3 content remained elevated despite significant reductions in FFA levels, but in the presence of increased 4-HNE levels. Thus, although elevated plasma FFA levels may explain the relatively high UCP3 levels in pair-fed rats, high FFA levels are not involved in increasing UCP3 content in zymosan-treated rats. Interestingly, an increased UCP3 protein content in the absence of increased plasma FFA levels has previously been observed in cancer cachexia [13]. This indicates that under conditions of cachectic stress other processes are involved which induce maintenance of high UCP3 levels. One such process may be an increased production of ROS and 4-HNE, although so far it has not been examined if 4-HNE is able to increase UCP3 protein content.

Not only has increased oxidative stress been reported in cachexia, but the resultant ROS have also been shown to result in lipid peroxidation products, as indicated by increased adducts of the lipid peroxidation byproduct 4-HNE in tumour-bearing cachectic rats [21]. This is in line with our present observation of increased 4-HNE in zymosan-treated rats. Interestingly, 4-HNE has recently been identified as one of the few UCP3 activators [20]. Thus, as indicated above, it is tempting to suggest that besides activating UCP3, 4-HNE also induces an increased UCP3 protein expression in the zymosan-treated rats at days 6 and 11. Activation of UCP3 by 4-HNE has been shown to result in a reduced mitochondrial proton gradient [20]. Reduction of the proton gradient in a process referred to as mild uncoupling results in lowered production of reactive oxygen species (ROS) [33]. Thus, a unifying physiological role for increased UCP3 content in cachectic conditions can be hypothesized; UCP3 increases in a process at least partly driven by increased plasma FFA levels during early cachexia to deal with increased ROS and lipid peroxides. In a feed-forward loop, 4-HNE may activate and possibly upregulate UCP3 to facilitate efflux of fatty acid anions or lipid peroxides from the mitochondrial matrix. This process has a dual effect; lowering of the proton gradient serves to reduce ROS production and efflux of fatty acid anions and/or lipid peroxides serves to preserve mitochondrial integrity and mitochondrial function. In the present model, where zymosan injection induces critical illness with a 20% mortality rate during the first 2 days after injection [34], the increase in UCP3 protein content seems inadequate to deal properly with the rise in lipid peroxidation observed at day 6 and 11 after zymosan injection. This may indicate that under these extreme conditions, the rise in lipid peroxides exceeds the capacity of UCP3 to deal with this appropriately, possibly resulting in increased mitochondrial damage. In line with this, a previous study using the same model has shown mitochondrial morphological abnormalities and decreased mitochondrial protein synthesis [23].

In conclusion, the present study shows increased UCP3 protein content along with increased 4-HNE adducts in skeletal muscle of cachectic rats. These observations support the idea that increased UCP3 in cachectic conditions helps to modulate the cachexia-related oxidative stress and ameliorates lipotoxicity.

Acknowledgements: Dr. M. Hesselink is supported by a VIDI Research Grant for innovative research from the Netherlands Organization for Scientific Research (Grant 917.66.359). The research of Dr. P. Schrauwen is sponsored by a fellowship of the Royal Netherlands Academy of Arts and Sciences.

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