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SHORT COMMUNICATION

Autophagy-related and autophagy-regulatory genes are induced in human muscle after ultraendurance exercise

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Abstract The purpose of this study was to evaluate whether ultra endurance exercise changes the mRNA levels of the autophagy-related and autophagy-regulatory genes. Eight men (44 ± 1 years, range: 38–50 years) took part in a 200-km running race. The average running time was 28 h 03 min ± 2 h 01 min (range: 22 h 15 min–35 h 04 min). A muscle sample was taken from the vastus lateralis 2 weeks prior to the race and 3 h after arrival. Gene expression was assessed by RT-qPCR. Transcript levels of autophagy-related genes were increased by 49% for ATG4b ($P = 0.025$), 57% for ATG12 ($P = 0.013$), 286% for Gabarapl1 ($P = 0.008$) and 103% for LC3b ($P = 0.011$). The lysosomal enzyme cathepsin L mRNA was upregulated by 123% ($P = 0.003$). Similarly, transcript levels of the autophagy-regulatory genes BNIP3 and BNIP3l were both increased by 113% ($P = 0.031$ and $P = 0.007$, respectively). Since upregulation of these genes has been related with an increased autophagic flux in various models, our results strongly suggest that autophagy is activated in response to ultra endurance exercise.

Keywords Endurance exercise · Autophagy · ATG · BNIP3 · Gabarapl1 · LC3b

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Introduction

Skeletal muscle represents the largest protein pool in human body and thereby constitutes an important stock for supplying alternative energetic substrates during long periods of high energy demand like an ultramarathon (Finn and Dice 2006). Skeletal muscle is also known to be mechanically damaged and metabolically disturbed during such an exercise (Sandri 2010). Therefore, skeletal muscle cell needs an efficient system for removing damaged proteins and organelles and for releasing amino acids in the case of energetic stress. Until recently, most attention had been paid to the ubiquitin–proteasome pathway and less consideration had been given to the autophagic-lysosomal pathway (ALP), but there is now a body of evidence that both systems are coordinately regulated in catabolic situations (Zhao et al. 2008).

Experiments conducted in yeast in the 90s brought novel knowledge in the understanding of ALP regulation (Wang and Klionsky 2003). They discovered a series of genes implicated in the ALP that are now referred to as the 'autophagy-related genes' (ATG) (Klionsky et al. 2003). To date, several mammalian homologues of ATG proteins have been identified. Among them, the ATG1 homologues Ulk1 and Ulk2 (unc-51-like kinases) as well as the ATG6 homologue Beclin1 play a role in the signalling pathways that activate autophagy (Eskelinen and Saftig 2009; Lee and Tournier 2011). Two conjugation systems controlling autophagosome formation and substrate targeting have been described: (1) the ATG12-ATG5 complex and (2) the lipidated form of ATG8. In mammalian cells, two homologues of ATG8 are LC3b (microtubule-associated protein 1 light chain 3 beta) and Gabarapl1 (GABA(A) receptor-associated protein like 1) (Cabrera et al. 2010). ATG8 participates in autophagosome formation and in substrate

targeting under its active form, namely when conjugated with phosphatidylethanolamine (PE). This lipidation process involves among others the participation of ATG4. More recently, the major role of BNIP3 (BCL2/adenovirus E1B 19 kDa interacting protein 3) in autophagy has been observed. The over-expression of this gene enhances autophagy and mitochondrial fragmentation (Hamacher-Brady et al. 2007; Tracy and Macleod 2007).

Autophagy can be regulated in response to conditions such as energy deprivation (Mizushima et al. 2004), the unfolded protein response (Ogata et al. 2006) or oxidative stress (Dobrowolny et al. 2008). These three situations can be triggered by exercise (de Lange et al. 2007; Kim et al. 2011; Sahlin et al. 2010). While increased lysosomal enzyme activities and elevated number of autophagic vacuoles have been reported during the repair of exercise injuries due to strenuous endurance exercise in rodents (Salminen 1985; Salminen and Vihko 1984), no data are available concerning the possible regulation of ATG in response to an acute form of strenuous endurance exercise in human. There is evidence that autophagy-related as well as autophagy-regulatory genes are regulated during ALP activation (Mammucari et al. 2007). Therefore, the goal of this study was to evaluate whether an ultra endurance exercise can trigger up-regulation of the autophagy-related and autophagy-regulatory genes. For that purpose, we used muscle samples acquired in another study from athletes who ran a 200-km race (Kim et al. 2011).

Methods

Subjects, race and sample collection

Eight men (44 ± 1 years, range: 38–50 years) well experienced in ultra endurance exercise took part in a 200-km running race. The average running time was 28 h 03 min ± 2 h 01 min (range: 22 h 15 min–35 h 04 min). The event was held at sea level in Cheju Island (South Korea). Subjects were experienced and well-prepared ultramarathon runners so that all completed the distance within the cut-off time of 36 h. The local temperature ranged from 12.5 to 25.3°C (mean 15.9°C) with a relative humidity between 59.0 and 63.4% and a wind speed of 3–4.5 km/h.

The subjects were informed about the experimental procedure before their written consent was obtained. The experiment was approved by the Ethical Committee of the Korea National Sport University. All the procedures used were in accordance with the WMA's Declaration of Helsinki on ethical principles for medical research involving human subjects.

The subjects reported to the laboratory in the morning, 2 weeks prior to the race. They had been told to refrain from exercise for a week prior to sample collecting and ate their usual breakfast 3 h prior to the biopsy. A muscle sample was taken from the mid portion of the vastus lateralis with a 5-mm Bergström biopsy needle. Local skin anesthesia was performed with 2% lidocaine. Samples were quickly frozen in liquid nitrogen and stored at −80°C before further analysis. During the race and the recovery, the runners were allowed to drink and eat ad libitum. Three hours after arrival, a second muscle biopsy was taken from the same leg about 1 cm apart from the first biopsy site.

RNA extraction and quantitative Real-Time PCR

Frozen muscle samples (∼30 mg) were pulverized using a mortar and a pestle, transferred in a pre-cooled micro-tube and homogenized in 1 ml TRIzol® reagent (Invitrogen, Vilvoorde, Belgium). Total RNA was then isolated following the manufacturer's instructions. Briefly, after centrifugation of the homogenate (12,000*g*, 10 min, 4°C), 0.2 ml chloroform was added to the supernatant The solution was centrifuged at 12,000*g* for 15 min at 4°C and the aqueous phase was removed. RNA was isolated using 0.5 ml 100% isopropanol. The pellet was washed with 1 ml of 75% ethanol and then dried on air. RNA was re-suspended in RNAase-free water and stored at −80°C. RNA quality was checked by 1.5% agarose gel electrophoresis. RNA quantity was measured by Nanodrop® spectrophotometry. Reverse transcription was performed by MyIQ2 thermocycler (Bio-Rad, Nazareth, Belgium). 10 µl RT Buffer and 1 µl 20× Enzyme Mix (Bio-Rad) were added to 1.5 µg RNA and RNase-free water to a final volume of 20 µl. Samples were run for 60 min at 37°C followed by 5 min at 95°C. Primers used for quantitative PCR are listed in Table 1. cDNA was amplified on MyIQ2 thermocycler, using the following conditions: 3 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 60°C and 30 s at 72°C. Triplicates containing 4.8 µl IQ SybrGreen SuperMix (Bio-Rad), 0.1 µl of each primer (final concentration: 100 nM) and 5 µl cDNA were analyzed. Melting curves were systematically performed for quality control. Beta-2-microglobulin (β2MG) was used as reference gene and was unchanged in response to exercise confirming previous results acquired in our laboratory with another exercise paradigm (Deldicque et al. 2008).

Statistics

Post-exercise mRNA expression levels are expressed in fold change (mean ± SEM), compared with their respective pre-exercise values. A paired *t*-test was conducted for statistical analysis. Pearson product moment correlations were applied to assess a possible relationship between magnitude of changes in autophagy markers and race time

Table 1 Primer sequences (5′-3′)

	Forward	Reverse
ATG4b	GAT GGA GGA AAT CAG AAG GTT G	CGC AGG GGA ATG AGA AGT A
ATG12	AGT AGA GCG AAC ACG AAC CAT C	CCA TCA CTG CCA AAA CAC TCA T
Beclin1	CAC ATC TGG CAC AGT GGA CA	CGG CAG CTC CTT AGA TTT GT
BNIP3	CTG AAA CAG ATA CCC ATA GCA TT	CCG ACT TGA CCA ATC CCA
BNIP3l	CCA AGG AGT TCC ACT TCA GAC A	AGT AGG TGC TGG CAG AGG GTG T
Cathepsin L	GTG AAG AAT CAG GGT CAG TGT G	GCC CAG AGC AGT CTA CCA GAT
Gabarap1 l	GTG CCC TCT GAC CTT ACT GTT G	CAT TTC CCA TAG ACA CTC TCA TC
LC3b	AAT CCC GGT GAT AAT AGA ACG A	GGA GAC GCT GAC CAT GCT GT
Ulk2	CTT CTC CAC CAT CCC TTC CA	ACT GCC CTC CAC ACA CCA A
***β*2MG**	ATG AGT ATG CCT GCC GTG TGA	GGC ATC TTC AAA CCT CCA TG

as well as magnitude of changes in autophagy markers and age. Statistical significance was set at $P < 0.05$.

Results

The mRNA level encoding for key proteins of the ALP were increased in response to the 200-km race (Fig. 1). Transcript levels of ATG4b increased by 1.49 ± 0.21 fold ($P = 0.025$), and transcripts of ATG12 similarly raised by 1.57 ± 0.23 fold ($P = 0.013$). Gabarapl1 and LC3b—two human homologues of ATG8—increased by 3.86 ± 0.92 ($P = 0.008$) and 2.03 ± 0.35 fold ($P = 0.011$), respectively. The lysosomal enzyme cathepsin L was up-regulated by 2.23 ± 0.33 fold ($P = 0.003$).

The mRNA expression level of genes encoding for proteins regulating the ALP are presented in Fig. 2. Transcript levels of the autophagy-regulatory genes BNIP3 and BNIP3l were similarly increased by 2.13 ± 0.51 ($P = 0.031$) and 2.13 ± 0.34 fold ($P = 0.007$), respectively, in response to exercise. The mRNA expression level of Beclin1 and Ulk2 remained unchanged.

There was no association between race time and magnitude of changes in autophagy markers or between age and magnitude of changes in autophagy markers.

Discussion

Already in 1984 and 1985, increase in lysosomal enzyme activities and an elevated number of autophagic vacuoles were reported subsequently to a strenuous endurance exercise (Salminen 1985; Salminen and Vihko 1984). Much more recently, the control of autophagy in response to endurance exercise was investigated in rat skeletal muscle. In that study, endurance exercise, 1 h treadmill 6 days a week for 8 weeks, induced an increase in the protein expression level of ATG7, Beclin1 and LC3 in rat soleus muscle (Feng et al. 2011). The results of the present investigation are the first showing an increase in transcripts

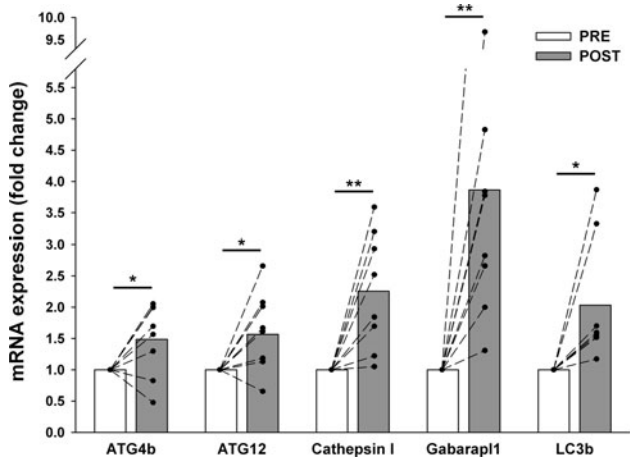

Fig. 1 mRNA expression levels of autophagy markers before (*white bars*) and 3 h after (*grey bars*) an ultramarathon. *Bars* represent mean fold change. Variability is represented by individual response (*black dots*) to exercise. *$P < 0.05$, **$P < 0.01$

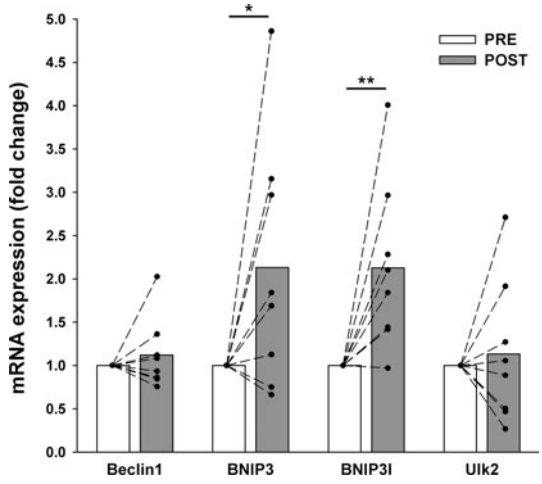

Fig. 2 mRNA expression levels of autophagy mediators before (*white bars*) and 3 h after (*grey bars*) an ultramarathon. *Bars* represent mean fold change. Variability is represented by individual response (*black dots*) to exercise. *$P < 0.05$, **$P < 0.01$

of autophagy-related and autophagy-regulatory genes in human skeletal muscle in response to an acute form of strenuous endurance exercise, namely an ultramarathon run.

These results are in agreement with those reported in previous studies which investigated in vivo modulation of autophagy-related and autophagy-regulatory genes in response to both atrophy models of denervation and food deprivation in rodents (Mammucari et al. 2007; Zhao et al. 2007). Three days of denervation induced a significant increase in the mRNA expression level of ATG4b, ATG12, Beclin1, BNIP3, BNIP3l, cathepsin L, Gabarapl1 and LC3b, followed by a later increase of Ulk2 after 7 days. The same pattern of gene expression was observed in response to 24-h food deprivation, except for Ulk2 and Beclin1 the mRNA levels of which were not modified. The similarity of our results with those obtained on the basis of denervation and food deprivation models of muscle atrophy supports the idea that extreme endurance exercise may also be considered as a catabolic model for skeletal muscle.

Recent data indicate that the transcription factor forkhead box O3 (FoxO3) is probably the major effector for autophagy regulation in skeletal muscle (Mammucari et al. 2008). Cell culture experiments have evidenced that FoxO3 stimulates autophagy through the transcriptional activation of several autophagy genes including ATG4b, ATG12, Beclin1, Gabarapl1 and LC3b (Zhao et al. 2007), which were all increased in response to the ultramarathon race.

The precise role of the observed transcript up-regulation remains to be elucidated. On the one hand, it is likely that increased expression of BNIP3 and BNIP3l regulates directly autophagy induction. Over-expression of one or the other is sufficient to induce autophagosome formation in skeletal muscle. Their respective inhibition reduces LC3 lipidation caused by a constitutively active form of FoxO3 (Mammucari et al. 2007). On the other hand, up-regulation of ATG could be essential for autophagic flux maintenance. LC3b and Gabarapl1 are degraded by lysosomes and must be constantly renewed to maintain the autophagic flux. LC3b over-expression does not increase autophagic flux while LC3b gene inhibition blocks the autophagic process (Mammucari et al. 2008). Thus, increased expression of LC3b and Gabarapl1 transcripts could be necessary for replenishing protein stocks.

Skeletal muscle response to exercise is highly specific and depends on the characteristics of exercise, including intensity and duration. Even if the predominant energetic substrates during endurance exercise remain carbohydrates and fats, 1–6% of the dissipated energy can originate from amino acid (AA) oxidation (Tarnopolsky 2004). Ultramarathon race represents an extreme exercise in which food and fluids intake cannot compensate energy and fluid needs. Not only endurance exercise *per se* but also a suboptimal energy intake as well as dehydration can induce an increase in AA oxidation (Tarnopolsky 2004). Alternatively, repeated eccentric contractions can cause severe injury to skeletal muscle (McCully and Faulkner 1985).

A limitation of the present study is that biopsies were taken in the context of a race, so that intensity and duration of exercise as well as food and water intake were impossible to control. This can probably induce variations in the responses of autophagic markers. As mentioned above, increase in autophagy-related and autophagy-regulatory gene expression is strongly dependent on dietary state (Finn and Dice 2006; Mammucari et al. 2007). The fact that subjects had time to eat between arrival and post-biopsy sampling must undoubtedly have partially blunted the level of the observed increases, but does not question the main outcomes of this study.

In conclusion, our results show an increase in autophagy-related and autophagy-regulatory gene expression in human, in response to ultra endurance exercise. As mentioned above, such an increase has been associated with ALP activation and catabolism (Mammucari et al. 2007). However, more research is needed to shed light on the exercise-dependent signalling pathways which regulate the expression of these transcripts.

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Conflict of interest The authors have no conflict of interest to declare.

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