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Modulation of autophagy and ubiquitin-proteasome pathways during ultra-endurance running

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Jamart C, Francaux M, Millet GY, Deldicque L, Frère D, Féasson L. Modulation of autophagy and ubiquitin-proteasome pathways during ultra-endurance running. *J Appl Physiol* 112: 1529–1537, 2012. First published February 16, 2012; doi:10.1152/japplphysiol.00952.2011.—In this study, the coordinated activation of ubiquitin-proteasome pathway (UPP), autophagy-lysosomal pathway (ALP), and mitochondrial remodeling including mitophagy was assessed by measuring protein markers during ultra-endurance running exercise in human skeletal muscle. Eleven male, experienced ultra-endurance athletes ran for 24 h on a treadmill. Muscle biopsy samples were taken from the vastus lateralis muscle 2 h before starting and immediately after finishing exercise. Athletes ran 149.8 ± 16.3 km with an effective running time of 18 h 42 min (±41min). The phosphorylation state of Akt (−74 ± 5%; $P < 0.001$), FOXO3a (−49 ± 9%; $P < 0.001$), mTOR Ser2448 (−32 ± 14%; $P = 0.028$), and 4E-BP1 (−34 ± 7%; $P < 0.001$) was decreased, whereas AMPK phosphorylation state increased by 247 ± 170% ($P = 0.042$). Proteasome β2 subunit activity increased by 95 ± 44% ($P = 0.028$), wheras the activities associated with the β1 and β5 subunits remained unchanged. MuRF1 protein level increased by 55 ± 26% ($P = 0.034$), whereas MAFbx protein and ubiquitin-conjugated protein levels did not change. LC3bII increased by 554 ± 256% ($P = 0.005$), and the form of ATG12 conjugated to ATG5 increased by 36 ± 17% ($P = 0.042$). The mitochondrial fission marker phospho-DRP1 increased by 110 ± 47% ($P = 0.003$), whereas the fusion marker Mfn1 and the mitophagy markers Parkin and PINK1 remained unchanged. These results fit well with a coordinated regulation of ALP and UPP triggered by FOXO3 and AMPK during ultra-endurance exercise.

muscle protein degradation; mitochondrial remodelling; mitophagy; LC3b; MuRF1

SPORT EVENTS LASTING >6 h are defined as ultra-endurance exercise (61). During such events, which can last up to several days, food and fluid intakes are not able to equilibrate energy expenditure, which leads to negative energy and protein balances (43). Although the main energy substrates during endurance exercise are carbohydrate and fat, 1–6% of the dissipated energy originate from amino acid (AA) oxidation (56). This percentage can be increased by a suboptimal energy intake as well as dehydration (56), which is the fate of ultra-endurance exercise (14, 26). Ultra-endurance exercise is also associated with stresses that lead to damaged proteins and organelles and/or to accumulation of misfolded proteins (24, 50). Prolonged running implies repeated eccentric contractions that may mechanically damage muscle cells (27). As a consequence, efficient systems that allow releasing amino acids as alternative energy substrate and that ensure removing or recycling of damaged/abnormal cell constituents are needed in skeletal muscle during long-lasting exercise. This is important for ensuring whole body capacity to cope with high energy demand and thereby its ability to sustain exercise. Moreover, this transitory catabolic state could also induce cell adaptations to exercise by, for example, initiating the first step of the mitochondrial remodeling, namely the fission.

Two main proteolytic pathways controlling protein degradation in skeletal muscle are the ubiquitin-proteasome pathway (UPP) and the autophagy-lysosomal pathway (ALP) (51). The UPP is responsible for degrading soluble and myofibrillar muscle proteins (53). Briefly, substrates are tagged by a polyubiquitin chain, which allows their recognition and subsequent cleaving by the catalytic sites localized inside the proteolytic room of proteasome 26S (47). The specificity of substrate tagging is ensured by ubiquitin ligases. For example, two muscle-specific ligases [namely muscle ring finger 1 (MuRF1) and muscle atrophy F-box (MAFbx)] have been shown to increase in numerous situations of catabolism (3). We recently showed that the mRNA expression level of these two ligases was increased in response to a 200-km run (24).

Much less is known about the potential implication of macroautophagy (here called autophagy) during long-lasting events. Autophagy starts with autophagosome formation: a double membrane is formed around a portion of the cytoplasm containing proteins and/or organelles. Autophagosome then fuses with lysosomes containing lysosomal enzymes such as cathepsin L (CATH L) that degrade the content of the vacuole (15). Autophagosome formation is under the control of the autophagy-related novel genes proteins (ATG). In particular, two ubiquitin-like conjugation systems have been described. The first one implicates the ATG12-ATG5 complex, which interacts with ATG16 to participate in the autophagosome membrane formation. The second implies ATG8, also known as microtubule-associated protein 1 light chain 3 (LC3) in mammals. Pro-LC3 is first cleaved by ATG4 to its mature form, LC3I. The latter is then processed through ATG7 and ATG3 to a lipidated form, LC3II, which is specific for autophagy (15). LC3II participates in the substrate selection for degradation and also plays a role in membrane fusion (40). Several LC3 isoforms exist, but LC3bII seems to be the best marker for autophagy activation since it is the only isoform that has been positively correlated with an increase in autophagosome number (1). Among proteins implicated in the control of the autophagy process, the ATG6 homolog Beclin-1 appears to play a central role in the initiation of sequestration (55, 57).

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More recently, an important role for BCL2/adenovirus E1B 19-kDa interacting protein 3 (BNIP3) in autophagy induction has been evidenced, since overexpression of this gene is sufficient to enhance autophagy in skeletal muscle (30).

BNIP3 also regulates mitophagy (62), which consists in a specific form of ALP aiming to eliminate dysfunctional mitochondria (49). Fusion and fission events regulate the remodelling of the mitochondrial network (7). The balance between these two processes seems essential for preventing accumulation of dysfunctional mitochondria that are generated under metabolic stress conditions. In rat skeletal muscle, endurance exercise has been reported to regulate the expression of key proteins implicated in fusion and fission events (12, 16).

The forkhead box O3 (FOXO3) transcription factor coordinately regulates the UPP and the ALP by inducing the transcription of autophagy-related genes such as LC3, ATG12, and BNIP3, as well as the muscle-specific ligases MuRF1 and MAFbx (31, 64), and is therefore believed to be a master regulator of proteolytic pathways. Under basal conditions, FOXO3 is phosphorylated through the serine/threonine kinase Akt and remains in the cytosol. Under catabolic signal such as food deprivation, Akt becomes dephosphorylated, which leads to FOXO3 dephosphorylation and subsequent nucleus translocation (51). Another key regulator of autophagy is the mammalian target of rapamycin (mTOR) (23). When phosphorylated by Akt or high intracellular amino acid content, mTOR suppresses autophagy. On the other hand, when the AMP-activated kinase (AMPK), a sensor for cell energy status, is phosphorylated, autophagy can be activated through mTOR inhibition (23).

To date, only little data are available about autophagy, mitophagy, and mitochondrial network remodeling in response to exercise. Therefore, the first purpose of this study was to assess the activation of protein markers for these processes during ultra-endurance running exercise in humans. A second goal was to evaluate whether this activation was coordinated with the UPP and to highlight the signaling pathways implicated in these regulations. For these purposes, we used muscle biopsy samples acquired in another study (37) from runners who exercised for 24 h on a treadmill and were taken before and immediately after the end of the run. We hypothesized that such an extreme and long-lasting exercise, which induces a negative energy balance, would repress muscle protein synthesis markers, activate muscle protein degradation markers, and initiate mitochondria remodeling by stimulating mitochondrial fission.

MATERIALS AND METHODS

Subjects. Eleven male athletes [age: 42.1 ± 7.8 yr; weight: 75.2 ± 7.6 kg; height: 178.6 ± 4.8 cm; maximal oxygen uptake ($\dot{V}O_{2max}$): 53.0 ± 5.4 ml·kg^{-1}·min^{-1}; velocity associated with $\dot{V}O_{2max}$ ($V_{\dot{V}O_{2max}}$): 18.4 ± 1.4 km/h] took part in the study. They were all experienced ultra-endurance runners. All of them had already completed a race longer than 24 h or >100 km. Their training history in running was 14.5 ± 7.3 yr, and they had 6.5 ± 4.1 yr of ultra-endurance experience. They reported to run an average of 78.1 ± 10.7 km/wk. The protocol was in accordance with the Declaration of Helsinki, had been approved by the local ethics committee (Comité de Protection des Personnes Sud-Est 1), and is registered in http://clinicaltrial.gov (no. NCT 00428779).

Protocol. Subjects reported to the laboratory 3–4 wk before the experiment for inclusion, medical examination, and preliminary testing. During this session, subjects gave their written consent after having been informed of the entire procedure. A progressive maximal test was performed on a motorized treadmill (Gymrol S2500, HEF Tecmachine) for determining $\dot{V}O_{2max}$ as well as $V_{\dot{V}O_{2max}}$ (37). Subjects ran at least three times on a motorized treadmill before participating in the 24-h treadmill protocol (24TR) and were asked to refrain from strenuous exercise for a week before the 24TR. The day of the experiment, all subjects ate the same lunch at noon. Two hours before starting the 24TR, a first muscle biopsy was taken under local anesthesia from the superficial portion of the left vastus lateralis, using a percutaneous technique (20). Samples were quickly frozen in liquid nitrogen and stored at −80°C before further analysis. Athletes were asked to rest between muscle sample collecting and beginning of exercise. Subjects started to run between 4:30 PM and 6:00 PM. They exercised on a motorized treadmill (Gymrol S2500, HEF Tecmachine and ProForm 585 Perspective, Health & Fitness) with a null slope. Athletes ran at a freely chosen speed that was set by an investigator and that could be modified at will. Subjects were cooled with fans to avoid hyperthermia. They were fed ad libitum with meals containing mainly carbohydrates, energetic bars, and drinks. Food and water intakes were recorded by an investigator throughout the 24TR. Caloric intake was subsequently analyzed for each subject using the Nutrilog software. Subjects stopped running every 4 h for neuromuscular function test sessions (32) and blood sample collection. A second biopsy sample was taken from the superficial portion of the right vastus lateralis within the 10 min following the end of exercise and processed as described above.

Blood samples. Peripheral venous blood samples were harvested from an antecubital vein of runners before, every 4 h during, and after the 24TR. Samples were collected in EDTA tubes under sterile conditions and centrifugated at 1,000 *g* for 10 min at room temperature. Plasma insulin concentrations were determined by radioimmunoassay (Bi-insulin-irma kit purchased from Cisbio bioassays). Due to technical problems, only four plasma samples were available at 16 h. Therefore, this time point is not presented in the results.

Protein extraction. Muscles were cut into small pieces with razor blades then homogenized on ice with a Tenbroeck Tissue Grinder in ice-cold buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA and 1 mM DTT. Homogenates were centrifuged for 30 min at 10,000 *g*, 4°C. Supernatants were stored at −80°C. Protein content was determined using the DC protein assay kit (Bio-Rad) with BSA as a standard.

SDS-PAGE and immunoblotting. Proteins (20–30 μg) were combined with Laemmli sample buffer. Proteins were separated by SDS-PAGE for 2 h at a constant intensity of 40 mA and transferred on polyvinylidene fluoride membranes at 80 V for 2.5 h. Membranes were blocked 1 h in 0.1% Tween-20 Tris-buffered saline (TBST) and 5% no-fat dry milk, then incubated overnight at 4°C with the primary antibody. ATG3, ATG7, ATG12, Beclin-1, LC3b, phospho-Akt (Ser473), phospho-AMPKα (Thr172), phospho-dynamin-related protein 1 (DRP1) (Ser616), phospho-eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1) (Thr37/46), phospho-FOXO3a (Thr32), phospho-mTOR (Ser2448), phospho-mTOR (Ser2481), and Ubiquitin antibodies were purchased from Cell Signaling Technology. MuRF1 and Mfn1 were from Santa Cruz Biotechnology. MAFbx was from ECM Biosciences. BNIP3, Parkin, PTEN-induced putative kinase 1 (PINK1), and GAPDH were from Abcam. Membranes were washed three times with TBST and incubated for 1 h at room temperature with a secondary antibody conjugated to horseradish peroxidase. Three washings were made again before detection by chemiluminescence with ECL-Plus Western blotting kit (Amersham Biosciences). Films were scanned on an ImageScanner using the Labscan software, and bands were quantified with the Image Master 1D Image Analysis Software (Amersham Biosciences). Expression levels were normalized to GAPDH, and its expression was unaffected by exercise. Since the specificity of the MAFbx and MuRF1 antibodies has previously been questioned, we performed additional Western

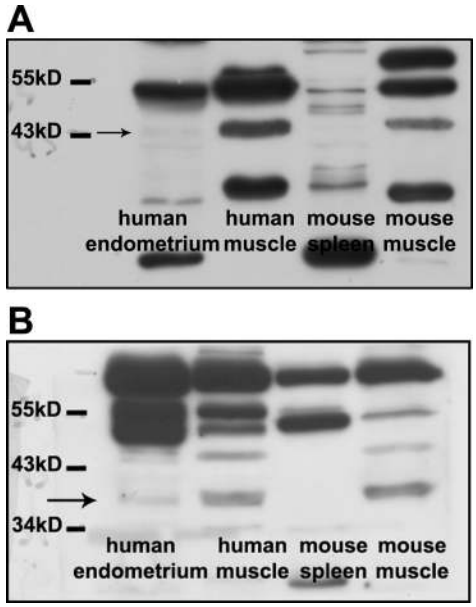

Fig. 1. Representative Western blots for the muscle-specific ligases MAFbx (*A*) and MuRF1 (*B*) proteins. Arrays show the expected band.

blots to provide compelling arguments regarding the specificity of the antibodies that we used. We loaded a gel with the extract of a human muscle biopsy used for our study, an extract of human endometrium, an extract of mouse spleen, and an extract of mouse gastrocnemius. As expected, the band representing MAFbx was considerably reduced with the endometrium extract when compared with muscle extracts and was totally absent with the spleen extract (Fig. 1*A*). We used the same procedure to validate the MuRF1 antibody. We identified a 45-kDa band and another one at 40 kDa that could potentially represent MuFR1 since they were absent with the spleen extract (Fig. 1*B*). According to the UniProt website (http://www.uniprot.org), MuRF1 has a calculated weight of ~40 kDa. When rats were made septic by cecal ligation and puncture, the same antibody was able to show that a 39-kDa representing MuRF1 was increased by threefold compared with controls (45). Taken together, these results indicate that the 40-kDa band represents MuRF1.

Enzymatic activity assays. Enzymatic activities were determined fluorometrically using specific substrates and specific inhibitors. Each sample was assessed in triplicate, with one replicate containing a specific inhibitor to the activity studied. 26S proteasome activities were determined by adding 100 μM Z-LLE-AMC (Peptide International), LSTR-AMC (Bachem), or Suc-LLVY-AMC (Calbiochem) for β1, β2, and β5 subunits, respectively, to 15 μg of proteins in a reaction buffer containing 50 mM Tris, pH 7.5, 1 mM EDTA, 150 mM NaCl, 5 mM $MgCl_2$, 0.5 mM DTT, and 100 μM ATP, ± inhibitor [40 μM Z-Pro-Nle-Asp-al (Alexis), 60 μM epoxomicin (Peptide Institute), or 20 μM epoxomicin, respectively]. CATH L activity was determined by adding 100 μM Z-Phe-Arg-AMC (Peptide Institute) to 15 μg of proteins in a buffer containing 100 mM sodium acetate, pH 5.5, 1 mM EDTA, 1 mM DTT ± 10 μM CATH L inhibitor I (Calbiochem). Fluorescence was monitored every 5 min for 105 min on a fluorometer (Fluostar Optima, BMG Labtech) at an excitation and emission wavelength of 370 and 460 nm, respectively. Enzymatic activities were calculated as the difference of the slope of the accumulation of fluorescence as a function of time in the absence of inhibitor and presence of inhibitor.

Statistical analysis. All values are expressed as means ± SE. One-way repeated-measures ANOVAs were applied to assess differences in plasma glucose and insulin concentration between exercise and control values, as well as differences in distance and caloric intake during exercise, with Bonferroni post hoc tests. For other variables, postexercise values (POST) were expressed as the percentage of change compared with the preexercise values (PRE). A normality test was first conducted for each variable. If normality test failed, a Wilcoxon signed rank test was applied to evaluate statistical significance of changes. If normality test passed, a paired *t*-test was conducted. Statistical significance was set at $P < 0.05$.

RESULTS

Performance, caloric intake, and last snack. Athletes ran a distance of 149.8 ± 16.3 km with an effective running time of 18 h and 42 min (±41 min). The average running speed was 7.9 ± 0.7 km/h, which represents 43.0 ± 2.4% of $V_{\dot{V}O_{2max}}$. The distance, registered over 4-h periods, decreased progressively over time ($P < 0.001$; Fig. 2*A*). Post hoc analysis revealed that this diminution was significant by 4–8 h after the beginning of exercise. There was a significant effect of time on caloric intake ($P < 0.001$; Fig. 2*B*), which declined progressively, reaching the statistical threshold of significance after 16 h. The time between the last snack and completion of the protocol was 140 ± 27 min.

Autophagy-lysosomal pathway, mitophagy, and mitochondrial network remodeling markers. Figure 3*A* shows protein expression levels of markers of the ALP. LC3bII increased by 554 ± 256% ($P = 0.005$), and the form of ATG12 conjugated to ATG5 (cATG12) increased by 36 ± 17% ($P = 0.042$). ATG7 and BNIP3 remained unaffected, whereas ATG3 and Beclin-1 did not change significantly. The relative activity of the lysosomal enzyme CATH L showed a large interindividual variability. As a consequence, the mean value was not altered compared with PRE (Fig. 3*B*). Mitochondrial fission and fusion as well as mitophagy markers are presented in Fig. 3*C*. The mitochondrial fission marker phospho-DRP1 increased significantly by 110 ± 47% ($P = 0.003$).

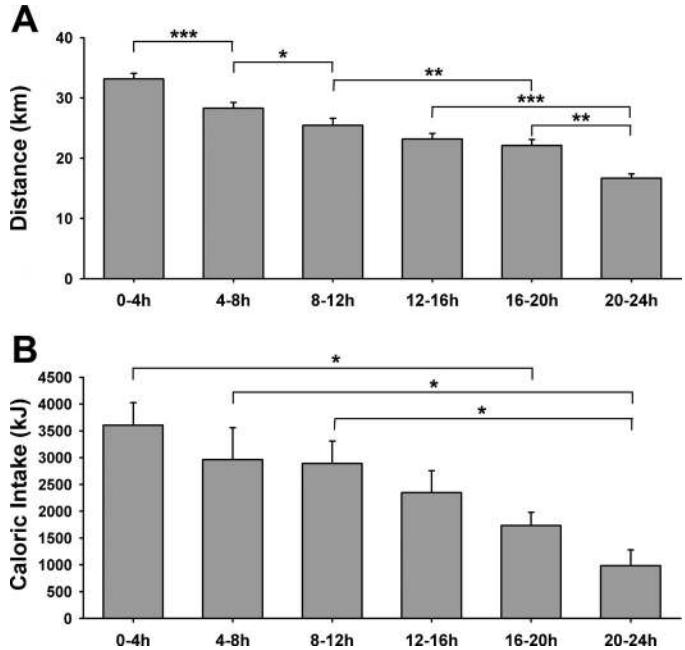

Fig. 2. Raced distance (*A*) and caloric intake (*B*) over time during the 24-h treadmill protocol (24TR). Values are means ± SE. Significant difference: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

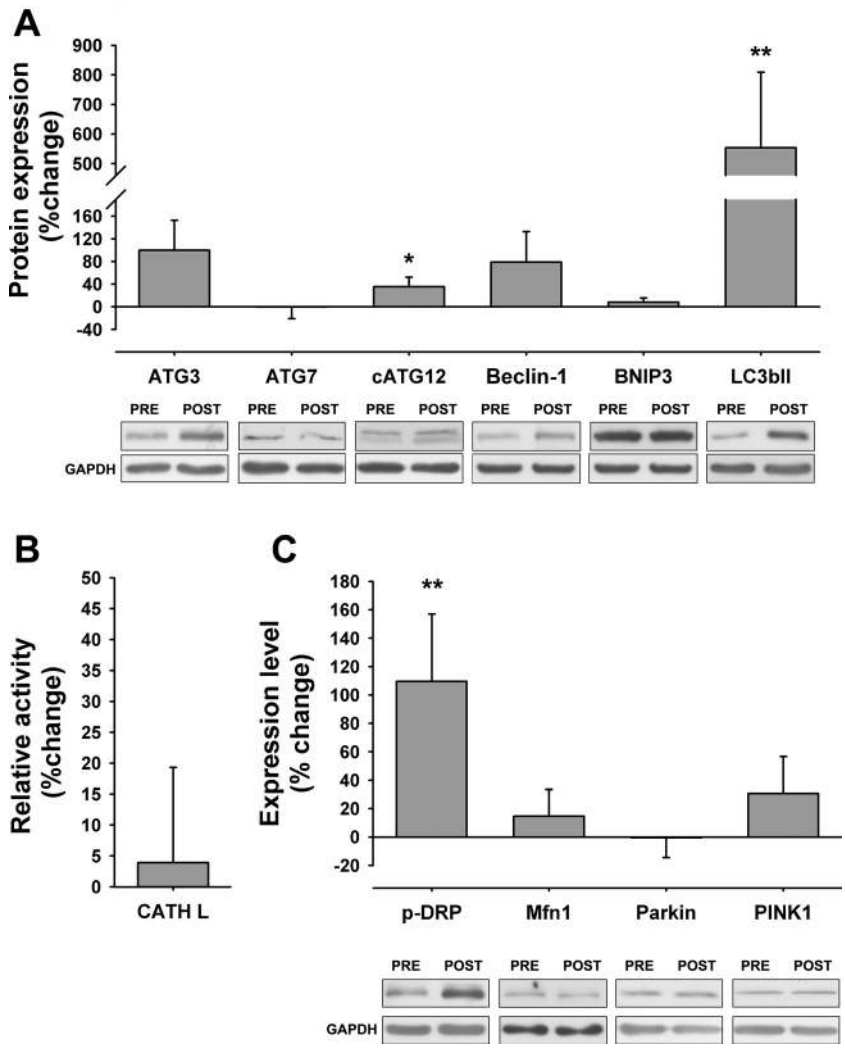

Fig. 3. Changes in ALP markers (*A*), CATH L relative activity (*B*), and mitochondrial remodeling markers (*C*). Values are expressed as percentage of change (means ± SE) compared with the preexercise condition. Representative Western blots with control for loading are added under each variable. Adjustment for contrast and brightness was applied to the entire figure. Significant difference: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Ubiquitin-proteasome pathway markers. Proteasome 26S relative activities are presented in Fig. 4*A*. The enzymatic activity associated with the β2 subunit increased by 95 ± 44% ($P = 0.028$), whereas the activities associated with the β1 and β5 subunits remained unchanged. At the protein expression level, MuRF1 increased by 71 ± 31% ($P = 0.023$), whereas MAFbx and the level of ubiquitin-conjugated protein (UbCP) did not change (Fig. 4*B*).

Signaling pathways. Immediately after exercise, the phosphorylation state of Akt was decreased by 74 ± 5% ($P < 0.001$; Fig. 5*A*). This exercise-induced dephosphorylation was also observed for both Akt downstream targets, namely FOXO3a (−49 ± 9%; $P < 0.001$) and mTOR at Ser2448 (−32 ± 14%; $P = 0.028$) (Fig. 5*A*). There was a tendency toward a decrease in mTOR phosphorylation level at Ser2481, but it did not reach the level of significance ($P = 0.062$; Fig. 5*A*). The phosphorylation state of 4E-BP1, a downstream target of mTOR, decreased by 34 ± 7% ($P < 0.001$) (Fig. 5*A*). AMPK phosphorylation state increased by 247 ± 170% ($P = 0.042$; Fig. 5*B*).

Insulin and glucose plasma levels. Plasma insulin concentration was 9.36 ± 1.62 μU/ml in the preexercise condition and decreased significantly after 8 h (3.69 ± 0.89 μU/ml; $P = 0.044$), 12 h (3.42 ± 1.62 μU/ml; $P = 0.024$), 20 h (3.75 ± 1.47 μU/ml; $P = 0.039$), and 24 h (2.93 ± 0.61 μU/ml; $P = 0.016$) compared with PRE (Fig. 6). Glucose plasma concentration remained unchanged.

DISCUSSION

Energy deficit occurs when caloric intake cannot balance energy expenditure. It can be due to either a decrease or even suppression in food intake (i.e., fasting) or an increase in physical activity. Skeletal muscle accounts for the largest protein pool in the body and must therefore be able to degrade proteins into amino acids for providing energy substrate during catabolic periods. Prolonged running exercise is known to mechanically and metabolically damage muscle cell components, which must be eliminated to ensure cellular homeostasis. These muscle damages are often reflected by the rise in the serum level of muscle-specific proteins like creatine kinase (32). In this study, we showed that prolonged endurance running was associated with an increase in protein markers of the two main proteolytic pathways described in skeletal muscle, namely UPP and ALP. These modulations were linked to a repression of the insulin-phosphoinositide-3-kinase (PI3K)-Akt signaling pathway.

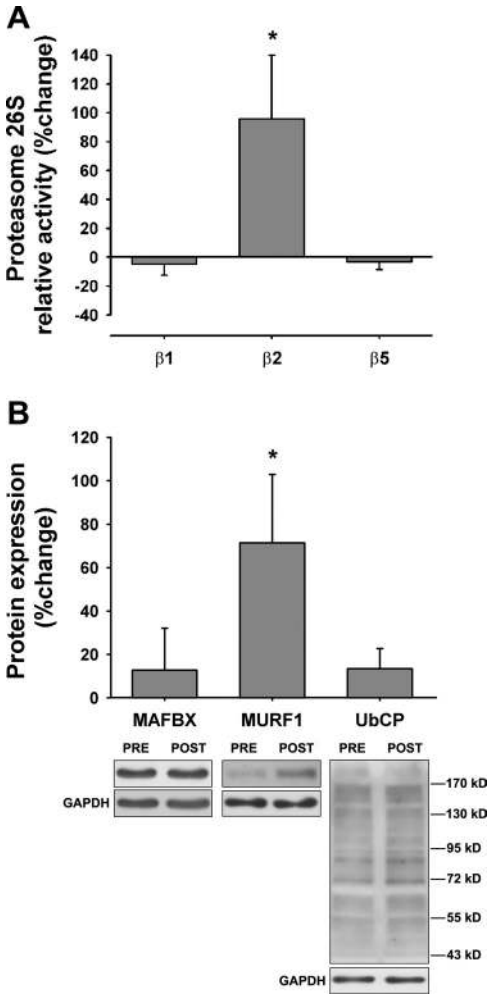

Fig. 4. Changes of proteasome 26S relative activities (C), protein expression level of MAFbx, MuRF1, and UbCP (B). Values are expressed as percentage of change (means ± SE) compared with the preexercise condition. Representative Western blots with control for loading are added under each variable. Adjustment for contrast and brightness was applied to the entire figure. *Significant difference ($P < 0.05$).

Caloric balance. Since standardizing caloric intake could severely compromise ultra-marathon completion, the runners participating in the present study had free access to food and beverage ingestion, which was monitored throughout the 24TR protocol. The caloric intake was subsequently calculated. On the basis of the distance, the effective running time, and an average running economy of 200 ml $O_2 \cdot kg^{-1} \cdot km^{-1}$ (36), the energy expenditure and therefore the caloric balance were estimated. Energy intake allowed covering ~30% of caloric needs over the entire 24TR. This ratio was higher during the first 16 h (~35%) than in the last 8 h (~20%). Clearly, the caloric balance was negative throughout the exercise protocol and has probably led to a negative muscle protein balance, which was not measured in the present study.

Autophagy-lysosomal pathway. The present study shows, for the first time, an increase in protein markers of autophagy in human skeletal muscle in response to ultra-endurance running. Up to now, only one animal study has investigated the modulation of protein markers of autophagy in skeletal muscle in response to endurance training. In that study, running 1 h on a treadmill 6 days a week during 8 wk induced a significant increase in the protein expression level of ATG7, Beclin-1, and LC3b I and II forms in rat soleus muscle (16).

Among LC3 mammalian isoforms, only LC3bII has been positively correlated with an increased number of autophagosomes and is therefore recommended as an accurate marker for Western blot analysis (1). The antibody used in the present study has been previously reported to recognize LC3bII only (13). Our data showed a large and significant increase in LC3bII. This increase supports the idea of an increased number of autophagosomes but does not allow determining whether this raise is the consequence of an enhanced autophagosome formation or a defect in autophagosome degradation. The latter can be due among others to impaired lysosomal proteolytic activity (1). In cell culture, a defect in the degradation of autolysosomes may be evidenced by assessing the failure of LC3bII to increase in the presence of ALP inhibitors (1). Using human tissue did not permit us to perform these control experiments. However, the fact that cathepsin L activity was not modified is an element in favor of an enhanced autophagosome formation. We also previously measured an increase in the transcripts of LC3b in response to a 200-km race (21), which has been interpreted as necessary for replenishing LC3b stocks, thereby ensuring the persistence of the autophagic process (30).

In the present experiment, ATG7 remained unchanged and Beclin-1 did not increase significantly. This discrepancy with previous work (16) could be due to the exercise paradigm, since our study investigated the effects of an acute model of ultra-endurance in humans, whereas Feng et al. investigated the chronic effects of endurance training in rats. The ATG5-ATG12 complex is the other conjugation system that is known to be implicated in autophagosome formation. Our data showed that cATG12 increased at the end of the 24-h run. These results are in line with the fact that ATG12 mRNA expression level is increased in muscle atrophy models such as denervation and food deprivation (30, 63) but also in response to a 200-km race (21). The fact that BNIP3 did not increase was unexpected as its gene is induced in various situations of catabolism (28). More research should be conducted to evaluate the modulation of BNIP3 at the post-transcriptional level.

Interpreting the role of an activation of autophagy in response to exercise remains hypothetical as the exact role of the process in skeletal muscle is still unclear. On one side, results from atrophy models showed that autophagy is a major actor in protein degradation and thereby in muscle wasting (30, 38, 63). In that point of view, extreme endurance exercise may be considered as a catabolic model for skeletal muscle. On the other side, basal levels of autophagy are necessary for cell survival since $ATG7^{-/-}$ as well as $ATG5^{-/-}$ mice undergo severe muscle atrophy and weakness (34, 46). This suggests that autophagy is not only responsible for removing proteins but also plays an adaptive role in muscle remodeling that is positive for muscle mass maintenance. More research using knockout animal models or drugs repressing autophagy should be conducted to determine the exact role of the pathway on skeletal muscle response to exercise.

Mitochondrial network remodeling and mitophagy. Mitochondrial network remodeling through fusion and fission events is essential for preventing accumulation of dysfunctional mitochondria. Endurance exercise requires high energy

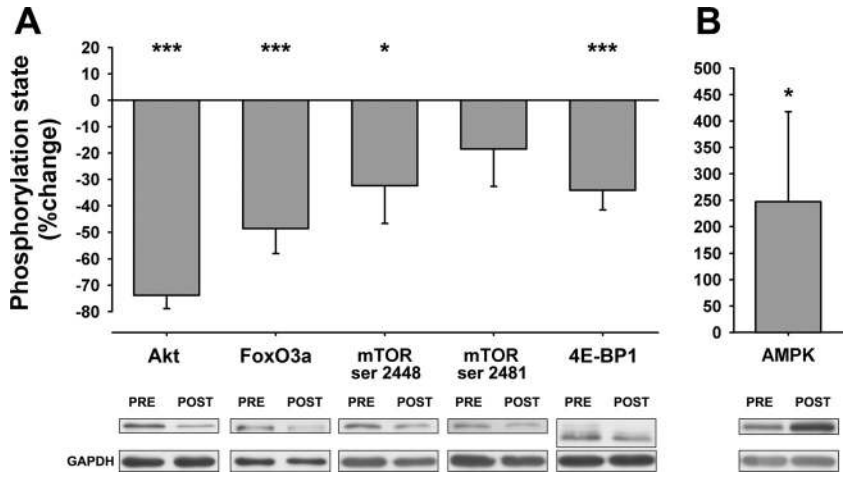

Fig. 5. Changes in phosphorylation state of kinases implicated in signaling pathways regulating protein balance. Values are expressed as percentage of change (means ± SE) compared with the preexercise condition. Representative Western blots with control for loading are added under each variable. Adjustment for contrast and brightness was applied to the entire figure. Significant difference: $*P < 0.05$; $***P < 0.001$.

production levels for a long time and must therefore induce a metabolic stress that causes damage to mitochondria. DRP1 stimulates mitochondrial fission when phosphorylated at Ser616 (10). Here, we showed a raise in the phosphorylation state of this protein in human skeletal muscle in response to exercise, whereas the fusion marker Mfn1 remained unchanged. These results are in accordance with the study of Feng et al. (16).

An increase in mitochondrial fission has been reported to be permissive for mitophagy (58). Therefore, we expected to measure a rise in the mitophagy markers Parkin and PINK1, but they were unaffected by the 24TR protocol. Parkin is an ubiquitin ligase that is recruited selectively through PINK1 to the damaged mitochondrial membrane and whose function is to promote mitochondria engulfment by the autophagosome (41). The mechanism controlling the interaction between these two proteins is not clearly established but could involve phosphorylation of Parkin by PINK1 (25, 41). Thus the phosphorylation state of Parkin could have been modified by PINK1 during the 24TR, thereby promoting mitophagy, without measuring alteration in protein expression level. Unfortunately, we could not test this possibility since no commercial antibody exists.

Ubiquitin-proteasome pathway. Although few results are available about autophagy modulation in response to endurance exercise, more research has been conducted on the UPP. However, most studies focused on mRNA ligases regulation and showed an increase in MuRF1 and MAFbx expression (16, 18, 19, 24, 29). The present study shows for the first time an increase in MuRF1 protein expression, whereas MAFbx remained unchanged. Few studies have evaluated the modulation of the proteasome activity pattern in response to an acute bout of ultra-endurance exercise. The caspase-like, trypsin-like, and chymotrypsin-like activities are the three main proteasomal activities. They are respectively localized inside the proteolytic room of proteasome in the β1, β2, and β5 subunits. The trypsin-like activity significantly increased after the 24-h run, whereas the caspase and chymotrypsin-like activities remained unchanged. These results are in contrast with our previous work in which chymotrypsin-like activity was decreased 3 h after the completion of a 200-km run (24). This difference could be explained by the fact that the 200-km runners were fed between the end of the race and the time of sampling biopsy, whereas the postexercise biopsy was taken just after the end of exercise in the 24-h runners. Indeed, it has been shown that chymotrypsin-like activity was decreased in extensor digitorum longus of adult rats in the postprandial state (9). Ubiquitin-conjugated protein level remained unchanged after exercise. This is not incompatible with an increased flux in UPP. Indeed, measuring ubiquitin-conjugated protein level determines the amount of ubiquitinated proteins at a given time but does not allow evaluation of a change in ubiquitin-conjugated protein turnover. Therefore, the UPP-dependent protein degradation process might still be increased. The increases in MuRF1 as well as in the β2-associated 26S activity of proteasome are elements that support that hypothesis.

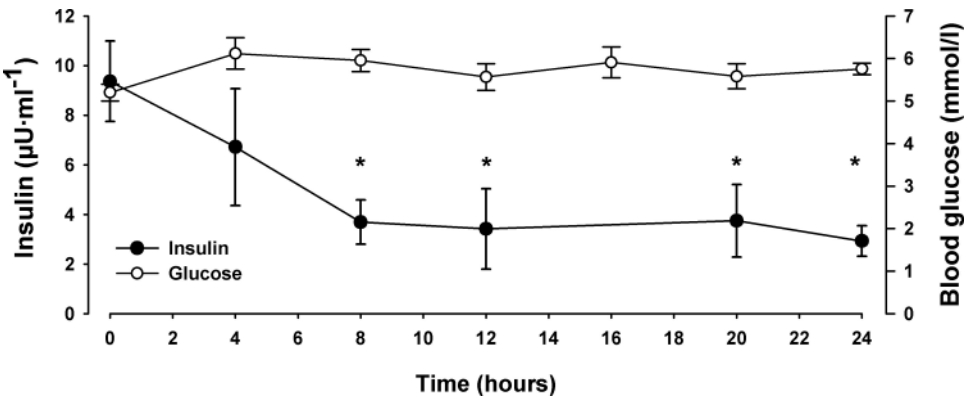

Fig. 6. Time course of plasma glucose and insulin concentration during the 24TR. *Signficant difference ($P < 0.05$).

Regulatory signaling pathways. The ALP and UPP are stimulated in a variety of stress conditions, including food deprivation. As discussed above, the exercise paradigm that we used was associated with a caloric deficit that reached ~80% during the last 8 h of exercise. Therefore, it is challenging to speculate about the respective contribution of exercise itself and caloric deficit in the modulation of proteolytic pathways markers that we observed. Nevertheless, our results point out a major implication of the insulin-PI3K-Akt signaling pathway, which is known to play a central role in protein balance regulation (54). The decrease in plasma insulin concentration during the 24-h run was expected since ultra-endurance exercise has already been associated with low plasma insulin levels (50). Nevertheless, glycemia remained unchanged during the same period of time. Epinephrine released during exercise is well known for inhibiting insulin secretion even when a potent stimulus (i.e., feeding) is applied (60).

Even if low plasma insulin concentration likely inactivates the Akt pathway (5), experiments have given contrasting results concerning the phosphorylation state of Akt in response to exercise (2, 6, 8, 11, 33). It should be kept in mind that Akt activity is indirectly dependent on dietary state through associated insulin secretion, whereas muscular contraction per se does not seem to affect the activity of the kinase (5). When runners were fed between the end of a race and the time of biopsy sampling was 3 h after a 200-km run, the phosphorylation state of Akt was unchanged compared with preexercise conditions (unpublished personal data). In our study, the biopsies were taken within the 10 min following the completion of exercise. In this condition, the phosphorylation state of Akt was drastically decreased and was associated with a major dephosphorylation of its downstream target FOXO3, a transcription factor known to control protein degradation via transcriptional activation of both UPP and ALP components. Transcripts of FOXO3 have been reported to increase in response to either an acute bout of endurance running (29) or excessive endurance training (16). However, regulation of transcripts does not allow reporting for posttranslational modifications that can alter protein activity. Here, we confirmed that FOXO3 was clearly dephosphorylated, which allows for subsequent nucleus translocation and activation of target genes.

The mTOR kinase is another downstream target of the insulin-PI3K-Akt signaling pathway and is considered to be a major sensor of nutritional status, cellular stress, and growth factor signals by both controlling protein synthesis and autophagy. mTOR is the central component of two multiprotein complexes known as mTORC1 and mTORC2, both of which control autophagy by distinct processes. mTORC2 has been shown to be a negative regulator of autophagy via Akt phosphorylation and FOXO3 inhibition, whereas reduced mTORC1 activity is responsible for autophagy induction (23). Our data showed that mTOR phosphorylation state was significantly decreased on Ser2448, which is known to be phosphorylated by Akt (42), and tended to diminish on Ser2481, an autophosphorylation site. Both of these sites have been shown to be stimulated in the presence of insulin (48, 52). The dephosphorylation of mTOR at Ser2448 and Ser2481 is thus in line with the decreased plasmatic insulin measured at the end of the 24-h run. The fact that 4E-BP1, a downstream substrate of mTOR, was dephosphorylated is another element that supports a decrease in mTOR activity.

AMPK plays a major role in homeostasis maintenance in response to energetic stresses, including exercise (59). It was therefore not surprising to observe an increase in the phosphorylation state of AMPKα on Thr172, which witnesses the full activation of the kinase (22) in response to the 24-h run. Moreover, AMPK could play a role in the increase in protein degradation markers that we observed since it has been reported that AMPK was implicated in the increase in ALP markers through inhibition of its substrate mTOR (35) and also in the increase of ligase expression through FOXO3 activation (17, 39) via an inhibition of Akt (4).

Although muscle glycogen concentration was not measured in our study, it is likely that the increased phosphorylation state of AMPKα was regulated by its depletion (44). Another study reported that muscle glycogen concentration was decreased by 60% after a 24-h exercise, which consisted in four blocks of kayaking, running, and cycling (14).

Limitations

The main limitation of this study is its descriptive character. Intensity and duration of exercise were monitored, as well as food and fluid intake, but were impossible to standardize without compromising the completion of the protocol. Also, the studied population and the duration of exercise were quite exclusive. It remains to be determined whether the reported changes are generic responses to any endurance exercise or whether they are specific to ultra-endurance exercise. Experiments on more conventional forms of endurance exercise with controlled intensity, duration, and food intake should be conducted for identifying the physiological mechanisms implicated in the modulation of autophagy markers that we observed. Another limitation is that this protocol does not allow determination of the respective contribution of the regulation pathways analyzed in the modulation of the protein degradation markers. Nevertheless, Mammucari et al. showed that the role of mTOR signaling in the control of autophagy in skeletal muscle seems to be less relevant than in other cell types and

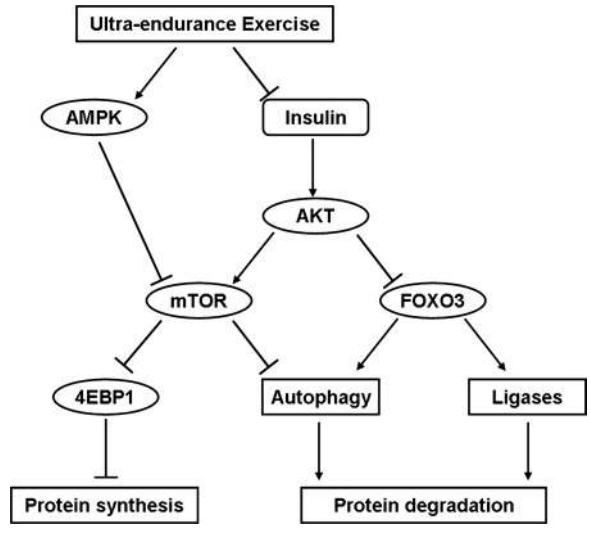

Fig. 7. Proposed model for signaling pathways regulating protein balance in skeletal muscle in response to ultra-endurance exercise.

that FOXO3 seems to be a major regulator of ALP component transcription (30). Moreover, Zhao et al. evidenced that FOXO3 is responsible for controlling transcription of both ALP and UPP components, which is not the fate of mTOR (63, 64).

In conclusion, to the best of our knowledge, this study is the first to give a picture of the regulation of pathways implicated in protein degradation in response to long-lasting endurance exercise. They are summarized in Fig. 7. The present results evidence that ultra-endurance running leads to a reduced plasmatic insulin concentration that is accompanied by a clear depression of the Akt pathway, including a decrease in FOXO3a phosphorylation state and a subsequent increase in both ALP and UPP markers. The mTOR kinase, another target of Akt, was also repressed with a subsequent decrease in 4E-BP1 phosphorylation state, a marker for protein synthesis. Even if the Akt-FOXO3 pathway is pointed out as the main regulator of ALP in skeletal muscle, mTOR has been implicated in autophagy regulation through the repression of its inhibitor AMPK, which can potentially activate FOXO3 and which is known to be triggered by exercise. We were not able to show an upregulation of mitophagy markers, a cellular process also regulated by AMPK. Only the phosphorylation state of DRP1, a regulator of mitochondrial fission, was increased. The upstream kinase of DRP1 potentially activated by exercise is presently unknown. Further studies should be conducted to determine the respective contribution of ALP and UPP in the regulation of the protein degradation markers in skeletal muscle in response to exercise as well as the molecular mechanisms regulating mitochondrial fission.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: C.J., M.F., G.Y.M., L.D., and L.F. conception and design of research; C.J., G.Y.M., L.D., D.F., and L.F. performed experiments; C.J., M.F., G.Y.M., L.D., D.F., and L.F. analyzed data; C.J., M.F., G.Y.M., L.D., and L.F. interpreted results of experiments; C.J. and M.F. prepared figures; C.J., M.F., L.D., and L.F. drafted the manuscript; C.J., M.F., G.Y.M., L.D., and L.F. edited and revised the manuscript; C.J., M.F., and L.D. approved the final version of the manuscript.

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