Lack of Activation of Mitophagy during Endurance Exercise in Human

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ABSTRACT

SCHWALM, C., L. DELDICQUE, and M. FRANCAUX. Lack of Activation of Mitophagy during Endurance Exercise in Human. *Med. Sci. Sports Exerc.*, Vol. 49, No. 8, pp. 1552–1561, 2017. **Purpose**: This study aimed to determine whether fission and mitophagy are activated by acute endurance exercise in human skeletal muscle and to investigate if this activation is dependent upon the nutritional state. **Methods**: Trained athletes (n = 7) cycled for 2 h at 70% $\dot{V}O_{2peak}$ in a fed or fasted state. Vastus lateralis muscle biopsies were obtained at baseline, before, immediately after, and 1 h after exercise. Protein and mRNA markers for mitophagy, mitochondrial biogenesis, fission, and fusion were analyzed using quantitative real-time polymerase chain reaction and Western blot. **Results**: Fission, assessed by phospho-DRP1^{Ser616} in the mitochondrial fraction, increased postexercise and 1 h postexercise only in the fed state. LC3bII and p62/SQSTM1 in the mitochondrial fraction were unchanged, whereas the LC3bII/LC3bI ratio was decreased only postexercise in the fasted state (P = 0.019), indicating a reduced mitophagy. Genes implicated in fission and mitophagy, such as *Drp1*, *Bnip3*, and *Bnip3L*, and proteins involved in fission (Fis1) or mitophagy (BNIP3) were all more expressed after exercise in the fed state ($P < 0.05$). As expected, the mRNA levels of *PGC1α*, *Tfam*, and *Hsp60*, all markers of mitogenesis, were increased after endurance exercise, but to a larger extent in the fed than that in the fasted state. **Conclusion**: The present study provides the very first evidence that mitophagy is not activated during and early after high-intensity endurance exercise in human, whatever the nutritional state, despite a selective activation of fission in the fed state. However, when nutrient availability is optimal, muscle cells seem capable of preparing mitochondria for lysosomal degradation. Thus, we may not exclude an activation of mitophagy at a later stage after exercise. **Key Words:** BNIP3, CYCLING, DRP1, FASTING, MITOCHONDRIA, PGC1A

BASIC SCIENCES

Mitochondria are organelles providing a large part of energy to cells via oxidative phosphorylation. They form a dynamic network, the abundance, size, distribution, and morphology of which are continuously rearranged through biogenesis, motility, fusion, and fission events. Fusion and fission are complementary quality control strategies, which help the cell to cope with metabolic and environmental disturbances, making possible the maintenance of optimal energetic capacity of the cell, together with the maintenance of a healthy mitochondria population (39,42). Fusion gathers individual mitochondria within an elongated interconnected mitochondrial network (42), whereas fission selectively fragments mitochondrial network into isolated mitochondria (42). The outer mitochondrial membrane protein mitofusin 2 (Mfn2) and the inner membrane protein optic atrophy 1 (OPA1) are both GTPases that play a key role in the fusion process (6). Fission is usually consecutive to fusion, giving birth to mitochondria harboring uneven membrane potential for a transitory period. Upon mitochondria depolarization, the cytosolic GTPase dynamin-related protein 1 (DRP1) is phosphorylated at serine 616 and translocates to the outer mitochondria membrane to initiate fission (25). Mitochondrial fission 1 protein (Fis1) is another fission protein embedded in the outer membrane of mitochondria, which is believed to interact with DRP1 (41).

Severely depolarized mitochondria are removed through mitophagy, namely, a selective form of autophagy (36). Autophagy traps targeted cell constituents in a double membrane vesicle called autophagosome, which fuses with lysosomes containing hydrolases, allowing the entire degradation of the autophagosome. At a molecular level, activation of autophagy is featured by increased conversion of microtubule-associated protein-1 light chain 3 (LC3b) I protein to its active form LC3b II, parallel to a lower p62/SQSTM1 protein level (1). Both proteins are cleaved on autophagosome membrane. Although LC3b II intervenes from autophagosome formation to their degradation (29), p62/SQSTM1 is a receptor for proteins aggregates, which is eliminated with autophagosome content after it fuses with lysosome (26). Both LC3b II and p62/SQSTM1 facilitate recruitment of damaged mitochondria to autophagosome, either by LC3b II interaction with Bcl-2/adenovirus E1B 19 kDa interacting protein 3 (BNIP3) or by p62/SQSTM1-dependent clustering of ubiquitinated mitochondria (5,22,24,30). Depolarized

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mitochondria might be specifically tagged by the E3-ubiquitin ligase Parkin, which ubiquitinates mitochondrial outer membrane proteins after recruitment by PTEN-induced kinase-1 to mitochondria (23). In addition, the complex BNIP3/BNIP3-Like (Nix) can interact with and bind to LC3 (24,30). Both Parkin and BNIP3 facilitate the engulfment of mitochondria into autophagic vesicles before fusion with lysosomes and subsequent proteolytic degradation.

Mitochondrial remodeling (i.e., fusion and fission) seems to be closely related to mitochondrial turnover (i.e., the balance between mitochondria biogenesis and removal). The fine tuning of these two processes has been proposed to participate at the basal integrity of the mitochondrial network and in the maintenance of muscle mass (31). Yet, little is known about the role of mitochondrial machinery in the regulation of mitochondrial homeostasis under stressful conditions, such as exercise. Most of the studies exploring the potential benefits of exercise on murine or human muscle metabolism mainly focused on mitochondria biogenesis by measuring, among others, the expression of the peroxisome proliferator-activated receptor gamma coactivator 1α (PGC1α) and mitochondrial transcription factor A (Tfam), two key regulators of this process (18,20). Nonetheless, mitochondria plasticity and clearance (mitophagy) are emerging as essential mechanisms in exercise-induced metabolic adaptations in skeletal muscle (19,40).

Nutrient deprivation may disturb cell homeostasis, leading to catabolism of cellular constituents, including mitochondria, to provide alternative sources of energy and sustain cell function. Although starvation has been reported to induce mitochondria fusion elongation *in vitro,* sparing these organelles from autophagic removal (9,28), increased mitophagy markers were found in skeletal muscle of fasted mice (13). In human, however, the regulation of mitophagy under these conditions has not been documented yet. Therefore, it remains to determine to what extent nutrient deficit can affect mitochondrial remodeling to face energetic challenges.

On the basis of the aforementioned background, we hypothesized that, in human skeletal muscle, mitophagy is transitorily activated during endurance exercise in a fission-dependent manner and that this transient mitophagy is preferentially activated when exercise is performed in a fasted state.

MATERIALS AND METHODS

Subject characteristics and preliminary testing. The samples analyzed in the present work were collected during a previous study (33). Here, we used the remaining muscle samples of the seven healthy young volunteers cycling at high-intensity (HI group: age = 24 ± 1 yr, body mass index = 21.3 ± 2.3 kg·m^{-2}, $\dot{V}O_{2peak}$ = 64.5 ± 1.7 mL·min^{-1}·kg^{-1}, W_{max} = 4.7 ± 0.1 W·kg^{-1}). The protocol was approved by the local ethical committee (Université catholique de Louvain) and conducted in accordance with the Declaration of Helsinki. The subjects were fully informed about the experimental procedure before their written consent was obtained. They were selected on a voluntary basis and were all experienced cyclists or triathletes. The minimal level of maximal oxygen consumption ($\dot{V}O_{2peak}$) for inclusion in the protocol was 50 mL·min^{-1}·kg^{-1}. Before the beginning of the study, subjects reported to the laboratory for a medical examination aiming to exclude any underlying pathology. Afterward, height, weight, and percentage of body fat were measured according to the Jackson and Pollock method (11). A maximal incremental exercise test was performed on a cycle ergometer (Cyclus II; RBM Electronics, Leipzig, Germany) for determining $\dot{V}O_{2peak}$ and maximal power output (W_{max}). The starting load was 70 W, incremented by 40 W every 3 min, until exhaustion. Heart rate (Polar Team System 2; Polar Electro, Kempele, Finland) and respiratory exchanges (Metalyzer 2; Cortex, Leipzig, Germany) were continuously monitored.

One week after the maximal test, subjects took part in a familiarization session to ensure they could handle the workload asked during the protocol in the fasted state. A 2-wk period separated the familiarization session and the first experimental session. Subjects were randomly chosen to perform exercise sessions either in a fed or in a fasted state. Subjects had to refrain from strenuous exercise 3 d before each experimental session.

Dietary control. The day before each experiment, a standardized carbohydrate-rich diet was given to the subjects (63% carbohydrate, 13% protein, and 24% fat). During the fed session, a standardized breakfast was given immediately after the first biopsy. During the exercise period in the fasted state, 150 mL of water was ingested every 15 min, whereas in the fed state, 150 mL of a 6% carbohydrate energetic drink was consumed every 15 min (Sports Drink Orange; 3 Action, Tessenderlo, Belgium). Participants were asked not to consume medication or dietary complement during the 3 d preceding each experiment.

Exercise protocol. Each subject undertook two experimental sessions, a fasted and a fed one, randomly assigned and interspersed with a 2-wk period (Fig. 1). For each session, they arrived at 6:00 AM to the laboratory after an 8-h overnight fast. A first biopsy sample, with the needle pointing proximally, was taken from the mid portion of the vastus lateralis muscle under local anesthesia (1 mL of Xylocaine 2%; AstraZeneca, Belgium) (baseline). The leg of the first biopsy was chosen at random. Samples were immediately frozen in liquid nitrogen then stored at −80°C before further analysis. Thereafter, the fed group received a standardized breakfast, whereas the fasted group only received water (see previous section). Ninety minutes after the first biopsy, a second muscle biopsy, with the needle pointing distally, was taken through the same incision as the first one (preexercise). Still under local anesthesia, a second incision was made 3 cm distally from the first incision site, to be able to take the third biopsy within 30 s at the end of the exercise. Fifteen minutes after, subjects started to cycle for 2 h at 70% of $\dot{V}O_{2peak}$. With the needle pointing proximally, a third muscle biopsy was taken from the second incision site made before the beginning of the exercise (postexercise). One hour after the end of the exercise bout, a last biopsy—with the needle pointing distally

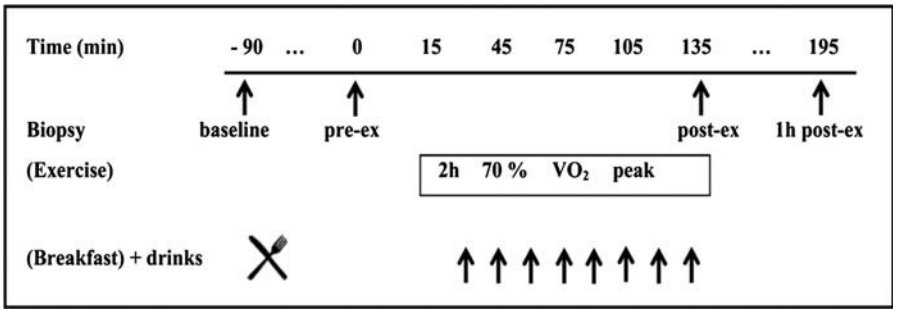

FIGURE 1—Schematic view of the experimental protocol. A first muscle biopsy was taken at baseline (−90 min) and a second biopsy 90 min after (preexercise, 0 min). Fifteen minutes after the second biopsy, subjects cycled for 2 h at 70% of $\dot{V}O_{2peak}$ (15 min). A third biopsy was taken at the end of the exercise period (postexercise, 135 min) and a last biopsy 1 h postexercise (195 min). Fed subjects received a breakfast immediately after baseline measurements as well as 150 mL of a 6% carbohydrate drink every 30 min during the exercise bout while fasted subjects received the same amount of water.

in the incision made before exercise—was taken (1 h postexercise). Pointing the needle in the opposite direction was intended to reduce potential activation of inflammatory signaling pathways as recommended by van Thienen et al. (37). All subjects underwent the same protocol 2 wk after the first session, receiving the crossover nutritional treatment (fasted vs fed).

Cytosolic and mitochondrial protein fractionation. Muscular biopsy sample (~30 mg) was dedicated to cytosolic and mitochondrial protein fractionation following an adapted protocol of Quadrilatero and Rush (27). Muscle samples were homogenized with a TissueLyser II (Quiagen, Hilden, Germany) for 2 × 3 min at 15 Hz in ice-cold buffer containing 250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 $MgCl_2$, 1 mM ethyleneglycotetraacetic acid, 1 mM ethylenediaminetetraacetic acid, pH adjusted to 7.4, supplemented with 200 mM sodium orthovanadate, 1 mM 1,4 dithiothreitol, and a protease inhibitor cocktail containing 1 mM ethylenediaminetetraacetic acid (Roche Applied Science, Vilvoorde, Belgium). Homogenates were centrifuged for 10 min at 10,000*g* at 4°C. Supernatants (S1) were collected and centrifuged for 20 min at 16,000*g* at 4°C. Resulting supernatants (S2) were removed from pellets (P1) to be centrifuged for 20 min at 16,000*g* at 4°C and stored at −80°C (cytosolic protein fraction). P1 were washed in ice-cold buffer and centrifuged for 2 × 20 min at 16,000*g* at 4°C, each time carefully removing supernatants. Resulting pellets (P2) were resuspended in ice-cold buffer supplemented with 0.1% Triton X-100 (mitochondrial protein fraction), sonicated with Ultrasonic Cleaner (VWR, Radnor, PA) and immediately stored at −80°C before further analysis. Cytosolic and mitochondrial protein contents were determined using the DC kit for protein dosage (Bio-Rad Laboratories, Hercules, CA). The sole presence of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and lysosome-associated membrane protein 2b (LAMP2b) protein in the cytosolic fraction and cytochrome *c* oxidase (COx IV) and mitochondrial 20 kDa outer membrane protein (TOM20) protein in the mitochondrial fraction were assessed to verify the purity of our extracts. GAPDH and LAMP2b were detected in the cytosolic fraction only, whereas COx IV and TOM20 were measured exclusively in the mitochondrial fraction, excluding any possible contamination between the two different fractions (Fig. 2).

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting. Muscle protein (~20 μg) was mixed with Laemmli sample buffer and warmed for 5 min at 95°C. Proteins were separated by *s*odium dodecyl sulfate–polyacrylamide gel electrophoresis during 2 h at 40 mA then transferred on polyvinylidene fluoride membranes for 2.5 h at 80 V. After blocking for 1 h in a Tris-buffered

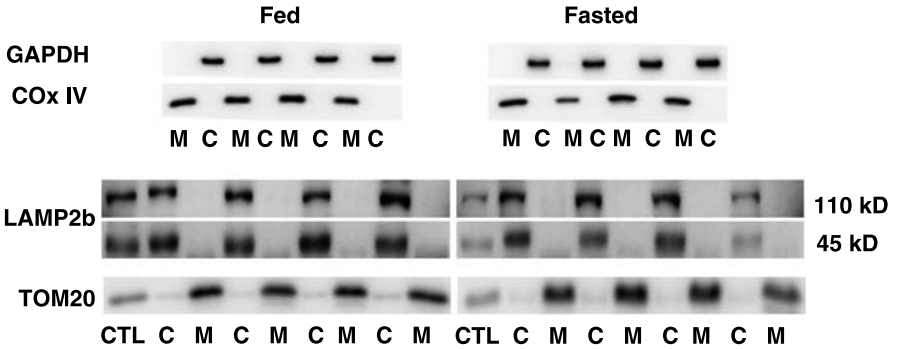

FIGURE 2—Assessment of the purity of cytosolic and mitochondrial fractions. GAPDH, COx IV, LAMP2b, and TOM20 were all measured in the cytosolic and mitochondrial extracts by western blotting to ascertain the absence of contamination between the different cellular fractions. GAPDH/LAMP2b and COx IV/TOM20 proteins were chosen as representative markers of the purity of cytosolic and mitochondrial fractions, respectively. For LAMP2b, two bands were detected at approximately 110 (glycosylated form) and 45 kDa (unglycosylated form) and are therefore displayed here (7). C, cytosolic fraction; M, mitochondrial fraction; CTL, whole cell lysate.

saline plus 0.1% Tween 20 containing 5% nonfat dry milk, membranes were incubated at 4°C overnight with one of the following antibodies: BNIP3 (ab10433), Parkin (ab77924), COx IV (ab14744), GAPDH (ab9492), LAMP2b (ab118959) (Abcam, Cambridge, UK), phospho-DRP1^{Ser616} (#3455), DRP1 total (#5391), heat shock protein 60 (*Hsp60*; #4870), Mitofusin-2 (#9482), TOM20 (#42406), OPA1 (#67589) (Cell Signaling Technology, Leiden, The Netherlands), Fis1 (SAB2702049), LC3b (L7543), or p62/SQSTM1 (P0067) (Sigma-Aldrich, St. Louis, MO). Membranes were washed thrice for 10 min in Tris-buffered saline plus 0.1% Tween 20 then incubated for 1 h at room temperature with a secondary antibody conjugated to horseradish peroxidase. Three additional washes were performed before chemiluminescence detection with the ECL-Plus Western blotting kit (Amersham Biosciences, GE Healthcare, UK). The bands were captured with the GeneSnap and quantified with the GeneTools software (G-Box; Syngene, Cambridge, UK). DRP1 was quantified in the cytosolic and in the mitochondrial fraction, whereas all other protein expressions were quantified in the mitochondrial fraction. Phosphorylated DRP1 was reported to total DRP1 as the latter was found to be unmodified in any condition. LC3b I, LC3b II, LC3b II/I, *Hsp60*, p62/SQSTM1, BNIP3, Parkin, OPA1, and Mfn2 expression were reported to COx IV (Abcam), which remained unchanged in all experimental conditions. An internal standard was used to limit variations in protein expression when protein samples were separated on different gels.

RNA extraction and quantitative real-time polymerase chain reaction. Muscle biopsy (~30 mg) was homogenized in 1 mL Trizol® reagent (Invitrogen, Merelbeke, Belgium), using the TissueLyser II from Qiagen (Venlo, Netherlands). RNA isolation was achieved according to the manufacturer's instructions. RNA quality and quantity were assessed by Nanodrop® spectrophotometry. Reverse transcription was performed from 1 μg RNA using the iScript™cDNA Synthesis Kit from Bio-Rad, following manufacturer's instructions. The following primers were used: *Bnip3* forward sequence 5′-CTGAAACAG-ATACCCATAGCATT-3′ and reverse sequence 5′-CCGACTTGACCAATCCCA-3′; *Bnip3L* forward sequence 5′-CCAAGGAGTTCCACTTCAGAC-3′ and reverse sequence 5′-AGTA-GGTGCTGGCAGAGGGTGT-3′; *Drp1* forward sequence 5′-CGCAGAACCCTAGCTGT-AATC-3′and reverse sequence 5′-CTGGAATAACCCTTCCCATCAA-3′; *Fis1* forward sequence 5′-GAACTACCGGCTCAAGGAATAC-3′ and reverse sequence 5′-CCCACGA-GTCCATCTTTCTTC-3′; *Gapdh* forward sequence 5′-CATGGTCGTCATGGGTGTGA AC-CA-3′ and reverse sequence 5′-AGTGATGGCATGGACT GTGGTCAT-3′; *Hsp60* forward sequence 5′-TCCAGGGTTT GGTGACAATAG-3′ and reverse sequence 5′-GATTCAGGG-TCAATCCCTCTTC-3′; *Mfn2* forward sequence 5′-CCTTCC TTGAAGACACGTACAG-3′ and reverse sequence 5′-GATG CCTCTCACTTTGGATAGG-3′; *Park2* forward sequence 5′-CCTTCTGCCGGGAATGTAAA-3′ and reverse sequence 5′-GGCTCTTTCATCGACT-CTGTAG-3′; *PGC1α* forward sequence 5′-TGAACTGAGGGACAGTGATTTC-3′ and reverse sequence 5′-CCCAAGGGTAGCTCAGTTTATC-3′ and *Tfam* forward sequence 5′-CTCAGAACCCAGATGCAAA-3′ and reverse sequence 5′-GCCACTCCGCCCTATAA-3′. Experiments were conducted 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. All samples were run in triplicate with an internal standard on each plate to correct for interplate variability. Each reaction was processed in a 10-μL volume containing 4.8 μL IQ SybrGreen SuperMix (Bio-Rad), 0.1 μL of each primer (100 nM final), and 5 μL cDNA at the appropriate dilution. Melting curves were systematically performed for quality control. Relative mRNA levels were normalized to *Gapdh*, the expression of which was unaffected by the nutritional state or exercise.

Statistical analysis. All values are expressed as the mean ± SEM. Statistical analyses were performed using GraphPad Prism 7.0. Data were analyzed by two-way repeated-measures ANOVA to test the main effect of exercise and nutrition as well as the interaction of these two factors. When a significant main effect was found, Fisher *post hoc* tests were performed to test potential differences between fed and fasted subjects at each time point (asterisks) as well as differences between preexercise and each other time point in the same nutritional state (dollar signs). Statistical significance was set at $P < 0.05$.

RESULTS

Acute endurance exercise and the nutritional state regulate markers for fission. Compared with preexercise, mitochondrial DRP1^{Ser616} increased postexercise ($P = 0.013$) and 1 h postexercise in the fed state ($P = 0.052$, Fig. 3A), whereas cytosolic DRP1^{Ser616} increased 1 h postexercise in the fasted state ($P = 0.019$, Fig. 3B). *Drp1* mRNA increased immediately postexercise ($P < 0.001$) and 1 h postexercise ($P = 0.006$) in the fed conditions only (Fig. 3C). Immediately postexercise, *Drp1* mRNA levels were twofold higher in fed compared with fasted state ($P = 0.018$). Similarly to *Drp1* mRNA, mitochondrial Fis1 protein expression increased postexercise in the fed conditions only ($P = 0.038$, Fig. 3D), with a ~50% higher level in fed compared with fasted state immediately postexercise ($P = 0.018$) and 1 h postexercise ($P = 0.009$). *Fis1* mRNA was neither impinged by exercise nor the nutritional state (Fig. 3E).

Canonical autophagic markers are not activated in the mitochondrial fraction after endurance exercise. Independently of the nutritional state, the mitochondrial protein content of LC3b I was approximately 1.7-fold more elevated immediately postexercise than preexercise ($P < 0.01$, Fig. 4A). In accordance with increased LC3b I and stable LC3b II levels postexercise (Fig. 4B), the LC3b II/I ratio was decreased immediately after exercise ($P < 0.001$, ANOVA; Fig. 4C). More specifically, the ratio decreased by a half postexercise compared with preexercise in the fasted conditions ($P = 0.019$). In addition, at preexercise, the LC3b II/I ratio tended to be different between the fed conditions, after having received a breakfast, and the fasted conditions

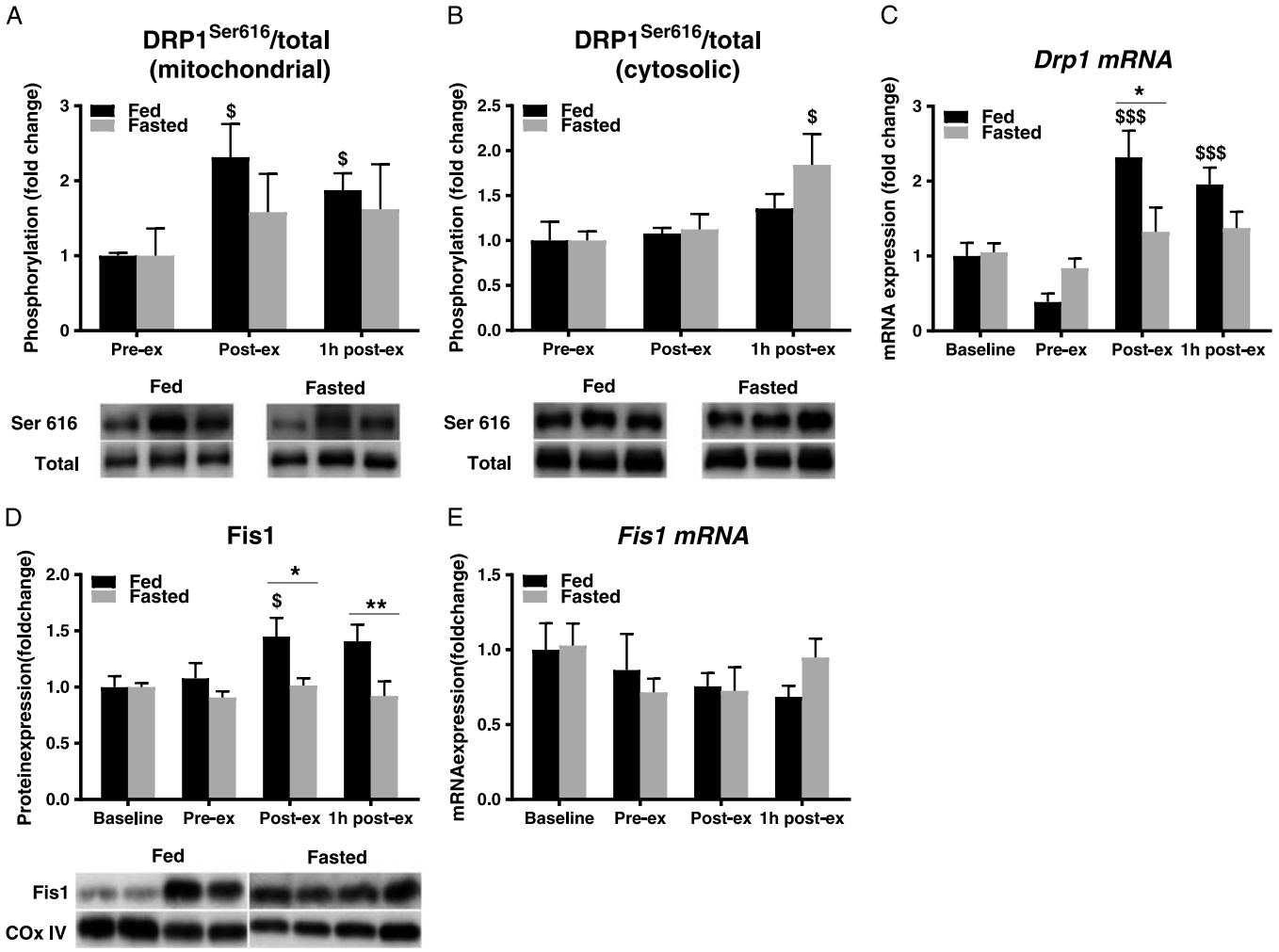

FIGURE 3—Regulation of fission markers by exercise and the nutritional state in human skeletal muscle. Phosphorylation of mitochondrial (A) and cytosolic DRP1^{Ser616} (B), mRNA levels of *Drp1* (C), protein expression of Fis1 (D), and mRNA levels of *Fis1* (E) in response to high-intensity concentric endurance exercise at baseline, preexercise, immediately postexercise, and 1 h after exercise in the fasted and fed states. Values are expressed as mean ± SEM. A and B, $n = 4$; C–E, $n = 7$. \$$P < 0.05$, \$\$\$$P < 0.001$ vs preexercise (time effect); *$P < 0.05$, **$P < 0.01$ vs fasted (nutrition effect).

(P = 0.095). The mitochondrial level of p62/SQSTM1, however, was unvarying throughout the trial (Fig. 4D).

BNIP3 expression is increased whereas Parkin is not affected in response to acute endurance exercise in the fed state. No changes in mitochondrial Parkin amount were detected as a result of exercise or the nutritional state (Fig. 4E). Nonetheless, in the fed conditions, *Park2* mRNA levels were approximately 50% higher at baseline and postexercise compared with preexercise ($P < 0.05$, Fig. 4F). The presence of BNIP3 in the mitochondrial fraction tended to increase postexercise in the fed conditions only (P = 0.080), resulting in a twofold higher expression in fed compared with fasted state (P = 0.003, Fig. 4G). Accrued mitochondrial content of BNIP3 was associated with elevated *Bnip3* and *Bnip3L* mRNA levels in the fed state postexercise ($P < 0.01$) and 1 h postexercise ($P < 0.05$), reaching a twofold difference with the fasted conditions postexercise ($P < 0.05$, Fig. 4H and I).

Markers for mitochondrial fusion are not modified by endurance exercise or the nutritional state. Preexercise *Mfn2* mRNA levels were lower compared with baseline in the fed state only (P = 0.019, Fig. 5B), whereas no variations in Mfn2 (Fig. 5A) and OPA1 (Fig. 5C) protein expression were observed in any condition, suggesting that fusion was not altered either by exercise or by the nutritional state.

The nutritional state influences endurance-induced mitochondrial biogenesis. An indirect marker of mitochondrial biogenesis is the *Hsp60*, which belongs to the matrix chaperones of the mitochondrial protein folding system (38). *Hsp60* protein expression was higher in the fed state than that in the fasted state immediately (P = 0.041) and 1 h (P = 0.020) after exercise (Fig. 5D). Similarly, *Hsp60* mRNA levels was higher in the fed state than that in the fasted state immediately after exercise (P = 0.003, Fig 5E). In addition, compared with preexercise levels, *Hsp60* mRNA was higher

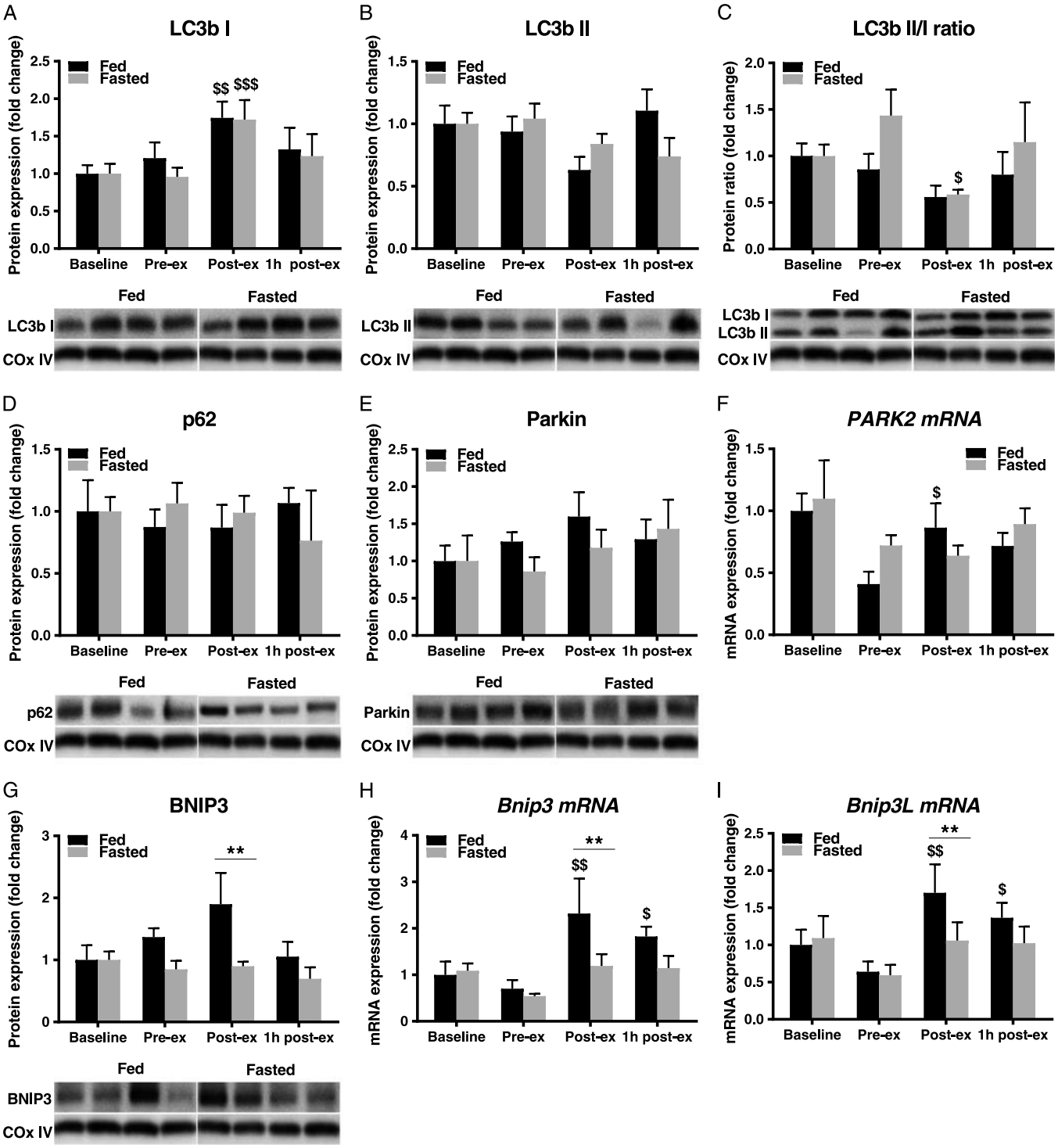

FIGURE 4—Regulation of mitophagy markers by exercise and the nutritional state in human skeletal muscle. Protein expression of LC3b I (A), LC3b II (B), LC3b II/I ratio (C), p62/SQSTM1 (D), Parkin (E), mRNA levels of *Park2* (F), protein expression of BNIP3 (G), and mRNA levels of *Bnip3* (H) and *Bnip3L* (I) in response to high-intensity concentric endurance exercise at baseline, preexercise, immediately postexercise, and 1 h after exercise in the fasted and fed states. Values are expressed as mean ± SEM. A–I, $n = 7$. $\$P < 0.05$, $\$\$P < 0.01$, $\$\$\$P < 0.001$ vs preexercise (time effect); $*P < 0.05$, $**P < 0.01$ vs fasted (nutrition effect).

immediately postexercise ($P < 0.001$) and 1 h postexercise ($P = 0.001$) in the fed state and 1 h postexercise in the fasted state ($P = 0.044$). *PGC1α* mRNA levels increased after exercise ($P < 0.05$), independently of the nutritional state (Fig 5F). Following a similar pattern of regulation as *Hsp60* mRNA, the mRNA levels of *Tfam* increased after exercise only in the fed group ($P < 0.05$, Fig. 5G). Together our results show that markers for mitogenesis were upregulated

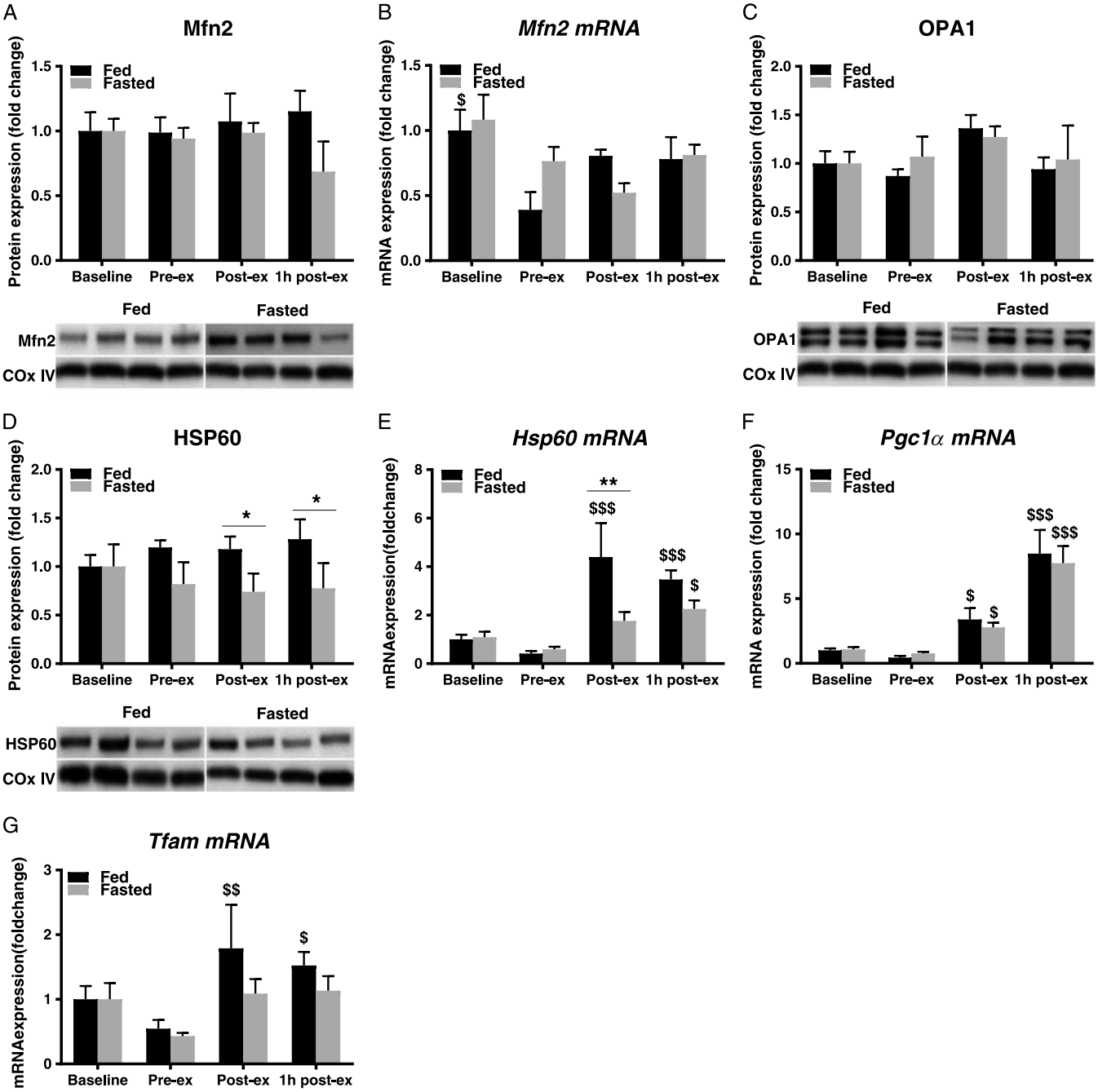

FIGURE 5—Regulation of fusion and mitogenesis markers by exercise and the nutritional state in human skeletal muscle. Protein expression of Mfn2 (A), mRNA levels of *Mfn2* (B), protein expression of OPA1 (C), *Hsp60* (D), mRNA levels of *Hsp60* (E), *PGC1α* (F), and *Tfam* (G) in response to high-intensity concentric endurance exercise at baseline, preexercise, immediately postexercise, and 1 h after exercise in the fasted and fed states. Values are expressed as mean ± SEM. A, B, D–G, $n = 7$; C, $n = 4$. $\$P < 0.05$, $\$\$P < 0.01$, $\$\$\$P < 0.001$ vs preexercise (time effect); $*P < 0.05$, $**P < 0.01$ vs fasted (nutrition effect).

after exercise, more in the fed state compared with the fasted state.

DISCUSSION

This study is the first to analyze the regulation of the fission, mitophagy, and fusion processes by endurance exercise in the mitochondrial fraction of human muscle biopsies. Contrary to our hypothesis, which was based on the activation of autophagy in human skeletal muscle during high-intensity endurance exercise (33), the results indicate that mitophagy is not activated during the same exercise protocol, and even inhibited while exercise is performed in a fasted state. This provides arguments in favor of a differential regulation of autophagy and mitophagy in skeletal muscle during exercise.

Regulation of fission. During an acute bout of high-intensity endurance exercise, increased fission markers were found in murine skeletal muscle, concurrently with decreased fusion markers (4). Forasmuch as these changes were all the

most evident when the trial was prolonged, a redistribution of the mitochondrial network was thought to occur within muscle cell in response to higher local energy needs. Here, we quantified DRP1^{Ser616} phosphorylation in the mitochondrial fraction to assess fission as activation of DRP1 is a mandatory process for mitochondrial fission to take place. Indeed, DRP1 controls the final step by splitting the membrane and leading to the birth of daughter mitochondria (36). In our experiment, mitochondrial DRP1^{Ser616} was increased postexercise and 1 h postexercise in the fed state only, which suggests that fission was activated during exercise itself in a nutrition-dependent way. Interestingly, 1 h postexercise, cytosolic DRP1^{Ser616} was upregulated only in the fasted state, which might underline a delayed effect of exercise on fission protein kinetics in that nutritional state. DRP1^{Ser616} is initially phosphorylated in the cytoplasm before migrating to the mitochondria (35). It can therefore be hypothesized that the regulation of fission in the fasted state is somewhat delayed compared with the fed state. Unfortunately, no biopsy was taken beyond 1 h postexercise, and this hypothesis cannot be tested. Supporting the increase in mitochondrial DRP1^{Ser616} during exercise in the fed state, we also observed an elevated mitochondrial content of Fis1 protein during the recovery period only in the fed state. Fis1 is known to instigate a molecular network favoring a fission process directed preferentially toward dysfunctional mitochondria rather than fragmented ones (10). Our results suggest therefore a role of endurance exercise in the mitochondrial quality control. Nevertheless, it is surprising to observe that Fis1 increases only when exercise is performed in a fed state. This could reflect a protective mechanism of the cell to avoid disposal of damaged mitochondria when the nutrient availability is reduced (fasting) and the energy expenditure increased (exercise). In summary, as phosphorylation of mitochondrial DRP1^{Ser616} and appearance of Fis1 in the mitochondrial compartment were concomitant in the fed state, our results support an increased mitochondrial fission during high-intensity exercise selectively in that nutritional state.

Regulation of mitophagy. Because we previously reported an activation of autophagy in the same condition (33), we hypothesized that mitophagy would be activated during endurance exercise and more in the fasted state compared with the fed state. The presence of autophagosomes in the mitochondrial compartment is depicted by LC3b II, whereas the LC3b II/I ratio is an indicator of autophagosome turnover, which must be interpreted together with p62/SQSTM1 changes. LC3b protein is involved in the turnover of autophagosomes from their formation to their lysosomal degradation, whereas p62/SQSTM1 is only associated with later stages of autophagy, i.e., with autophagosomes removal. Thus, the analysis of upstream and downstream markers of autophagy process helps to better understand whether autophagy is directed specifically to mitochondria. In our experiment, LC3b II did not change, whereas the LC3b II/I ratio was decreased immediately postexercise in the fasted state, p62/SQSTM1 remaining unchanged. This means that mitophagy was globally unchanged during our experiment except during exercise in the fasted state where it was decreased (decreased LC3b II/I and unchanged p62/SQSTM1). These conclusions do not corroborate our initial hypothesis but are compatible with the lack of fission activation as the latter is known to be permissive for mitophagy (36). Nevertheless, similarly to fission, we may not rule out any activation of mitophagy in the hours after the exercise.

The selectivity of mitophagy relies in particular on Parkin and BNIP3 effectors (32). Like Fis1, a higher content of BNIP3 in the mitochondrial compartment after exercise in the fed state constitutes an argument in favor of the formation of a selective molecular network dictated by the nutritional state.

In other words, even if mitophagy does not seem to be activated during and 1 h after a high-intensity endurance exercise in human, muscle cells seemed to prepare mitochondria for lysosomal degradation in conditions where nutrient availability was not impaired. This regulation in the fed state is reinforced by augmented *Drp1*, *Bnip3*, and *Bnip3L* mRNA levels after exercise, showing that the transcriptional mitophagic program was activated as well. Parkin is another key mediator of mitophagy. It has been advanced to be phosphorylated by PTEN-induced kinase 1 at serine 65 to onset mitophagy upon mitochondria membrane depolarization (16,34). Hence, we cannot exclude that the phosphorylation state of Parkin might have been modified without displaying changes in total protein content in the outer membrane of the mitochondria. Unfortunately, currently, no commercially available phosphoantibody is validated in human. Whether DRP1 and Parkin can regulate mitophagy independently is still under debate. Parkin is supposed to be dispensable for mitophagy in the presence of DRP1 (15), whereas both DRP1-dependent fission and Parkin recruitment have been shown to be involved in BNIP3-induced mitophagy in cardiac myocytes (17).

Forkhead box O 3a (FoxO3a) regulates the transcription of *Bnip3* and *Bnip3L* genes in skeletal muscle (21). As we previously documented that our protocol activated FoxO3a in the fasted state (33), we expected to observe increased mRNA levels of *Bnip3* and *Bnip3L* in similar conditions. Contrarily, an increase in *Bnip3* and *Bnip3L* mRNA with exercise was found after food intake, but no variations were spotted with fasting. These results contrast with those of Jamart et al. (12) in human, as the transcription of those genes was augmented after ultraendurance exercise, either ensuing from nutrient stocks in deficit or owing to muscle damages after eccentric muscle contractions. Here, any implication of high-energetic stress or muscle damage in the regulation of *Bnip3* and *Bnip3L* after exercise may be ruled out as the upregulation was specific to the fed conditions and cycling implies mainly concentric muscle contractions. It remains to determine the mechanisms responsible for the selective increased expression of *Bnip3* and *Bnip3L* in the fed state.

BNIP3 is needed for autophagosomes generation, helping the processing of LC3b I to LC3b II (21). Regardless of enhanced mitochondrial BNIP3 content after exercise in the fed state, mitochondrial LC3b II was not increased, and mitochondrial p62/SQSTM1 was not attenuated, suggesting that the basal autophagic flux might have been likely sufficient to

remove any undesirable mitochondria, if there is any. Besides, increased LC3b I amount in the mitochondrial fraction is somewhat surprising because this precursor form of LC3b is supposed to be located in the cytosol (14). This cannot originate from any contamination of the mitochondrial fraction by the cytosolic fraction because we confirmed the high purity of both subcellular extracts. Interestingly, we previously found that autophagy was activated in whole cell extracts following the same protocol (33). It seems therefore that the mitochondrial fraction does not reflect what happens at the whole cell level. These observations are compatible with Frank et al. (8), who evidenced that specific degradation of mitochondria did not rely on increased autophagic flux, but was triggered by fission activation. In that study, mitophagy was triggered by a mild and transient oxidative stress, resembling the one encountered during endurance exercise. Moreover, exercise-induced production of reactive oxygen species may account for the augmented presence of Fis1 protein we found in the mitochondrial fraction, Fis1 targeting preferentially mitochondria that become dysfunctional (10).

The nutritional state influences the exercise-induced mitochondrial biogenesis. Either no change or a marked decrease of fusion at the mRNA level during endurance exercise has been reported in murine skeletal muscle (4,13). The various exercise durations and intensities chosen probably account for these controversial data. Conversely, transcription of fusion-related genes was importantly and durably stimulated during the hours after the trial (4). Here, in human, cycling endurance exercise did not enhance Mfn2 or OPA1 expression at short term, regardless of the nutritional state. Mfn2 expression was not modified 24 h after a similar modality of exercise either (3). The only variations previously detected in mitofusins were found at the transcriptional level 24 h after exercise (3). This delayed activation of the fusion program after exercise was assumed to be controlled by mitochondrial biogenesis, via upregulation of *PGC1α* (3). Although we confirmed an increase in *PGC1α*, *Tfam*, and *Hsp60* mRNA early during the recovery period after exercise, we were not in position to corroborate the reliance of fusion on those markers of mitochondria biogenesis as we did not take samples a few hours or the day after exercise.

AMPK has been described to regulate PGC1α via SIRT1-dependent deacetylation of PGC1α during fasting and exercise (2). The increase in *PGC1α* mRNA observed during exercise, independently of the nutritional state, fits with AMPK activation previously confirmed in our exercise protocol (33). Although AMPK activation was not influenced by the nutritional state, the markers for mitogenesis were globally more increased when the exercise was performed in the fed state. We have no definitive explanation for this observation, but here again the nutritional state enhanced mitochondrial adaptation induced by exercise, which, together with the regulation observed at the fission level, seems to indicate a higher remodeling rate in the fed state. Although no ultimate conclusion may be drawn, this observation challenges the relevance of training in the fasted state for increasing muscle mitochondrial content, which is commonly used by athletes. It also brings an argument in favor of a decoupling between the processes regulating mitophagy and mitochondrial biogenesis. Indeed, in the light of our results, mitophagy does not seem mandatory to increase key markers of mitochondrial biogenesis. Importantly, no changes in the mRNA levels of cytochrome *c* oxidase subunit I, cytochrome *b*, and ATPase subunit 6 were detected in any condition, suggesting that the transcriptional regulation of mitochondrial biogenesis was not modified during the early recovery in our conditions (data not shown). These results were expected, as the first hour after exercise seems a too short period to observe any change in mitochondrial content.

In conclusion, the present study provides the very first evidence that mitophagy is not activated during and early after high-intensity endurance exercise in human, whatever the nutritional state and despite a selective activation of fission in the fed state. However, when nutrient availability is optimal, muscle cells seem capable of preparing mitochondria for lysosomal degradation. Thus, we may not exclude an activation of mitophagy at a later stage after exercise.

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