Higher activation of autophagy in skeletal muscle of mice during endurance exercise in the fasted state

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Activation of autophagy in skeletal muscle has been reported in response to endurance exercise and food deprivation independently. The purpose of this study was to evaluate whether autophagy was more activated when both stimuli were combined, namely when endurance exercise was performed in a fasted rather than a fed state. Mice performed a low-intensity running exercise (10 m/min for 90min) in both dietary states after which the gastrocnemius muscles were removed. LC3b-II, a marker of autophagosome presence, increased in both conditions, but the increase was higher in the fasted state. Other protein markers of autophagy, like Gabarapl1-II and Atg12 conjugated form as well as mRNA of Lc3b, Gabarapl1, and p62/Sqstm1 were increased only when exercise was performed in a fasted state. The larger activation of autophagy by exercise in a fasted state was associated with a larger decrease in plasma insulin and phosphorylation of AktSer473, AktThr308, FoxO3aSer256, and ULK1Ser757, AMPKαThr172, ULK1Ser317, and ULK1Ser555 remained unchanged in both conditions, whereas p38Thr180/Tyr182 increased during exercise to a similar extent in the fasted and fed conditions. The marker of mitochondrial fission DRP1Ser616 was increased by exercise independently of the nutritional status. Changes in mitophagy markers BNIP3 and Parkin suggest that mitophagy was increased during exercise in the fasted state. In conclusion, our results highlight a major implication of the insulin-Akt-mTOR pathway and its downstream targets FoxO3a and ULK1 in the larger activation of autophagy observed when exercise is performed in a fasted state compared with a fed state.

LC3b; mitophagy; signaling; fission; ER stress

EXERCISE DISTURBS MUSCLE CELL HOMEOSTASIS by modifying the intra- and extracellular milieu, impairing energetic status and stretching membranes. These stressors lead to muscle remodeling by regulating transcriptional and translational events aiming at coping with further exercise-induced homeostatic disturbances. These adjustments give rise to beneficial effects of exercise for health and also increase in sports performance. However, remodeling implies that protein degradation is, at least transiently, activated. Several enzymatic systems are involved in muscle protein degradation. Besides the role of calpains, caspases, and metalloproteins, the activation of the ubiquitin-proteasome pathway in skeletal muscle during endurance exercise was the subject of particular attention during the past few years (16, 18). More recently, endurance exercise has also been identified as a stimulus that induces autophagy in this tissue (13, 14, 16, 27).

Macroautophagy, here called autophagy, is a catabolic cellular process that provides cellular constituents encapsulated inside a double-membrane vesicle called an autophagosome (AP) to lysosomes, the latter taking in charge of the degradation. Autophagy can process numerous cellular constituents, including soluble proteins, protein aggregates, and mitochondria (2, 22). Identification of autophagy genes and their related proteins (Atg) in mammals highlighted the molecular mechanisms responsible for AP formation (for review see Ref. 23).

Autophagy was initially considered a nonselective bulk degradation process. There is now evidence that autophagy can selectively target protein aggregates and organelles. Several proteins are known to be required for selective removal of specific substrates, including p62/sequestosome 1 (SQSTM1) (36). Mitophagy refers to the selective degradation of mitochondria through autophagy and is necessary for maintaining a healthy mitochondrial network through selective targeting of old/damaged mitochondria (52). Mitochondrial network dynamics are governed by fusion and fission events. These processes enable the cell to possess an interconnected mitochondrial network. An increase in mitochondrial fission events seems to precede mitophagy (52).

Autophagy is activated in skeletal muscle by numerous catabolic stimuli such as food deprivation, denervation, or sepsis (28, 34, 54). However, evidence for the necessity of a basal autophagy level in the maintenance of myofibrillar integrity has counterbalanced the vision of a system only implicated in muscle wasting (29). Very recently, activation of the autophagy-lysosomal pathway has emerged as an essential process for skeletal muscle adaptation after endurance training (27). Therefore, it seems important to study the physiological conditions of exercise in which autophagy activation is optimized. In this context, we hypothesized that autophagy is activated to a larger extent when endurance exercise is performed in a fasted rather than in a fed state. The secondary purpose of the present study was to elucidate the molecular mechanisms behind the regulation of exercise-induced autophagy in both aforementioned nutritional states.

MATERIALS AND METHODS

Animal care and groups. Thirty-six female C57BL6 mice (12 wk old) were obtained from Janvier (Le Genest-Saint-Isle, France). Animals were housed at 22°C in a 14-h light (7AM-9PM)-10h dark cycle with free access to food and water. All procedures were accepted by the Committee for Ethical Practices in Animal Experiments of the Université catholique de Louvain. The housing conditions were in accordance with the Belgian Law of April 6, 2010 on the protection of laboratory animals (Agreement no. LA-1220548). Animals were divided into four groups (n = 9 per group), following nutritional and exercise status: 1) fed at rest (Fed+Rest), 2) fed and exercised (Fed+Run), 3) fasted at rest (Fasted+Rest), and 4) fasted and exercised (Fasted+Run).

Exercise protocol. Animals performed three preliminary exercise bouts at 8 m/min for 10 min to be familiarized with treadmill running.
The delay between two familiarization sessions was at least 2 days, and the last familiarization session was organized 3 days before experiment. The day of the experiment, exercised groups ran for 90 min at a speed of 10 m/min, which corresponds to low intensity for this mouse strain (~55% of VO2 max.) (53). This duration has been associated with a plateau phase, i.e., no further increase, in the accumulation of autophagosomal number in different skeletal muscle groups of mice submitted to exercise running. (14). The fasted groups were deprived of food access at 11:30 PM, i.e., during the dark cycle. Mice from the Fasted+Run group started the exercise 8 h after the beginning of food deprivation.

_Euthanasia and sample collection_. Mice were given an intraperitoneal injection of a lethal dose of a mix of ketamine (200 mg/kg) and xylazine (20 mg/kg). Depth of anaesthesia was checked by the absence of eyelid and peda withdrawal reflexes. Exercised animals were euthanized immediately after the completion of exercise. Fasted mice were euthanized after 9.5 h of food withdrawal. Left and right gastrocnemius muscles were excised, blotted, and immediately frozen in liquid nitrogen. Venous blood was collected from the inferior vena cava using a 26-G syringe and conserved on ice in an EDTA tube until centrifugation. Plasma samples were harvested after centrifugation at 10,000 g for 5 min. All samples were conserved at ~80°C before further analysis.

_Plasma insulin concentration_. Plasma insulin concentration was determined by ELISA using the ultrasensitive mouse insulin kit from Mercodia (Uppsala, Sweden), following the manufacturer’s instructions. Briefly, 25 µl plasma of each samples was put down on a mouse monoclonal anti-insulin coated plate. Peroxidase-conjugated mouse monoclonal anti-insulin (100 µl) was added, followed by incubation with gentle shaking for 2 h at room temperature. Wells were subsequently washed six times to remove unbound antibodies. Bound conjugates were detected by reacting with 200 µl of tetramethylbenzidine (TMB) substrate. After a 15-min incubation with TMB, 50 µl of a stop solution containing 0.5 M H2SO4 was added. The plate was shaken for 5 s before reading absorbance at 450 nm. Samples were assessed in duplicate, and concentration was determined based on kit calibrators.

_Protein extraction_. Muscles were crushed in liquid nitrogen using a mortar and pestle. Powdered muscle was homogenized in ice-cold buffer containing 20 mM Tris, pH 7.0, 270 mM sucrose, 5 mM EGTA, 1 mM EDTA, 1% Triton X-100, 1 mM sodium orthovanadate, a mortar and pestle. Powdered muscle was homogenized in liquid nitrogen using a mortar and pestle. Left and right gastrocnemius muscles were excised, blotted, and immediately frozen in liquid nitrogen. Venous blood was collected from the inferior vena cava using a 26-G syringe and conserved on ice in an EDTA tube until centrifugation. Plasma samples were harvested after centrifugation at 10,000 g for 5 min. All samples were conserved at ~80°C before further analysis.

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**SDS-PAGE and immunoblotting**. Proteins (20–40 µg) were mixed with Laemmli sample buffer. For protein carbonyl measurements, 10 µg of proteins was derivatized with 2,4-dinitrophenyl hydrazine before electrophoresis, as described in the protein oxidation kit from Merck Millipore (Billerica, MA). Proteins were separated by SDS-PAGE for 2 h at a constant intensity of 40 mA and transferred to PVDF membranes at 80 V for 2.5 h. After blocking for 1 h in 0.1% Tween 20, Tris-buffered saline (TBST), and 5% nonfat dry milk, membranes were incubated overnight at 4°C with one of the following primary antibodies: dinitrophenyl (Sigma Aldrich, Diegem, Belgium), phospho-AktSer473, phospho-AktThr308, Akt, phospho-5′-AMP-activated protein kinase-αThr172 (AMPKαThr172), AMPKα, Atg5b, Atg12, Beclin-1, phospho-dynamin-related protein-1Ser616 (DRP1Ser616), DRP1, phospho-eukaryotic initiation factor (eIF)4E-binding protein-1Ser70/46 (4-E-BP1Ser70/46), 4-E-BP1, eukaryotic elongation factor 2 (eEF2; phospho-eEF2Ser57), eEF2a, phospho-Forhead box-containing proteins O-subclass 3aThr32 (FoxO3aThr32), FoxO3a, microtubule-associated protein-1 light chain 3b (LC3b; phospho-LC3bThr10), Cln-1, phospho-dynamin-related protein-1Ser616 (DRP1Ser616), p38 (not specific to a determined isoform), phospho-p70 ribosomal protein S6 kinase-αThr389 (S6K1Thr389), phospho-UNC51-like kinase-1 (ULK1Ser757, ULK1Ser553, ULK1Ser173, ULK1 (Cell Signaling Technology, Leiden, The Netherlands), Bcl-2/E1B-19kD interacting protein-3 (BNIP3), γ-aminobutyric acid receptor-associated protein (Gabarap)-like 1 (Gabarap1), Parkin (Abcam, Cambridge, UK), S6K1 (Santa Cruz Biotechnology, Heidelberg, Germany), or p62/SQSTM1 (Progen Biotechnik, Heidelberg, Germany). Membranes were washed three times with TBST and incubated for 1 h at room temperature with a secondary antibody conjugated to horseradish peroxidase (Sigma-Aldrich). Three more washes were carried out before detection by chemiluminescence with an ECL-Plus Western blotting kit (Amer sham Biosciences, Diegem, Belgium). Membranes were then photographed with the GBox system (Syngene, Cambridge, UK) using the software GeneSnap. Signal intensity was densified using the Gene-Tool program (Syngene, Cambridge, UK). Expression levels were normalized to eEF2, the expression of which was unaffected by treatments. The phosphorylated proteins were corrected to the total forms. When not mentioned, the total forms of phosphoproteins remained unchanged in all experimental conditions.

**RNA extraction and quantitative real-time PCR**. Muscles were crushed in liquid nitrogen using a mortar and pestle. Powdered muscles were homogenized in 1 ml TRizol reagent (Invitrogen, Merelbeke, Belgium). RNA isolation was performed according to the manufacturer’s instructions. RNA quality and quantity were assessed.

| Table 1. Sequences of primers (5′-3′) |
|-----------------|-----------------|
| **Forward**     | **Reverse**     |
| Atf4            | GAGCTTCTGGAACAGGGAGAGT | TGGCCACCTCTCAGAATGATCATC |
| Bnip3           | TAAAGCCCGAGGCGGACAC | GCCCTCTCAATGAATATCCCCA |
| Chop            | CTTAGATCTGGCTGAGACAAGG | CAGAGGAGATATCAATCTGCTG |
| Drp1            | TGAGCGCTGTTGGGGAGAGAACGAG | CAGAGAAGATAAATCTCCTGCTG |
| Gabarap1L       | GAGGACACCCCCCTCTGAAATTC | CATGAGAGTGGCGAGGGA |
| Lc3b            | ATCCGCTGCGAGAAGACCTT | ATCATCGGATCTGGTGGG |
| Mfn1            | GCCGAGACAGATGAGCCAA | CTTGCGTCTAAGTGATGC |
| Mfn2            | TTCCTCTGAAGAGACCCACAG | GGGTATGACAACTCAG |
| p62/Sqstm1      | GGGACTCAGGCTTCTCAATGGA | TGGTCCGATCCTCTGTTCC |
| Parkin          | CGTGGACGATCTGAGCTG | CTTGCGTCTCCTTTGTTGG |
| Pgc1a           | TCGGCTGCTGTTGGAGCTC | TCGGCTGCTCCTTTGTTGG |
| Rpl19           | GAAGCTGAAAGGGATATTGCTA | CCTGCGTCTCCTGAGG |
| Xbp1 s          | TGAGAAGAAGAATGTTAAGACAGC | CTTGCGATCGGAGGAG |

Atf4, activating transcription factor 4; Bnip3, Bcl-2/E1B-19kD interacting protein-3; Chop, CCAAT/enhancer-binding protein (C/EBP) homologous protein; Drp1, dynamin-related protein-1; Gabarap1L, γ-aminobutyric acid receptor-associated protein-like 1; Lc3b, protein-1 light-chain 3b; Mfn1, mitofusin 1; Mfn2, mitofusin 2; p62/Sqstm1, p62/sequestosome 1; Pgc1a, peroxisome proliferator-activated receptor-γ coactivator-1α; Rpl19, ribosomal protein L19; Xbp1 s, splicing of transcription factor X box-binding protein-1.
Fig. 1. Changes in autophagy-related proteins in response to treadmill running performed in the fed or fasted state. Values are means ± SE. *P < 0.05, **P < 0.01, ***P < 0.001 vs. rest (exercise effect); †P < 0.05, ‡P < 0.01, §§P < 0.001 vs. fed (fasting effect). Representative Western blots with control for loading are added under each variable. Adjustment for contrast and brightness was applied to the entire figure.
RESULTS

Regulation of autophagy-lysosomal pathway. We first investigated whether autophagy-related proteins (Fig. 1) and autophagy-related genes (Fig. 2) were increased more when exercise was combined with fasting. The lipidated form of LC3b (LC3b-II) has been extensively used as a valuable marker of the presence of AP in the cell (1), while determination of the ratio of LC3b-II on the nonlipidated form (LC3b-I) is a reliable assay for AP synthesis (23). LC3b-II was higher after exercise or fasting, and the combination of the two stimuli led to a further increase of LC3b-II (Fig. 1B). The nonlipidated form of LC3b (LC3b-I) was drastically lowered with fasting and was not affected subsequently by exercise (Fig. 1A). As a consequence, the LC3b-II/I ratio dramatically increased in response to fasting (Fig. 1C). The LC3b mRNA increased as a consequence of exercise in a fasted, but not in a fed state (Fig. 2A). Although Gabarapl1 mRNA is elevated in various atrophy models associated with increased autophagy (28, 54), determination of the protein expression is less common. Like the LC3 subfamily, the attachment of the Gabarap subfamily members to the AP membrane depends on their conjugation to phosphatidylethanolamine (17). Contrarily to LC3b, the increase of the Gabarapl1-II/I ratio in the Fasted+Run group (Fig. 1F) was only due to the depletion of the nonlipidated form (Fig. 1D), whereas the lipidated form remained unaltered (Fig. 1E). Similarly to LC3b, Gabarapl1 mRNA was the highest when fasting and exercise were combined (Fig. 2B).

Drp1 and mitophagy markers. Exercise induced the phosphorylation of Drp1Ser616, thereby potentially promoting mitochondrial fission through Drp1 translocation to the mitochondrial membrane (43) (Fig. 3A). Drp1 mRNA was unchanged by exercise but was decreased in the fasted condition (Fig. 3D). BNIP3 promotes mitophagy by disrupting mitochondrial membrane potential (41). BNIP3 protein accumulated as a consequence of fasting in resting animals, but this increase was completely reversed by exercise (Fig. 3B). As Bnip3 mRNA was also higher in the fasted state and remained elevated after exercise (Fig. 3E), it is likely that fasting increased the expression of BNIP3, which was degraded during exercise by a higher mitophagy rate. Similarly, Parkin, which is a ubiquitin ligase mediating ubiquitination of mitochondria (52), was higher in the Fasted+Run group, whereas exercise caused removal of this protein (Fig. 3C). Parkin mRNA remained unaffected in all conditions (Fig. 3F). The master regulator of mitochondrial biogenesis Pgc1α was higher after exercise but was not affected by the nutritional status (Fig. 3G). The mitochondrial fusion markers Mfn1 and Mfn2 were affected neither by the exercise protocol nor by the dietary status of the animals (Fig. 3, H and I).

Signal transduction. The secondary purpose of the present study was to elucidate the molecular mechanisms behind the

Fig. 2. Changes in autophagy-related genes in response to treadmill running performed in the fed or fasted state. Values are means ± SE. *P < 0.05 vs. rest (exercise effect); §P < 0.05, §§P < 0.01, §§§P < 0.001 vs. fed (fasting effect).
higher activation of autophagy when exercise is performed in a fasted state. Autophagy is transcriptionally activated by FoxO3a and posttranslationally inhibited by the mammalian target of rapamycin complex 1 (mTORC1) (19, 28). Both proteins are under the control of the insulin-phosphoinositide 3-kinase (PI3K)-Akt pathway. Exercise strongly reduced plasma insulin concentration (Fig. 4A), which was undetectable when exercise and fasting were combined, meaning that the concentration was lower than 4.35 pM, as specified by the manufacturer (Mercodia). Similar to the insulin pattern, the Akt phosphorylation state on both Ser473 (Fig. 4B) and Thr308 (Fig. 4C) was the lowest in the Fasted/H11001 Run group. Akt prevents the nuclear translocation of FoxO3a by phosphorylating multiple residues of this transcription factor, among which is Thr32 (11). The increase in FoxO3aThr32 observed in the Fast/H11001 Rest group (Fig. 4D) was associated with an upregulation of the total form of the transcription factor with fasting (Fig. 4E). Consequently, the ratio between FoxO3aThr32 and its total form was decreased when the exercise was performed in a fasted state \( P < 0.001 \); data not shown), supporting a nuclear translocation of FoxO3a in this condition. To evaluate mTORC1 activity, we measured the phosphorylation states of S6K1 and 4E-BP1, two downstream targets of mTORC1. S6K1Thr389 was drastically decreased as a consequence of exercise and fasting (Fig. 4F), and the 4E-BP1Thr37/46 phosphorylation state was the lowest in the Fasted/H11001 Run group (Fig. 4G), suggesting that mTORC1 activity was decreased more in these animals. mTORC1 is also a kinase for ULK1Ser757, the activity of which regulates the induction of autophagy (19). ULK1Ser757 was largely dephosphorylated in response to exercise combined with fasting (Fig. 4H).
Fig. 4. Changes in signal transduction components in response to treadmill running performed in the fed or fasted state. Values are means ± SE. *P < 0.05, **P < 0.01, ***P < 0.001 vs. rest (exercise effect); §P < 0.05, §§P < 0.01, §§§P < 0.001 vs. fed (fasting effect). When appropriate, representative Western blots with loading control or total form are presented under each variable. Adjustment for contrast and brightness was applied to the entire figure.
The total form of ULK1 was upregulated by exercise and fasting, but the two stimuli were not additive (Fig. 4I). Autophagy can also be activated by the energy sensor AMPK, which among others phosphorylates ULK1 at multiple sites (12, 19). In our experiment, the phosphorylation state of AMPKaThr172 was unchanged (Fig. 4J), as well as two AMPK-dependent phosphorylation sites of ULK1, namely ULK1Ser555 (Fig. 4K) and ULK1Ser317 (Fig. 4L).

Cellular stresses. Others signals transducing cellular stresses could potentially account for the observed increase in autophagy. p38 MAPK is activated in a variety of stresses, among which is exercise. p38 MAPK has been shown to link oxidative stress to autophagy activation (30). Phosphorylation of p38Thr180/Tyr182 was higher as a consequence of exercise in the fed state but did not further increase when the exercise in the fasted state was performed (Fig. 5A), while protein carbonyls remained unaltered (Fig. 5B).

Endoplasmic reticulum (ER) stress is activated in skeletal muscle in response to endurance exercise (18, 49). To cope with ER stress, the cell activates the unfolded protein response (UPR). The downstream effectors of this pathway, among which is eIF2α, induce the expression of transcription factors such as activating transcription factor 4 (ATF4) and CCAAT/enhancer-binding protein (C/EBP) homologous protein (CHOP), as well as the splicing of the transcription factor X box-binding protein-1 (XBP1s). ATF4 is known to play a role in the transcription of LC3 and Atg12, making the link between ER stress and autophagy (24, 38). Exercise increased the Atf4 mRNA level, but the post hoc tests within each nutritional condition were not significant (Fig. 6A). Phosphorylation of eIF2αSer51 increased when the exercise was performed in a fed state but not in a fasted state (Fig. 6B). Exercise increased Xbp1s mRNA in the fed but not significantly in the fasted mice (Fig. 6C). Chop mRNA was lower in response to exercise in a fed state, whereas fasting lowered Chop in both the rested and exercised groups (Fig. 6D).

DISCUSSION

In this study, we show that performing a low-intensity endurance exercise after a night of fasting causes a larger increase of autophagy in skeletal muscle of mice than performing the same exercise in a fed state. This larger increase of autophagy seems to be mediated by the insulin-Akt-mTOR pathway and its downstream targets FoxO3a and ULK1.

Autophagy and mitophagy. Ninety minutes of low-intensity exercise running in the fed state clearly increased LC3b-II and thereby AP presence in the cell. These results are in agreement with those reported by Grumati et al. (13) and He et al. (14) but differ from the unchanged level observed by Kim et al. (21). The stable p62/SQSTM1 level suggests that the lysosomal degradation of AP was not (yet) activated at the end of the exercise performed in a fed state. When exercise was performed in a fasted state, LC3b-II was further increased. The decreasing trend of p62/SQSTM1 combined with the significant alteration of several autophagy markers, i.e., Gabarapl1-I, Gabarapl1-II/I ratio, and cATG12, as well as p62, LC3b, and Gabarapl1, were arguments in favor of a higher autophagic flux. Taken together, these results show that the combined effects of fasting and exercise lead to a higher activation of autophagy in skeletal muscle. Whether fasting and exercise always have additive effects for activating autophagy is difficult to evaluate. A huge increase of LC3b-II (120-fold) has been observed in plantaris muscle of rats after 3 days fasting (35). In this condition, it is unlikely that the fourfold increase reported in the present study after endurance exercise may have a significant influence on the activation of autophagy.

Increasing mitochondria volume is essential for muscle oxidative capacity enhancement, but efficient removal of dysfunctional mitochondria also seems crucial for sustaining op-
timal mitochondrial respiration in skeletal muscle. Although most of the attention has been paid to mitochondrial biogenesis, it has been suggested that mitochondrial network remodeling (fission and fusion events) and mitophagy also contribute to the benefits of exercise on muscle health (50).

Fission seemed to be inversely controlled by exercise and fasting. Fasting repressed the expression of \( \text{Drp1} \) mRNA, a regulation that goes against the fission process. Conversely, exercise upregulated the initiation of fission by increasing the phosphorylation state of DRP1Ser616. This confirms our previous observations in exercising humans (16). Nutritional status did not regulate the DRP1 phosphorylation state, showing that exercise is required and fasting not sufficient for inducing fission, a process that likely participates in the remodeling of the mitochondrial network. Increased mitochondrial fission is supposed to be permissive for mitophagy (44). Key proteins regulating mitophagy are BNIP3, Parkin, and \( \text{p62/SQSTM1} \) (37). In a previous study, we did not observe any change in BNIP3 and Parkin protein levels in response to ultraendurance exercise in human skeletal muscle (16). Here, both Parkin and BNIP3 were increased as a consequence of fasting, and this rise completely disappeared after exercise. These observations suggest that mitophagy was activated during the exercise in the fasted state. As Parkin and BNIP3 are anchored in the mitochondrial membrane, their restoration to basal levels after exercise performed in a fasted state are likely due to an increased mitophagic flux, with consequent degradation of these proteins together with mitochondria. The higher mRNA expression of \( \text{Bnip3} \) and phosphorylation of DRP1, as well as the lower level of \( \text{p62/SQSTM1} \) in the Fasted+Run compared with the Fasted+Rest group, are other elements in favor of this hypothesis.

Our exercise protocol activated the master regulator of mitochondrial biogenesis, \( \text{Pgc1} \), but the fusion markers \( \text{Mfn1} \) and \( \text{Mfn2} \) remained unchanged. Other authors, using an incremental exercise protocol in rats, reported a decrease in \( \text{Mfn1} \) and \( \text{Mfn2} \) mRNA during exercise and an increase in the hours after exercise completion (6). This suggests that mitochondrial fusion is repressed or unchanged during exercise and activated during recovery.

Whether increased mitophagy contributes to a more favorable remodeling of mitochondria that would be beneficial to muscle remains unknown, but experimental evidence supports this hypothesis. PINK1-deficient mice, unable to activate mitophagy, show deep mitochondrial dysfunctions in skeletal muscle. At the opposite, training in a fasted state leads to better improvements in fat metabolism and oxidative capacity than the same program performed in a fed state (46). Clearly, if further research is able to confirm this hypothesis, exercise in a fasted state could reveal an efficient paradigm of exercise to fight against the deleterious metabolic consequences of diseases like obesity and insulin resistance.

Signaling pathways and cellular stresses. Autophagy activation in response to endurance exercise is related to its main homeostatic functions, namely 1) supply of alternative energy substrate and 2) removal of damaged cellular constituents (26). Our results support the idea that low-intensity endurance running increases autophagy in an insulin-Akt/mTOR-ULK1-dependent fashion. When the same exercise is performed in a fasted state, a further increase in autophagy is observed, which relies on transcriptional regulation through FoxO3a activation. ULK1 activity is under the control of both mTORC1 and AMPK (19). mTORC1 prevents ULK1 activation by phosphorylating the kinase at Ser757 (19). Under low-energy con-
dition, mTORC1 dissociates from ULK1, and the latter phosphorylates Atg13 and FIP200 with consequent autophagy activation (55). To the best of our knowledge, this is the first study reporting a dephosphorylation of ULK1\(^{\text{Ser759}}\) as well as an increase in the total form of ULK1 in skeletal muscle in response to exercise. AMPK is also able to control ULK1 activity either directly by phosphorylating the kinase at multiple sites or indirectly by inhibiting mTORC1 (9, 39, 40). Nevertheless, the phosphorylation state of AMPK\(^{\text{Thr172}}\) remained unchanged in all our experimental conditions. These results are in accord with previous studies showing no alterations in AMPK\(^{\alpha}\) phosphorylation state or activity following fasting or low-intensity endurance exercise (8, 10, 42). This was confirmed by the unchanged phosphorylation state of the AMPK-dependent sites on ULK1 (Ser\(^{117}\) and Ser\(^{555}\)). Therefore, our results suggest that AMPK was not essential for activation of the ULK1 complex; this activation relied rather on mTORC1 inhibition in our conditions.

FoxO3a is the main transcriptional regulator of autophagy in skeletal muscle (28, 54), the nuclear localization of which is mainly regulated by its phosphorylation state (45). FoxO3a\(^{\text{Thr32}}\) was lower when exercise was performed in a fasted state, and this decrease was associated with a higher expression of transcriptional targets of FoxO3a, such as LC3b and Gabarap1. Taken together, these data support the idea that exercise in a fasted state further increases autophagy through transcriptional regulations mediated by FoxO3a. Nevertheless, loss-of-function experiments would allow one to clearly determine the function of FoxO3a in the regulation of autophagy-related genes.

Other signaling pathways potentially contributed to the increase in LC3b-II in response to endurance running. He et al. showed that disruption of the interaction between B-cell lymphoma 2 (Bcl-2) and Beclin-1 accounts for the metabolic benefits of autophagy activation in response to exercise (14). Interaction between Bcl-2 and Beclin-1 is regulated by several mechanisms. Phosphorylation of Bcl-2 by the c-Jun NH\(_2\)-terminal (JNK) MAPK (47) is an attractive hypothesis, as MAPKs are known to be regulated by exercise (25). We measured a significant increase of JNK at Thr\(^{183}\)/Tyr\(^{185}\) but it remained unchanged (data not shown). BNIP3 can compete with Beclin-1 by binding Bcl-2 through their BH3 domain (3). Therefore, the increase in BNIP3 in the Fasted+Rest group could account for the higher LC3b-II through disruption of Bcl-2/Beclin-1 interaction.

Another kinase that is known to regulate autophagy in skeletal muscle is p38 MAPK (7, 30). Phosphorylation of p38 increases in a variety of cellular stresses, including exercise (25) and starvation (20). We measured a significant increase of p38\(^{\text{Thr180}/\text{Tyr182}}\) in Fed+Run and Fasted+Rest groups, but saw no further increase when exercise was combined with fasting. While the implication of p38 in autophagy-related gene expression and AP presence has been evidenced (7, 30), its functional role and the signaling pathways implicated still need to be fully elucidated. Possibly, p38 links oxidative stress to autophagy (30). However, in our experiment we did not measure any increase in protein carbonyls.

Autophagy is conceivably upregulated as a consequence of ER stress to help remove mis- or unfolded proteins. The UPR induces autophagy by controlling transcription of various Atg (4). For example, the transcription factor ATF4 elicits the expression of LC3 and Atg12 (24, 38). Our results indicate that ATF4 could contribute to the activation of autophagy during exercise independently of dietary status, but ATF4 is not a specific marker of UPR, since other regulatory mechanisms of ATF4 transcription have been described (5). Activation of eIF2\(\alpha\) during exercise performed in a fasted state also favors the implication of ER stress in LC3 conversion during exercise (24). Unexpectedly, eIF2\(\alpha\)\(^{\text{Ser51}}\), CHOP, and XBPIs remained unchanged when exercise was performed in a fasted state, showing that in this circumstance ER stress is not activated by exercise and therefore cannot contribute to the activation of autophagy. The mechanisms responsible for the lack of UPR activation during exercise in a fasted state are currently unknown.

**Conclusions.** Low-intensity exercise is effective for enhancing autophagy in skeletal muscle of mice. This increase is larger when exercise is performed in a fasted state. In a fed state, autophagy activation seems to rely on the combination of various signals originating potentially from mitochondrial fission, ATF4, p38 MAPK, and the insulin-Akt-mTORC1-ULK1 pathway, at least. The higher activation of autophagy during exercise performed in a fasted state is the consequence of a larger repression of the insulin-Akt pathway compared with exercising fed, with consequent transcriptional regulation of autophagy by FoxO3a.

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**DISCLOSURES**

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

**AUTHOR CONTRIBUTIONS**

Author contributions: C.J., H.G., and M.F. conception and design of study; C.J., H.G., and M.F. performance of experiments; C.J., H.G., and M.F. analysis of data; C.J., H.G., and M.F. writing of the manuscript; and C.J., H.G., and M.F. final approval of the manuscript.

**REFERENCES**

7. Doyle A, Zhang G, Abdel Fattah EA, Eissa NT, Li YP. Toll-like receptor 4 mediates lipopolysaccharide-induced muscle catabolism via...
to exercise in skeletal muscle through a PGC-1alpha/ATF6alpha complex. 

50. **Yan Z, Lira VA, Greene NP.** Exercise training-induced regulation of 

51. **Yao Z, Gandhi S, Burchell VS, Plun-Favreau H, Wood NW, Abramov 
AY.** Cell metabolism affects selective vulnerability in PINK1-associated 

52. **Youle RJ, van der Bliek AM.** Mitochondrial fission, fusion, stress. 

53. **Zbinden-Foncea H, Raymackers JM, Deldicque L, Renard P, 
Francaux M.** TLR2 and TLR4 activate p38 MAPK and JNK during endurance 

SH, Goldberg AL.** FoxO3 coordinately activates protein degradation by 
the autophagic/lysosomal and proteasomal pathways in atrophying muscle 

55. **Zhao M, Klionsky DJ.** AMPK-dependent phosphorylation of ULK1 