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Activation of autophagy in human skeletal muscle is dependent on exercise intensity and AMPK activation

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ABSTRACT **In humans, nutrient deprivation and extreme endurance exercise both activate autophagy. We hypothesized that cumulating fasting and cycling exercise would potentiate activation of autophagy in skeletal muscle. Well-trained athletes were divided into control ($n = 8$), low-intensity (LI, $n = 8$), and high-intensity (HI, $n = 7$) exercise groups and submitted to fed and fasted sessions. Muscle biopsy samples were obtained from the vastus lateralis before, at the end, and 1 h after a 2 h LI or HI bout of exercise. Phosphorylation of ULK1^{Ser317} was higher after exercise ($P < 0.001$). In both the fed and the fasted states, LC3bII protein level and LC3bII/I were decreased after LI and HI ($P < 0.05$), while p62/SQSTM1 was decreased only 1 h after HI ($P < 0.05$), indicating an increased autophagic flux after HI. The autophagic transcriptional program was also activated, as evidenced by the increased level of *LC3b*, *p62/SQSTM1*, *GabarapL1*, and *Cathepsin L* mRNAs observed after HI but not after LI. The increased autophagic flux after HI exercise could be due to increased AMP-activated protein kinase α (AMPKα) activity, as both AMPKα^{Thr172} and ACC^{Ser79} had a higher phosphorylation state after HI ($P < 0.001$). In summary, the most effective strategy to activate autophagy in human skeletal muscle seems to rely on exercise intensity more than diet.—Schwalm, C., Jamart, C., Benoit, N., Naslain, D., Prémont, C., Prévet, J., Van Thienen, R., Deldicque, L., Francaux, M. Activation of autophagy in human skeletal muscle is dependent on exercise intensity and AMPK activation. *FASEB J.* 29, 000–000 (2015). www.fasebj.org**

Key Words: cathepsin · cycling · fasting · LC3b · p62/SQSTM1

REGULAR PHYSICAL EXERCISE IS WIDELY recognized as a primary prevention tool to fight against many chronic diseases (1). Acute physical exercise induces a powerful but brief stress that challenges the whole body's homeostasis. An appropriate recovery period leads to adaptations aiming at reducing homeostasis disturbances when the same stimulation is applied again, as it is the case during a training program. Over the past decades, intensive work has been dedicated to unravel the molecular mechanisms behind exercise training–induced health benefits, specifically in skeletal muscle as a result of its major role during exercise (2, 3).

Among the cellular processes contributing to the maintenance of homeostasis, macroautophagy—hereafter called autophagy—has emerged as a crucial mechanism for maintaining muscle structure and preserving muscle mass (4). Autophagy is a bulk degradation process through which intracellular components such as soluble proteins, protein aggregates, and organ-elles are wrapped inside double membrane vesicles called autophagosomes, which fuse with endosomes and lysosomes, the latter containing hydrolases necessary for autophagosome degradation. The products of this proteolytic process are then transferred back to the cytoplasm, where they can be reused.

The autophagic flux is featured by autophagic initiation and resolution events, namely LC3b (microtubule-associated protein-1 light chain 3b) I lipidation associated to p62/SQSTM1 (p62/sequestosome 1) degradation (5). LC3b II, the lipidated form of LC3b I, directly reflects the presence of autophagosomes (5): LC3b II is recruited both at the inner and outer membrane during vesicle elongation, where it remains bound until autophagosomes fuse with lysosomes (6). Thus, the LC3b II/LC3b I ratio is recognized as a reliable marker of autophagosomes synthesis (7). p62/SQSTM1 binds both to aggregated proteins and to LC3b II and is degraded with the autophagosome content after fusion with lysosome (8). Because LC3b II is involved both in early and later stages of autophagy, from autophagosomes formation to lysosomal degradation, the assessment of additional autophagic markers acting upstream or downstream LC3b II is helpful to better interpret changes in autophagic flux (5).

Autophagy is constitutively active in skeletal muscle cell and plays a predominant role in cellular remodelling by controlling quality and clearance of intracellular altered proteins and organelles (9, 10). Besides its role in basal homeostasis, autophagy is also part of a cytoprotective

Abbreviations: 4E-BP1, factor 4E binding protein 1; ACC, acetylCoA carboxylase; AMPKα, AMP-activated protein kinase α; FoxO1/3, forkhead box containing protein O subclass 1/3; GabarapL1, GABA(A) receptor-associated protein-like 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HI, high intensity; LC3b, microtubule-associated protein-1 light chain 3b; LI, low intensity;

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process in the myofiber response to intracellular and extracellular stresses by supplying alternative sources of energy and preventing accumulation of damaged and/or aberrantly folded proteins and organelles (6). Recently, strenuous endurance running has been evidenced as a stress leading to autophagy activation and higher autophagic flux in mouse skeletal muscle (11, 12), which is possibly dependent on the eccentric component of the contractions (13). Contrary to studies reporting a chronic increase of autophagy in various muscle atrophy models (14–17), studies dealing with exercise rather consider the potential beneficial effects of a transitory increase of autophagy for muscle metabolism. Indeed, those studies suggest that activation of autophagy in skeletal muscle by running probably aims at eliminating the damaged and/or aberrantly folded proteins generated to prevent accumulation of toxic waste that would challenge cellular homeostasis (13, 18, 19) and that autophagy is required for improving insulin sensitivity, mitochondrial biogenesis, and angiogenesis (11, 20).

Most of the work on endurance exercise–induced autophagy has been performed in mice. Animal studies are generally carried out on small, immature mammals with a high basal metabolic rate and a weak capacity to maintain homeostasis. These studies are difficult to transfer to human adults, whose body weight is supposed to remain stable over years, with consequently slower protein turnover. In human, 2 studies from our laboratory showed that an ultramarathon activates autophagy both by transcriptional and posttranslational regulatory mechanisms in human skeletal muscle (18, 19). The aforementioned studies evidenced autophagy activation in response to strenuous running, the nature of which involves eccentric contractions and consequently muscle damage. We are unaware of any experiment showing the activation of autophagy in human muscle in response to endurance exercises, like cycling, which implicate only concentric contractions. Indeed, beyond muscle damage, autophagy can be activated while the energetic status of the cell is impaired or in response to changes in hormonal environment (21, 22). This suggests that eccentric contractions and subsequent muscle damage might not be required to activate autophagy during exercise.

In the present study, we thoroughly evaluated how endurance exercise regulates short-term autophagy in human skeletal muscle by the use of a low- and high-intensity cycling protocol. Because we previously showed in mice that cumulating both fasting and exercise running led to a higher activation of autophagy (23), we tested the hypothesis that autophagy activation could be potentiated by the association of fasting with exercise in humans. Finally, we aimed to elucidate the signaling mechanisms implicated in the regulation of autophagy after such a concentric exercise protocol in human skeletal muscle.

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mTORC1, mammalian target of rapamycin complex 1; p62/SQSTM1, p62/sequestosome 1; TBST, Tris-buffered saline plus 0.1% Tween-20; ULK1, unc-51-like kinase 1

MATERIALS AND METHODS

Subject characteristics and preliminary testing

Twenty-three healthy young men whose characteristics are described in **Table 1** were recruited for the study. The protocol was approved by the local ethical committee of the Université catholique de Louvain and conducted in accordance with the Declaration of Helsinki. Subjects were all 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 ($Vo_{2\ peak}$) for inclusion in the protocol was 50 $ml \cdot min^{-1} \cdot kg^{-1}$. Before the study began, subjects reported to the laboratory for a medical examination that aimed to exclude subjects with any underlying pathology. Afterward, height, weight, and percentage of body fat were measured using the Jackson and Pollock method (24). A maximal incremental exercise test was performed on a cycle ergometer (Cyclus III; RBM Electronics, Leipzig, Germany) for determining $Vo_{2\ peak}$ 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.

Participants were randomly divided into 3 groups: a low-intensity exercise group (LI, $n = 8$), a high-intensity exercise group (HI, $n = 7$), and a nonexercising control group ($n = 8$). One week after the maximal test, subjects took part in a familiarization session to ensure that they could handle the workload requested of them during the protocol in the fasted state. A 2 wk period separated the familiarization session and the first experimental session. Subjects had to refrain from strenuous exercise 3 d before each experimental session.

Dietary control

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

Exercise protocol

Each subject undertook 2 experimental sessions, fasted and fed, interspersed by a 2-wk period (**Fig. 1**). For each session, they arrived at 6 AM to the laboratory after an 8 h overnight fast. A flexible catheter was placed into the antecubital vein, from which a first venous blood sample of 5 ml was collected (time 0). Then, 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 Pharmaceuticals, Wilmington, DE, USA) (baseline). The leg on which the first biopsy was performed was chosen at random. Samples were immediately frozen in liquid nitrogen, and then stored at −80°C before further analysis. Ninety minutes later, a second venous blood sample was collected. A second muscle biopsy, with the needle pointing distally, was performed, with the sample taken through the same incision as the first preexercise sample. Still under local anesthesia, a second incision was made 3 cm distally

TABLE 1. *Subject characteristics*

Group	Age (y)	Height (cm)	Weight (kg)	Body fat (%)	$V_{O_2\ peak}$ (L/min)	$V_{O_2\ peak}$ (ml/min/kg)	W_{max} (W)	W_{max} (W/kg)
Control (n = 8)	23 ± 1	182 ± 2	72.7 ± 2.2	10.2 ± 1.6	4.44 ± 0.17	61.2 ± 2.5	328 ± 15	4.5 ± 0.2
LI (n = 8)	22 ± 1	182 ± 2	77.2 ± 3.8	10.7 ± 1.2	4.78 ± 0.16	62.5 ± 2.7	347 ± 12	4.5 ± 0.2
HI (n = 7)	24 ± 1	179 ± 3	68.5 ± 4.1	8.0 ± 1.3	4.44 ± 0.33	64.5 ± 1.7	323 ± 20	4.7 ± 0.1

from the first incision site, to be able to perform the third biopsy within 30 s of the end of exercise. The exercise bout then began (time 105). The LI group cycled for 2 h at 55% of $V_{O_2\ peak}$, whereas the HI group cycled for 2 h at 70% of $V_{O_2\ peak}$. A venous blood sample was taken immediately before the start of, every 30 min during, and during the last 5 min of the exercise period (times 135, 165, 195, and 225). With the needle pointing proximally, a third muscle biopsy was performed, with the sample taken from the second incision site made before the beginning of the exercise. One hour after the end of the exercise bout, a last venous blood sample was taken and the last biopsy—with the needle pointing distally in the incision made before exercise—performed. Pointing the needle in the opposite direction was intended to reduce potential activation of inflammatory signaling pathways, as recommended by Van Thienen *et al.* (25). All subjects underwent the same protocol 2 wk after the first session, receiving the crossed-over nutritional treatment (fasted *vs.* fed).

Protein extraction

A total of ~30 mg of muscular biopsy sample was homogenized with an Ultra-Turrax homogenizer (Ika, Staufen, Germany) for 2 × 10 s at 13,500 rpm in an 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, 50 mM sodium β-glycerophosphate, 5 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM DTT, and a protease inhibitor cocktail containing 1 mM EDTA (Roche Applied Science, Vilvoorde, Belgium). Homogenates were centrifuged for 10 min at 10,000 *g* at 4°C. Supernatants were collected and immediately stored at −80°C before further analysis. Protein contents were determined using the DC kit for protein dosage (Bio-Rad, Hercules, CA, USA).

SDS-PAGE and immunoblotting

A total of ~50 μg muscle proteins were mixed with Laemmli sample buffer and warmed for 5 min at 95°C. Proteins were separated by SDS-PAGE during 2 h at 40 mA, then transferred on PVDF membranes for 2.5 h at 80 V. After blocking for 1 h in Tris-buffered saline plus 0.1% Tween-20 (TBST) containing 5% nonfat dry milk, membranes were incubated at 4°C overnight with one of the following antibodies: phospho-4E-BP1$^{Thr37/46}$ (4E-BP1, factor 4E binding protein 1) (#2855), 4E-BP1 total (#9644), phospho-ACC^{Ser79} (ACC, acetylCoA carboxylase) (#11818), ACC total (#3676), phospho-Akt^{Ser473} (#9271), phospho-Akt^{Thr308} (#9275), Akt total (#2920), phospho-AMPKα^{Thr172} (AMPKα, AMP-activated protein kinase α) (#2535), AMPKα total (#2603), phospho-FoxO1/3a$^{Thr24/32}$ (FoxO1/3, forkhead box containing protein O subclass 1/3) (#9464), FoxO3a total (#24975), phospho-ULK1^{Ser317} (ULK1, unc-51-like kinase 1) (#12753), phospho-ULK1^{Ser757} (#6888), ULK1 total (#8054) (Cell Signaling Technology, Danvers, MA, USA), LC3b (L7543), or p62/SQSTM1 (P0067) (Sigma-Aldrich, St. Louis, MO, USA). Membranes were washed 3 times for 10 min in TBST, then incubated for 1 h at room temperature with a secondary antibody conjugated to horseradish peroxidase. Three additional washes were done before chemiluminescence detection with the ECL-Plus Western blot kit (Amersham Biosciences, GE Healthcare, Waukesha, WI, USA). The bands were captured with GeneSnap and quantified with the GeneTool software (G-Box; Syngene, Cambridge, United Kingdom). When the electrophoretic migration of a protein leaded to 2 bands, both of them were considered in the quantification process. Phosphorylated proteins were reported to their respective total form; LC3b results were reported as LC3b II/I ratio; and FoxO3a total, LC3b II/I, p62/SQSTM1, and ULK1 total expression were reported to glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Abcam, Cambridge, United Kingdom), 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 SDS-PAGE gels. If not mentioned, the total forms of the phosphoproteins were unchanged by exercise or diet.

RNA extraction and quantitative real-time PCR

A total of ~30 mg of muscle biopsy sample was homogenized in 1 ml Trizol reagent (Invitrogen, Merelbeke, Belgium) using the TissueLyser II from Qiagen (Venlo, The Netherlands). RNA isolation was achieved according to the manufacturer's instructions. RNA quality and quantity were assessed by NanoDrop

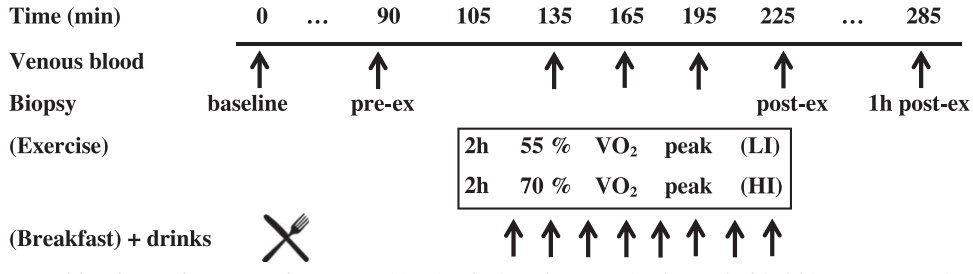

Figure 1. Schematic view of experimental protocol. One muscle biopsy was performed and 1 venous blood sample was taken at time 0 (baseline). After 90 min, a second biopsy was performed and 1 venous blood sample was taken. Subjects then either rested for 2 h or cycled for 2 h at 55% (LI) or at 70% of $V_{O_2\ peak}$ (HI). Venous blood samples were taken every 30 min during the exercise bout. A third biopsy was performed and 1 venous blood sample was taken at the end of the exercise period. One hour later, a last biopsy was performed and 1 venous blood sample was taken. 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 quantity of water.

spectrophotometry. Reverse transcription was performed from 1 μg RNA using the iScriptcDNA Synthesis Kit from Bio-Rad, following the manufacturer's instructions. The following primers were used: *Cathepsin L* forward sequence 5′-GTGAAGAATCAGGGTCAGTGTG-3′ and reverse sequence 5′-GCCCAGAGCAGTCTACCAGAT-3′; *GabarapL1* (GABA(A) receptor-associated protein-like 1) forward sequence 5′-GTGCCCTCTGACCTTACTGTTG-3′ and reverse sequence 5′-CATTTCCC-ATAGACACTCTCATC-3′; *Gapdh* forward sequence 5′-CATGGTCGTCATGGGTGTGAACCA-3′ and reverse sequence 5′-AGTGATGGCATGGACTGTGGTCAT-3′; *LC3b* forward sequence 5′-AATCCCGGTGATAATAGAACGA-3′ and reverse sequence 5′- GGAGACGCTGACCATGCTGT-3′; and *p62/Sqstm1* forward sequence 5′-CCTCTGGGCATTGAAGTTG-3′ and reverse sequence 5′-TATCCGACTCCATCTGTTCCTC-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 expression level were normalized to *Gapdh*, the expression of which was unaffected by diet or exercise.

Blood sample analysis

Samples were collected in heparin tubes and kept on ice before centrifugation for 6 min at 3000 *g* at 4°C. Supernatants were collected and stored at −20°C before further analysis. Plasma insulin concentration was measured using ELISA kits (Mercodia, Uppsala, Sweden), following the manufacturer's instructions.

Statistical analysis

All values are expressed as mean ± SEM. A mixed ANOVA model was used with the subjects as a random variable and groups (control, LI, and HI) and conditions (fasted, fed) as fixed independent variables. When appropriate, contrast analyses were performed to compare means. Statistical significance was set at $P < 0.05$.

RESULTS

Plasma insulin levels

Baseline plasma insulin concentrations were approximately 3 to 5 mU/L and were not different among the 3 groups and the nutritional conditions (**Table 2**). In the fasted conditions, insulin levels remained stable throughout the experiments, independent of resting or exercise intensity. Fed subjects displayed an increased plasma insulin concentration after breakfast (90 min) in each group ($P < 0.001$). Compared to control conditions, in which insulin levels remained elevated until 165 min, insulin levels in LI and HI rapidly returned to levels near to basal ($P < 0.05$). No effect of exercise intensity was observed at any time point.

mTORC1-dependent control of autophagy is not regulated by cycling exercise

An increase in plasma insulin levels activates Akt, which acts both as an activator of protein synthesis *via* its downstream target mTORC1 (mammalian target of rapamycin complex 1) and as a repressor of protein degradation through the ubiquitin–proteasome and autophagy–lysosomal pathways (22). After the peak in insulin concentration after breakfast, the phosphorylation state of Akt^{Ser473} (**Fig. 2*A***, Supplemental Fig. S1*A*) and Akt^{Thr308} (Fig. 2*B*, Supplemental Fig. S1*B*) was more than doubled after food intake and remained higher in the fed compared to the fasting group until the end of the trial in control ($P < 0.001$). Compared to control, feeding-induced increase in Akt^{Ser473} phosphorylation was reduced immediately after exercise in both LI ($P < 0.05$) and HI ($P < 0.001$). In the fasted state, exercise did not modify Akt^{Ser473} or Akt^{Thr308} phosphorylation in HI, but an increased Akt^{Thr308} phosphorylation was observed after exercise in LI ($P < 0.01$).

We then evaluated mTORC1 activity by measuring the phosphorylation state of 2 of its downstream targets, the translation repressor 4E-BP1 at $Thr^{37/46}$ (26) and the autophagic inductor ULK1 at Ser757 (27). Phospho-4E-$BP1^{Thr37/46}$ was decreased when exercise was performed in the fasted state ($P < 0.001$), and even more in HI (−80%) than in LI (−44%) ($P < 0.01$, Fig. 2*C*, Supplemental Fig. S1*C*). In fed subjects, a dephosphorylation of 4E-$BP1^{Thr37/46}$ was observed after exercise as well, but only in HI, thus providing an argument for decreased mTORC1 activity. Feeding had no effect on 4E-$BP1^{Thr37/46}$ phosphorylation. Similar to Akt, an increase in $ULK1^{Ser757}$ was measured after feeding ($P < 0.001$), but contrary to Akt, $ULK1^{Ser757}$

TABLE 2. *Plasma insulin concentrations*

Group	State	Time (min) 0	90	135	165	195	225	285
Control	Fasted	4.2 ± 0.4	3.7 ± 0.2	4.5 ± 0.3	6.1 ± 0.4	5.6 ± 0.3	4.4 ± 0.3	3.4 ± 0.2
	Fed	3.8 ± 0.2^{g}	36.8 ± 2.2^{f}	35.9 ± 1.1^{f}	39.1 ± 2.8^{f}	22.2 ± 0.8^{a,g}	24.1 ± 2.0^{f,g}	5.0 ± 0.2^{d,g}
LI	Fasted	4.6 ± 0.3	4.5 ± 0.3	4.1 ± 0.1	3.6 ± 0.1^{a}	2.3 ± 0.1^{b}	1.9 ± 0.2^{a}	2.8 ± 0.2
	Fed	4.3 ± 0.3^{g}	23.8 ± 1.4^{f}	11.4 ± 1.2^{f,g,c}	11.8 ± 1.5^{f,g,c}	6.7 ± 0.5^{e,g,a}	6.0 ± 0.6^{b,g,e}	4.3 ± 0.3^{g}
HI	Fasted	5.2 ± 0.7	4.8 ± 0.3	2.4 ± 0.3^{a}	1.7 ± 0.3^{b}	1.1 ± 0.3^{b}	0.5 ± 0.2^{a}	2.4 ± 0.4
	Fed	4.6 ± 0.6^{g}	34.0 ± 3.9^{f}	6.8 ± 0.8^{d,g,c}	9.0 ± 2.1^{e,g,c}	4.5 ± 1.0^{d,g,b}	3.5 ± 0.7^{g,c}	10.7 ± 3.1^{f,g,b}

Values are presented as means ± SEM of the control, LI and HI and high exercise groups, measured during fasted or fed sessions at 0, 90, 135, 165, 195, 225 and 285 min. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ *vs.* control group (exercise effect); $^{d}P < 0.05$, $^{e}P < 0.01$, $^{f}P < 001$ *vs.* fed (nutrition effect; $^{g}P < 0.001$ *vs.* pre-exercise (time effect).

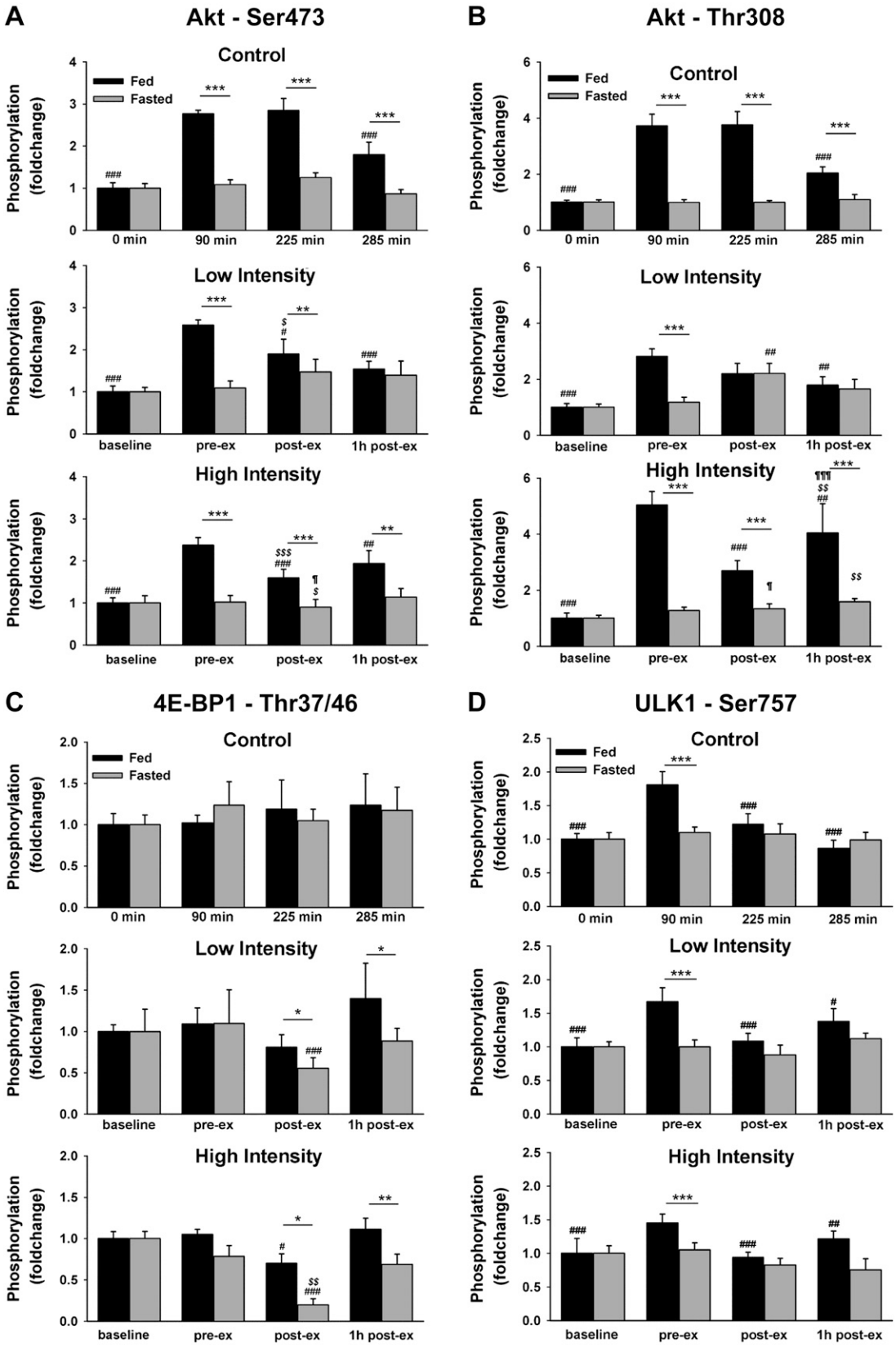

Figure 2. Phosphorylation changes in the insulin-dependent autophagy pathway in response to concentric endurance exercise as a function of intensity, nutritional state, and time. Phosphorylation of (*A*) Akt^{Ser473}, (*B*) Akt^{Thr308}, (*C*) 4E-$BP1^{Thr37/46}$, and (*D*) $ULK1^{Ser757}$ at baseline, before, immediately after, and 1 h after cycling exercise at LI (55% $Vo_{2\ peak}$) or HI (70% $Vo_{2\ peak}$) or in control conditions. Values are presented as mean ± SEM. $^{\$}P < 0.05$, $^{\$\$}P < 0.01$, $^{\$\$\$}P < 0.001$ *vs.* control group (exercise effect); $^{¶}P < 0.05$, $^{¶¶¶}P < 0.001$ *vs.* LI (intensity effect); $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* fed (nutrition effect); $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs.* preexercise (time effect).

returned to baseline within 2 h independently of exercise, as the control group showed the same pattern of phosphorylation as the exercised groups (Fig. 2*D*, Supplemental Fig. S1*D*).

AMPK-dependent autophagy pathway is regulated by cycling exercise

In addition to its regulation by insulin, autophagy is also controlled by AMPK (27), a master regulator of cellular energy homeostasis activated by nutrient deprivation and strenuous exercise, among others (21). The phosphorylation state of AMPKα^{Thr172} was increased immediately after exercise, especially when performed at HI ($P < 0.01$ in LI and $P < 0.001$ in HI) but was independent of nutritional state (**Fig. 3*A***, Supplemental Fig. S1*E*). To ascertain the activation of AMPK after exercise, we measured the phosphorylation state of ACC at Ser^{79}, a downstream target site for AMPK (21). Similar to AMPKα^{Thr172}, phosphorylation of ACC^{Ser79} was increased after exercise, but contrary to AMPK, the effect seems to be more persistent. Indeed, ACC^{Ser79} was still doubled 1 h after exercise compared to preexercise levels in HI ($P < 0.001$, Fig. 3*B*, Supplemental Fig. S1*F*). The phosphorylation state of ACC was independent of the nutritional state. ULK1 has multiple AMPK-dependent sites of phosphorylation, among which the Ser^{317}, thereby entailing autophagy initiation (27). Exercise increased phosphorylation of $ULK1^{Ser317}$ in both fed and fasted subjects ($P < 0.05$, Fig. 3*C*, Supplemental Fig. S1*G*). Similar to ACC^{Ser79}, exercise-induced $ULK1^{Ser317}$ was higher and more persistent in HI than in LI, as 1 h after exercise $ULK1^{Ser317}$ was still more phosphorylated than before exercise ($P < 0.01$). The combination of HI with fasting potentiated the phosphorylation of $ULK1^{Ser317}$ because a 2-fold increase *vs.* a 50% increase in the fed state was observed immediately after exercise ($P < 0.01$). Interestingly, compared with preexercise levels in HI, $ULK1^{Total}$ expression was higher immediately after HI exercise in both fasted and fed subjects ($P < 0.01$) and lower at 1 h after exercise, but only in fasted subjects ($P < 0.05$, Supplemental Figs. S1*G* and S2*A*). No variations in $AMPK^{Thr172}$, ACC^{Ser79}, or $ULK1^{Ser317}$ were measured in control rested conditions. These findings collectively indicate that cycling endurance exercise up-regulates the AMPK-dependent autophagy pathway in human skeletal muscle, especially when performed at HI, without any additional effect of fasting.

Autophagic flux is activated by cycling exercise in an intensity-dependent way

In light of our previous findings, we investigated whether the autophagic flux was activated, which is featured by an enhanced LC3b II protein level and LC3b II/ I ratio associated to a decreased p62/SQSTM1 protein content (5). To have better interpretation of changes in autophagic flux, changes in LC3b II, LC3b II/I ratio, and p62/SQSTM1 were referred to phosphorylation state of $ULK1^{Ser317}$, which acts upstream of LC3b II. In fasted control conditions, the LC3b II/I ratio, recognized as a reliable marker of autophagosomes synthesis (7), was gradually increased throughout the trial ($P < 0.05$), while in fed conditions this ratio decreased after breakfast ($P < 0.001$) and returned to baseline level 3 h later ($P < 0.001$, **Fig. 4*A***, Supplemental Fig. S1*H*). Compared with control, the LC3b II/I ratio in LI and HI was lower immediately after exercise in fasted conditions (approximately -70%, $P < 0.05$) and 1 h after exercise in both fasted and fed conditions (approximately -70% and -75%, respectively, $P < 0.01$). This exercise-induced decrease in LC3b II/I ratio was independent of the intensity and was due to a decreased LC3b II expression rather than a change in LC3b I (data not shown). p62/SQSTM1 binds both to aggregated proteins and to LC3b, and it is degraded with the autophagolysosome content (8). In control subjects, p62/SQSTM1 expression was about 10% less in fasted than in fed conditions at time 225 min, corresponding to the end of the nutrition protocol for the fed subjects ($P < 0.05$) and approximately 60% less in fasted *vs.* fed 1 h after ($P < 0.01$, Fig. 4*B*, Supplemental Fig. S1*I*). In LI p62/SQSTM1 was not modified by any condition, while in HI its expression 1 h after the end of the exercise was lower than before exercise as well as lower than in LI conditions at the same time, both in fed and fasted conditions ($P < 0.05$). Taking into account AMPK-dependent autophagy activation, these findings support the notion that HI is the most potent way to increase autophagic flux during cycling exercise in human skeletal muscle, independent of nutritional condition.

Autophagy-related genes expression is activated by cycling exercise in an intensity-dependent way

To gain further insight on the regulation of autophagy at the transcriptional level with concentric endurance exercise, we measured the phosphorylation state of the transcription factor FoxO1/3a, which, when dephosphorylated, translocates from the cytosol to the nucleus and promotes the transcription of autophagic genes (28). Overall, FoxO1/$3a^{Thr24/32}$ decreased over time in fasted conditions, being different from preexercise levels in control ($P < 0.05$) and HI ($P < 0.01$) groups and from fed conditions immediately after exercise ($P < 0.05$) and 1 h after exercise ($P < 0.01$) in the LI group (Supplemental Fig. S2*B*, *D*). No changes were observed among fed subjects. It is of note that a decrease of FoxO3 total was measured 1 h after HI exercise in both fed and fasted subjects ($P < 0.01$, Supplemental Fig. S2*C*, *D*). The changes observed at the level of FoxO1/$3a^{Thr24/32}$ were only partially followed by changes in mRNA expressions of the autophagy-related genes *LC3b* (**Fig. 5*A***), *p62/Sqstm1* (Fig. 5*B*), *GabarapL1* (Fig. 5*C*), and *Cathepsin L* (Fig. 5*D*), as the latter were increased over time only in HI exercise subjects ($P < 0.001$). In addition, contrary to what we expected from FoxO1/$3a^{Thr24/32}$ results, higher *LC3b* ($P < 0.001$) and *p62/Sqstm1* ($P < 0.001$) mRNA levels were observed in fed than in fasted subjects after exercise. However, fasting potentiated *GabarapL1* mRNA expression after exercise ($P < 0.01$ after exercise and $P < 0.001$ 1 h after exercise), indicating a different impact of diet on the transcription of those autophagy-related genes. Taken together, these results indicate a transcriptional activation of autophagy in human skeletal muscle with concentric endurance exercise performed at HI, yet only

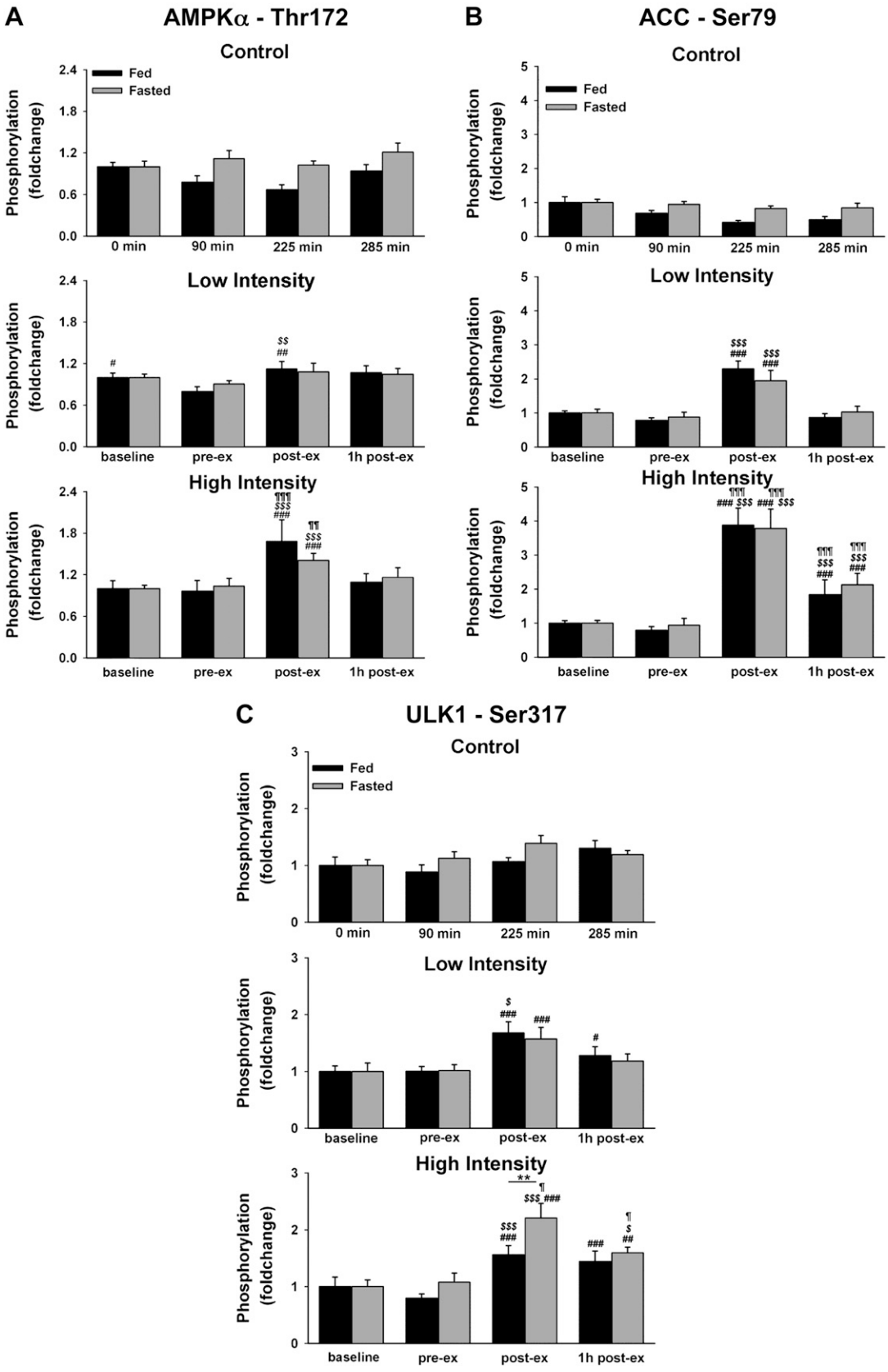

Figure 3. Phosphorylation changes in the AMPK-dependent autophagy pathway in response to concentric endurance exercise as a function of intensity, nutritional state, and time. Phosphorylation of (*A*) AMPK^{Thr172}, (*B*) ACC^{Ser79}, and (*C*) ULK1^{Ser317} at baseline, before, immediately after, and 1 h after cycling exercise at LI (55% $Vo_{2\ peak}$) or HI (70% $Vo_{2\ peak}$) or in control conditions. Values are presented as mean ± SEM. $^{\$}P < 0.05$, $^{\$\$}P < 0.01$, $^{\$\$\$}P < 0.001$ *vs.* control group (exercise effect); $^{¶}P < 0.05$, $^{¶¶}P < 0.01$, $^{¶¶¶}P < 0.001$ *vs.* LI (intensity effect); $^{**}P < 0.01$, *vs.* fed (nutrition effect); $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs.* preexercise (time effect).

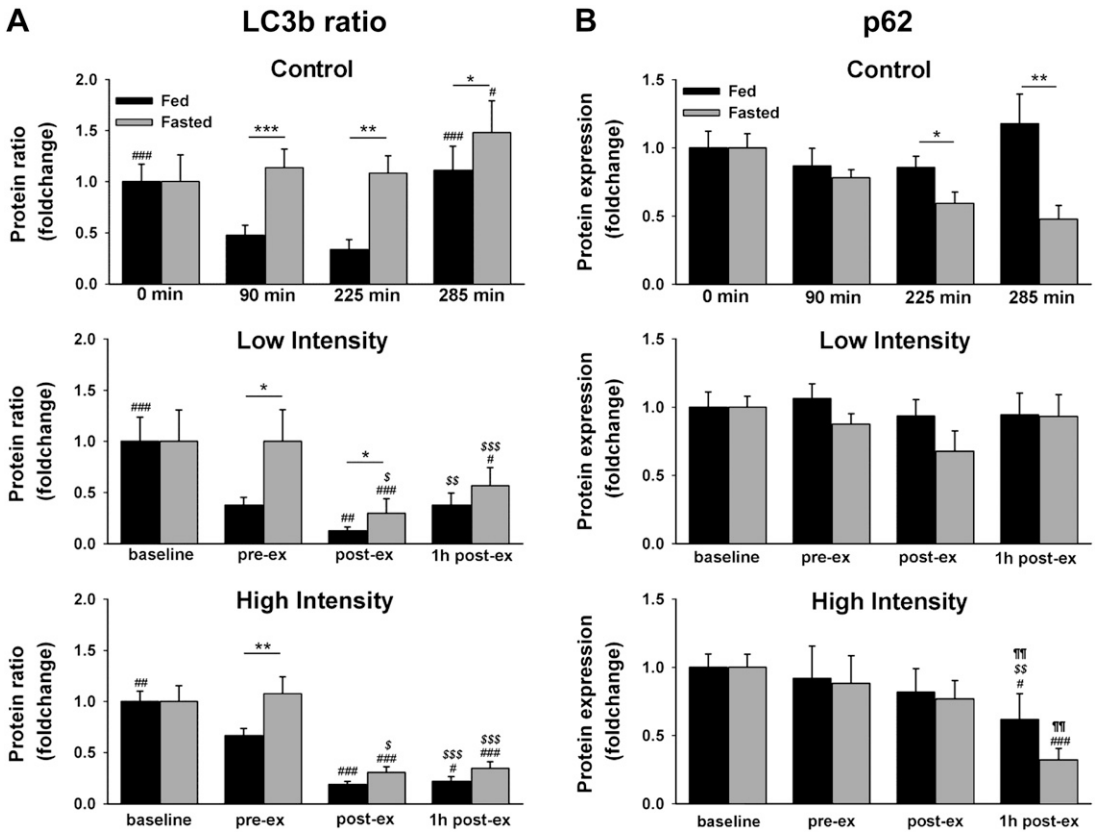

Figure 4. Changes in the expression of autophagic flux–related proteins in response to concentric endurance exercise as a function of intensity, nutritional state, and time. Phosphorylation of (*A*) LC3b II/I ratio and (*B*) protein expression of p62/SQSTM1 at baseline, before, immediately after, and 1 h after cycling exercise at LI (55% $Vo_{2\ peak}$) or HI (70% $Vo_{2\ peak}$) or in control conditions. Values are presented as mean ± SEM. $^{\$}P < 0.05$, $^{\$\$}P < 0.01$, $^{\$\$\$}P < 0.001$ *vs.* control group (exercise effect); $^{¶¶}P < 0.01$, *vs.* LI (intensity effect); $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* fed (nutrition effect); $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs.* preexercise (time effect).

partially regulated by FoxO1/3a$^{Thr24/32}$ and without additional effect of fasting.

DISCUSSION

Food deprivation and exercise are commonly identified as major stimuli of autophagy, although this has only been demonstrated in murine models and in human skeletal muscle after ultraendurance running, which is characterized by extensive muscle damage (13, 18, 19, 23, 29, 30). Hence, the possible activation of autophagy in human skeletal muscle during other kind of exercise is still uncharted. In this study, we used cycling to show for the first time that autophagy is also activated by concentric muscle contractions, probably *via* the activation the AMPK pathway and its downstream effector, ULK1. This process seems to rely more on the intensity of exercise than the nutritional state.

The strength of the present study is the inclusion of a control resting group, whether fasted or fed. Many studies dealing with the regulation of autophagy, most of which were performed in mice, measured the changes in autophagic markers before and after exercise as well as during the recovery period. Without a resting group, it is not possible to isolate the effects due to exercise itself, the effects due to the nutritional state, and those combined. As mentioned above, nutritional state and exercise can each regulate autophagy independently, but both have never been tested together in a single study in humans. The present methodology allows conclusions to be drawn for the first time on the activation of autophagy in human skeletal muscle by exercise and even on the intensity of exercise, the nutritional state, and the combination of both. Thanks to the rested conditions, the effect of the nutritional status on the regulation of autophagy could be clearly interpreted and isolated from the effect of exercise.

On the basis of the literature (31), the lowest level of autophagy was expected in the fed conditions, in which high phosphorylation states were measured at the level of Akt^{Ser473}, Akt^{Thr308}, and ULK1^{Ser757}, paralleling the elevated insulinemia after feeding. In this condition, the unchanged level of ULK1^{Ser317} and p62/SQSTM1, together with a lower LC3b II/I ratio, were arguments in favor a repressed autophagic flux due to food intake. Opposite results were hypothesized in fasted conditions, as reduced Akt phosphorylation and higher autophagosome number has been previously documented in skeletal of mice in response to fasting (30, 32). However, in the present study, insulin concentration and Akt phosphorylation in rested conditions remained stable over time. At the end of the experimental trial at rest and in the fasted state, an increase in

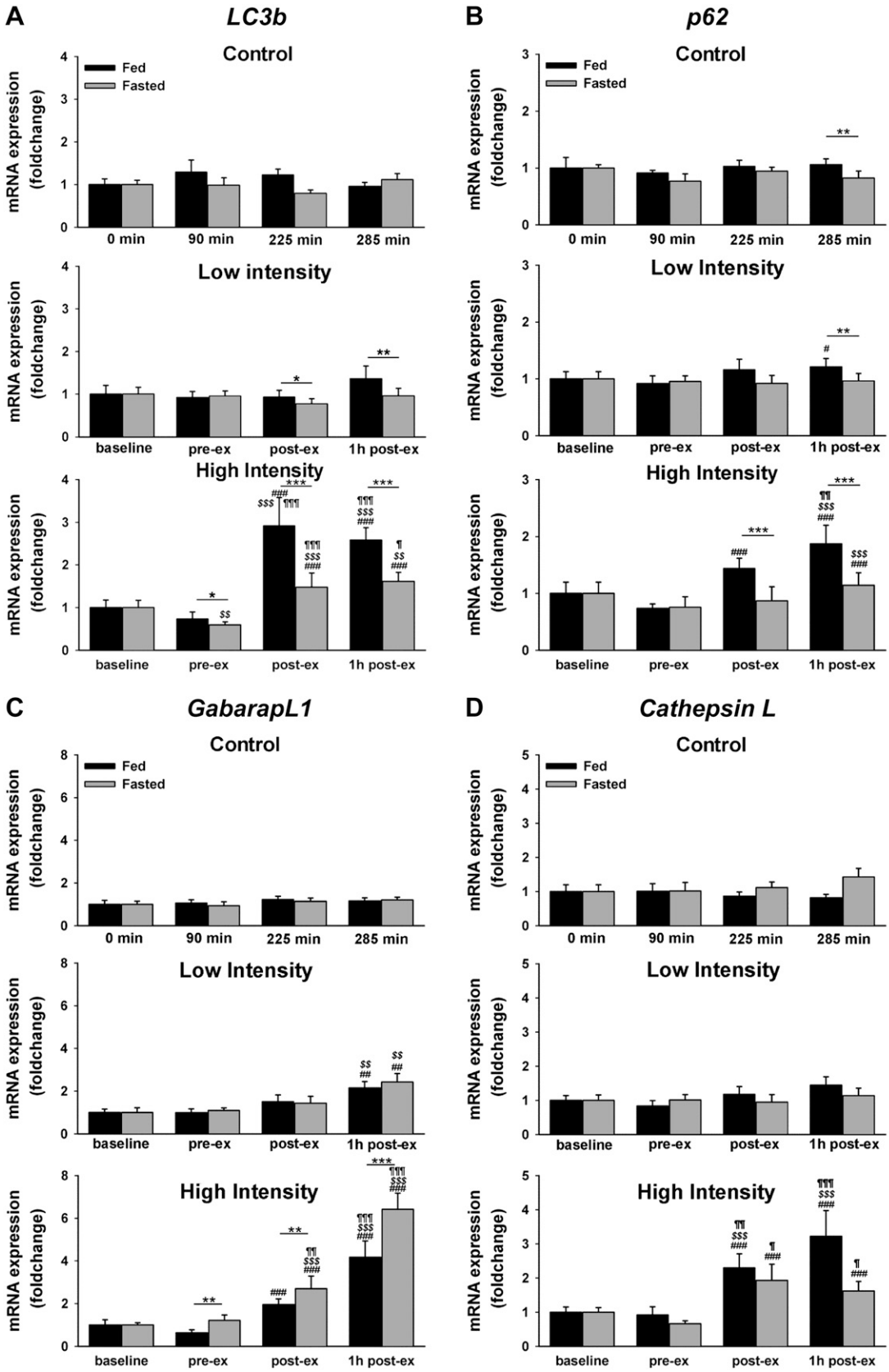

Figure 5. Changes in autophagy-related gene expression in response to concentric endurance exercise as a function of intensity, nutritional state, and time. mRNA levels of (*A*) *Lc3b,* (*B*) *p62/sqstm1,* (*C*) *GabarapL1,* and (*D*) *Cathepsin L* at baseline, before, immediately after, and 1 h after cycling exercise at LI (55% $Vo_{2\ peak}$) or HI (70% $Vo_{2\ peak}$) or in control conditions. Values are presented as mean ± SEM. $^{\$\$}P < 0.01$, $^{\$\$\$}P < 0.001$ *vs.* control group (exercise effect); $^{¶}P < 0.05$, $^{¶¶}P < 0.01$, $^{¶¶¶}P < 0.001$ *vs.* LI (intensity effect); $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* fed (nutrition effect); $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs.* preexercise (time effect).

the LC3b II/I ratio and a tendency of the p62/SQSTM1 level to decrease can be interpreted as an accelerated autophagic flux over time even if the phosphorylation state of ULK1^{Ser317} remained stable. Interestingly, the subjects were fasted for 8 h already, which could have been postulated as sufficient to activate autophagy. On the basis of our data, it seems that autophagy in skeletal muscle can still be further activated during the 5 h after an overnight fast and that this occurs independently of the Akt and AMPK pathways, as neither was modified in rested fasted conditions. Of note, neither AMPK nor ACC phosphorylation was changed in rested conditions, indicating that it is unlikely that autophagy relies on AMPK-dependent signal transduction in nonexercised conditions. Taken together, our results suggest that feeding can repress autophagy in rested human skeletal muscle, likely through the insulin/Akt pathway, while autophagy can be further activated after 8 h of fasting by a mechanism that remains to be determined but that is independent of the Akt and AMPK pathway.

A single session of LI concentric endurance exercise led to a decrease of LC3b II and LC3b II/I ratio in all subjects, indicating a reduction of autophagosomes synthesis in skeletal muscle cells, but did not alter p62/SQSTM1 level, suggesting that autophagosomes degradation did not occur. However, we cannot exclude the notion that activation of autophagy was delayed during LI exercise and therefore was not observable in the studied time frame. Two previous studies concluded that autophagic flux increased after acute LI exercise in mice, despite the opposite fluctuation of LC3b II level and no significant changes in p62/SQSTM1 content (23, 29). When the exercise was performed at HI, the decreased LC3b II level and LC3b II/I ratio suggests *a priori* a lower autophagic flux. However, it must be kept in mind that LC3b II depicts the presence of autophagosomes in the cell and that the LC3b II/I ratio is an indicator of autophagosome synthesis, which, taken alone, do not allow us to assert that the autophagic flux is activated or inhibited. In order to get a better picture of the changes in autophagic flux, which cannot be measured *per se* in humans, LC3b measurements need to be combined with other markers of autophagy like ULK1, which initiates this process and p62/SQSTM1, which reflects autophagosome degradation. Clearly, 1 h after intensive exercise, the ULK1^{Ser317} phosphorylation state was increased and p62/SQSTM1 was decreased, thus providing an argument for increased autophagic flux. Together, these data support the idea that eccentric contractions are not required to activate autophagy in skeletal muscle during exercise.

Our data in humans are somewhat different from previous work in mice, as the LC3b II/I ratio was found to be enhanced after acute exhausting exercise (13). Interestingly, these autophagic markers appeared to be poorly affected by the nutritional state during exercise, contrary to our recent findings carried out in mice, which showed a higher autophagic flux in the fasted state compared to the fed state (23). One could postulate that exercise added to food deprivation probably results in more intense cellular stress in mice than in humans, likely as a result of their higher metabolism rate and insulin sensitivity. Overnight fasting is thus likely not long enough to enhance exercise-induced autophagy in human skeletal muscle. These findings support the notion that HI is the most potent stimulus to increase the autophagic flux during cycling exercise in human skeletal muscle compared to nutritional state.

We then tried to understand further how autophagy was regulated by exercise and nutritional state by analyzing the posttranslational autophagic initiation, mainly controlled by ULK1 (27). ULK1 is activated by AMPK, which phosphorylates the latter at several sites, including Ser^{317}, while ULK1 is inhibited by mTORC1, which phosphorylates it at Ser^{757}, thereby preventing ULK1 activation by AMPK. Initiation of autophagy can therefore be regulated by AMPK-dependent and mTORC1-dependent pathways. Because mTORC1 is highly regulated by insulin, the latter pathway is also called the insulin-dependent pathway. Although we found that phosphorylation of AMPKα^{Thr172} was dispensable for LI exercise–induced autophagy in the skeletal muscle of mice, which instead relied on the insulin/Akt/mTORC1 signaling (23), the present study is to our knowledge the first to show that in humans, HI cycling exercise induces autophagy concomitantly with an increase in AMPKα^{Thr172} and ULK1^{Ser317} phosphorylation, indicating a likely involvement of the AMPK-dependent pathway. In accordance with previous studies conducted in skeletal muscle, we confirmed that AMPKα^{Thr172} phosphorylation was increased with exercise intensity (11, 33, 34) but was not affected by fasting (35), as supported by substantial ACC^{Ser79} and ULK1^{Ser317} phosphorylation state after HI exercise, whatever the diet. Importantly, this effect was long-lasting, as phosphorylation of ACC and ULK1 were still higher 1 h after exercise completion. Of note, the insulin/mTORC1 pathway did not contribute to the activation of autophagy during endurance exercise, as ULK1^{Ser757} was not regulated differently in the 2 exercised groups than in the control group. Without a control group, we could have concluded that exercise reduced ULK1^{Ser757} in both LI and HI fed groups. But a closer look at the control rested group in the fed state reveals that ULK1^{Ser757} showed exactly the same pattern of regulation, indicating that exercise was not involved in the decrease in ULK1^{Ser757} phosphorylation between the preexercise and postexercise time points. The increase between baseline and preexercise values and the subsequent return to baseline 1 h after seems to be attributable to the normal kinetics of regulation of ULK1^{Ser757} after feeding. This result highlights the real added value of having a nonexercised group. Our results show that concentric endurance exercise–induced autophagy relies mostly on AMPK activation, which is dictated by the intensity of exercise rather than by nutrient depletion in human skeletal muscle.

Autophagy can be regulated at the posttranslational level, as mentioned above, but transcriptional mechanisms participate in the regulation of autophagy as well (36). Both Akt and AMPK play a key role in the transcriptional modulation of autophagy as antagonist regulators of a common downstream substrate, FoxO3a (32, 37). Expression of autophagic genes was previously stated to be enhanced further to fasting or diverse exercise conditions in both mice and human skeletal muscle (18, 23, 32, 38). Having found that HI cycling exercise promoted autophagic genes expression, particularly in the fed condition, we addressed the question of which one of the AMPK or Akt pathways was brought into play to induce autophagy at the transcriptional level. FoxO3a activation was reported

to directly stimulate autophagy *via LC3b* and *GabarapL1* transcription in rested fasted mice (32, 38). Unexpectedly, the lower phosphorylation of FoxO1/3a$^{Thr24/32}$, and thereby the higher activity of FoxO1/3a (28), with fasting failed to increase the expression of *LC3b* and *GabarapL1* in control conditions. Combination of fasting with HI exercise also reduced FoxO1/3a$^{Thr24/32}$ levels, but compared to control conditions, the activation of FoxO1/3a was probably high enough to induce, or at least to contribute to, transcription of *LC3b* and *GabarapL1*. Although Akt is a known kinase for FoxO1/3a at Thr$^{24/32}$ (39), another undefined kinase (or phosphatase) should be responsible for the decrease in FoxO1/3a phosphorylation, as Akt phosphorylation at Ser^{473} remained stable during the 3 trials in the fasted state.

Those observations in the fasted state suggest that fasting *per se* is insufficient to trigger FoxO1/3a-dependent autophagy, yet additional cell stresses, such as HI exercise, are necessary to allow transcriptional autophagy to occur in human skeletal muscle. Interestingly, our results show that other transcription factors should activate the transcriptional autophagic program in fed conditions, as FoxO1/3a$^{Thr24/32}$ was not modified in these conditions. Possible candidates are NF-κβ (40) or unknown transcription factors downstream of p38 mitogen-activated protein kinase (41), or we could have missed phosphorylation of FoxO1/3a by AMPK. Indeed, FoxO3a is controlled by AMPK *via* different phosphorylation sites than Akt (39, 42). Measuring the AMPK-dependent activity of FoxO3a would have given us helpful clues to figure out and clarify the underlying mechanisms of transcriptional autophagy activation in our experimental protocol. A potential sensitivity of FoxO3a to HI exercise would be an attractive hypothesis to link, at least in part, AMPK activation to a larger autophagic genes expression with HI exercise. However, no FoxO3a antibodies specific to the sites phosphorylated by AMPK are currently available. In addition, it must be kept in mind that cytosolic levels of FoxO3a are also under proteasome control (43). Therefore, the decrease in the total form of FoxO3a, which ensued 1 h after HI exercise completion, may be interpreted as a higher proteosomal degradation of the latter after its acetylation, which we did not assess. Our findings, although strongly evidencing a short-term transcriptional activation of autophagy with HI concentric endurance exercise, emphasize the necessity of further investigations to decipher the molecular mechanisms responsible for autophagy transcriptional regulation in human skeletal muscle during exercise.

In summary, the present study is to our knowledge the first to provide evidence that exercise-induced autophagy relies on the activation of the AMPK pathway in human skeletal muscle. In addition, our findings indicate that the most effective strategy to activate autophagy depends on exercise intensity rather than dietary supply, thus providing interesting clues to improve the understanding of remodeling in both healthy and pathologic human skeletal muscles. FJ

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