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# Effects of aerobic training on markers of autophagy in the elderly

**Yubisay Mejías-Peña · Paula Rodriguez-Miguelez · Rodrigo Fernandez-Gonzalo · Susana Martínez-Flórez · Mar Almar · José A. de Paz · María J. Cuevas · Javier González-Gallego**

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**Abstract** Autophagy is a molecular process essential for the maintenance of cellular homeostasis, which appears to (i) decline with age and (ii) respond to physical exercise. In addition, recent evidence suggests a crosstalk between autophagy and toll-like receptor (TLR)-associated inflammatory responses. This study assessed the effects of aerobic exercise training on autophagy and TLR signaling in older subjects. Twenty-nine healthy women and men (age, 69.7 ± 1.0 year) were randomized to a training (TG) or a control (CG) group. TG performed an 8-week aerobic training program, while CG followed their daily routines. Peripheral blood mononuclear cells were isolated from blood samples obtained before and after the intervention, and protein levels of protein 1 light chain 3 (LC3), sequestosome 1 (p62/SQSTM1), beclin-1, phosphorylated unc-51-like kinase (ULK-1), ubiquitin-like autophagy-related (Atg)12, Atg16, and lysosome-associated membrane protein (LAMP)-2 were measured. TLR2 and TLR4 signaling pathways were also analyzed. Peak oxygen uptake increased in TG after the intervention. Protein expression of beclin-1, Atg12, Atg16, and the LC3II/I ratio increased following the training program ($p < 0.05$), while expression of p62/SQSTM1 and phosphorylation of ULK-1 at $Ser^{757}$ were lower ($p < 0.05$). Protein content of TLR2, TLR4, myeloid differentiation primary response gen 88 (MyD88), and TIR domain-containing adaptor-inducing interferon (TRIF) were not significantly modified by exercise. The current data indicate that aerobic exercise training induces alterations in multiple markers of autophagy, which seem to be unrelated to changes in TLR2 and TLR4 signaling pathways. These results expand knowledge on exercise-induced autophagy adaptations in humans and suggest that the exercise type employed may be a key factor explaining the potential relationship between autophagy and TLR pathways.

**Keywords** Elderly · Autophagy · High-intensity interval training · TLR

Y. Mejías-Peña · S. Martínez-Flórez · M. Almar · J. A. de Paz · M. J. Cuevas · J. González-Gallego (✉)
Institute of Biomedicine (IBIOMED), University of León, Campus Vegazana s/n, 24071 León, Spain
e-mail: jgonga@unileon.es

P. Rodriguez-Miguelez
Divisions of Clinical and Translational Sciences, Georgia Prevention Institute, Department of Pediatrics, Augusta University, Augusta, USA

R. Fernandez-Gonzalo
Department of Physiology and Pharmacology, Karolinska Institutet, Stockholm, Sweden

## Introduction

Aging is characterized by the accumulation of damage in cellular components that progressively leads to deterioration of different biological functions, ultimately increasing frailty and risk of death (López-Otín et al. 2013). Autophagy is a fundamental cellular homeostatic process required in different pathophysiological conditions for the degradation and recycling of damaged

cellular organelles and proteins (Choi et al. 2013). Recent evidence indicates that autophagy is compromised during senescence, leading to the accumulation of dysfunctional organelles and harmful protein aggregates. Dysfunction of the autophagic systems may also be behind some cell-specific and tissue-specific functions that fail with age (Cuervo and Macian 2014).

The regulation of autophagy is a complex process controlled by the coordinated actions of autophagy-regulated genes. In this process, several complexes are necessary for autophagy initiation. The first one is the Atg12/Atg5/Atg16 complex, which is essential for the formation of the autophagosome membrane (Walczak and Martens 2013). Other two complexes containing the unc-51-like kinase (ULK-1) and the Bcl-2-interacting protein (beclin)-1 also contribute to promote this initial assembly (Pagano et al. 2014). The second step involves the microtubule-associated protein 1 light chain 3 (LC3). Pro-LC3 is first cleaved to its mature LC3I form and subsequently conjugated to the lipid phosphatidylethanolamine (PE) of the forming autophagosomal membrane. The new PE-LC3I conjugate, also named LC3II, plays a crucial role for membrane fusion and substrate selection for degradation (Nakatogawa et al. 2007). Then, the autophagy adapter p62/SQSTM1 (sequestosome 1), which contains multiple protein-protein interaction domains, interacts with LC3, allowing the entry of ubiquitinated cargo into the autophagosome (Pankiv et al. 2007). In a final step, the autophagosome fuses with the lysosome and the lysosome-associated membrane protein (LAMP)-2A, a glycoprotein, takes on a critical role in this event (Sanchez et al. 2014).

Autophagy is known to negatively regulate the induction of inflammation that occurs in various settings including senescence (Netea-Maier et al. 2015) through changes in toll-like receptor (TLR)-induced proinflammatory responses (Into et al. 2012). Upon stimulation, these receptors lead to the recruitment of different signaling adaptors such as myeloid differentiation primary response gene 88 (MyD88) and TIR domain-containing adaptor-inducing interferon (TRIF). Both processes result in the activation of a number of downstream signaling pathways, which control inflammatory and immune responses (Rodriguez-Miguelez et al. 2015). It has been described that several autophagy-related proteins have an inhibitory effect on TLR signaling, and it is known that p62/SQSTM1 is involved in the suppression of TLR4-induced activation of mitogen-activated protein kinases (Into et al. 2010). In addition, deficiencies of LC3 or beclin-1 result in more susceptible responses of macrophages to TLR4 stimulation with LPS (Into et al. 2012). However, a complex cross-talk between autophagy and TLRs seems to exist, and different studies have shown that activation of TLRs facilitates pathogen elimination by autophagy via the adaptor proteins MyD88 and TRIF (Oh and Lee 2013).

In the last few years, a link between autophagy and regular physical activity has been established, although the underlying molecular mechanisms are still a matter of intense investigation. In skeletal muscle, the activation of autophagy during muscle contraction is important for maintaining cellular energy homeostasis as well as for efficient organelle and protein turnover following exercise (Vainshtein et al. 2014). Thus, autophagy biomarkers measured in skeletal muscle biopsies are upregulated after ultraendurance running (Jamart et al. 2012) or high-intensity cycling (Schwalm et al. 2015). Moreover, both interval and continuous aerobic exercise training promote autophagy in CD4 lymphocytes (Weng et al. 2013). However, although one study has indicated that exercise attenuates age-related impairment of autophagy in extensor digitorum longus and gastrocnemius muscles in rodents (Kim et al. 2013), no information exists showing such adaptations in humans. In immune cells, decreased autophagy with age may not only impair their ability to kill pathogens but also cause dysregulated activation of the inflammasome and subsequently increase the production of inflammatory cytokines, likely contributing to the age-associated inflammaging phenotype (Cuervo and Macian 2014). Thus, the well-documented anti-inflammatory effects of exercise in the elderly could be potentially related to changes in the autophagic capacity. In fact, several investigations have reported the effect of aerobic and resistance exercise training modalities on TLR2 and TLR4 signaling pathways (Gleeson et al. 2006; Fernandez-Gonzalo et al. 2012, 2014), and we have recently shown that resistance or whole-body vibration training may improve the anti-inflammatory status in peripheral blood mononuclear cells (PBMCs) from elderly subjects through an attenuation of MyD88- and TRIF-dependent TLR signaling pathways (Rodriguez-Miguélez et al. 2014, 2015).

To this background, the aim of the current study was to evaluate whether the main autophagic biomarkers are modified in response to an 8-week aerobic exercise training in peripheral blood mononuclear cells from

healthy old subjects. Furthermore, we investigated the effects of the exercise program intervention on the TLR2 and TLR4-dependent pathways. Our hypothesis was that aerobic exercise would counteract age-related decline of autophagy in the elderly and that this effect would be linked to changes in TLR signaling.

## Methods

### Design

Two different experiments were performed in the present research. To confirm if autophagy declines with age, we initially investigated differences in several autophagy biomarkers between young ($n = 15$) and old ($n = 29$) subjects at rest. The main experimental part of the study was carried out exclusively by old participants ($n = 29$) and was completed in 10 weeks. Subjects performed an aerobic exercise training program during 8 weeks and blood samples were collected 1 week prior (pre) and after (post) the exercise protocol.

### Subjects

Twenty-nine healthy old (8 males, 21 females; 69.7 ± 1.0 year) and 15 healthy young (7 males, 8 females; 20.6 ± 0.8 year) subjects volunteered to participate in the study. The inclusion criteria specified not to take any medication known to affect the inflammatory status in the 6 months prior to or during the study. None of the female participants were taking any hormonal treatment, either before or at the time of the study. Participants did not have any experience in aerobic exercise training, and they were asked to maintain their physical activity routines during the study period. For the main experimental part, before any other activity, a medical screening including anthropometric analysis, the physical activity readiness questionnaire (PAR-Q), a risk factor quiz, blood pressure measurements, and a basal electrocardiogram test were performed in all the elderly participants. Then, subjects were randomly assigned to a training group (TG; $n = 16$) or to a control group (CG; $n = 13$). Age, height, weight, and body mass index were 69.6 ± 1.0 year, 1.61 ± 0.12 m, 70.6 ± 3.1 kg, and 26.9 ± 0.7 kg/m$^2$, respectively, for TG and 70.0 ± 0.9 year, 1.58 ± 0.09 m, 68.1 ± 2.5 kg, and 27.0 ± 0.8 kg/m$^2$, respectively, for CG. Participants from TG followed an 8-week aerobic exercise training program, whereas the control group kept their normal daily routines. All volunteers were informed of the objectives and possible risks of the intervention before individual written consent for participation was obtained. The study followed the principles of the Declaration of Helsinki, and the local ethics committee approved all procedures.

### Estimated oxygen uptake

Peak oxygen uptake ($VO_{2peak}$) was estimated during a submaximal cycle ergometer (Ergoline-Ergo-metrics 900, Berlin, Germany) test in all elderly subjects. Briefly, analysis of expired gases (Medical Graphics CPX/MAX®, St. Paul, USA) was performed during a submaximal incremental test (initial load 20 W; increments of 20 W every minute) that ended when the subject reached 80–85 % of the theoretical maximal heart rate. Then, the relationship between heart rate and oxygen uptake was established for each subject, and $VO_{2peak}$ was extrapolated from this individual relationship considering the theoretical maximum heart rate. Subjects were monitored by 10-lead electrocardiogram (ECG) to rule out cardiac complications during this test.

### Aerobic exercise training

Elderly subjects from the TG completed 16 aerobic exercise training sessions over a period of 8 weeks (2 sessions per week), with at least 48 h between sessions. All sessions consisted on 25–30 min of cycling on a stationary ergometer (Tunturi Bike F35, Almere, the Netherlands). The first and last 5 min were employed for warming up and cooling down, respectively. During the central part of the sessions (15–20 min), subjects were requested to exercise at an intensity of 70–75 % of maximum heart rate. Short periods of intense activity (1 min; 90–95 % of maximum heart rate) were progressively introduced across sessions (see Table 1). Subjects wore a heart rate monitor (Polar®, Kempele, Finland) at all times during training.

### Blood sample preparation

Venus blood samples (30 mL) were collected from the brachiocephalic vein using the EDTA anticoagulant Vacutainer™ systems (BD, Franklin Lakes, NJ, USA), 5–6 days before and after the training period. In the case of the young subjects, the blood samples were collected

**Table 1** Aerobic exercise training program

| Week | Session | Duration of session Min | Intensity %HRmax | Warm-up Min | Interval 1 Min | Time between intervals Min | Interval 2 Min | Time between intervals Min | Interval 3 Min | Time between intervals Min | Interval 4 Min | Time between intervals Min | Cool down Min |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1 | 25 | 70 % | 5 | | | | | | | | | 5 |
| | 2 | 25 | 70 % | 5 | | | | | | | | | 5 |
| 2 | 3 | 25 | 70 % | 5 | | | | | | | | | 5 |
| | 4 | 25 | 70 % | 5 | | | | | | | | | 5 |
| 3 | 5 | 26 | 75 % | 5 | 1 | 7 | 1 | 7 | | | | | 5 |
| | 6 | 25 | 75 % | 5 | 1 | 6 | 1 | 6 | 1 | | | | 5 |
| 4 | 7 | 27 | 75 % | 5 | 1 | 7 | 1 | 7 | 1 | | | | 5 |
| | 8 | 25 | 75 % | 5 | 1 | 6 | 1 | 6 | 1 | | | | 5 |
| 5 | 9 | 23 | 75 % | 5 | 1 | 5 | 1 | 5 | 1 | | | | 5 |
| | 10 | 28 | 75 % | 5 | 1 | 5 | 1 | 5 | 1 | 5 | | | 5 |
| 6 | 11 | 29 | 75 % | 5 | 1 | 5 | 1 | 5 | 1 | 5 | 1 | | 5 |
| | 12 | 28 | 75 % | 5 | 1 | 5 | 1 | 5 | 1 | 4 | 1 | | 5 |
| 7 | 13 | 27 | 75 % | 5 | 1 | 5 | 1 | 5 | 1 | 4 | 1 | | 5 |
| | 14 | 27 | 75 % | 5 | 1 | 4 | 1 | 5 | 1 | 4 | 1 | | 5 |
| 8 | 15 | 26 | 75 % | 5 | 1 | 4 | 1 | 4 | 1 | 4 | 1 | | 5 |
| | 16 | 30 | 75 % | 5 | 1 | 4 | 1 | 4 | 1 | 4 | 1 | 4 | 5 |

Each session was divided in an initial warm-up of 5 min, a central part at medium intensity (70–75 % of $HR_{max}$) with maximum (90–95 % $HR_{max}$) interval efforts of 1 min, and a cool-down of 5 min

only once at rest. To avoid circadian effects, all samples were collected between 08:00 and 09:00 am. Participants were required to fast for 12 h before the blood test and to avoid any intense exercise during the previous 5–6 days. No caffeine or alcohol was allowed during this time. Food frequency questionnaires (FFQs) were performed all through the study, and participants were asked to replicate their eating habits from pre- to post-training blood collection. Total blood was centrifuged to isolate plasma and peripheral blood mononuclear cells using density gradient centrifugation on Ficoll separation solution (Biochrom AG, Berlin, Germany) (Cuevas et al. 2005).

### Western blot analysis

For Western blot analysis, PBMCs were suspended on 150 mL of 0.25 mM sucrose, 1 m MEDTA, 10 mM Tris, and a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) and physical disrupted using a sonicator. Lysate proteins were fractionated by SDS-PAGE. Polyacrylamide gels (8 % for beclin-1, Atg12, Atg16, LAMP-2, phospho-ULK-1, β-actin; 9 % for TLR2, TLR4 and TRIF; 12 % for MyD88; 13 % for LC3II/I, p62/SQSTM1) were run containing 40 μg of samples, and then transferred to a polyvinylidene fluoride (PVDF) membrane by a Trans-Blot® Turbo™ Transfer System (Bio-Rad®, Hercules, CA, USA). Non-specific binding was blocked by preincubation of the PVDF membranes in PBS containing 2.5 % non-fat milk for 1 h. Then, membranes were incubated overnight at 4 °C with corresponding antibodies against Atg12 (21 kDa), Atg16 (63/71 kDa), beclin-1 (60 kDa), LAMP-2 (120 kDa), TLR2 (90–100 kDa), TLR4 (95 kDa) and MyD88 (33 kDa) (Santa Cruz Biotechnology, CA, USA), LC3I/II (14–16 kDa), p62/SQSTM1 (60 kDa) and phospho-ULK-1 ($Ser^{757}$) (140–150 kDa) (Cell Signaling Technology®, Beverly, MA, USA), and TRIF (66 kDa) (Abcam®, Cambridge, UK), and β-actin (42 kDa) (Sigma-Aldrich). Bound primary antibody was detected using an appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (Dako, Glostrup, Denmark) using a chemiluminescent HRP substrate (Luminol Reagent, Santa Cruz Biotechnology). Finally, blots were exposed to autoradiography films and developed (San-Miguel et al. 2014, 2015). The density of the specific bands was quantified with an imaging densitometer (ImageJ, Bethesda, MD, USA).

### Statistical analysis

Data are presented as mean ± standard error of means (SEMs). Saphiro-Wilk test was used to verify normal data distribution; when data were skewed, log transformation was used. Comparisons between young and old groups were performed by using Student's $t$ test. The effects of exercise on autophagic and TLR response in the elderly were analyzed using a two-way analysis of variance (ANOVA) with repeated measures for group (CG and TG) and time (pre and post). Bonferroni post hoc analysis was used where appropriate. Differences were considered significant when $p < 0.05$. All statistical analyses were performed using SPSS version 18 (SPSS Inc., Chicago, IL, USA).

## Results

In order to confirm that the main markers of autophagy were reduced in elderly persons, LC3I, LC3II, and beclin-1 protein levels were compared between young and old subjects at rest. The LC3II/LC3I ratio (Fig. 1a) and beclin-1 (Fig. 1b) was markedly lower in PBMCs from old participants when compared to the young group (LC3II/LC3I ratio, $p < 0.04$; beclin-1, $p < 0.03$). Upon confirmation of declined autophagy with age, we proceeded to analyze the effects of aerobic exercise training on the autophagic and inflammatory (i.e., TLR2 and 4) response in elderly subjects.

$VO_{2peak}$ increased in TG after 8 weeks of training (31.0 ± 1.30 vs 37.5 ± 1.7 mL/kg/min; $p < 0.03$), while it remained unaltered in subjects from CG (30.5 ± 1.3 vs 30.7 ± 1.5 mL/kg/min). Thus, $VO_{2peak}$ was higher ($p < 0.03$) in TG than in CG after the intervention period. These data indicate that the aerobic exercise training protocol employed was effective in inducing classical aerobic adaptations.

To investigate the effect of aerobic exercise on several markers of autophagy in the elderly, LC3II, LC3I, p62/SQSTM1, and LAMP-2 protein content were measured in PBMCs from old participants. A training effect was identified in the LC3II/LC3I ratio, as illustrated in Fig. 2a; this ratio increased in response to the aerobic exercise protocol ($p < 0.04$), with TG showing greater values than CG after the intervention ($p < 0.04$). In contrast, TG showed a significant downregulation in p62/SQSTM1 expression ($p < 0.05$) (Fig. 2b) after 8 weeks of training. p62/SQSTM1 values were lower

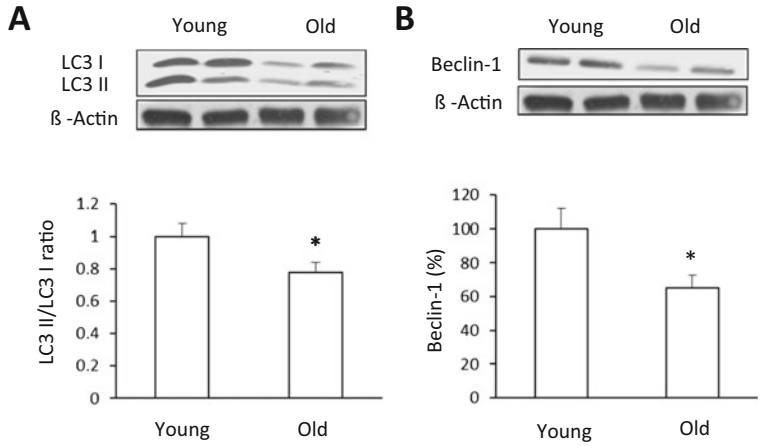


**Fig. 1** Densitometric quantification and representative Western blot of LC3I, LC3II (**a**), and beclin-1 (**b**) in PBMCs from young and old subjects at rest. Values are means ± SEM.*$p < 0.05$ vs young

in TG than in CG at post ($p < 0.05$). Figure 2c shows the protein content of LAMP-2 after 8 weeks of aerobic training (TG) or daily routines (CG). LAMP-2 expression was unaltered in any of the groups.

To further confirm changes in autophagy, expression of different proteins involved in the initiation of the autophagic process (Atg12, Atg16, beclin-1, and the $Ser^{757}$ phosphorylated form of ULK-1) were measured. As observed in Fig. 3a, b, the aerobic exercise training triggered a significant increase in Atg12 and Atg16 PBMC protein concentration compared to basal values ($p < 0.03$ and $p < 0.05$, respectively) and to values from CG at post ($p < 0.03$ and $p < 0.05$, respectively). Figure 3c shows how the exercise intervention induced a reduction of ULK-1 $Ser^{757}$ phosphorylation (i.e., decreased autophagic inhibition; $p < 0.04$), resulting in significant differences between TG and CG after the intervention ($p < 0.04$). The decreased ULK-1 phosphorylation was accompanied by an increase of beclin-1 in the TG ($p < 0.04$), which was significantly different than values from CG at post ($p < 0.04$) (Fig. 3d).

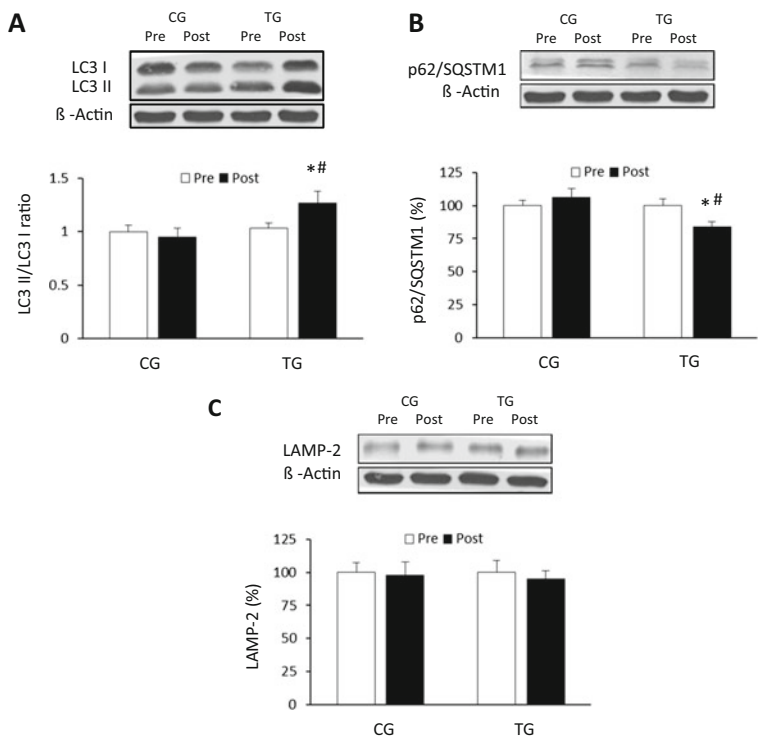


**Fig. 2** Densitometric quantification and representative Western blot of LC3II, LC3I (**a**), p62/SQSTM1 (**b**), and LAMP-2 (**c**) in PBMCs in response to 8 weeks of aerobic training for TG and the same period of normal daily routines for CG. Values are means ± SEM.*$p < 0.05$ vs CG; #$p < 0.05$ vs Pre within a group

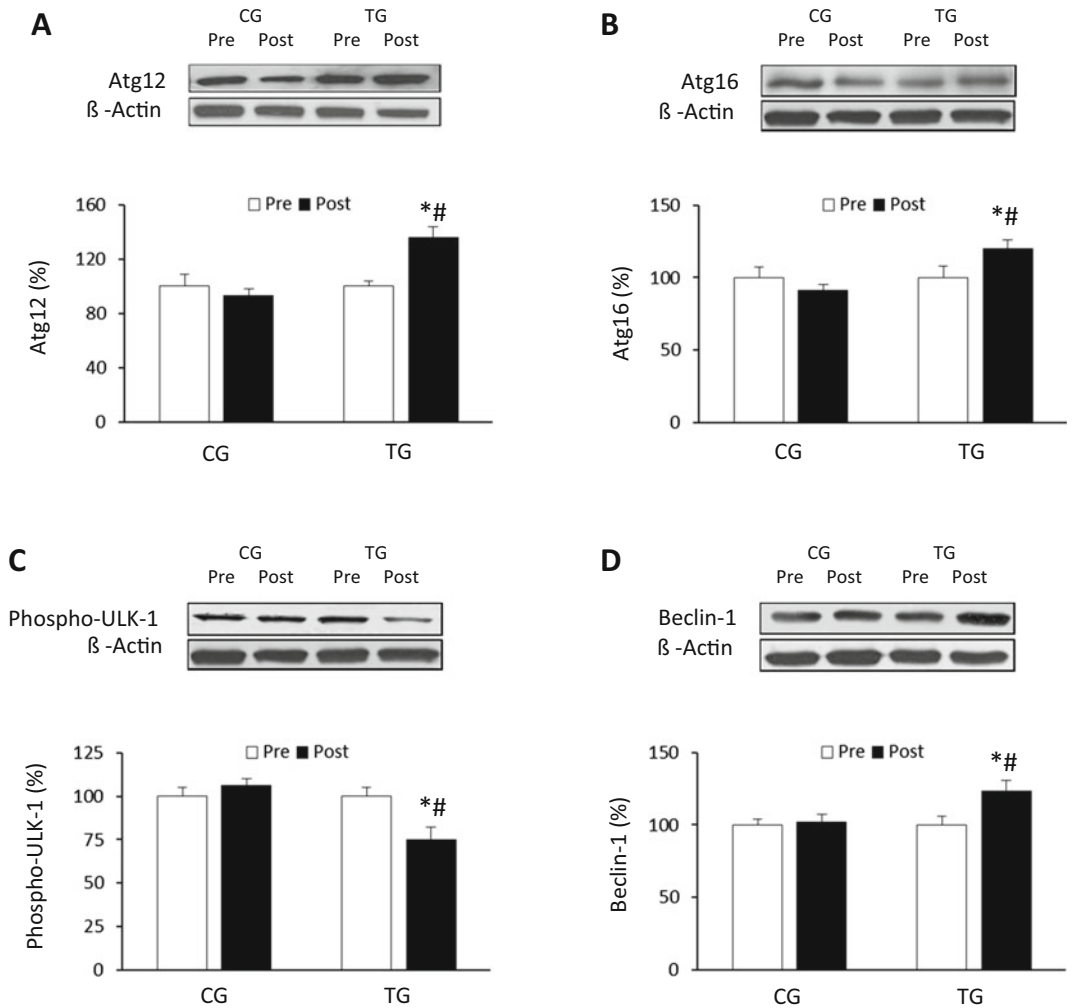


**Fig. 3** Densitometric quantification and representative Western blot of Atg12 (**a**), Atg16 (**b**), $Ser^{757}$ phospho-ULK-1 (**c**), and beclin-1 (**d**) in PBMCs in response to 8 weeks of aerobic training for TG and the same period of normal daily routines for CG. Values are means ± SEM.*$p < 0.05$ vs CG; #$p < 0.05$ vs Pre within a group

TLR2 and TLR4 protein content is shown in Fig. 4a, b. The expression of these receptors remained constant in both TG and CG, without differences across groups. Similar results were observed for MyD88 and TRIF protein concentrations, which did not change in any of the groups (Fig. 4c, d).

## Discussion

Malfunctioning of autophagy occurs in many organs and tissues with age. This may contribute to loss of cellular function and often to cell death by limiting the cell's ability to sustain a healthy proteome and organelles, making them more vulnerable to cellular stressors and pathogens and rendering them incapable to adapt to energetically demanding conditions (Tam and Siu 2014). In fact, a progressive decline of the autophagic activity and a reduced expression of different autophagy-regulating genes represent some of the hallmarks of the aging process (López-Otín et al. 2013). Results from the current study confirm the notion of hindered autophagy response in the elderly. Interestingly, 8 weeks of aerobic exercise training were able to attenuate the loss of autophagic activity in PBMCs from elderly subjects, through an increase in the LC3II/I ratio, reduced p62/SQSTM1 protein levels, and changes in the expression of autophagy regulatory proteins, including beclin-1, phosphorylated ULK-1, Atg12, and Atg16. However, contrary to our hypothesis, these effects do

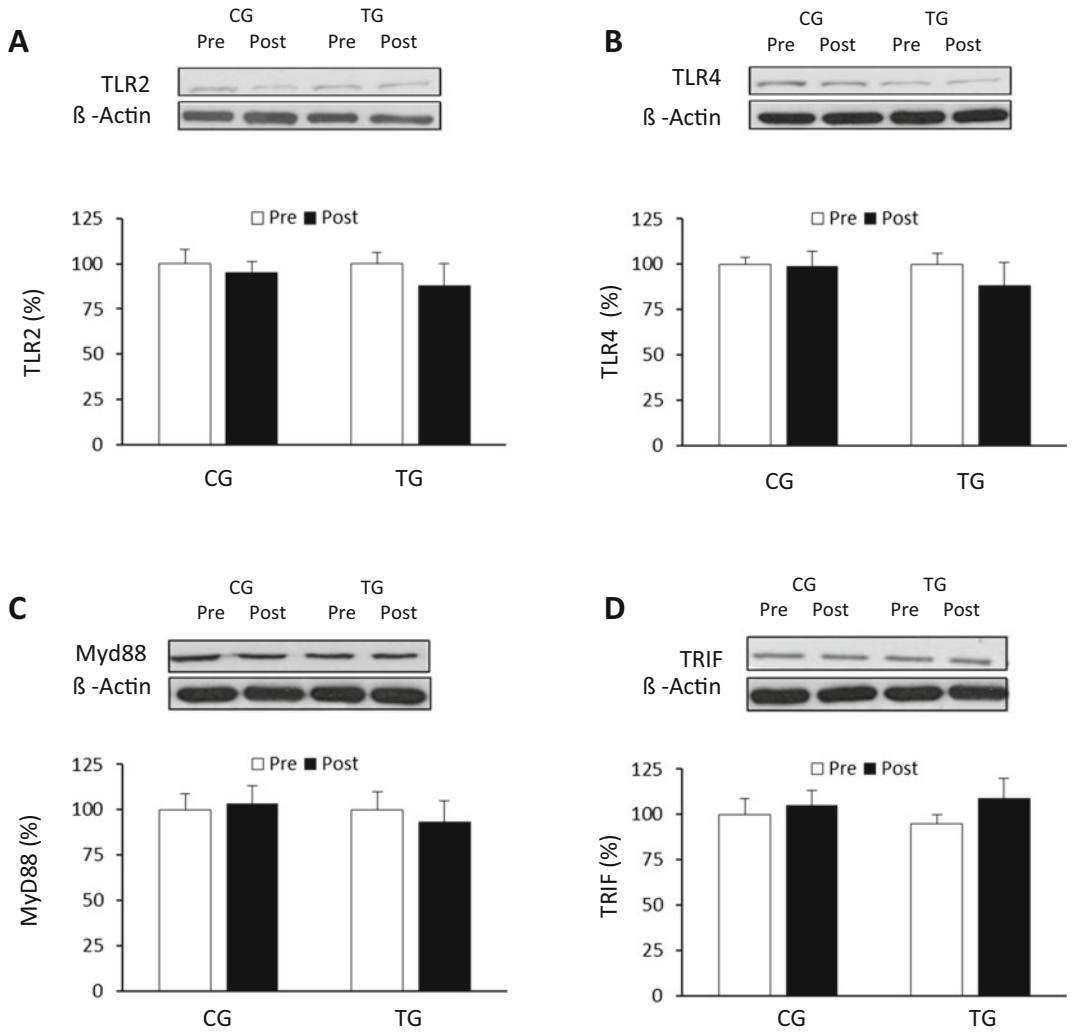


**Fig. 4** Densitometric quantification and representative Western blot of TLR2 (**a**), TLR4 (**b**), MyD88 (**c**), and TRIF (**d**) in PBMC in response to 8 weeks of aerobic training for TG and the same period of normal daily routines for CG. Values are means ± SEM

not seem to be related to a parallel downregulation of TLR2 and TLR4 MyD88 or TRIF-dependent pathways, since we could not identify any significant change in these markers after the training period. Since other training modalities (i.e., resistance exercise) induced TLR2 and TLR4 adaptations (Fernandez-Gonzalo et al. 2012, 2014; Rodriguez-Miguélez et al. 2014, 2015), the lack of an apparent relationship between autophagy and TLRs may be due to the exercise training mode currently employed, at least to some extent.

In the present study, the LC3II/I ratio and beclin-1 protein concentration were significantly lower in PBMCs from old subjects compared to young individuals. These data confirm the reduction in the autophagic machinery that has been previously reported over the course of aging in different organs and tissues, including blood cells (Huang et al. 2012). Among interventions aimed to prevent or reverse these undesirable effects, the role of exercise is growing (Vainshtein et al. 2014). Indeed, both acute and chronic endurance exercise seem to induce adaptations in the autophagy pathway (Sanchez et al. 2014; Jamart et al. 2012; Schwalm et al. 2015; Weng et al. 2013). However, it should be noted that most of the available data related to the effects of exercise on autophagy proteins in different tissues have been obtained from rodent studies, where long training periods induce a significant increase in the protein expression of beclin-1 and LC3I/II associated to a lowered expression of p62/SQSTM1 in muscles (He et al. 2012; Kim et al. 2013; Lira et al. 2013; Tam et al.

2015) or cerebral cortex (Bayod et al. 2014). Nevertheless and as far as we know, few investigations have studied the effects of exercise on the age-related impairment of autophagy using a murine model. Kim et al. reported lower expression of beclin-1 and Atg7 in skeletal muscle of old compared with young animals, but these factors were upregulated in old mice after an 8-week aerobic training program (Kim et al. 2013). In addition, 9 weeks of resistance exercise training prevented the loss of muscle mass and improved muscle strength, accompanied by reduced LC3-II/LC3-I ratio, reduced p62 protein levels, and increased levels of autophagy regulatory proteins, including Beclin 1, Atg5/12, and Atg7 in skeletal muscles from aged rats (Luo et al. 2013). In any case, the present work is the first investigation addressing the effects of exercise training on the decline of the autophagy machinery in older human subjects.

Among autophagic proteins, LC3II, which is an important mediator in the substrate selection for degradation and that also plays a role in membrane fusion, is commonly used as a marker of autophagy activation (Barth et al. 2010). Findings from our study indicate that expression of both LC3I and LC3II increases in response to the exercise program, with a significant elevation of the LC3II/I ratio, supporting a possible enhanced number of autophagosomes. However, whether this raise is the consequence of an increased autophagosome formation or a defect in their degradation through the lysosome remains to be elucidated (Pagano et al. 2014). To further understand the changes in the markers of autophagy, LC3II measurements should be combined with other markers of autophagy. p62/SQSTM1, a ubiquitin-binding scaffold protein that binds with LC3, may serve to link ubiquitinated substrates to the autophagic machinery and is itself degraded by the lysosome (Sanchez et al. 2014). Thus, because p62/SQSTM1 accumulates when autophagy is inhibited, and decreases when autophagy is induced, modulation of p62/SQSTM1 levels may be used as a marker to monitor autophagy (Klionsky et al. 2012). Our data show that aerobic exercise induces a reduction in p62/SQSTM1, suggesting an increase in the expression of autophagy regulatory proteins after the training program. These results are in line with previous studies which showed an increase in LC3II and a decrease in p62/SQSTM1 expression after exercise in different rodent (He et al. 2012; Lira et al. 2013; Bayod et al. 2014) and human models (Schwalm et al. 2015), hence supporting the notion that high-intensity exercise induces an increased autophagy.

To further confirm activation of the autophagic machinery by exercise training, we also measured the expression of different proteins contributing to promote the initial assembly of the autophagosomal membrane. The first ubiquitin-like conjugation system implicated in the autophagosome formation is the Atg12/Atg5/Atg16 (Walczak and Martens 2013). Previous data have shown that Atg12 is enhanced at the end of a 24-h run (Jamart et al. 2012) and in muscle atrophy models such as denervation and food deprivation (Zhao et al. 2007). Results from the present study indicate that protein expression of both Atg12 and Atg16 increases following exercise training in the elderly. The current exercise training employed also induced a reduction in ULK-1 phosphorylation at $Ser^{757}$ and a higher beclin-1 expression. It should be highlighted that ULK-1 plays a critical role in the initiation of autophagy. Thus, phosphorylation of ULK-1 at $Ser^{555}$ is an important stimulus of autophagy, whereas phosphorylation at $Ser^{757}$ promotes an inhibitory effect (Kim et al. 2011). Regulation of ULK-1 phosphorylation by feeding has been associated with an activation by AMP-activated protein kinase α in humans (Schwalm et al. 2015), and short-term aerobic exercise seems to increase the expression of ULK-1 phosphorylated at $Ser^{555}$ (Moller et al. 2015). In mice, LC3I is lipidated in response to running exercise, and this is accompanied by decreased ULK-1 $Ser^{757}$ phosphorylation (Pagano et al. 2014). The decreased expression in the autophagy-inhibitory $Ser^{757}$ phosphorylated ULK-1 described in the current study supports the notion of an exercise-induced autophagic stimulation.

Beclin-1 is a protein playing an essential role in the initiation of sequestration, being involved in a step upstream of autophagosome synthesis that is important for the recruitment of other Atg proteins (Sanchez et al. 2014). Although no changes have been reported in skeletal muscle following ultraendurance running (Jamart et al. 2012), beclin-1 levels increased in rodents following treadmill training (Kim et al. 2013). Our results are similar to a previous study where hypoxia hindered autophagy in CD4 lymphocytes, while both interval and continuous exercise training for 5 weeks effectively upregulated beclin-1 expression and thus attenuated the hypoxia-induced autophagic reduction (Weng et al. 2103).

An additional interesting finding concerns the expression of LAMP-2, a glycoprotein required for the

fusion of autophagosomes with lysosomes to create autophagolysosomes, involved in the lysosomal-autophagic degradation process (Wohlgemuth et al. 2010). In the present study, LAMP-2 levels were not affected by exercise training, a fact that has been previously reported both in skeletal muscle from young and old trained mice (Kim et al. 2013). Although increases in LAMP-2 mRNA levels have been described in obese adults performing moderate intensity exercise, the changes observed were not solely due to the effects of exercise but to a combination with caloric restriction (Wohlgemuth et al. 2011).

Despite that an attenuation of the several autophagy markers, such as Beclin-1, Atg7, LC3-II, and LAMP2a, is observed with age in both oxidative and glycolytic muscles (Kim et al. 2013), the increase in autophagy after aerobic exercise in animal models seems to take place when an oxidative phenotype is reached (Lira et al. 2013). Endurance exercise requiring high-energy production levels is likely to induce reactive oxygen species (ROS) production and metabolic disturbances that may damage the mitochondria, especially when high intensities are employed (Sanchez et al. 2014). Thus, selective autophagic elimination of flawed mitochondria (i.e., mitophagy pathway) may be involved in the removal and recycling of defective mitochondria during exercise, improving the resistance to oxidative stress, and potentially the inflammation (Sanchez et al. 2014; Peeri and Amiri 2015; Li et al. 2016). Nevertheless, resistance training, which has minimum impact on mitochondrial function, is also capable of reversing the undesirable effects of age on the autophagy regulatory proteins (Luo et al. 2013). These results support previous investigations showing increased mRNA levels of LC3B and Atg7, and a tendency for LAMP2, in vastus lateralis muscle from older and obese women after an intervention of caloric restriction and moderate-intensity combined resistance and endurance exercise (Wohlgemuth et al. 2011). Therefore, it seems both aerobic and resistance exercise training trigger beneficial adaptations in autophagy response, and that to further potentiate these effects in the elderly, future investigations should carry out dietary interventions concurrently with exercise training. Despite the exercise mode used, exercise-induced autophagy adaptations in PBMCs of elderly subjects may have positive consequences for the elimination of pathogens, decreasing the susceptibility to infections (Cuervo and Macian 2014). Such adaptations may also involve the delivery of specific endogenous or exogenous molecules to intracellular compartments, including modulation of the activity of the inflammasome, control of cytokine secretion, regulation of phagocytosis, and control of mitochondrial ROS and danger-associated molecular pattern (DAMP) production (Shi et al. 2012; Bonilla et al. 2013). Further studies are needed to clarify the precise functions of autophagy in PBMCs after exercise, and the possible involvement of mitophagy or other forms of autophagy.

TLR signaling is finely tuned to prevent harmful immune responses or severe inflammation, and increasing evidence support that autophagy has also important effects on the induction and modulation of the inflammatory reaction mediated by TLRs, with a potential crosstalk between these two pathways (Into et al. 2012). To test this idea, we decided to investigate whether the exercise-induced activation of autophagy was associated to a concomitant downregulation of TLR2 and TLR4-dependent pathways. Contrary to our initial hypothesis, the current aerobic exercise training did not modify the expression of neither TLR2 nor TLR4. Likewise, MyD88 and TRIF protein concentrations remained unchanged after the intervention. Other investigations have previously failed to show aerobic exercise-induced changes in TLR2 and TLR4 signaling pathways after a 15-day endurance program in diabetic patients (Reyna et al. 2013) or after 10 weeks of endurance training in obese athletes (Nickel et al. 2011). Yet, the current data contrast with previous results from our group indicating that other exercise modalities (resistance exercise or whole-body vibration training) downregulate TLR expression in old subjects, resulting in a less proinflammatory state (Rodriguez-Miguélez et al. 2014, 2015). Thus, despite the improved exercise capacity induced by the training protocol employed in the present study, results indicate that the current training failed to induce significant alterations in TLR2 and TLR4 pathways. Although our analyses seem to indicate a lack of association between autophagy and inflammatory adaptations to exercise, other mechanisms connecting both processes should not be excluded. Indeed, novel inflammasome components, such as receptors like Nod (NLR), have been recently proposed as the link between aerobic exercise-induced autophagy adaptations and inflammatory response in rats (Li et al. 2016). Clearly, human studies testing this, and other potential mechanisms explaining the autophagy-inflammatory response to exercise, should be conducted.

In summary, our study shows that aerobic exercise training induces a higher expression of several markers of autophagy in PBMCs from elderly subjects. These results expand on the already established benefits of exercise training in older population. However, it remains to be elucidated whether the reported changes in autophagy are generic responses to muscle activation (i.e., any form of exercise) or specific to the aerobic exercise protocol employed. In addition, the results of the current investigation indicate that aerobic exercise-induced autophagy adaptations are not accompanied by a downregulation of the TLR2 and TLR4 signaling pathways. Thus, it appears that the exercise training model used (e.g., resistance vs aerobic exercise) may be a key factor controlling the response of TLRs. Our data should serve as a platform for further studies designed to reveal the mechanisms by which exercise training upregulates the age-related attenuation of autophagy, and how these effects contribute to the resulting metabolic benefits of exercise. Finally, we believe that novel and effective training protocols should be developed to prevent both the age-related decline in autophagy and the chronic low-grade inflammation.

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