Hepatic LC3 II/I Ratio Is Not Modulated in Exercised Mice

Bruno Brieda MARAFON[1], Ana Paula PINTO[2], Alisson Luiz da ROCHA[2], Rafael Lemes ROVINA[1], José Rodrigo PAULI[3], Leandro Pereira DE MOURA[3], Dennys Esper CINTRA[3], Eduardo Rochete ROPELLE[3], Adelino Sanchez Ramos DA SILVA[1,2]

[1]School of Physical Education and Sport of Ribeirão Preto, University of São Paulo (USP), Ribeirão Preto, São Paulo, Brazil, [2]Postgraduate Program in Rehabilitation and Functional Performance, Ribeirão Preto Medical School, University of São Paulo (USP), Ribeirão Preto, São Paulo, Brazil. [3]Laboratory of Molecular Biology of Exercise (LaBMEx), School of Applied Sciences, University of Campinas (UNICAMP), Limeira, São Paulo, Brazil

Summary

Autophagy plays an essential role in body homeostasis achievement. One of the main proteins involved in this process is the LC3I, which, after lipidation, leads to the formation of LC3II that participates in the formation and maturation of autophagosome. This descriptive study verified the responses of LC3II to LC3I proteins, as well as the time-course of this ratio in mice livers after different types of acute physical exercise protocols. Eight-week-old male C57BL/6 mice were maintained three per cage with controlled temperature (22±2 °C) on a 12:12-h light-dark normal cycle with food (Purina chow) and water *ad libitum*. Mice were randomly divided into four groups: control (CT, sedentary mice), resistance (RE, submitted to a single bout of resistance exercise), endurance (EE, submitted to a single bout of endurance exercise), and concurrent (CE, submitted to a single bout of endurance combined with resistance exercise). The mice livers were extracted and used for the immunoblotting technique. The hepatic LC3B II/I ratio for the RE and EE groups were not altered during the different time-points. For the CE group, there was a decrease in this ratio 12h after exercise compared to time 0 and 18h. Also, the hepatic LC3B II/I ratios were not different among the acute physical exercise protocols along the time-course. The hepatic LC3B II/I ratio was not influenced by the endurance and resistance protocols but decreased in response to the concurrent protocol at 12h after the stimulus.

Corresponding author

Adelino S. R. da Silva. Address: Avenida Bandeirantes, 3900, Monte Alegre, 14040-907, Ribeirão Preto, São Paulo, Brazil. E-mail: adelinosanchez@usp.br

Introduction

Physical exercise is an activity performed systematically to improve cardiorespiratory and cardiovascular functions, structural adaptations, sports performance, as well as energy transfer and distribution (Guerreiro *et al.* 2016, Neufer *et al.* 2015). An acute bout of exercise can induce powerful stress defying cellular homeostasis. Therefore, exercise-induced positive adaptations require an optimal recovery period for stress adaptation and homeostasis unbalance reestablishment. One of the cellular processes involved in the maintenance of homeostasis is autophagy (Schwalm *et al.* 2015). Autophagy is defined as a process in which macromolecules or cell organelles are degraded for the repair and recycling of cytosolic materials, playing an important role in body homeostasis achievement (Cui *et al.* 2013, Mizushima 2007).

The main types of autophagy are macroautophagy, microautophagy, and chaperone-mediated autophagy (Cecconi and Levine 2008, Klionsky 2005). While microautophagy and chaperone-mediated autophagy involve the direct participation of lysosomes, macroautophagy (hereafter referred to as autophagy) is responsible for the turnover of organelles or portions of
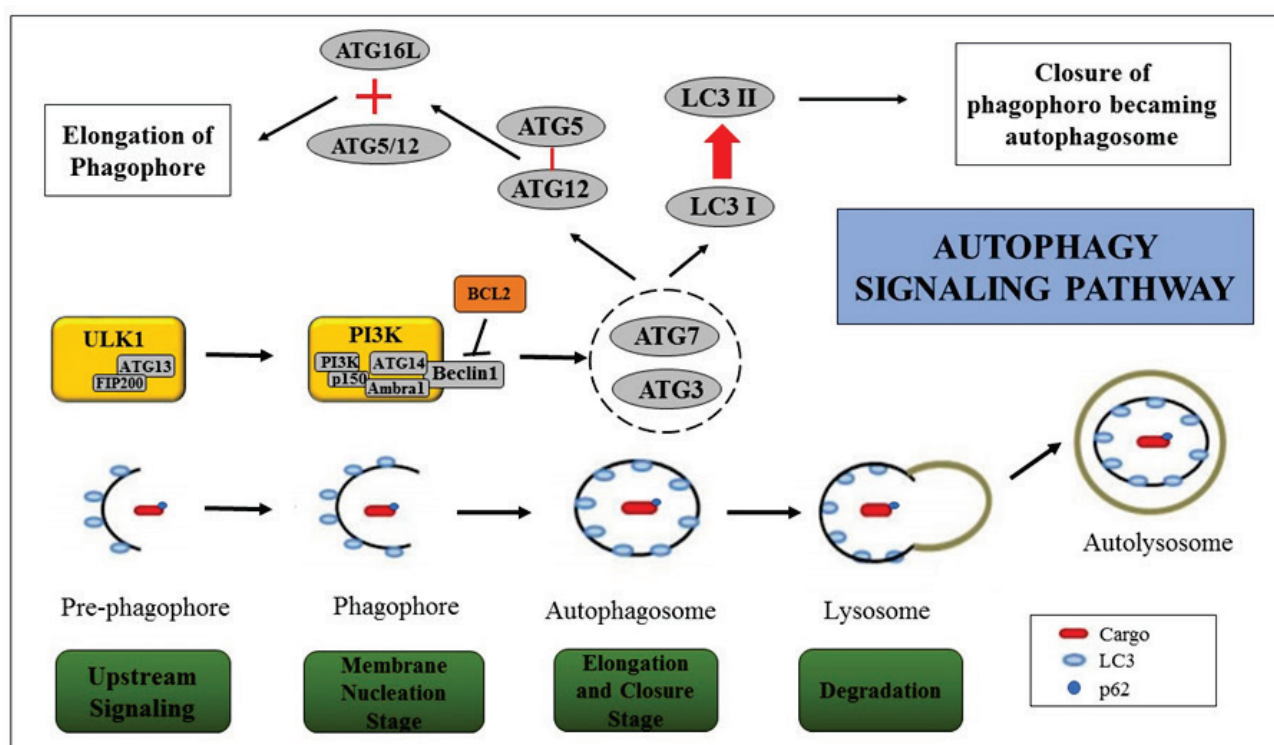
Fig. 1. Schematic model of the autophagy mechanism. The process begins with the formation of the ULK1 complex (ATG13 + FIP200), which leads to the initiation of the signaling pathway and activation of a second complex, PI3K (Beclin1 + PI3K + ATG14 + p150 + Ambra 1). At this stage, BCL-2 is dissociated from Beclin1, and the PI3K complex is conducted to the phagophore membrane and initiates nucleation. ATG7 activation occurs, which will catalyze the conjugation of ATG5 and ATG12. The ATG5/ATG12 complex binds to ATG16L generating phagophore elongation. The ATG7 and ATG3 proteins generate the lipidation of LC3 I forming LC3 II, which conjugates to phosphatidylethanolamine and lead to the closure of phagophore, turning into an autophagosome. Finally, fusion with lysosomes occurs to form autolysosomes for degradation.

the cytosol surrounded by a double membrane vesicle, called autophagosome. At the end of the signaling pathway, this vesicle merges with the lysosome leading to degradation of its content (Cecconi and Levine 2008, Papackova and Cahova 2014). One of the main proteins involved in this process is the microtubule-associated protein 1 light chain 3 (LC3I), which after lipidation, leads to the formation of LC3II that participates in the formation and maturation of autophagosome (Vainshtein and Hood 2016). Figure 1 summarizes the autophagy process.

When the accumulation of dysfunctional organelles occurs, the proper functioning of autophagy is necessary for the degradation and release of the cell nutrients (Schneider and Cuervo 2014). The degradation generates free fatty acids, amino acids, and carbohydrates, which can be recycled to synthesize new cellular components or to produce adenosine triphosphate (Deter *et al.* 1967). These processes are particularly important for highly metabolically active organs such as the liver, which participates in the synthesis, metabolism, storage and redistribution of lipids, carbohydrates, and proteins. Under stress conditions, such as fasting and/or

physical exercise, autophagy participates in the energy generation (He *et al.* 2012, Nonaka *et al.* 2017). Physical exercise can attenuate rates of lipogenesis and increase gene expressions linked to oxidative metabolism concomitant with whole-body fat oxidation enhancement (Townsend *et al.* 2019). The first evidence documented about autophagy and exercise was noted in 1980s. An increase in the number and size of autophagy vacuoles was observed in the liver and skeletal muscle of exercised animals (Salas *et al.* 1980). However, molecular functions of exercise-induced autophagy have recently been investigated.

Kristensen *et al.* (2018) verified that a 1-hour treadmill running was able to increase the liver LC3 II/I ratio immediately after exercise. After 2 hours of exercise, the LC3 II/I ratio returned to baseline values. To the best of our knowledge, the effects of different acute physical exercise protocols on the hepatic LC3 II/I ratio were not previously addressed. Therefore, this investigation aimed to verify the impact of resistance, endurance, and concurrent sessions on the LC3 II/I ratio in mice livers at different time-points.

Methods

Animals

Eight-week-old male C57BL/6 mice were provided by the Central Animal Facility of the Ribeirão Preto campus of the University of São Paulo (USP). The mice were maintained in sterile micro-insulators, three animals per cage with controlled temperature (22±2 °C) on a 12:12-h light-dark normal cycle with food (Purina chow) and water *ad libitum*. The experimental procedures were conducted according to the Brazilian College of Animal Experimentation (COBEA) and approved by the Committee on Ethics in the Use of Animals (CEUA) (n°:2017.5.30.90.8) of the School of Physical Education and Sport of Ribeirão Preto (EEFERP) from University of São Paulo. Mice were randomly divided into four groups: control (CT, sedentary mice), resistance (RE, submitted to a single bout of resistance exercise), endurance (EE, submitted to a single bout of endurance exercise), and concurrent (CE, submitted to a single bout of concurrent exercise).

Experimental procedures

Mice from the RE and CE groups were submitted to a 1-week adaptation period on a ladder-climbing (INSIGHT[TM], Ribeirão Preto, SP, Brazil) with and without external load (Kim HJ, 2015). The ladder had 1110 mm of height, 80° of inclination, and 85 steps with a distance of 6 mm between each. Also, mice from the EE and CE groups were submitted to a 1-week adaptation period on a treadmill (INSIGHT[TM], Ribeirão Preto, SP, Brazil) for five days, 10min/day, at a speed of 6m.min^{-1}.

Incremental load test

The incremental load test was performed 48 hours after the adaptation protocol, and started at an initial velocity of 6m/min, at 0 % of inclination with increments of 3 m/min every 3 min until voluntary exhaustion. Maximum power (Pmax), defined as the animals' exhaustion velocity (m/min), was used to prescribe the intensities for the EE and CE groups (Kuipers 1985).

Acute physical exercise protocols

For the RE group, mice first performed one climb without external load to warm-up. After that, mice performed ten climbs with a 2-minute recovery between each climb. An external load corresponding to 75 % of body weight was applied at the base of the tail of each animal (Wang *et al.* 2015). Mice from the EE group ran at 60 % of the Pmax without inclination for 60 min (Ferreira *et al.* 2007). Mice from the CE group performed one climb without external load to warm-up. After that, mice climbed five sets with 75 % of body weight with a 2-minute recovery between each and ran at 60 % of the Pmax at 0 % of inclination for 30 min. The animals were euthanized immediately, 6, 12, and 18h after the acute physical exercise protocols. Figure 2 illustrates the schematic representation of the experimental procedures.

Glucose levels

The blood from the tail was collected before and immediately after the acute physical exercise protocols, and the glucose levels were measured by the Accu-Check Active glucometer (Accu-Chek[TM] Active model, Roche, Santo André, SP, Brazil).
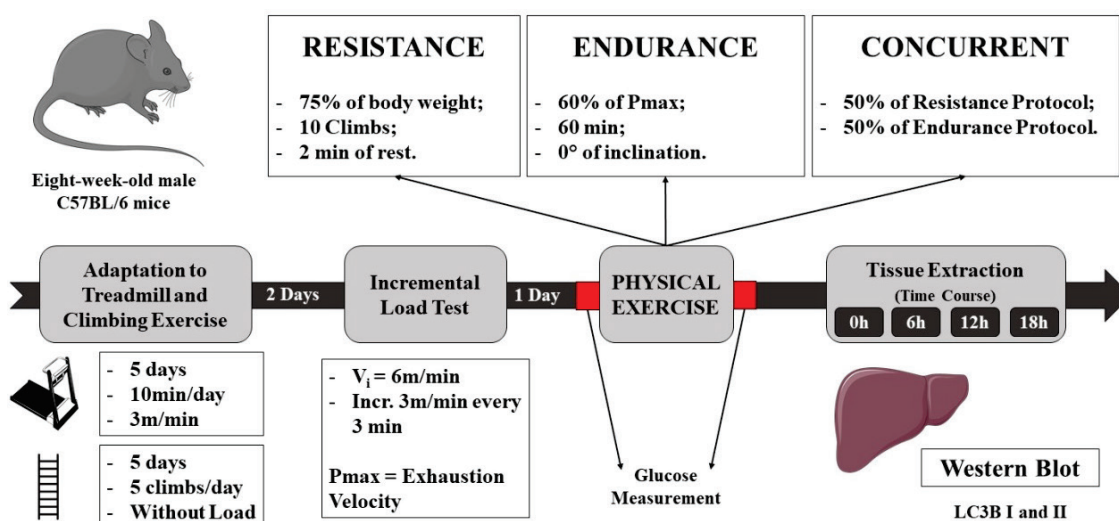

Fig. 2. Schematic representation of the experimental procedures.

Liver extraction

Immediately, 6, 12, and 18h after the acute physical exercise protocols, the mice were anesthetized by an intraperitoneal administration of xylazine (10 mg/kg body weight) and ketamine (100 mg/kg body weight). As soon as the loss of pedal reflexes confirmed the effect of anesthesia, the livers were removed, washed with saline, and used for the immunoblotting technique.

Immunoblotting technique

The immunoblotting technique was performed as previously described (da Rocha *et al.* 2015, da Rocha *et al.* 2016, Morais *et al.* 2018, Pereira *et al.* 2015a, Pereira *et al.* 2015b). Specific antibodies [anti-LC3B (3868S), and anti-alpha tubulin (2144S)] from Cell Signalling Technology (Cell Signalling Technology, MA, USA) were used. Routine chemical reagents were obtained from Sigma Chemical Corporation (St. Louis, MO, USA). The primary antibodies were utilized at a dilution of 1:1.000, and the secondary antibody (7074S) from Cell Signaling Technology (Cell Signaling Technology, MA, USA) was utilized at a dilution between 1:10.000 and 1:20.000. Images were acquired by the C-Digit Blot Scanner (LI-COR, Lincoln, Nebraska, USA) and quantified using the software Image Studio for C-DiGit^{TM} Blot Scanner.

Statistics analysis

Results are expressed as the mean±standard error of the mean (SE). The Shapiro–Wilk's W-test was used to verify data normality, and Levene's test was used to test the homogeneity of variances. One-way analysis of variance (ANOVA), followed by post-hoc Bonferroni's test when necessary, or Kruskal-Wallis test, followed by post-hoc Dunn's test when necessary, were used to verify the effects of acute physical exercise protocols at different time-points. All analyzes were bilateral, and the significance level was set at $p < 0.05$. Statistical analyses were performed using GraphPad Prism 8 software for Windows (IBM, Chicago, IL).

Results

Body weight, training load, and exhaustion time

Table 1 shows the body weight, external load for the RE and CE groups, as well as the maximum power for the EE and CE groups. Regarding body weight, there was no difference between the experimental groups.

Basal and post-exercise glycemia

Table 2 shows that the basal and post-exercise glycemia was not different between the experimental groups. The CE group presented a reduction of glycemia after exercise compared to its basal level.

Effects of the different acute physical exercise protocols on the hepatic LC3B II/I ratio

The LC3B II protein is essential for autophagosome formation. During the autophagy process, the lipidation of LC3B I to LC3B II occurs. When the

Table 1. Body weight, external load, and maximal power for the experimental groups.

Parameters	CT	RE	EE	CE
Body weight (g)	23.46±1.23	23.64±1.02	22.93±1.14	21.7±0.98
External load (g)	-	18.3±1.06	-	16.9±0.81
Maximum power (m/min)	-	-	20.6±3.47	22.8±2.11

Data correspond to the mean±standard error of the mean (SE) of n=5 mice. CT: sedentary mice, RE: mice submitted to a single bout of resistance exercise, EE: mice submitted to a single bout of endurance exercise, CE: mice submitted to a single bout of concurrent exercise.

Table 2. Basal and post-exercise glycemia for the experimental groups.

Glycemia	CT	RE	EE	CE
Basal (mg/dl)	157.4±5.51	173.6±7.94	164.4±5.59	154.6±6.05
Post-exercise (mg/dl)	-	187.2±12.25	147.8±24.14	126.0±10.4*

Data correspond to the mean ± standard error of the mean (SE) of n=5 mice. CT: sedentary mice, RE: mice submitted to a single bout of resistance exercise, EE: mice submitted to a single bout of endurance exercise, CE: mice submitted to a single bout of concurrent exercise. *p<0.05 *versus* basal glycemia for the same group.
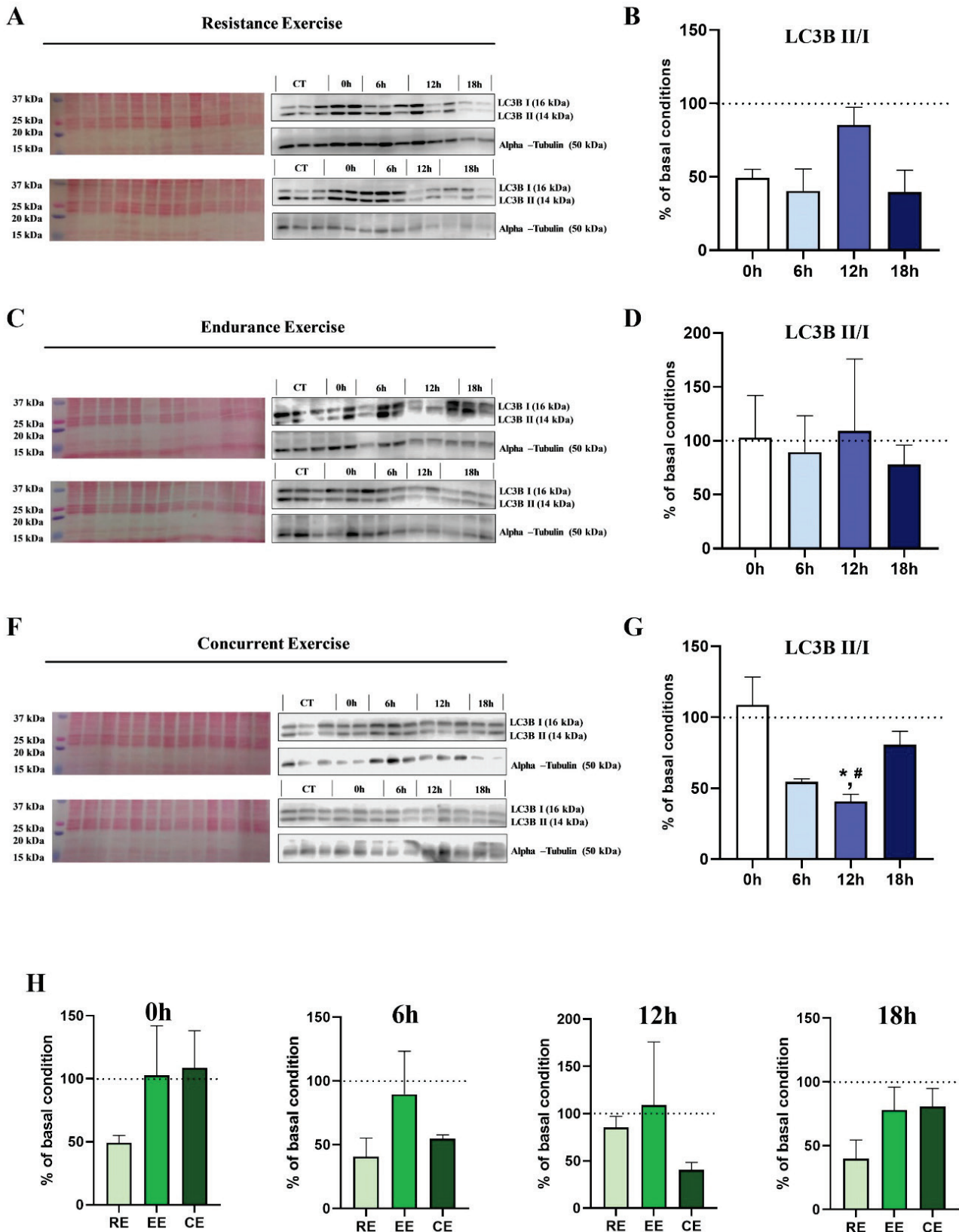
Fig. 3. A, C, E) Representative Ponceau and original bands with kDa for each protein. **B**) Percentage to the basal condition of the LC3B II to LC3B I ratio in the liver after the resistance exercise, **D**) endurance exercise, and **F**) concurrent exercise at 0, 6, 12, and 18 hours post-exercise. **H**) Comparison of the different types of acute physical exercise protocols on the LC3B II/I ratio at 0, 6, 12, and 18 hours post-exercise. *p < 0.05 *versus* 0h, #p < 0.05 *versus* 18h. Data correspond to the mean ± SE of n=5/mice.

LC3B II/I ratio is up or down-regulated, we can suggest that autophagy is present or absent, respectively. Figures 3A, 3C, and 3F demonstrate the representative Ponceau and original bands with kDa of the hepatic LC3B II/I ratios immediately, 6, 12, and 18h after the acute physical exercise protocols. Figure 3B and 3D show that the hepatic LC3B II/I ratio for the RE and EE groups were not altered during the time-course. Figure 3G shows that the hepatic LC3B II/I ratio for the CE group decreased significantly 12h after exercise compared to 0 and 18h. Figure H displays the hepatic LC3B II/I ratios were not different among the acute physical exercise protocols at any time-point.

Discussion

The main finding of this study was that hepatic LC3B II/I ratio evaluated 12h after the CE protocol was attenuated compared to the 0 and 12h, while there was no difference for this ratio among the EE and RE groups along the time-course. This is the first investigation showing the effects of RE and CE on the hepatic protein contents of the LC3B II/I ratio. Regarding the glycemia, the EE group did not exhibit a difference between basal and post-exercise situations. In agreement with our study, other investigations visualized that a single bout of endurance exercise did not change glycemia, probably because the hepatic glucose production is higher during the exercise session, compensating the higher glucose uptake (Adams 2013). On the other hand, in response to resistance exercise, glycemia was attenuated in some studies (Black *et al.* 2010, Liu *et al.* 2019, Yardley *et al.* 2013), differently from our RE group that did not present statistical difference, probably because the exercise training was not intense enough. For the concurrent group, the glycemia was lower after exercise compared to basal levels, the same as demonstrated by Yardley *et al.* (2012). One possible explanation is that the first bout of exercise (i.e., RE) reduced blood glucose levels by stimulating glucose transport (Krisan *et al.* 2004). When the second bout of exercise (i.e., EE) started, the hepatic glucose production did not overcome the glucose uptake.

Kristensen *et al.* (2018) verified an increase in the hepatic LC3B II/I ratio immediately after 1 hour of treadmill running at 15m/min with 10° of inclination, which was linked to enhanced autophagy. After 2 hours of exercise, the LC3B II/I ratio returned to the basal levels, indicating a transient increase in exercise-induced autophagy. In accordance, Kwon *et al.* (2019) verified

that five days of moderate-intensity exercise increased LC3B II/I ratio in mice livers. On the other hand, our EE protocol did not lead to significant changes in the hepatic LC3B II/I ratios at 0, 6, 12, and 18h post-exercise. Comparing our endurance exercise protocol with those used by Kristensen *et al.* (2018) and Kwon *et al.* (2019), we can conclude that the treadmill inclination and total exercise volume contributed to the lack of significant results in our investigation.

He *et al.* (2012) visualized an increase in autophagosome numbers in skeletal and cardiac muscles after 30 minutes of running, reaching a plateau at 80 minutes. This increase was caused by lipidation of LC3I and degradation of p62 protein. Also, the authors concluded that autophagy after acute endurance exercise was present in other organs involved in energy homeostasis such as the pancreas, adipose tissue, and liver. In the same study, the treadmill was also inclined at 10°, being a possible explanation for the increase in autophagosome and autophagy induction. Fry *et al.* (2012) demonstrated that an acute resistance exercise decreased the LC3B II/I ratio in the skeletal muscle of adolescent and elderly subjects due to a reduction in the LC3II since LC3I was unchanged. Once the conversion of LC3I to LC3II is a marker of increased autophagy, the authors concluded that this protocol led to an autophagic flux reduction (Fry *et al.* 2012). On the other hand, Pinto *et al.* (2020) did not find significant changes in the LC3B II/I 2h after an acute resistance exercise protocol, as well as Glynn *et al.* (2010) did not observe a modulation of the LC3II protein content after 1 hour of resistance exercise. Regarding the hepatic tissue, the combination of fasting, acute resistance exercise, and protein ingestion also did not change the LC3B II/I ratio (Pinto *et al.* 2020).

To the best of our knowledge, this is the first study to investigate the hepatic LC3B II/I ratio after an acute bout of concurrent exercise. Interestingly, we found a significant decrease of this autophagic marker 12h after the exercise stimulus compared to 0 and 18h. This decrement may be explained by the reduction of LC3II protein levels at 12h compared with the other time-points. Smiles *et al.* (2016) demonstrated a decrease in LC3II protein levels 2 and 8h after a concurrent exercise in skeletal muscle of individuals who ingested alcohol and carbohydrate. Also, the authors did not observe a significant change in the AMP-activated protein kinase protein, suggesting that autophagy could be repressed. Further investigations regarding the effects of concurrent training on hepatic autophagy are necessary for a better

comprehension of this relationship.

Although not significant, the hepatic LC3B II/I ratios of the RE group were lower in comparison with the EE and CE groups at 0 and 18h. These data may be linked to the lower volume of the RE (≈20min) compared to the EE (≈150min) and CE (≈40min) sessions. A limitation of the present study was the difference in our exercise protocols compared to those described by other authors (He *et al.* 2012, Kristensen *et al.* 2018, Kwon *et al.* 2019), which indicated that treadmill inclination or total exercise volume contributed to their significant alterations in the hepatic LC3B II/I ratios. For future investigations, it would be interesting to insert two groups performing the same volume of endurance exercise but with different inclinations (i.e., 0° *versus* 10° treadmill inclination).

In conclusion, one acute session of resistance or endurance was not able to generate significant changes in the hepatic LC3B II/I ratios at different time-points. On the other hand, the hepatic LC3B II/I decreased 12h after the concurrent protocol compared to 0 and 18h. Although the hepatic LC3B II/I ratios were not different among the acute physical exercise protocols at any time-point, further studies should verify the chronic effects of these exercise protocols and their relationships with hepatic functional and morphological adaptations.

Conflict of Interest

There is no conflict of interest.

Author's contributions

The substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data: Marafon B.B, Pinto A.P, da Rocha A.L, Rovina R.L and da Silva A.S.R. Involvement in drafting the manuscript or revising it critically for important intellectual content: Marafon B.B, Pinto A.P, da Rocha A.L, Rovina R.L, Pauli J.R, de Moura L.P, Cintra D.E, Ropelle E.R, and da Silva A.S.R. Final approval of the version to be published: Marafon B.B, Pinto A.P, da Rocha A.L, Rovina R.L, Pauli J.R, de Moura L.P, Cintra D.E, Ropelle E.R, and da Silva A.S.R.

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