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Original Research

Effect of Acute Aerobic Exercise and Rapamycin Treatment on Autophagy in Peripheral Blood Mononuclear Cells of Adults With Prediabetes

James J. McCormick PhD [a,*]; Kelli E. King MS [a]; Karol Dokladny PhD [b]; Christine M. Mermier PhD [a]

[a] Department of Health, Exercise, and Sports Sciences, University of New Mexico, Albuquerque, New Mexico, United States
[b] Department of Internal Medicine, University of New Mexico, Albuquerque, New Mexico, United States

Key Messages

- Autophagy may be impaired after an acute aerobic exercise bout in peripheral blood mononuclear cells from people with prediabetes.
- Autophagy may be impaired after acute rapamycin treatment in peripheral blood mononuclear cells from people with prediabetes.

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ABSTRACT

Objectives: Recently, a malfunction of the autophagic pathway has been implicated with impaired glucose metabolism and progression from prediabetes to type 2 diabetes. The aims of this study were to investigate the effect of exercise and rapamycin (RAPA) treatment on the autophagic process in peripheral blood mononuclear cells (PBMCs) from people with prediabetes compared with control subjects.
Methods: Two groups matched for age and sex served as participants and included 6 participants with prediabetes (42.4±11.7 years) and 6 control subjects (44.4±11.9 years). Participants exercised at 50% of maximal oxygen consumption for 60 min with 5 min of rest interspersed every 20 min. PBMCs were isolated pre-exercise, immediately postexercise and 4 h after exercise recovery. Additional PBMCs were incubated for 24 h and either exposed to bafilomycin, rapamycin with bafilomycin (RAPA), or no treatment with vehicle (dimethyl sulfoxide). Proteins and mRNA were analyzed via western blot and quantitative real-time polymerase chain reaction, respectively.
Results: Exercise increased autophagy immediately postexercise and recovered 4 h after exercise in control participants but not in participants with prediabetes. Autophagy increased in PBMCs from people with prediabetes and control participants after RAPA treatment; however, a significantly impaired autophagic response was observed in people with prediabetes when compared with control subjects.
Conclusions: Our results indicate an impairment in autophagic flux in PBMCs from people with prediabetes when compared with control subjects in response to both exercise and RAPA treatment. Future methods of autophagic upregulation should be investigated to spare malfunctions in autophagy in people with prediabetes.

Mots clés :
autophagie
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prédiabète
rapamycine

RÉSUMÉ

Objectifs : Récemment, il a été montré qu'un dysfonctionnement de la voie autophagique a été impliqué dans une altération du métabolisme du glucose et une progression d'un prédiabète en diabète de type 2. Les objectifs de cette étude étaient d'étudier l'effet de l'exercice et d'un traitement à la rapamycine (RAPA) sur le processus autophagique dans les cellules mononucléées du sang périphérique (CMSP) de personnes prédiabétiques comparativement aux sujets témoins.

* Address for correspondence: James J. McCormick PhD, Department of Health, Exercise, and Sport Science, University of New Mexico, Johnson Center B143, 200 Cornell Dr, Albuquerque, New Mexico 87131, United States.
E-mail address: jeremy.mccormick.j@gmail.com

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Méthodes : Les participants étaient répartis en deux groupes appariés pour l'âge et le sexe et comprenaient 6 participants atteints de prédiabète (42.4±11.7 ans) et 6 sujets témoins (44.4±11.9 ans). Les participants ont fait de l'exercice à 50 % de la consommation maximale d'oxygène pendant 60 minutes avec 5 minutes de repos toutes les 20 minutes. Les CMSPs ont été isolées avant l'exercice, immédiatement après l'exercice physique et 4 heures après la récupération de l'exercice. D'autres CMSPs ont été incubées pendant 24 h et exposées soit à la bafilomycine, à la rapamycine avec de la bafilomycine (RAPA) ou sans traitement avec véhicule seul (diméthylsulfoxyde). Les protéines et l'ARNm ont été analysés respectivement par immunobuvardage de type Western et par réaction en chaîne par polymérase en temps réel quantitative.
Résultats : L'exercice a augmenté l'autophagie immédiatement après l'exercice puis celle-ci s'est rétablie 4 h après l'exercice chez les participants témoins, mais pas chez les participants atteints de prédiabète. L'autophagie a augmenté dans les CMSPs chez les personnes atteintes de prédiabète et chez les participants témoins après le traitement à la RAPA; cependant, une réponse autophagique significativement altérée a été observée chez les personnes atteintes de prédiabète comparativement aux sujets témoins.
Conclusions : Nos résultats indiquent une diminution du flux autophagique dans les CMSPs chez les personnes atteintes de prédiabète par rapport aux sujets témoins en réponse à l'exercice et au traitement à la RAPA. Les futures méthodes promouvant l'autophagie devraient être étudiées pour éviter des dysfonctionnements de l'autophagie chez les personnes atteintes de prédiabète.

Introduction

Type 2 diabetes is a disease with increasing prevalence that has become a global health problem that affects people of all socioeconomic classes. According to the International Diabetes Federation Atlas (1), the estimated diabetes prevalence in 2017 was 425 million, with >641 million people predicted to be living with type 2 diabetes worldwide by the year 2040. It is well established that type 2 diabetes is associated with increased risk of developing microvascular disorders, including nephropathy, neuropathy and retinopathy (2). Furthermore, individuals with type 2 diabetes have increased morbidity and mortality because of increased risk of cardiovascular disorders, such as coronary artery disease, hypertension and stroke (3,4). However, prior to the development of type 2 diabetes, a period of prediabetic state occurs, which may be characterized by impaired glucose and insulin tolerance, mild to moderate obesity and fluctuations in normoglycemic and hyperglycemic states (5).

Exercise is known to play a fundamental role in the prevention and treatment of type 2 diabetes, in part because of improvements in insulin sensitivity and increased maximal oxygen consumption (V_{O_2}max) (6). Additionally, exercise-induced adaptations include an increased abundance of proteins involved with insulin signalling (e.g. insulin receptor substrate 1) and glucose metabolism important in cellular homeostasis (7,8). However, these metabolic adaptations have been reported to be impaired in patients with type 2 diabetes and patients with prediabetes, indicating a potential resistance to the beneficial effects of exercise (9).

Macroautophagy (herein referred to as autophagy) is a catabolic cellular process, which involves the degradation of protein aggregates and damaged organelles, and is crucial in maintaining cellular homeostasis (10). Autophagic flux refers to the complete process of autophagy, including the amount and rate of cargo sequestered and degraded (11). Stimulation of autophagic flux has been shown to be crucial in the development of cellular adaptations to exercise (12). However, disruption of autophagy in mice has been shown to impair endurance exercise performance and glucose metabolism during an acute exercise bout (13). Furthermore, impaired autophagic functioning may contribute to the development of prediabetes and type 2 diabetes through the development of insulin resistance (14) and other comorbidities including neurodegenerative disorders (15,16), cardiomyopathy (17) and cancer (18). Although the beneficial effects of exercise in prediabetes and type 2 diabetes have been well established (19), little is known about the effect of exercise on autophagy in people with prediabetes and people with type 2 diabetes. It has recently been demonstrated that an acute bout of endurance exercise (1 to 2 h) is sufficient to stimulate autophagic signalling in skeletal muscle of young healthy adult men (20,21). Recent evidence suggests that the acute autophagic response may be intact in human skeletal muscle of people with type 2 diabetes in response to 60 min of cycling exercise at 70% of V_{O_2}max (22). This may be because of an autophagic adaptation to chronic hyperglycemic conditions; however, it is unknown if people with prediabetes exhibit this adaptive response (23). Therefore, the autophagic response to exercise in individuals with prediabetes remains to be established.

The aim of this study was to investigate the response of autophagy in response to an acute bout of exercise and subsequent recovery in peripheral blood mononuclear cells (PBMCs) of individuals with prediabetes compared with age- and sex-matched control subjects. Because PBMCs come into contact with every cell in the human body and are sensitive to various physiological, pathologic and environmental changes, they were chosen as a representation of the whole body autophagy response (24). To further elucidate the mechanism of autophagic modulation, we treated PBMCs from people with prediabetes with the known autophagy inducer, rapamycin (RAPA).

Methods

Human participants

The study was approved by the University of New Mexico's Human Research Review Committee (HRPO-14-200), and all participants provided written informed consent. Twelve adult participants (6 men and 6 women) between the ages of 27 and 61 years and who were free of known illness or disease were recruited. This sample size was determined via an a priori power analysis (G*Power; Universität Düsseldorf, Düsseldorf, Germany) which estimated 12 participants were required based on the variable *microtubule-associated light chain 3 beta* (*LC3*)-*II* (25). Participants falling outside of the age ranges, currently taking glucose-lowering or statin medications or experiencing an acute illness were excluded. Participants were categorized into the group with prediabetes, which was determined via a glycated hemoglobin (A1C) blood test value >5.7% and <6.4% (26). Control participants were age and sex matched with the group with prediabetes and categorized by having an A1C value <5.7%.

Maximal exercise test

Prior to the experimental trial, all participants were asked to refrain from rigorous exercise for at least 48 h, to abstain from alcohol for at least 24 h and to abstain from caffeine for 12 h. A small blood sample was drawn for the measurement of A1C. Waist circumference, height and weight were recorded, and body fat percentage was estimated using a bioelectrical impedance analyzer (HBF-306; Omron Healthcare, Bannockburn, Illinois, United States) immediately prior to the measurement of V_{O_2}max. All V_{O_2}max tests were conducted on a cycle ergometer (Excalibur sport; Lode BV, Groningen, The Netherlands) and included a 5-min self-selected warm-up. A maximal exercise test was performed using a ramp protocol in which power was increased every 60 s at an individualized rate, based on participant's size, exercise and health history to induce fatigue within 8 to 12 min. Expired air was analyzed breath-by-breath during the exercise test using a metabolic cart (TrueOne 2400; Parvo Medics, Sandy, Utah, United States) and calibrated as recommended by the manufacturer.

Aerobic exercise session

Participants were asked to refrain from vigorous exercise for at least 48 h and to abstain from caffeine and alcoholic beverages for at least 24 h before the experimental trial. The participants ingested a standardized breakfast (240 kcal of total energy intake, 62% carbohydrate, 17% protein and 21% fat) 2 h before the start of exercise. Once the participants arrived at the laboratory, they rested for 20 min in a seated position before the initial blood draw and exercise session.

The exercise session consisted of a 5-min warm-up while cycling at an intensity corresponding to 20% of the peak power achieved in the V_{O_2}max test. The workload was then increased to correspond with 50% of V_{O_2}max, as confirmed by the metabolic cart. Participants continued to exercise for 60 min (3 sets of 20 min separated by 5-min intervals of passive rest). Water was provided ad libitum throughout the exercise trials.

PBMC collection

Venous blood was collected via venipuncture from an antecubital vein prior to the aerobic exercise session and after 20 min in a seated position. Venous blood was also drawn immediately after the 60-min exercise session and 4 h after termination of the exercise session. PBMCs were immediately isolated from whole blood suspended in Histopaque-1077 (Sigma-Aldrich) and centrifuged at 1.1 relative centrifugal force (RCF) for 30 min. Cells were then washed 3 times with phosphate-buffered saline (P4417; Sigma-Aldrich) and frozen at −80°C until analyzed.

Cell experiments

Immediately prior to the aerobic exercise session, PBMCs were isolated from whole blood suspended in Histopaque-1077 (Sigma-Aldrich), counted to ensure equal cell densities between all participants (approximately 4×10^{-6} cells) and transferred to cell culture plates in duplicate. Cells were then incubated at 37°C for a period of 24 h in cell culture medium containing MegaCell RPMI-1640 (F4135; Sigma-Aldrich), 1% L-glutamine (25030081; Thermo Fisher Scientific), 1% Penicillin-Streptomycin (15140122; Thermo Fisher Scientific) and 5% fetal bovine serum (F4135; Sigma-Aldrich) to allow for cell homogenization. PBMCs were then treated with dimethyl sulfoxide (DMSO) vehicle (0.25% DMSO; 472301; Sigma-Aldrich), bafilomycin (BAF) A1 in DMSO (tlrl-bafl; InvivoGen) (100 nM) or BAF and RAPA in DMSO (tlrl-rap; InvivoGen) (0.5 nM) for 2 h at 37°C. BAF was used to block the formation of the autophagolysosome preventing *LC3-II* protein degradation, therefore enabling the quantification of autophagy via *LC3-II* accumulation (11,27). Treatment with RAPA simulated starvation-induced autophagy via upstream inhibition of *mammalian target of rapamycin* (*mTOR*). Cells were immediately harvested, washed 3 times in phosphate-buffered saline and stored at −80°C until analyzed.

Immunoblot analysis

Cells were lysed in a modified radioimmunoprecipitation assay (RIPA) buffer (Tris-HCl 8.0 pH; 15568-025; Invitrogen), 0.5 M EDTA (15568-020; Invitrogen), Sodium chloride 1.5M (S9888; Sigma-Aldrich), 1% Triton X100 (Sigma-Aldrich) and freshly added protease (78430; Thermo Fisher Scientific) and phosphatase (7842; Thermo Fisher Scientific) inhibitors. *LC3, p62/SQSTM1* and *beta actin* were resolved by electrophoresis in a 12% polyacrylamide gel (456-144; Bio-Rad, Touch, California, United States). Total protein concentration was measured (DC Protein Assay, 5000112; Bio-Rad), and equal amounts of protein (40 μg) were transferred to nitrocellulose membranes (162-0094; Bio-Rad) and then blocked in Tris-buffered saline (150 mM NaCl, pH 8.0) containing 0.2% polysorbate (Tween 20; 170-6531; Bio-Rad) detergent and 5% powdered milk (170-6404; Bio-Rad). Membranes were then incubated in Tris-buffered saline containing 0.2% polysorbate detergent and 5% bovine serum albumin (A9418; Sigma-Aldrich) with the following primary antibodies: *LC3* (1:1,000, L7543; Sigma-Aldrich), *p62/SQSTM1* (1:1,000, ab56416; Abcam) and *β actin* (A5441; Sigma-Aldrich). Primary antibodies were detected by horseradish peroxidase-labelled secondary antibody (goat anti-rabbit, 7074s; Cell Signaling and goat anti-mouse, 70076s; Cell Signaling) binding, which was detected using Western Blotting Luminol Reagent (Santa Cruz Biotechnology, Santa Cruz, California, United States) and imaged with ChemiDoc Touch Imaging System (Bio-Rad).

Quantitative reverse transcription–polymerase chain reaction

Total RNA was isolated from human PBMCs using the QIAshredder (79654; QIAGEN) and RNeasy Mini Kit (74104; QIAGEN). It was then reverse transcribed using the cDNA Reverse Transcription Kit (64869866001; Roche Life Science). Complementary DNAs (cDNAs) were amplified in a StepOnePlus Lightcycler (Applied Biosystems, Grand Island, New York, United States) using the following amplification conditions: polymerase chain reaction initial activation step, 95°C for 10 min; 2-step cycling, 40 cycles of denaturation, 95°C for 15 s and combined annealing/extension, 60°C for 1 min. The sequence-specific primers used in the reactions were *MAP1LC3B* (Hs00792944_s1; Applied Biosystems) and *p62/SQSTM1* (Hs01061917_g1; Applied Biosystems). Gene expression was normalized to reference gene *β actin* (internal control gene) (4326315E; Applied Biosystems). Relative quantification of *MAP1LC3B* and *p62/SQSTM1* genes were calculated relative to

Table 1
Clinical and metabolic characteristics at study entry

Characteristic	Control group (n=6)	Prediabetes group (n=6)
Age, years	42.4±11.7	44.4±11.9
BMI, kg/m^2	23.7±2.5	24.2±5.6
Body fat, %	23.5±7.2	25.6±5.9
Waist circumference, cm	80.8±3.5	83.9±15.3
V_{O_2}max, mL/kg/min	38.6±9.1	36.8±6.8
A1C, %	5.3±0.1	6.0±0.2*

A1C, Glycated hemoglobin; *BMI*, body mass index; *V_{O_2}max*, maximal oxygen consumption.
Note: Data are presented as mean ± SD.
* $p<0.05$ compared with control subjects.

β actin, and the mean fold change in expression of the *MAP1LC3B* and *p62/SQSTM1* genes were calculated using the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

All data are presented as mean ± SD. Significant interactions between groups and within group comparisons were performed by a repeated-measures 2-way analysis of variance, followed by a Tukey posttest, using SPSS version 19 software (IBM). Single statistical comparisons of clinical and metabolic characteristics between people with prediabetes and control subjects were performed with a 2-tailed Student *t* test. All reported levels of significance are indicated as $p<0.05$.

Results

Clinical and metabolic characteristics

Participants with prediabetes had significantly higher A1C levels ($p=0.001$) than control participants but showed a similar $V_{O_2}max$ ($p=0.36$), indicating minimal differences in cardiorespiratory fitness between groups. Furthermore, no differences were observed in body mass index ($p=0.47$), body fat percentage ($p=0.56$) or waist circumference ($p=0.49$) between participants with prediabetes and control participants (Table 1).

Effect of aerobic exercise on markers of autophagy

The protein abundance of important autophagy markers was examined between PBMCs from participants with prediabetes and PBMCs from control subjects. *LC3-II* and *p62/SQSTM1* are common autophagic markers that are degraded during periods of increased autophagy in most cells. There was a significant increase in *LC3-II* protein accumulation ($p=0.03$) after 4 h of recovery in the control group. Lipidation of *LC3-I* to *LC3-II* is a commonly used indicator of autophagosome abundance (28). A significant decrease in the *LC3-II/LC3-I* ratio was observed immediately postexercise in the control group ($p=0.04$), but not in participants with prediabetes, which was restored after 4 h of recovery (Figure 1C).

The mRNA levels of *MAP1LC3B* and *p62/SQSTM1* were also examined. No significant differences were observed under any condition; however, participants with prediabetes showed an observed lower expression of all genes compared with control subjects under all conditions.

Effect of RAPA treatment on markers of autophagy

RAPA treatment was used to block *mTORC1*, which is a known autophagy negative regulator (11). Additionally, BAF was used to block autophagic degradation. A significant increase in *LC3-II* protein was observed after BAF treatment in the control group ($p<0.01$) but not the group with prediabetes ($p=0.12$) (Figure 2A). After RAPA treatment, a significant increase in *LC3-II* protein abundance was observed in both the group with prediabetes ($p=0.04$) and the control group ($p=0.01$); however, the increase in the control group was significantly higher than the group with prediabetes ($p=0.02$) (Figure 2A). A significant reduction in *p62/SQSTM1* protein abundance compared with no treatment was observed after BAF treatment in the group with prediabetes ($p=0.01$) but not the control group, with a significant reduction

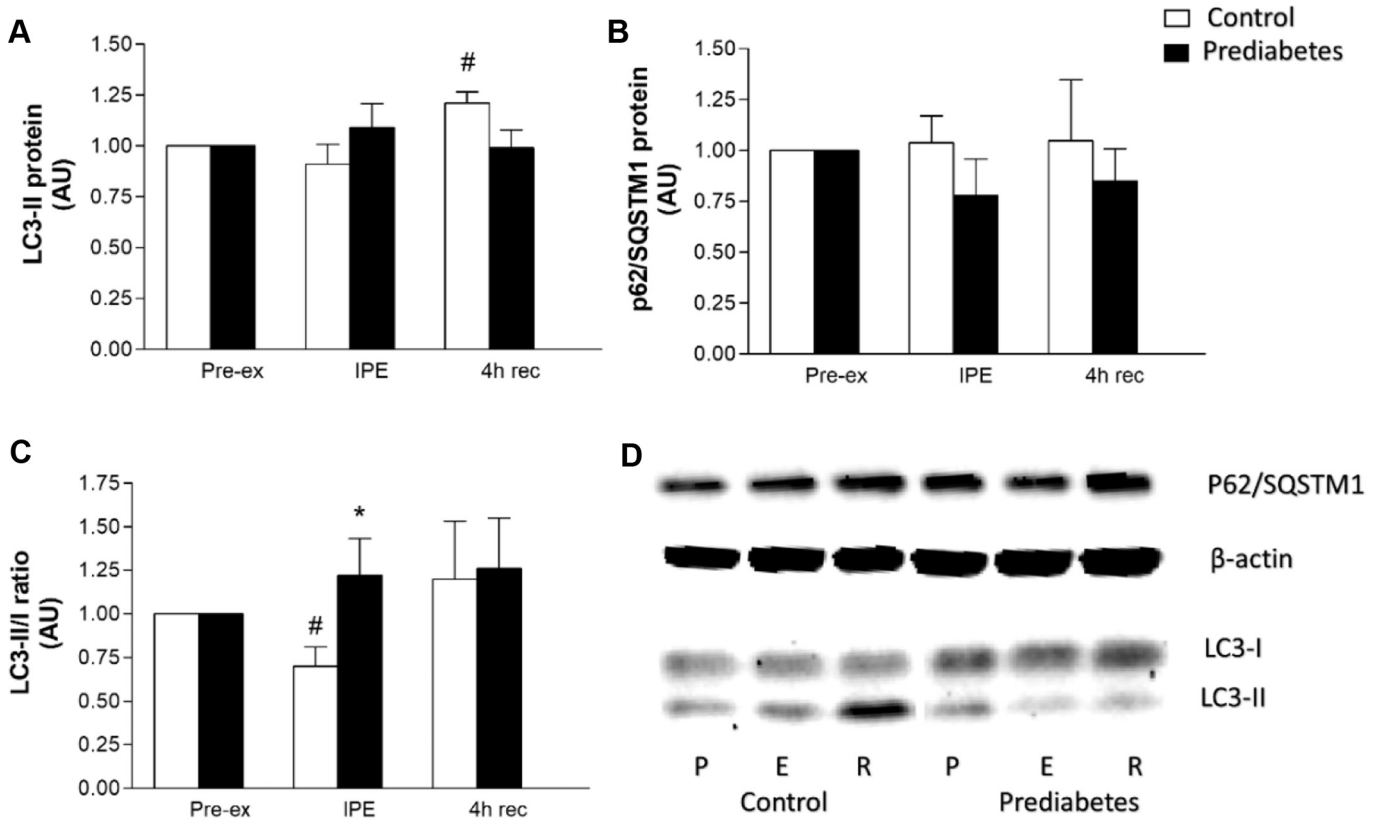

Figure 1. Exercise-mediated regulation of protein markers of autophagy. Protein content of (A) *LC3-II*, (B) *p62/SQSTM1* and (C) *LC3-II/LC3-I* in peripheral blood mononuclear cells of individuals with prediabetes (black bars) and control individuals (white bars) before exercise, immediately after exercise and 4 h into recovery. (D) Representative blots. Data are mean ± SEM. *$p<0.05$ compared with respective control subjects; #$p<0.05$ compared with preexercise. *4h rec*, 4 h into recovery; *E*, postexercise; *IPE*, immediately after exercise; *LC3*, light chain 3 beta; *P*, pre-exercise; *pre-ex*, before exercise; *R*, 4 h into recovery.

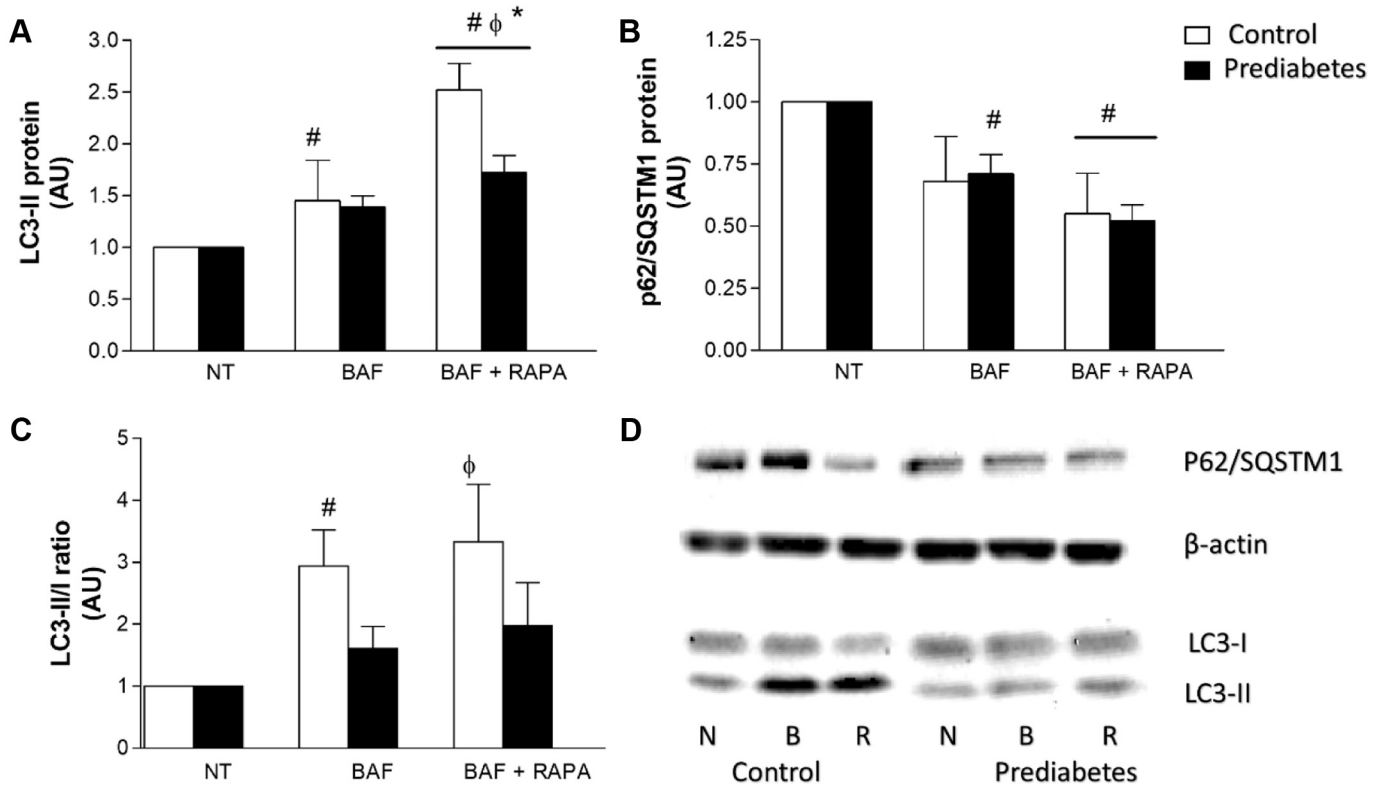

Figure 2. Rapamycin-mediated regulation of protein markers of autophagy. Protein content of (A) *LC3-II*, (B) *p62/SQSTM1* and (C) *LC3-II/LC3-I* in peripheral blood mononuclear cells of individuals with prediabetes (black bars) and control individuals (white bars) with no treatment, bafilomycin treatment and bafilomycin with rapamycin treatment. (D) Representative blots. Data are mean ± SEM. *p<0.05 compared with respective control subjects; #p<0.05 compared with no treatment; ϕp<0.05 compared with bafilomycin. *B*, bafilomycin treatment; *BAF*, bafilomycin treatment; *BAF + RAPA*, bafilomycin with rapamycin; *LC3*, light chain 3 beta; *N*, no treatment; *NT*, no treatment; *R*, bafilomycin with rapamycin treatment.

of *p62/SQSTM1* in both groups (p<0.01 and p=0.03 for the group with prediabetes and control group, respectively) compared with no treatment after RAPA treatment (Figure 2B). A significant increase in the *LC3-II/LC3-I* protein ratio was observed under BAF and RAPA conditions for the control group (p<0.01 and p=0.03, respectively) but not the group with prediabetes (p=0.18 and p=0.25 for BAF and BAF+RAPA conditions, respectively) (Figure 2C).

When examining differences in gene expression, no changes were observed in *MAP1LC3B* expression between groups under BAF or RAPA conditions. A significant increase was observed in p62/SQSTM1 expression after RAPA treatment compared with no treatment in both control subjects (p=0.02) and people with prediabetes (p<0.01) (Figure 3D).

Discussion

In this study, we examined the impact of an acute bout of moderate intensity (50% V_{O_2}max) endurance exercise on markers of autophagy response in PBMCs of individuals with prediabetes and age- and sex-matched control subjects. To further understand differences in autophagy between people with prediabetes and control subjects, we treated PBMCs from the same participants with prediabetes and control subjects with RAPA. Our findings suggest a minor impairment of the autophagic response in people with prediabetes immediately postexercise. In control individuals, a recovery of autophagic flux was observed within 4 h of exercise recovery that was not observed in participants with prediabetes. Furthermore, we found a blunted autophagic response in the group with prediabetes compared with control subjects when exposed to RAPA treatment.

In response to acute endurance exercise, there was a decrease in the *LC3-II/LC3-I* ratio immediately after exercise cessation in the control group but not the group with prediabetes, indicating greater autophagic flux (11); however, levels were restored within 4 h of recovery. This is further evidenced by the significant increase in *LC3-II* protein accumulation observed in the control group 4 h postexercise, but not in the group with prediabetes. The observed drop in the *LC3-II/LC3-I* protein ratio in the control group is similar to previous findings that found a drop in the *LC3-II/LC3-I* protein ratio immediately after acute endurance exercise in human skeletal muscle of young, healthy men (20,22). However, previous findings by Kruse et al (22) did not show any differences in autophagic initiation between patients with type 2 diabetes and control subjects after acute endurance exercise in human skeletal muscle. Because autophagy measurements were only made in PBMCs in the present study, these differences may be explained by the highly tissue-specific nature of the autophagic response to stress (29). Previous findings have reported significantly reduced *MAP1LC3B* mRNA expression after exercise and 3 h of recovery in patients with type 2 diabetes when compared with control subjects and no differences in *p62/SQSTM1* in skeletal muscle (22). Our present findings show no differences in *MAP1LC3B* or *p62/SQSTM1* mRNA expression, suggesting transcriptional regulation of autophagy remains intact in people with prediabetes.

Increased levels of *LC3-II* and decreased levels of *p62/SQSTM1* are typically indicative of increased autophagic flux (11). We found no differences in *p62/SQSTM1* protein abundance in response to exercise in either group. These results are similar to previous findings, which showed no difference in *p62/SQSTM1* protein regulation after acute exercise in healthy, young men (20,21) or in response to acute exercise in muscle from patients with type 2

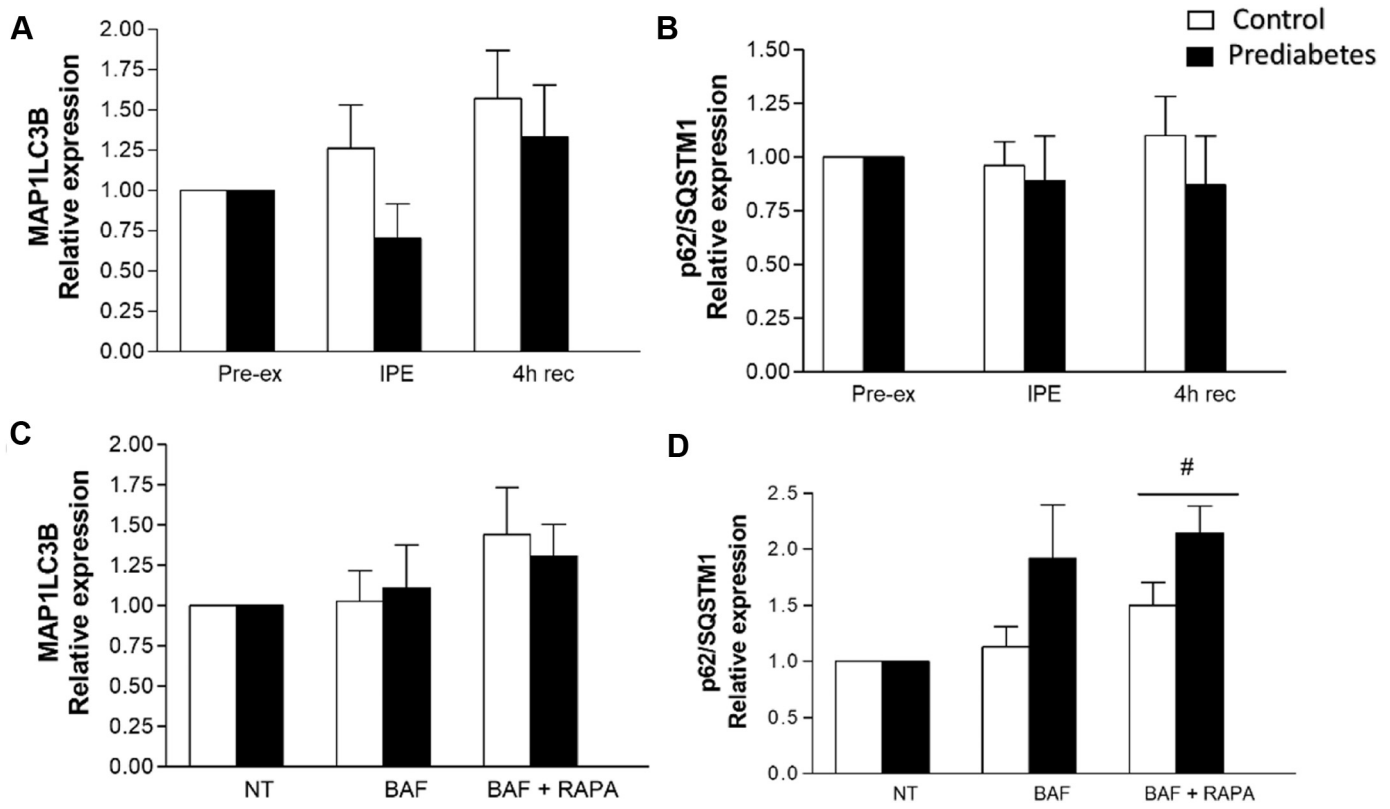

Figure 3. Exercise and rapamycin-mediated transcriptional regulation of autophagy. mRNA expression of genes (A) *MAP1LC3B* and (B) *p62/SQSTM1* in peripheral blood mononuclear cells (PBMCs) in response to acute exercise in patients with prediabetes (black bars) and control individuals (white bars) before exercise, immediately after exercise and 4 h into recovery. mRNA expression of genes (C) *MAP1LC3B* and (D) *p62/SQSTM1* in PBMCs in response to no treatment, bafilomycin treatment and bafilomycin with rapamycin treatment. Data are mean ± SEM. #p<0.05 compared with no treatment. *4h rec*, 4 h into recovery; *BAF*, bafilomycin treatment; *BAF + RAPA*, bafilomycin with rapamycin; *IPE*, immediately after exercise; *NT*, no treatment; *pre-ex*, before exercise.

diabetes (22). This may suggest an inhibition of autophagosome formation independent of cargo protein *p62/SQSTM1*. Previous findings in patients with type 2 diabetes have shown *p62/SQSTM1* accumulation (23) or no change (11) after strong levels of autophagy induction in skeletal muscle, suggesting *p62/SQSTM1* responses may be tissue specific, as observed by others (23,30). It is plausible that the lack of degradation in *p62/SQSTM1* in the present study was independent of changes in autophagic flux. This may be through mechanisms involving *tumor protein p53 inducible nuclear protein 2*, which may act as an autophagic cargo protein independent of *p62/SQSTM1* (31). Taken together, our findings support the interpretation that *LC3-II/LC3-I* reduction in the absence of *p62/SQSTM1* regulation may indicate increased autophagic turnover (lysosomal degradation) after exercise, as observed in our control participants.

Autophagy induction via RAPA treatment induced much more pronounced differences in autophagic activation between control subjects and people with prediabetes. BAF was used to block autophagolysosome formation, therefore enabling the accumulation of *LC3-II* protein with increased autophagy. After RAPA treatment, *LC3-II* protein was significantly higher in control PBMCs than in those with prediabetes, which is a key marker of autophagosome formation (11). This was accompanied by a significant increase in the *LC3-II/LC3-I* ratio in control subjects, indicating increased autophagic flux that was not observed in individuals with prediabetes. The findings in control subjects are similar to those found by Dokladny et al (32), where a marked increase in *LC3-II* abundance was observed after RAPA treatment in A549 cells. This is unsurprising because RAPA treatment blocks *mTORC1*, a potent inhibitor of autophagy (33), in a similar manner to starvation. There is increasing evidence supporting the hypothesis of dysregulated autophagy in obesity and type 2 diabetes (23). The present findings suggest a reduction in autophagosome formation in response to RAPA treatment in people with prediabetes, further supporting autophagic dysregulation found in type 2 diabetes (23). Although the mechanism of autophagy reduction in people with prediabetes is presently unknown, impaired insulin signalling, mitochondrial dysfunction and altered protein metabolism are all known characteristics of prediabetes and type 2 diabetes (8,34,35), which may contribute to disruptions in autophagy (36).

Interestingly, despite an impaired response in *LC3-II* protein abundance and the *LC3-II/LC3-I* ratio, a significant decrease in *p62/SQSTM1* was observed in both individuals with prediabetes and control subjects. This may suggest an inhibition of autophagosome formation independent of cargo protein *p62/SQSTM1* in people with prediabetes, indicating a preservation of the selective autophagy response (37). Previous research has reported no changes in *p62/SQSTM1* accumulation after insulin treatment in muscle biopsies of young, healthy individuals (38). Because RAPA directly inhibits *mTORC1* downstream of the insulin-signalling pathway, it is possible that observed changes in *p62/SQSTM1* were independent of autophagic flux. However, because transcription of *p62/SQSTM1* increased in both control subjects and people with prediabetes in the presence of decreased protein abundance, it is more likely indicative of an increased autophagic response rather than an independent mechanism as described by others (22,23). This is an important response because it enables the autophagic process to sort and remove vacuolar enzymes and aggregate-prone proteins and unwanted organelles that may contribute to the development of insulin resistance (39,40).

The limitations of this study include the small sample size and lack of additional markers of autophagy regulation and upstream signalling. Additionally, our interpretation of starvation-induced autophagy would have benefited from the inclusion of a RAPA-only control condition. Furthermore, we have no data on the course of autophagy regulation beyond 4 h of recovery post-exercise. We identified quantitatively small but significant changes in autophagy responses, which are consistent with findings presented in other studies (22,32), reflecting the highly conserved nature of the autophagic response under physiological conditions, because large changes in this process may have damaging cellular consequences. Finally, our findings are limited to PBMCs, which may not coincide with responses observed in skeletal muscle.

In summary, our findings demonstrate a small inhibition in the autophagic response to exercise in individuals with prediabetes. Furthermore, we show autophagic inhibition after RAPA treatment in PBMCs, indicating a reduced capacity to upregulate autophagy under *mTORC1*-limiting conditions in people with prediabetes. Future studies are required using different exercise intensities and measurements beyond 4 h of recovery to gain a more complete understanding of the relationship between exercise and autophagy in prediabetes.

Acknowledgments

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Author Disclosures

Conflicts of interest: None.

Author Contributions

JJM, KD, and CMM conceptualized and designed the research. JJM and KEK performed data collection and analysis. JJM drafted the manuscript and all authors edited, revised, and approved the final version.

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