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Single bout of running exercise changes LC3-II expression in rat cardiac muscle

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ABSTRACT

Macroautophagy (autophagy) is an intracellular catalytic process. We examined the effect of running exercise, which stimulates cardiac work physiologically, on the expression of microtubule-associated protein 1 light chain 3 (LC3)-II, an indicator of autophagy, as well as some autophagy-related proteins in rat cardiac muscle. The left ventricles were taken from rats immediately (0 h), and at 0.5 h, 1 h or 3 h after a single bout of running exercise on a treadmill for 30 min and also from rats in a rest condition. In these samples, we evaluated the level of LC3-II and p62, and the phosphorylation level of mammalian target of rapamycin (mTOR), Akt and AMP-activated protein kinase alpha (AMPKα) by Western blotting. The exercise produced a biphasic change in LC3-II, with an initial decrease observed immediately after the exercise and a subsequent increase 1 h thereafter. LC3-II then returned to the rest level at 3 h after the exercise. A negative correlation was found between the LC3-II expression and mTOR phosphorylation, which plays a role in inhibiting autophagy. The exercise increased phosphorylation of AMPKα, which stimulates autophagy via suppression of mTOR phosphorylation, immediately after exercise. The level of p62 and phosphorylated Akt was not altered significantly by the exercise. These results suggest for the first time that a single bout of running exercise induces a biphasic change in autophagy in the cardiac muscle. The exercise-induced change in autophagy might be partially mediated by mTOR in the cardiac muscle.

1. Introduction

Macroautophagy (autophagy) is a catalytic process occurring in cells. In this process, cytosolic proteins and organelles are sequestered by an isolation membrane structure to form autophagosome. The autophagosome then fuses with lysosome, where the inclusions of autophagosome are degraded to amino acid or peptide [1,2]. Autophagy constitutively takes place at lower levels in most cells, but it is stimulated markedly under some pathophysiological and pathological conditions. In the cardiac muscle, for example, autophagy is induced by starvation [3,4] and by ischemia [5,6].

Previous reports have described that pharmacological manipulation of cardiac work also affects autophagy in the heart. For example, the autophagic vacuole volume fraction in rat cardiac muscle was increased by suppression of cardiac work using propranolol or verapamil [7] and was decreased by augmentation of cardiac work using isoproterenol [8]. However, it remains uncertain whether the physiological change in the cardiac work might also influence autophagy in the cardiac muscle.

In the current study, therefore, we examined the effect of a single bout of running exercise, which augments cardiac work transiently and physiologically [9,10], on the expression of microtubule-associated protein 1 light chain 3 (LC3)-II, an indicator of autophagy [11], in the rat cardiac muscle. In addition, we evaluated phosphorylation of some signaling proteins regulating autophagy and the expression of p62, a lysosome degradation marker [12], in the same organ.

2. Materials and methods

2.1. Ethical approval and animals

The experimental protocol was approved by the Committee on Animal Research at the University of Tsukuba, and the Institute for Animal Experimentation at the St. Marianna University Graduate School of Medicine. The male Sprague–Dawley rats ($n = 29$, 10-weeks old) used for this study were housed on a 12:12-h light–dark cycle. They received food and water ad libitum.

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2.2. Running exercise

The exercise protocol, which physiologically increases cardiac work, has been described in earlier reports [10,13]. For 2 days before the experiment, the animals were familiarized with running exercise on a motor-driven treadmill at 15 m min^{-1} without incline for 10 min each day. On the day of the experiment, the animals were divided into rest control (n = 5, body weight 392 ± 5 g), and exercise groups (n = 24). Rats in exercise groups were run on a treadmill for 30 min at 30 m min^{-1} and were then killed immediately (0 h, n = 6, 383 ± 4 g), 0.5 h (n = 6, 375 ± 4 g), 1 h (n = 6, 386 ± 7 g), and 3 h (n = 6, 392 ± 3 g) after cessation of the exercise. Rest control animals were kept for 30 min on the treadmill without exercise. The left ventricle was quickly excited under anesthesia with diethyl ether, frozen in liquid nitrogen, and stored at −80 °C. A possible circadian influence was mitigated by averaging data obtained from animals killed at different times of the day. In the pilot experiment with identical exercise protocol to this study, we confirmed that the glycogen concentration was significantly (P < 0.01) decreased immediately after running exercise in the rat cardiac muscle, but it returned to basal level after 3 h of the exercise (basal: 21.3 ± 1.5 μmol glucose g muscle wet weight (MWW)$^{-1}$, immediately after the exercise: 8.2 ± 2.5 μmol glucose g MWW^{-1}, 3 h after the exercise: 24.8 ± 1.8 μmol glucose g MWW^{-1}, n = 6/group).

2.3. Protein extraction

Frozen samples were homogenized (1:7 w/v) in ice-cold buffer (20 mM Tris–HCl, 1 mM EDTA, 150 mM NaCl, 2% Triton X-100, pH 7.5) with phosphatase inhibitors (Phosstop tablet; Roche Diagnostics Corp., Indianapolis, USA) and protease inhibitors (Complete tablet; Roche Diagnostics Corp.) [14]. The homogenates were incubated at 4 °C for 30 min, then centrifuged at 12,000g for 20 min at 4 °C. Then the supernatant was collected. Protein concentrations of the supernatant were measured using the bicinchoninic acid method (Thermo Fisher Scientific Inc., Waltham, USA) with bovine serum albumin as a standard.

2.4. Western blotting

Identical amounts of proteins were separated using SDS–PAGE and were transferred to PVDF membranes (Bio-Rad Laboratories Inc.). The membranes were blocked using PVDF blocking reagent (Toyobo Co., Ltd., Osaka, Japan) and then 2% blocking agent (GE Healthcare UK Ltd., Buckinghamshire, UK) in tris-buffered saline with 0.1% Tween-20 (T-TBS) for 1 h each at room temperature. The membranes were probed with primary antibodies in primary antibody solutions (NKB-201; Toyobo Co., Ltd.) with 2% blocking agent (GE Healthcare) for 2 h at room temperature. After washing, the membranes were probed with rabbit horseradish peroxidase-conjugated secondary antibody (1:30,000) in secondary antibody solution (NKB-301; Toyobo Co., Ltd.) with 2% blocking agent for 1 h at room temperature. Protein was visualized using an enhanced chemiluminescence reagent (ECL Advance; GE Healthcare) and captured (LAS-3000; Fuji Photo Film Co., Ltd., Minato-ku, Japan). Each band was evaluated using specialized software (Image gauge Ver. 4.23; Fuji Photo Film Co., Ltd.). Data were normalized using mean values for the rest control animals.

2.5. Antibodies

Antibodies used for this study were LC3-II (LC3B, diluted to 1:2000, No. 2775), Akt (1:2000, No. 4691), phospho-Akt at Ser473 (1:2000, No. 4060), AMP-activated protein kinase alpha (AMPKα, 1:2000, No. 2603), phospho-AMPKα at Tyr172 (1:2000, No. 4188), mammalian target of rapamycin (mTOR, 1:2000, No. 2983), phospho-mTOR at Ser2448 (1:2000, No. 2971), and p62 (1:3000, PM045). ß-Tubulin (1:3000, No.5346) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:10000, No. 3683) were used to confirm equal loading for LC3-II and p62, respectively, because of their close molecular weight. All antibodies except for p62 were purchased from Cell Signaling Technologies (Beverly, USA). Then p62 was obtained from Molecular Biological Laboratories Co., Ltd. (Nagoya, Japan).

2.6. Statistics

Data are expressed as means ± SEM each protein expression was compared using one-way ANOVA with the Tukey–Kramer test. Correlation between LC3-II expression and mTOR phosphorylation at Ser2448 was evaluated using the least-squares method in exercised animals (i.e. 0, 0.5, 1 and 3 h). All statistical analyses were performed using a statistical software package (Prism 5.0d; GraphPad Software Inc., La Jolla, CA, USA). A probability level of P < 0.05 was considered statistically significant.

3. Results

Fig. 1A and B shows the effect of a single bout of exercise on the LC3-II and p62 expression in the rat cardiac muscle, respectively. The LC3-II expression immediately after the exercise (i.e. 0 h) was significantly lower than in rest animals (P < 0.01). Then, LC3-II expression was increased significantly at 1 h compared to the level in rest animals (P < 0.05). The LC3-II expression was significantly higher at 0.5 h (P < 0.01), 1 h (P < 0.01), and 3 h (P < 0.01) than at 0 h. The running exercise did not change the p62 expression in the rat cardiac muscle.

ANOVA revealed a significant effect of time after exercise on phosphorylation of mTOR at Ser2448 in the rat cardiac muscle (Fig. 2A). Although the phosphorylation levels of mTOR in rest and exercised animals did not differ significantly, a tendency was observed: an increase immediately after exercise was followed by a decrease at 0.5 and 1 h. The phosphorylation level of mTOR was significantly lowered at 0.5 h (P < 0.05) and at 1 h (P < 0.01) compared with that at 0 h. Although the trend of decreasing in phosphorylation of mTOR was found at 3 h after the exercise, it did not reach the statistically significant level. A significant negative correlation was found between phosphorylation of mTOR at Ser2448 and LC3-II expression in exercised animals (r = −0.7696, P < 0.0001, Fig. 2B).

Fig. 3A and B shows the phosphorylation of AMPKα at Thr172 and that of Akt at Ser473 in the rat cardiac muscle, respectively. The phosphorylation of AMPKα at Thr172 was significantly higher immediately after the exercise compared to the level of the rest condition (P < 0.05). The phosphorylation of AMPKα at Thr172 was reduced after the exercise and was significantly lowered at 1 h (P < 0.05) and 3 h (P < 0.05) than the level at 0 h. On the other hand, no significant difference was found in phosphorylation of Akt at Ser473.

4. Discussion

Results show that a single bout of running exercise produced a biphasic change in LC3-II expression in rat cardiac muscle, with a transient decrease occurring immediately (0 h) after the running session, with a subsequent increase observed 1 h thereafter. The expression of LC3-II reflects the degree of autophagy in cells including cardiac myocytes [3,11,15,16]. Therefore, our results suggest that the running exercise produced a biphasic change in cardiac autophagy. The initial decrease in autophagy resembled that observed after isoproterenol stimulation of the heart [8], suggest-