Cardiac Kinetophagy Coincides with Activation of Anabolic Signaling

YOUNGIL LEE[1], EUN-BUM KANG[2], INSU KWON[1], LUDMILA COSIO-LIMA[1], PETER CAVNAR[3], and GULNAZ T. JAVAN[4]

[1]Molecular and Cellular Exercise Physiology Laboratory, Department of Exercise Science, University of West Florida, Pensacola, FL; [2]Exercise Biochemistry Laboratory, Korea National Sport University, Seoul, SOUTH KOREA; [3]Department of Biology, University of West Florida, Pensacola, FL; and [4]Department of Physical Sciences, Alabama State University, Montgomery, AL

ABSTRACT

LEE, Y., E.-B. KANG, I. KWON, L. COSIO-LIMA, P. CAVNAR, and G. T. JAVAN. Cardiac Kinetophagy Coincides with Activation of Anabolic Signaling. *Med. Sci. Sports Exerc.*, Vol. 48, No. 2, pp. 219–226, 2016. **Purpose**: Growing evidence has shown that endurance exercise is a strong inducer of autophagy in various tissues. Thus, we define here endurance exercise-induced autophagy as "kinetophagy" derived from the Greek terms "kineto" (movement), "auto" (self), and "phagy" (eating). Currently, the exact cellular mechanisms responsible for kinetophagy remain unclear; hence, we examined kinetophagy signaling transduction pathways occurring during acute endurance exercise (AEE). **Methods**: C57BL/6 mice were randomly assigned to either AEE ($n = 7$) or control sedentary group (CON, $n = 7$). After 5 d of treadmill running acclimation, mice performed 60 min of a single bout of treadmill running at 12 m·min^{-1} on a 0% grade. Hearts were excised immediately 1 h after exercise and homogenized for Western blot analyses. **Results**: Our data showed that AEE promoted kinetophagy flux (an increase in LC3-II to LC3-I ratio and LC3-II levels and a reduction in p62 levels) with Beclin-1 levels suppressed but Atg7 levels elevated compared with those in the sedentary group. We also observed that AEE increased lysosome-associated membrane protein and cathepsin L, linked to the termination process of autophagy, and that AEE augmented potent autophagy inducers (i.e., adenosine monophosphate kinase phosphorylation, BNIP3, and HSP70). Moreover, we found that exercise-mediated BNIP3 upregulation is associated with hypoxia-inducing factor 1α rather than FoxO3a. Intriguingly, we found for the first time that kinetophagy parallels with anabolic signaling activation (Akt and mammalian target of rapamycin). **Conclusions**: Our findings provide evidence that AEE results in kinetophagy without a time-associated elevation in Beclin-1 but with the presence of Akt-mTOR activation and that AEE-induced activation of anabolic signaling is not associated with kinetophagy promotion. **Key Words**: EXERCISE, AUTOPHAGY, KINETOPHAGY, HIF-1α, BNIP3, mTOR

Cardiac myocytes are postmitotic (terminally differentiated) cells and lack regenerative capacity. Thus, maintaining healthy, viable adult cardiac myocytes throughout the life span is of essence to prevent cell death (i.e., apoptosis). Discovery of autophagy, by which long-lived proteins and dilapidated organelles (e.g., mitochondria) are removed, has provided an important insight into protective mechanisms of the heart in that inactivation of autophagy compromises cell function and often results in cell death.

Notably, recent evidence has shown that acute endurance exercise (AEE) is a potent inducer of autophagy (12,23) in skeletal muscles and the heart. However, the cellular and molecular mechanisms of exercise-mediated autophagy remain to be elucidated. Because autophagy in response to exercise is universally observed elsewhere, hereafter, we defined exercise-induced autophagy as kinetophagy (i.e., "kineto," a Greek definition of movement; "auto," a Greek definition of self; and "phagy," a Greek definition of eating) and explored possible molecular mechanisms of cardiac kinetophagy. We specifically focused on whether cardiac kinetophagy is associated with a canonical autophagic pathway typically exhibited under starvation interventions or noncanonical pathways.

Evidence has shown that the upregulation of Beclin-1 is linked to autophagy induction because Beclin-1 mediates membrane nucleation in autophagy (8). On the contrary,

some studies have shown that autophagy can occur without the modulation of Beclin-1 levels under certain conditions (13,21). In addition, Atg 7, another important autophagy rate-limiting protein involved in autophagosome formation has received new attention in cardiac myocytes, as Atg7 overexpression could elicit upregulation of autophagy (3). It currently remains unclear whether modulation of Beclin-1 and Atg7 in response to endurance exercise is related to cardiac kinetophagy.

The BCL2/adenovirus E1B 19-kDa interacting protein 3 (BNIP3) is a potent inducer of autophagy in many cell types and tissues (11,16). The proposed roles of BNIP3 in autophagy are to promote dissociation of Beclin-1 from BCL2, leading to autophagy induction (2), and to inactivate mammalian target of rapamycin (mTOR) upon excessive oxidative stress (4). Although many studies showed that BNIP3 expression rises in skeletal muscle in response to endurance exercise concordant with autophagy, it is unknown whether this phenomenon happens in the heart and what transcription factors are responsible for this protein expression. Studies have reported that Forkhead box O3a (FoxO3a) (30) or hypoxia-inducing factor 1α (HIF-1α) (10) induces BNIP3 upregulation. However, no studies have investigated which transcription factor is responsible for exercise-induced BNIP3 expression in the heart. Heat shock protein 70 (HSP70), which is crucial for cellular protection under stress conditions, plays a novel role in enhancing autophagy flux by retaining the lysosomal proteases and cathepsin in the lysosome (9).

A potent anabolic signaling molecule, mTOR has been known to interfere in autophagy, whereas inactivation of mTOR via adenosine monophosphate kinase (AMPK) activation under nutrient deficiency or ischemia enhances autophagy (8). By contrast, recent studies have reported that autophagy can be elicited in the absence of mTOR alterations (13,21), which indicates possible noncanonical (or mTOR-independent) autophagy. Because molecular pathways of kinetophagy remain to be elucidated, we explored in this study a potent question of whether kinetophagy is associated with canonical or noncanonical autophagy pathways.

MATERALS AND METHODS

Animals. C57BL/6 male mice ($n = 14$) were maintained at a 12:12 dark–light cycle, housed at 22°C ± 2°C with 50% relative humidity and had free access to standard chow diet (Purina Mills, Seoul, Korea) *ad libitum*. Mice were handled in an accredited Korea Food and Drug Administration animal facility in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care International Animal Care Policies (accredited unit, Korea Food and Drug Administration: unit number 000996).

Treadmill running exercise. Male mice ($n = 14$) were divided into the following two groups: sedentary control group (CON, $n = 7$) and AEE group (AEE, $n = 7$). Before treadmill exercise, mice from the AEE group were acclimatized to running on a treadmill (Daemyung Scientific Co., Ltd., Korea) at a speed of 5 m·min^{-1} for 10 min·d^{-1} for 5 d, whereas animals in a sedentary group stayed on a nonmoving treadmill with the same amount of time to eliminate possible confounding factors that can be caused by being in the treadmill *per se*. After the familiarization of the treadmill exercise, mice performed treadmill running at a speed of 12 m·min^{-1} for 60 min (i.e., two sets of 30-min running with 5-min rest between sets) on a 0% grade as previously described (15).

Heart tissue collection and storage. Mice, after treadmill running, were allowed to recover from the running in their cage for an hour, after which, mice were sacrificed. Hearts from exercise-trained and sedentary groups were immediately excised, rinsed with ice-cold phosphate-buffered saline (pH, 7.4) and stored at −80°C until needed.

Western blotting. Heart tissues were homogenized in ice-cold lysis buffer containing 50-mM Tris–HCl, pH 7.4, 1-mM EDTA, 1-mM EGTA, 1% Trition X-100 plus complete protease inhibitor and phosphatase inhibitor (Roche Applied Science) and incubated on ice for 30 min. Then, tissue lysates were collected by centrifugation at 14,000 rpm for 20 min. Proteins were separated by SDS-PAGE under reduced conditions and transferred to nitrocellulose membranes. The membranes were blocked with 5% bovine serum albumin with Tris-buffered saline containing 0.1% Tween 20 for 1 h at room temperature and then incubated with designated antibodies over night at 4°C. Antibodies used in the study were as follows: BNIP3 (#3769, 1:1000), p62 (#5114, 1:1000), Beclin-1 (#3738, 1:1000), LC3 (#2775, 1:1000), Atg 7 (#2631, 1:1000), phospho-Akt at Ser473 (#9271, 1:1000), Akt (#9272, 1:1000), phospho-mTOR at Ser2481 (#2974, 1:1000), mTOR (#2983, 1:1000), phospho-p70s6k at Thr389 (#9234, 1:1000), p70s6k (#2708, 1:1000), phospho-AMPK at Thr172 (#2535, 1:1000), AMPK (#2532, 1:1000), phospho-FoxO1/3a at Thr32 (#9464, 1:1000), FoxO3a (#12829, 1:1000) from Cell Signaling (Danvers, MA); HSP70 (sc-24, 1:500), HIF1-α (sc-10790, 1:500), Tubulin (sc-5286, 1:500) from Santa Cruz Biotechnology (Santa Cruz, CA); Cathepsin L (ab133641, 1:1000) from Abcam (Cambridge, MA). After washing 1° antibodies, membranes were incubated with designated 2° antibodies (goat antimouse or rabbit HRP conjugated) for 1 h at room temperature. Blots were analyzed with a Bio-Rad QuantityOne (Hercules, CA) and quantified with LI COR Image Studio (Li-COR Biosciences, Lincoln, NE) and National Institutes of Health imageJ (National Institutes of Health, Bethesda, MD). Protein expression was normalized to α-tubulin.

TABLE 1. Animal characteristics.

	Gender	n	Age (months)	Body Weight (g)	Heart Weight (g)
Control	Male	7	6	25.17 ± 1.47	0.11 ± 0.01
Exercise	Male	7	6	24.70 ± 0.84	0.11 ± 0.01

No statistical differences were found in body weight and heart weight between groups. Values are mean ± SEM.

Statistical analysis. All values were expressed as means ± SEM. Statistical analysis was performed using student *t*-test (unpaired, one tail) to identify statistical significance between groups. Statistical significance was set at *P* < 0.05.

RESULTS

Animal characteristics. Animal characteristics are presented in Table 1. Body weight and heart weight were not different between groups.

AEE enhances kinetophagy flux without a time-associated elevation of Beclin-1 elevation.

First, we examined the levels of autophagy flux by assessing the ratio of LC3-II to LC3-I and LC3-II contents in the heart of trained and sedentary animals. Because the blockage of fusion process between autophagosomes and lysosomes can display false autophagic flux, we also measured p62 (an autophagsome adaptor protein) that is irreversibly degraded by autophagy (24). We observed that AEE elevated the ratio of LC3-II/LC3-I (Fig. 1A and B) concurrent with an increase in LC3-II expression (Fig. 1A and C) along with reduced levels of
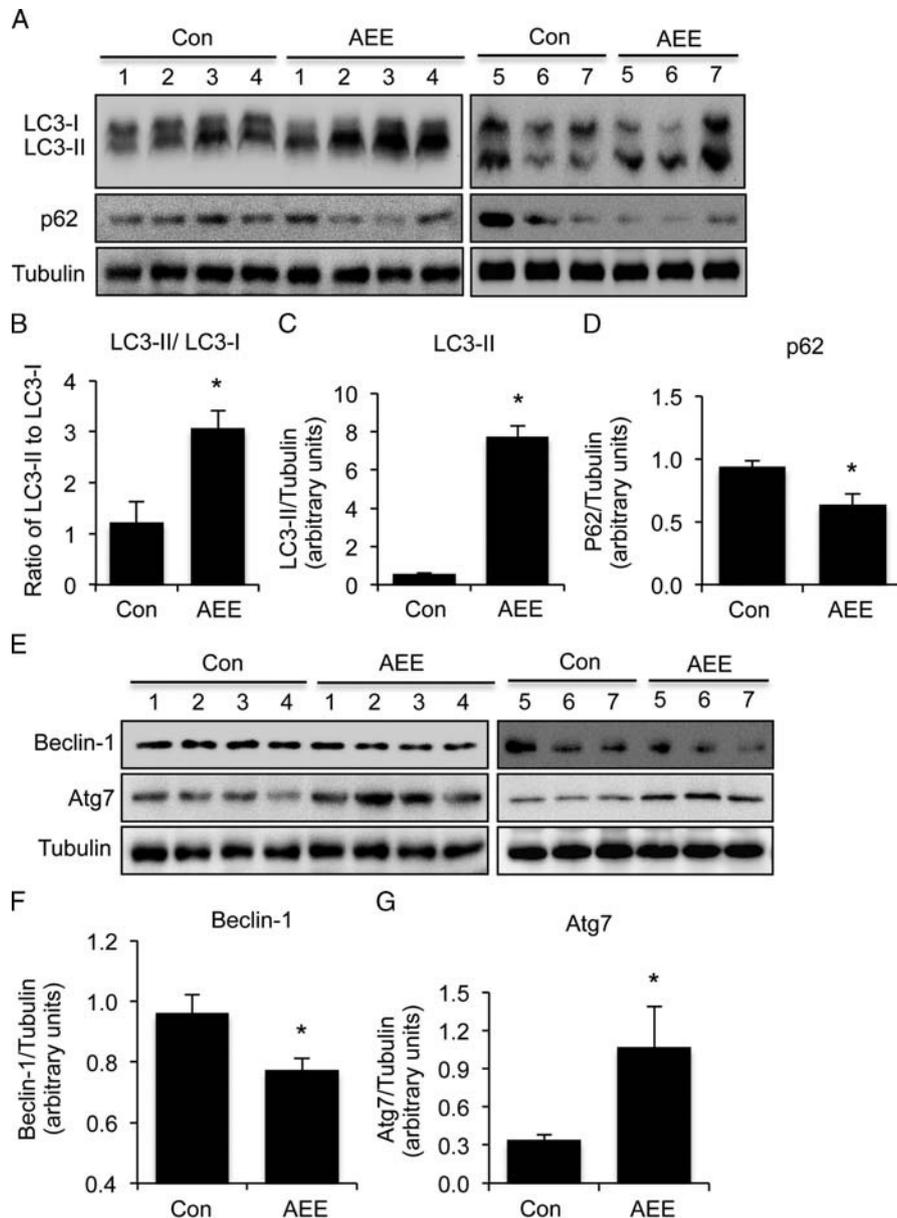

FIGURE 1—Cardiac kinetophagy occurs in the absence of Beclin-1 upregulation but associates with Atg7. Adult C57BL/6 mice ran on treadmill for 60 min followed by 5 d of acclimation, whereas control sedentary mice stayed in their cages. Hearts were excised after 1 h of exercise, and autophagy proteins were measured. A. Representative Western blot images for autophagy flux proteins. AEE increases LC3-II/LC3-I ratio and total LC3-II and decreases p62. B, C, and D. Quantitation of LC3-II/LC-I ratio, total LC3-II, and p62, respectively. E. Representative Western blots for autophagy-regulating proteins. AEE downregulates Beclin-1 but upregulates Atg7. F and G. Quantitation of Beclin-1 and Atg7, respectively. Each protein expression was normalized by loading control, *α*-Tubulin. *Denotes statistical difference from sedentary control (*P* < 0.05, *n* = 7).
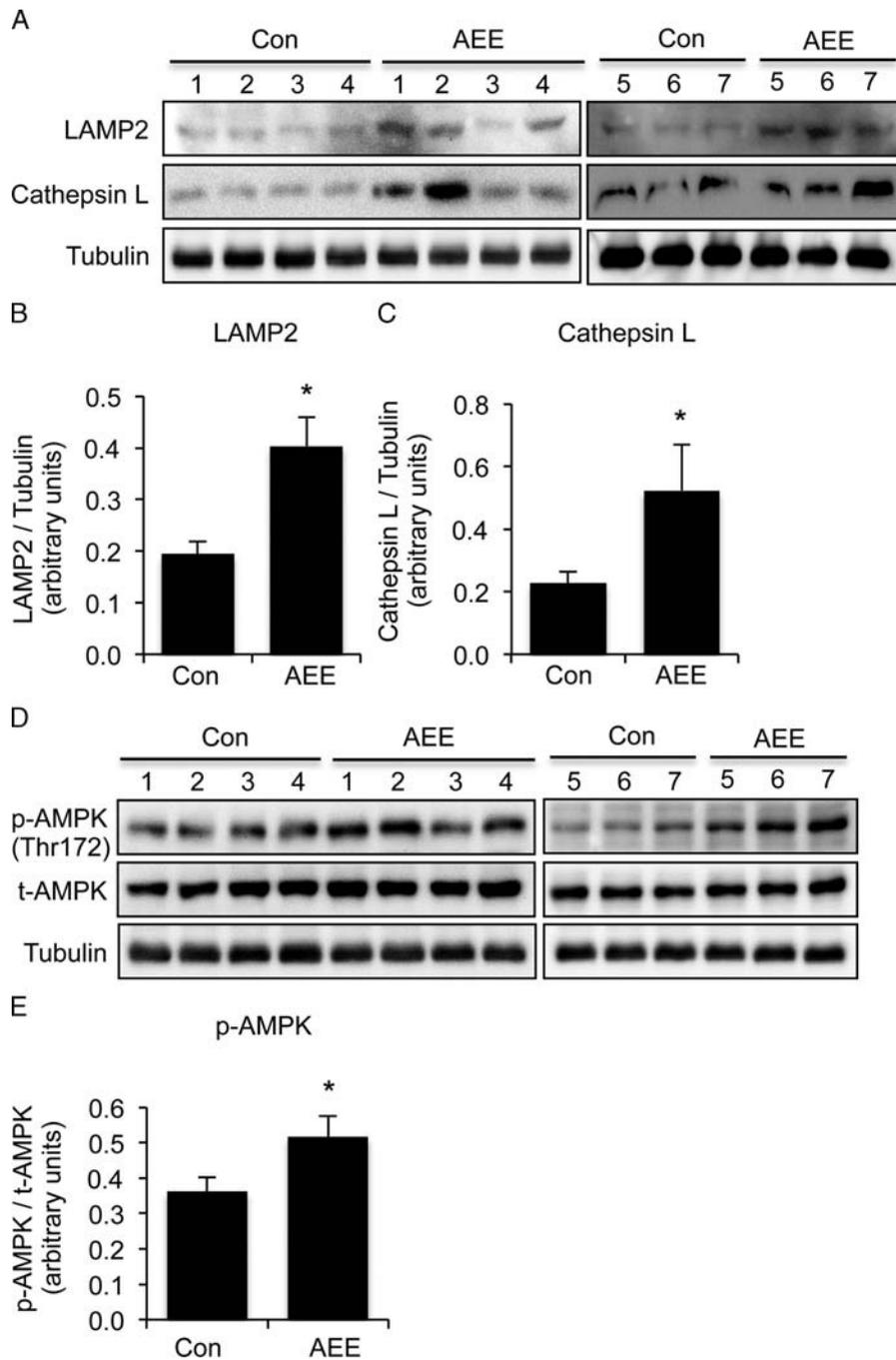
FIGURE 2—Endurance exercise enhances cardiac lysosomal biogenesis and activates AMPK. A. Representative Western blot images for lysosomal proteins. Exercise upregulates LAMP2 and cathepsin L. B and C. Quantitation of LAMP2 and cathepsin L. D. Representative Western blot images of AMPK. AEE increases AMPK phosphorylation. E. Quantitation of p-AMPK/t-AMPK ratio. Each protein expression was normalized by loading control, α-Tubulin. *Denotes statistical difference from sedentary control ($P < 0.05$, $n = 7$).

p62 (Fig. 1A and D). Next, we examined the relation of cardiac kinetophagy with Beclin-1. Interestingly, despite robust kinetophagy promotion, AEE reduced levels of Belcin-1 compared with the sedentary control group (Fig. 1E and F). In contrast to Beclin-1, immunoblotting analysis showed that AEE upregulated levels of Atg7 proteins compared with the sedentary control group (Fig. 1E and G).

AEE promotes lysosomal biosynthesis and activates ampk. Given the enhanced kinetophagy flux, we

next examined whether AEE-mediated alterations in lysosomal proteins are related to kinetophagy. We assessed lysosome-associated membrane protein (LAMP2) and a lysosomal protease, cathepsin L, as key factors of lysosomal biosynthesis. Immunoblotting data demonstrated that AEE upregulated the levels of LAMP2 proteins (Fig. 2A and B). In addition, we observed an increase in cathepsin L in the trained heart compared with that in the untrained heart (Fig. 2A and C). These findings prompted us to explore a possible
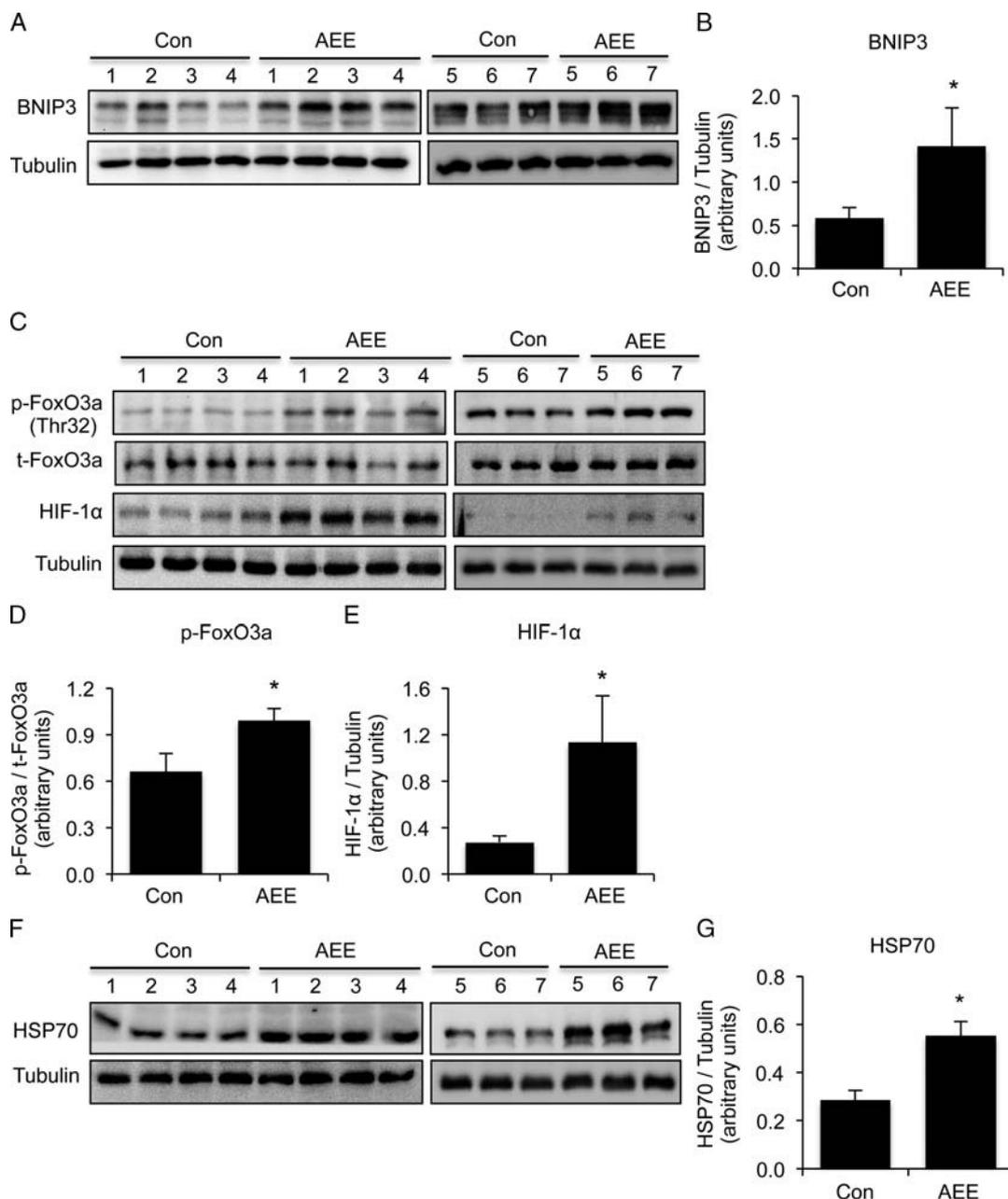
FIGURE 3—Endurance exercise-induced upregulation of BNIP3 is associated with HIF-1α rather than FoxO3a. A. Western blot images of BNIP3. B. Quantitation of BNIP3. C. Representative Western blot images of transcription factors FoxO3a and HIF-1α. AEE induces FoxO3a phosphorylation (inactive form) but instead upregulates HIF-1α. D and E. Quantitation of p-FoxO3a/t-FoxO3a ratio and HIF-1α, respectively. F. Representative Western blot images of HSP70. AEE significantly increases HSP70. G. Quantitation of HSP70. The protein expression was normalized by loading control, α-Tubulin. *Denotes statistical difference from sedentary control ($P < 0.05$, $n = 7$).

signaling molecule responsible for kinetophagy. Because AMPK is a potent autophagy activator and its activity is regulated by phosphorylation, we assessed the levels of AMPK phosphorylation and total AMPK. Our data revealed that AEE resulted in increased phospho-AMPK relative to total AMPK expression (Fig. 2D and E).

AEE upregulates a potent inducer and enhancer of kinetophagy: BNIP3 and HSP70. BNIP3 is a potent inducer of autophagy. To explore whether BNIP3 is related to cardiac kinetophagy, we conducted an immunoblotting analysis and found that AEE upregulated BNIP3 expression (Fig. 3A and B), which might reflect the potential association with kinetophagy. Because FoxO3a, a potent transcription factor, has been involved in BNIP3 gene expression (7), we next measured both phosphorylated (inactive) and nonphosphorylated form (active) of FoxO3a and found that FoxO3a was inactivated by AEE, evidenced by elevated levels of phosphorylation (Fig. 3C and D). Given that HIF-1α is also a crucial transcription factor regulating BNIP3 gene expression (28), we measured levels of HIF-1α protein and observed that

AEE significantly upregulated HIF-1α compared with the control group (Fig. 3C and E). Furthermore, AEE elevated levels of HSP70 proteins (Fig. 3F and G).

Acute cardiac kinetophagy occurs concurrently with elevated anabolic signaling. Exercise potentiates anabolic signaling (i.e., phosphorylation of Akt and mTOR) in the heart (17), which is a well-accepted mechanism that inhibits autophagy under starvation, but this creates paradox because exercise also promotes kinetophagy. Therefore, we explored the question of whether kinetophagy occurs in parallel with mTOR activation. Surprisingly, we found that AEE resulted in increased levels of mTOR phosphorylation (Fig. 4A and C). We further observed elevated phosphorylation levels of Akt, an upstream signaling molecule of mTOR (Fig. 4A and B). Lastly, we confirmed AEE-induced mTOR activation by measuring phosphorylation levels of p70S6K, a downstream target of mTOR (Fig. 4A and D).

DISCUSSION

In the present study, we examined molecular signaling pathways of acute cardiac kinetophagy, evaluated potential inducers of kinetophagy, and finally explored the association of kinetophagy with the canonical autophagic pathways. Four key findings emerged from the present study. First, AEE increases cardiac kinetophagy flux evidenced by the upregulation of lysosomal proteins. Second, cardiac kinetophagy occurs without a time-associated elevation in Beclin-1 but with Atg7 elevation. Third, exercise-induced upregulation of BNIP3 is associated with the transcription factor HIF-1α but not FoxO3a. Fourth, cardiac kinetophagy coincides with anabolic signaling (Akt-mTOR) activation. Taken together, these results suggest that a signaling pathway of acute cardiac kinetophagy may depend on noncanonical autophagic pathways. A detailed discussion of these findings follows.

AEE promotes cardiac autophagy that reaches its maximum between 30 and 80 min after exercise (12,23). Consistent with these data, our results also reveal that a single bout of endurance exercise enhances cardiac autophagy 1 h after exercise. As reported from a study of He et al. (12), our study shows that the kinetophagy is not just due to accumulation of autophagosomes but also due to augmented autophagic flux, evidenced by both increased LC3-II levels and LC3-II/LC3-I ratio and decreased p62 levels. Contrary to our observation, Ogura et al. (23) failed to show an increase in autophagy flux (no changes in p62) despite the similar exercise protocol. Furthermore, a study from Quindry et al. (26) and others using rats have reported that neither three consecutive days nor 36 wk of treadmill running (1) does not modulate autophagy levels (i.e, Beclin-1 and LC3-II/LC3-I ratio). Although very little is known about how exercise modulates autophagy, it can be presumed that autophagy by endurance exercise may be an early adaptive response; for example, studies showed that an autophagic response declines fairly quickly (approximately 60–80 min after exercise) (12,23). Thus, measuring time points of autophagy may yield variable results. In addition, conflicting results seem to be linked to the difference in experimental models (mice vs rats).
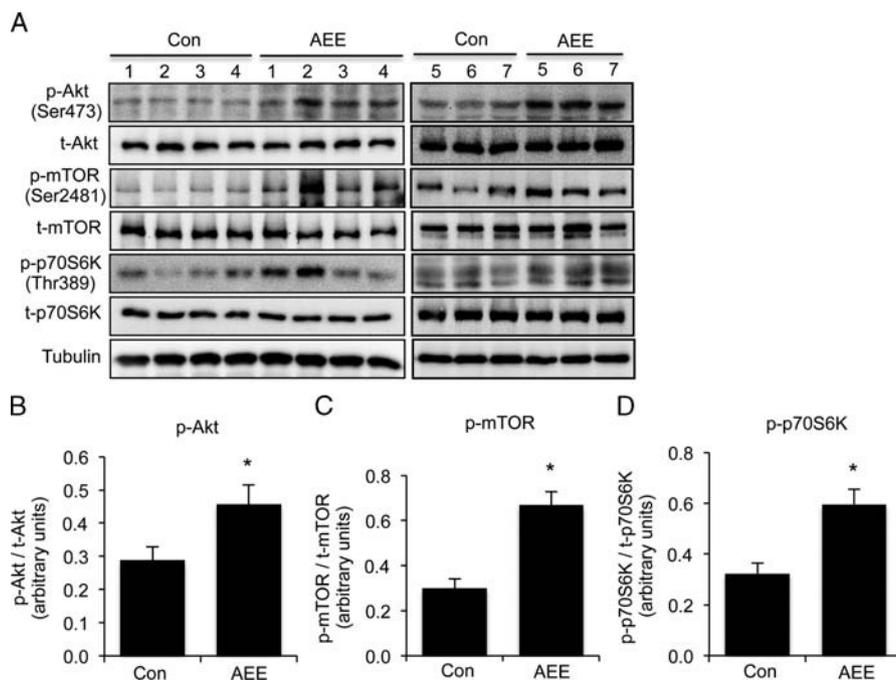
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FIGURE 4—Cardiac kinetophagy coincides with mTOR activation. A. Representative Western blot images of a mTOR upstream factor, Akt, and a downstream factor, p70S6K. AEE facilitates phosphorylation (activation) of cell survival signaling molecules. **B, C, and D.** Quantitation of p-Akt/t-Akt ratio, p-mTOR/t-mTOR ratio, and p-p70S6K/t-p70S6K ratio, respectively. The protein expression was normalized by loading control, α-Tubulin. *Denotes statistical difference from sedentary control ($P < 0.05$, $n = 7$).

Currently, no explanation is available to describe this conflicting observation, and thus, future studies are required to resolve this discrepancy.

Elevated Beclin-1 levels are linked to nutrient deficiency-induced autophagy in cardiomyocytes (21) and in skeletal muscle undergoing long-term endurance running exercise (18). Although little attention has been given to cardiac Beclin-1 in exercise model studies, a study by Quindry et al. showed that three consecutive days of treadmill exercise does not modulate cardiac Beclin-1 content (26). On the contrary, our results show that a single bout of endurance exercise downregulates Beclin-1 despite enhanced kinetophagy. We believe that measuring time points (1 h after exercise vs overnight after exercise) may generate discrete outcomes. Despite the discordant findings, we believe that exercise-induced Beclin-1 suppression is important to explain exercise-mediated enhancement in autophagic flux on the basis of recent exquisite studies. For example, Ma et al. (19,20) discovered that downregulation of Beclin-1 augments LAMP2 expression, enhances autophagic turnover (i.e., an increase in autolysosomes), and restores cell viability. Furthermore, a recent study supports our observation by demonstrating enhanced cardiac autophagy in Beclin-1$^{-/+}$ mice during reperfusion after an ischemic insult (21). Thus, our data provide important evidence that the absence of a time-associated elevation of Beclin-1 in acute kinetophagy may be an important adaptive process, facilitating lysosomal degradation for the proper orchestration of autophagic turnover.

Atg7 is another critical rate-limiting autophagy protein. The physiological importance of Atg7 in autophagy was well characterized in several studies where Atg7 hematopoietic conditional knockout mice exhibited loss of autophagy and consequently induced cell death (6), whereas cardiac-specific inducible Atg7 transgenic mice displayed upregulated levels of autophagy and conferred protection against cardiac proteinopathy without an exhibition of Beclin-1 elevation (3). Similarly, our results show that AEE upregulates Atg7, which parallels with autophagy augmentation. Given the study by Bhuiyan et al. (3) where overexpression of cardiac specific Atg7 promotes autophagy regardless of significant modulation of other autophagic proteins, our data suggest that exercise-mediated Atg7 elevation may be a primary element for kinetophagy.

A recent cardiac study showed that AMPK with activities that are regulated by phosphorylation status serves as a potent activator of autophagy under nutrient deficiency or ischemia (21). In the present study, our data show that acute exercise elevates AMPK phosphorylation concurrent with kinetophagy. Similarly, He et al. (12), using an AEE model, observed that kinetophagy occurs in the presence of AMPK phosphorylation. Although we did not conduct a thorough mechanistic experiment to examine the functional role of AMPK in kinetophagy, AMPK is known to initiate autophagy by inhibiting mTOR by activating tuberous sclerosis complex 2 (8). Thus, we postulated that AEE would inactivate mTOR to promote kinetophagy; surprisingly instead, we observed mTOR activation, confirmed by activation of upstream (phospho-Akt) and downstream (phospho-p70S6K) target of mTOR despite increased AMPK activation. Currently, a possible explanation for this novel observation is not available because very little research has been investigated in cardiac kinetophagy. Nevertheless, our finding may be supported by recent studies demonstrating that endurance exercise elicits activation of a serious anabolic signaling including mTOR (25) and that autophagy rises in the presence of mTOR activation (22) or in absence of mTOR modulation (13). Furthermore, a study by Matsui et al. (21) showed that cardiac autophagy during reperfusion increases in parallel with mTOR activation. Collectively, our data suggest that cardiac kinetophagy in the context of AEE coincides with activation of anabolic signaling.

Overexpression of BNIP3 strongly induces autophagy in various types of cells and tissues (14,16), and BNIP3 upregulation was observed in skeletal muscle in response to endurance exercise (18). Our study also provides the first evidence that AEE upregulates BNIP3 in cardiac tissue. We next sought to determine a transcription factor responsible for BNIP3 expression in response to exercise. Two well-known transcription factors for BNIP3 expression are HIF-1α and FoxO3a (5,27). In our study, we discovered that exercise-induced BNIP3 upregulation is associated with HIF-1α (i.e., increased protein content) rather than FoxO3a (i.e., reduced activation form, phosphorylation). Despite clear association of BNIP3 with kinetophagy, at this point, the direct mechanistic link between BNIP3 upregulation and enhanced cardiac kinetophagy still remains unknown. Thus, future studies using genetic animal models are warranted. In addition to BNIP3, our study shows that AEE enhances HSP70 expression. Although no research in cardiac kinetophagy is available to relate HSP70 to kinetophagy, recent studies indicate a possible involvement of HSP70 in autophagy induction and termination processes. For example, acetylated HSP70 promotes autophagosome formation by triggering VPS34 SUMOylation (29) and secures lysosomal protease in lysosomes for optimal lysosomal catabolic function (9). These potential mechanisms may be linked to improved kinetophagy flux, but further mechanistic studies are warranted.

In summary, our study demonstrates that acute cardiac kinetophagy occurs during the early recovery periods and that an increase in kinetophagy is due to the enhanced turnover rate (flux) of kinetophagy. We show that acute cardiac kinetophagy occurs without a time-associated elevation in Beclin-1 and reveal that kinetophagy concurs with mTOR activation. Taken together, our study suggests that the duel activation (anabolic signaling and kinetophagy) via regular endurance exercise may be an important mechanism of exercise-induced cardiac health.

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