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Original Contribution

Calorie restriction combined with resveratrol induces autophagy and protects 26-month-old rat hearts from doxorubicin-induced toxicity

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ABSTRACT

The multiple beneficial effects of calorie restriction (CR) on several organs, including the heart, are widely known. Recently, the plant polyphenol resveratrol has been shown to possess several beneficial effects similar to those of CR. Among the host of effects on cardiac muscle, a cellular self-eating process called autophagy has been shown to be induced by both CR and resveratrol. Autophagy is vital in removing dysfunctional organelles and damaged proteins from the cell, thereby maintaining cellular quality control. In this study, we explored whether short-term moderate CR (20%), either alone or in combination with resveratrol, can induce autophagy in the hearts of 26-month-old Fischer 344 × Brown Norway rats. Autophagy stimulation was investigated by measuring the protein expression levels of the autophagy proteins beclin-1, Atg5, and p62 and the LC3-II/LC3-I ratio. We found that 20% CR or resveratrol alone for 6 weeks could not induce autophagy, but 20% CR in combination with 50 mg/kg/day resveratrol resulted in an induction of autophagy in the hearts of 26-month-old rats. Although oxidative stress has been proposed to be an inducer of autophagy, treatment with the chemotherapeutic drug doxorubicin was unable to stimulate autophagy. The enhanced autophagy due to CR + resveratrol was associated with protection from doxorubicin-induced damage, as measured by cardiac apoptotic levels and serum creatine kinase and lactate dehydrogenase activity. We propose that a combinatorial approach of low-dose CR and resveratrol has the potential to be used therapeutically to induce autophagy and provides protection against doxorubicin-mediated toxicity.

Autophagy is a cellular self-digestion process whereby cells degrade dysfunctional proteins and organelles, thereby playing a major role in mediating cellular quality control. This vital housekeeping process has been shown to protect against several pathological conditions such as infections, neurodegenerative disorders, inflammatory diseases, and cancer [1]. Cardiac-specific loss of autophagy, by the conditional deletion of autophagy protein 5 (Atg5), has confirmed that this housekeeping process is essential for optimal cardiac functioning and survival [2,3]. Abrogation of the autophagic pathway in the adult heart by conditional inactivation of Atg5 or Atg7 causes rapid onset of cardiac abnormalities characterized by cardiac hypertrophy, left-ventricular dilatation, and decreased cardiac output [2,3]. In addition, an age-related decline in the efficiency of the autophagic process can lead to the accumulation of damaged cellular components, which can further lead to cardiac functional deterioration [4]. Although several inducers of autophagy have been discovered under cell culture conditions, few studies have investigated interventions to induce autophagy in vivo, especially starting at an older age. Kanamori et al. [5] reported that starving transgenic mice expressing green fluorescent protein fused to microtubule-associated light chain 3 (GFP–LC3) for 12 h led to an increase in fluorescent autophagic puncta, which was further confirmed by electron microscopic visualization of autophagic vacuoles. Starvation periods longer than 12 h led to a more robust autophagic stimulation and enhanced the expression of downstream lysosomal enzymes, such as cathepsin D, suggesting an overall enhancement of autophagic flux [5]. Consistent with the study of Kanamori et al., our group has previously shown that lifelong 40% calorie restriction (CR) can increase the appearance of autophagic vacuoles in the hearts of Fischer 344 rats and enhance the expression of autophagy proteins Atg7 and Atg9, and the lysosomal enzyme procathepsin D [6]. Similar effects also have been observed in the skeletal muscle of 40% CR animals [7]. However, despite the autophagy-enhancing capability of

Abbreviations: AL, ad libitum; AMPK, 5′ AMP-activated protein kinase; Atg, autophagy protein; CK, creatine kinase; CR, calorie restriction; FBN, Fischer 344 × Brown Norway; LC3, microtubule-associated light chain 3; LDH, lactate dehydrogenase, ROS, reactive oxygen species; rpS6, ribosomal protein S6; SIRT1, sirtuin 1; SQSTM1, sequestosome 1; TD-NMR, time-domain nuclear magnetic resonance

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lifelong 40% CR, a severe and prolonged dietary restriction is not practical in humans. In addition to infeasibility, it can lead to undesirable and potentially harmful changes if started at a vulnerable stage of the life cycle, such as during adolescence, at a very old age, or during pregnancy. Hence, in this study, we investigated whether a moderate CR (20%) regimen, beginning in late middle age, could have a similar effect of inducing autophagy in Fischer 344 × Brown Norway (FBN) rat hearts.

In addition to a mild CR regimen, we investigated the natural polyphenol resveratrol as a potential inducer of autophagy. Resveratrol is produced by a wide variety of plants, such as grapes, berries, and peanuts, in response to environmental stress and has been extensively investigated in numerous clinical studies [8]. Resveratrol has been implicated in the "French paradox," an observation that French people, despite their high consumption of saturated fats, have a low incidence of age-associated cardiovascular disorders. It is believed that this protective effect is due to their moderately high consumption of red wine, which contains resveratrol. In animals, resveratrol was shown to have numerous beneficial effects, such as promoting vasodilation in models of coronary heart disease and enhancing the expression of antioxidant enzymes [9]. Additionally, resveratrol can protect the heart against ischemia–reperfusion injury [10], improve endothelial function [11], and prevent platelet aggregation [12]. Notably, resveratrol has been shown to be a potent inducer of autophagy in several cell culture models, including cardiomyocytes [13–16]. Studies investigating whether resveratrol can induce autophagy in animals, however, are limited. In this study, we investigated whether CR or resveratrol alone, or a combination of the two, can stimulate autophagy in the hearts of 26-month-old FBN rats.

In addition to exploring interventions to induce autophagy in rodent hearts, we examined whether such autophagy interventions can protect against cardiac damages induced by the oxidative stressor doxorubicin. Although doxorubicin is a highly effective chemotherapeutic agent used in the treatment of solid tumors and hematologic malignancies [17], its cardiotoxic effects severely limit its dosage and, hence, its chemotherapeutic efficacy [17,18]. Although a complete understanding of the mechanisms involved in doxorubicin's toxicity remains elusive, the generation of reactive oxygen species (ROS) in cells has been proposed to be a major player [19,20]. The generation of ROS by doxorubicin can lead to cellular oxidative damage, resulting in cytotoxic effects [21], and the housecleaning process of autophagy can be hypothesized to be beneficial under such circumstances.

Our study showed that 50 mg/kg/day of resveratrol combined with 20% CR for 6 weeks can enhance autophagic flux in the hearts of 26-month-old FBN rats. Interestingly, CR or resveratrol alone was found to have no effect on autophagy. Induction of autophagy using the combinatorial approach helps protect rat hearts against doxorubicin-mediated toxicity. We therefore propose that induction of autophagy in late-middle-aged rat hearts could potentially be developed as a therapeutic target for mitigating oxidative stress-induced damage.

Materials and methods

Animals and dietary intervention

Male FBN rats at 25 months of age were purchased from the National Institute on Aging and were singly housed at the University of Florida animal facility in a temperature- (20 ± 2.5 °C) and light-controlled (12-h/12-h light/dark cycle) environment with unrestricted access to water. After arrival, the animals were acclimated for a period of 3 weeks and were then randomly assigned into one of six experimental groups: (1) ad libitum (AL), (2) 20% CR (CR), (3) 5 mg/kg/day resveratrol (Resv-5), (4) 50 mg/kg/day resveratrol (Resv-50), (5) CR with 5 mg/kg/day resveratrol (CR + Resv-5), and (6) CR with 50 mg/kg/day resveratrol (CR + Resv-50). Animals were maintained on the interventions for 6 weeks. Animals on the CR, CR + Resv-5, and CR + Resv-50 groups received 20% less food from a 125% fortified diet to ensure that all groups received equal amounts of proteins, vitamins, and minerals. Both AL and CR diets were based on the AIN-93M chow, formulated for the maintenance of mature rodents, with lower levels of proteins and fats to reduce the incidence of kidney stones in older rodents [22]. Supplementary Table S1 summarizes the composition of the AL and CR diets. Resveratrol (Sigma, St. Louis, MO, USA) was given as highly palatable supplementation pellets. Control supplementation tablets (without resveratrol) were given to AL rats. The supplementation pellets were similarly formulated, with the AL or CR diet as the base chow, with or without resveratrol, and were prepared by Custom Animal Diets (Bangor, PA, USA) using an effective bacon-flavor masking capability. The pellets were made as 1-g tablets containing either 0.5 or 5 mg resveratrol. To ensure that every rat received the proper dose of resveratrol, the number of pellets given on each day was calculated based on the body weight of individual animals. The consumption of tablets was visually inspected daily. Throughout the entire study, the amount of food given to the CR rats was adjusted weekly based on the food consumed by the AL rats. Body weights of rats were recorded at least once per week.

Induction of oxidative stress by doxorubicin

At the end of the intervention period, animals received a single intraperitoneal injection of 10 mg/kg doxorubicin (Sigma) or saline control. The dose was chosen based on our previous observation that 10 mg/kg/day of doxorubicin can activate caspase-3 and induce apoptosis in the hearts of Fischer 344 rats [23]. Twenty-four hours after injection, animals were sacrificed by rapid decapitation. Serum and plasma were collected by trunk blood processing and stored at −80 °C until further analysis. Hearts were dissected out, separated into individual compartments, weighed, and saved at −80 °C. All experiments and procedures were approved by the Institutional Animal Care and Use Committee at the University of Florida.

Body composition analysis

Fat and lean mass percentages of rats were determined at baseline levels (before the start of interventions) and after 6 weeks of CR and resveratrol interventions using time-domain nuclear magnetic resonance (TD-NMR) analyzer (Minispec, Bruker Optics, The Woodlands, TX, USA). TD-NMR provides an accurate, fast, and easy-to-use method for determining fat and lean tissue in rodents without the need for anesthesia. The validation of the TD-NMR methodology has been provided elsewhere [24].

Plasma resveratrol and resveratrol conjugates concentration analysis

Resveratrol and resveratrol conjugates were quantified in plasma using LC/MS/MS analysis at the Biomedical Mass Spectrometry Core at the University of Florida. Rat serum samples (30 μl) were loaded onto an Accela Open autosampler (Thermo Fisher Scientific, San Jose, CA, USA) connected to an Accela 600 UPLC and LTQ Velos mass spectrometer (Thermo Fisher Scientific). Separation was achieved on a BetaBasic C18 HPLC column (150 × 2.1 mm, 5 μm, Thermo Fisher Scientific).