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Nobiletin attenuates adverse cardiac remodeling after acute myocardial infarction in rats via restoring autophagy flux

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ABSTRACT

Background: Our previous study showed that autophagy flux was impaired with sustained heart ischemia, which exacerbated adverse cardiac remodeling after acute myocardial infarction (AMI). Here we investigated whether Nobiletin, a citrus polymethoxylated flavonoids, could restore the autophagy flux and improve cardiac prognosis after AMI. AMI was induced by ligating left anterior descending (LAD) coronary artery in rats. Nobiletin improved the post-infarct cardiac dysfunction significantly and attenuated adverse cardiac remodeling. Meanwhile, Nobiletin protected H9C2 cells against oxygen glucose deprivation (OGD) in vitro. The impaired autophagy flux due to ischemia was ameliorated after Nobiletin treatment by testing the autophagy substrate, LC3BII and P62 protein level both in vivo and in vitro. GFP-mRFP-LC3 adenovirus transfection also supported that Nobiletin restored the impaired autophagy flux. Specifically, the autophagy flux inhibitor, chloroquine, but not 3 MA, alleviated Nobiletin-mediated protection against OGD. Notably, Nobiletin does not affect the activation of classical upstream autophagy signaling pathways. However, Nobiletin increased the lysosome acidation which also supported that Nobiletin accelerated autophagy flux.

Taken together, our findings suggested that Nobiletin restored impaired autophagy flux and protected against acute myocardial infarction, suggesting a potential role of autophagy flux in Nobiletin-mediated myocardial protection.

1. Introduction

Despite progress in cardiovascular research and evidence-based therapies, acute myocardial infarction (AMI) is a leading cause of morbidity and mortality in industrialized countries [1]. Our previous study showed that autophagy was activated shortly after AMI, whereas sustained ischemia impaired cardiomyocyte autophagy flux, which exacerbated the post-infarct adverse cardiac modeling [2]. Accumulating evidences indicated that autophagy as a crucial modulator of ischemic diseases promote cell survival by ways of replenishing of substrates form degraded cellular constituents and eliminating the damaged organelles [3–5]. Therefore, modulating the activities of autophagy degradation system may be a promising therapeutic strategy for AMI.

Nobiletin, a citrus polymethoxylated flavonoids, is abundantly present in the peel of Citrus with versatile health-promoting properties including anti-inflammatory [6], antioxidant [7] and anticarcinogenic activities [8]. Additionally, Nobiletin also displays anti-atherogenic [9], anti-diabetic [10], hepatic-protective effects [11]. In recent years, interests have been growing in the neuro-cardiovascular protective effect of Nobiletin. Animal studies demonstrated that Nobiletin improves brain ischemia-induced learning and memory deficits and protects against cerebral ischemia/reperfusion injury [12–15]. These studies indicated that Nobiletin is neuroprotective against cerebral ischemic disease. However, whether Nobiletin could exert myocardial protection against ischemia is still unknown. In the present study, we investigated the effect of Nobiletin in AMI and explored the

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mechanism(s), if any, in particular focusing on the autophagy machinery.

2. Materials and methods

2.1. Animals and experimental protocols

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH publication no. 85–23, revised 1996). The protocol was approved by the Animal Research Committee, Guangzhou Medical University, Guangzhou, China. Adult male Sprague-Dawley rats (body weight around 200–220 g) were obtained from Medical Experimental Animal Center of Guangdong Province and received humane care. The left anterior descending (LAD) coronary artery was ligated as we have previously described [2,16,17]. Briefly, rats were anesthetized with 10% Chloral hydrate (0.3 ml/100 mg) intraperitoneally and artificially ventilated with a respirator, and all efforts were made to minimize suffering. Myocardial infarction was performed by LAD ligation by a 6-0 silk suture 2 mm below the tip of the left atrial appendage. Sham-treated animals underwent the same surgical procedure without ligating LAD coronary artery.

Rats were randomized into 3 groups: (i) sham group (Sham, n = 15); (ii) AMI group, AMI rats received the same volume of saline alone (AMI, n = 15); (iii) AMI + Nobiletin, AMI rats received Nobiletin intraperitoneally for 21 days (15 mg/kg/day; Cat#962600, Sigma-Aldrich, St. Louis, MO) (AMI + Nob, n = 15).

2.2. Echocardiography

Transthoracic echocardiography was performed with a VisualSonics (Vevo 2100; VisualSonics Inc., Ontario, Canada) equipped with a 30 MHz imaging transducer as we have described previously [17]. Briefly, rats were anaesthetized with 2% isoflurane gas with an inflow rate of 2–2.5 ml/min. The left ventricle was analyzed through the parasternal long- and short-axis views. The body temperature was maintained at 37 °C and the heart rate was maintained around 350 beats/min. The cardiac output (CO), left ventricle ejection fraction (LVEF), left ventricle fractional shortening (LVFS) and other parameters were calculated according to the guidelines of the Vevo 2100.

2.3. Determination of infarct size

The rats were anaesthetized with sodium pentobarbital (100 mg/kg) and assessed to be fully anaesthetized and sacrificed. As we have described previously [2], the ventricles were sliced transversely and incubated in 1% 2, 3, 5-triphenyl tetrazolium chloride (TTC; pH 7.4) for 20 min at 37 °C. The infarct area was shown as the area unstained by TTC and was measured by Image-Pro plus 5.0 (Media Cybernetics Inc., MD, USA).

2.4. H9C2 cell culture and oxygen-glucose deprivation (OGD)

The H9C2 cells were cultured and subjected to OGD as described previously [2]. Briefly, the cells were rinsed twice with serum-free, glucose and sodium pyruvate free DMEM (Cat#D5030, Sigma-Aldrich, St. Louis, MO) and cultured in the same medium at 37 °C in an anoxia chamber (InVivo 500, Ruskinn Life Science) saturated with 94%N_2/5%CO_2/1%O_2 for 12 h. Incubation of Nobiletin (20 μM), 3 MA (5 mM, Cat#M9281, Sigma-Aldrich, St. Louis, MO) or Chloroquine (20 μM, Cat#C6628, Sigma-Aldrich, St. Louis, MO) was 2 h before OGD treatment.

2.5. MTT assay

As described previously, the cells were incubated with MTT reagent (KeyGEN, China) for 4 h, followed by measurement of absorbance at 570 nm using a spectrophotometer [2].

2.6. Flow cytometry detecting FITC-Annexin V positive apoptotic cells

The cell apoptosis was detected by the FITC-Annexin V Apoptosis Detection Kit (Cat#556547, BD Pharmingen) according to the manufacturer's protocol. Briefly, the cells with indicated treatment were stained with FITC-Annexin V and propidium iodide (PI). Both early (Annexin V+/PI-) and late (Annexin V+/PI+) apoptotic cells were sorted by fluorescence-activated cell sorting (FACS) (Beckman Coulter Inc., Brea, CA). Cell apoptosis was reflected by the Annexin V positive cell percentage.

2.7. mRFP-GFP-LC3 adenovirus transduction and confocal microscopy

The mRFP-GFP-LC3 adenoviral particles were purchased from HanBio Inc (Shanghai, China). Cells were infected with adenoviral particles at 50 MOI. 24 h after adenovirus transduction, the cells were washed and fixed with 4% paraformaldehyde. Confocal sections were collected with Nikon A1 laser scanning confocal microscope (Nikon America Inc., Melville, NY) under uniform settings. The number of GFP and mRFP dots was determined by manual counting of fluorescent puncta from at least 4 different myocyte preparations with a 60× objective. At least 40 cells were scored in each experiment. The number of dots/cell was obtained by dividing the total number of dots by the number of nuclei in each microscopic field.

2.8. LysoTracker staining

Lysosome abundances were assessed by LysoTracker Red DND-99 (L7528, Thermo Fisher Scientific, Waltham, MA) staining. Cells were incubated with LysoTracker Red DND-99 (50 nM final concentration) at 37 °C for 30 min. Following fixation in 4% paraformaldehyde, the LysoTracker signal was excited at 562 nm, and emission was read at 595 nm using Nikon A1 laser scanning confocal microscope.

2.9. Western blot analysis

Western blot analysis was performed as we described previously [18]. The border zone of the infarct hearts was separated and homogenized. Equal amounts of protein (20 μg) were separated on SDS–polyacrylamide gels (10%) and electrotransferred to polyvinylidene difluoride membranes (Roche, Switzerland). The membranes were incubated with rabbit anti-LC3 (Cat#L7543, Sigma), rabbit anti-P62(Cat#5114), rabbit anti-phospho mTOR (Cat#5536), rabbit anti-mTOR (Cat#2972), rabbit anti-AMPK (Cat#5831), rabbit anti-Phospho-AMPK (Cat#2535), rabbit anti-PI3KIII (Cat#4263) and rabbit anti-Beclin1 (Cat#3495) from Cell signaling technology, goat anti-GAPDH (Cat# SC-48166, Santa Cruz Biotechnology) at1:1000 dilution at 4 °C overnight, and incubated with either goat anti-rabbit (1:5000 dilution; Santa Cruz Biotechnology) for 1 h at room temperature. Blots were developed using a chemiluminescent substrate and molecular band intensity was determined by densitometry.

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