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Short communication

Evaluating mitochondrial autophagy in the mouse heart

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ABSTRACT

Mitochondrial autophagy plays an important role in mediating mitochondrial quality control. Evaluating the extent of mitochondrial autophagy is challenging in the adult heart *in vivo*. Keima is a fluorescent protein that emits different colored signals at acidic and neutral pHs. Keima targeted to mitochondria (Mito-Keima) is useful in evaluating the extent of mitochondrial autophagy in cardiomyocytes *in vitro*. In order to evaluate the level of mitochondrial autophagy in the heart *in vivo*, we generated adeno-associated virus (AAV) serotype 9 harboring either Mito-Keima or Lamp1-YFP. AAV9-Mito-Keima and AAV9-Lamp1-YFP were administered intravenously and mice were subjected to either forty-eight hours of fasting or normal chow. Thin slices of the heart prepared within cold PBS were subjected to confocal microscopic analyses. The acidic dots Mito-Keima elicited by 561 nm excitation were co-localized with Lamp1-YFP dots (Pearson's correlation, 0.760, $p < 0.001$), confirming that the acidic dots of Mito-Keima were localized in lysosomes. The area co-occupied by Mito-Keima puncta with 561 nm excitation and Lamp1-YFP was significantly greater 48 h after fasting. Electron microscopic analyses indicated that autophagosomes containing only mitochondria were observed in the heart after fasting. The mitochondrial DNA content and the level of COX1/GAPDH, indicators of mitochondrial mass, were significantly smaller in the fasting group than in the control group, consistent with the notion that lysosomal degradation of mitochondria is stimulated after fasting. In summary, the level of mitochondrial autophagy in the adult heart can be evaluated with intravenous injection of AAV-Mito-Keima and AAV-Lamp1-YFP and confocal microscopic analyses.

1. Introduction

Autophagy is an important mechanism for degradation, where cytosolic proteins and organelles are sequestered in double-membrane vesicles called autophagosomes and delivered to lysosomes for degradation [1]. Although autophagy can degrade bulk intracellular materials in a relatively non-specific manner, it can also specifically target malfunctioning organelles. Activation of a series of events, including stabilization of Pink1 on depolarized mitochondria, phosphorylation of Mfn2, recruitment of Parkin to mitochondria, and recognition of depolarized mitochondria by autophagosomes, specifically eliminates damaged mitochondria; this process is termed mitochondrial autophagy or mitophagy [2, 3]. We have shown that dynamin-related protein1 (Drp1) also plays an important role in mitochondrial autophagy in cardiomyocytes (CMs) at baseline and in response to energy stress [4].

Increasing lines of evidence suggest that myocardial injury and heart failure are accompanied by mitochondrial dysfunction [5]. Dysfunctional mitochondria can be a major source of reactive oxygen species and trigger cell death, including apoptosis and necrosis. Furthermore, depolarized mitochondria fuse to healthy mitochondria and possibly decrease the overall health of mitochondria. Since mitochondrial autophagy or mitophagy is a major mechanism eliminating damaged mitochondria and maintaining mitochondrial function [2], mitochondrial autophagy is expected to be activated in the heart under stress and protect the heart against it.

Conventionally, mitochondrial autophagy is detected by the presence of autophagosomes containing mitochondria, as evaluated with electron microscopic analyses [2]. Alternatively, mitochondrial autophagy is also identified by co-localization of established markers of autophagosomes, such as LC3, or autolysosomes, such as Lamp1 and Lamp2, and mitochondrial proteins, including COX1. In addition, stimulation of mitochondrial autophagy is often accompanied by decreases in mitochondrial mass, as evidenced by decreases in mitochondrial DNA content or COX1/GAPDH, although decreases in mitochondrial mass can be compensated by concomitant increases in mitochondrial

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biogenesis [6], and, thus, they are not obvious in some conditions. Therefore, the evaluation of mitochondrial autophagy should proceed using multiple methods. Unfortunately, the aforementioned methods are generally laborious and some methods, including electron microscopic analyses, are challenging to quantify. These problems are particularly true when one conducts analyses using *in vivo* samples and they generally make the analyses of mitochondrial autophagy challenging and, in some cases, less convincing.

Mito-Keima is a mitochondria-associated, pH-sensitive fluorescent protein and it emits a different color when it moves to a subcellular environment with low pH, namely lysosomes [7]. We have shown recently that Mito-Keima is an extremely convenient and accurate measure of mitochondrial autophagy in cardiomyocytes *in vitro* [4]. Here, we describe *in vivo* application of Mito-Keima to evaluate mitochondrial autophagy in the adult heart. Using adeno-associated virus harboring Mito-Keima and confocal microscopic analyses of thin-sliced myocardial sections, we here successfully demonstrate activation of mitochondrial autophagy in the adult heart *in vivo* in response to fasting, a well-established stress for inducing mitochondrial autophagy in cardiomyocytes.

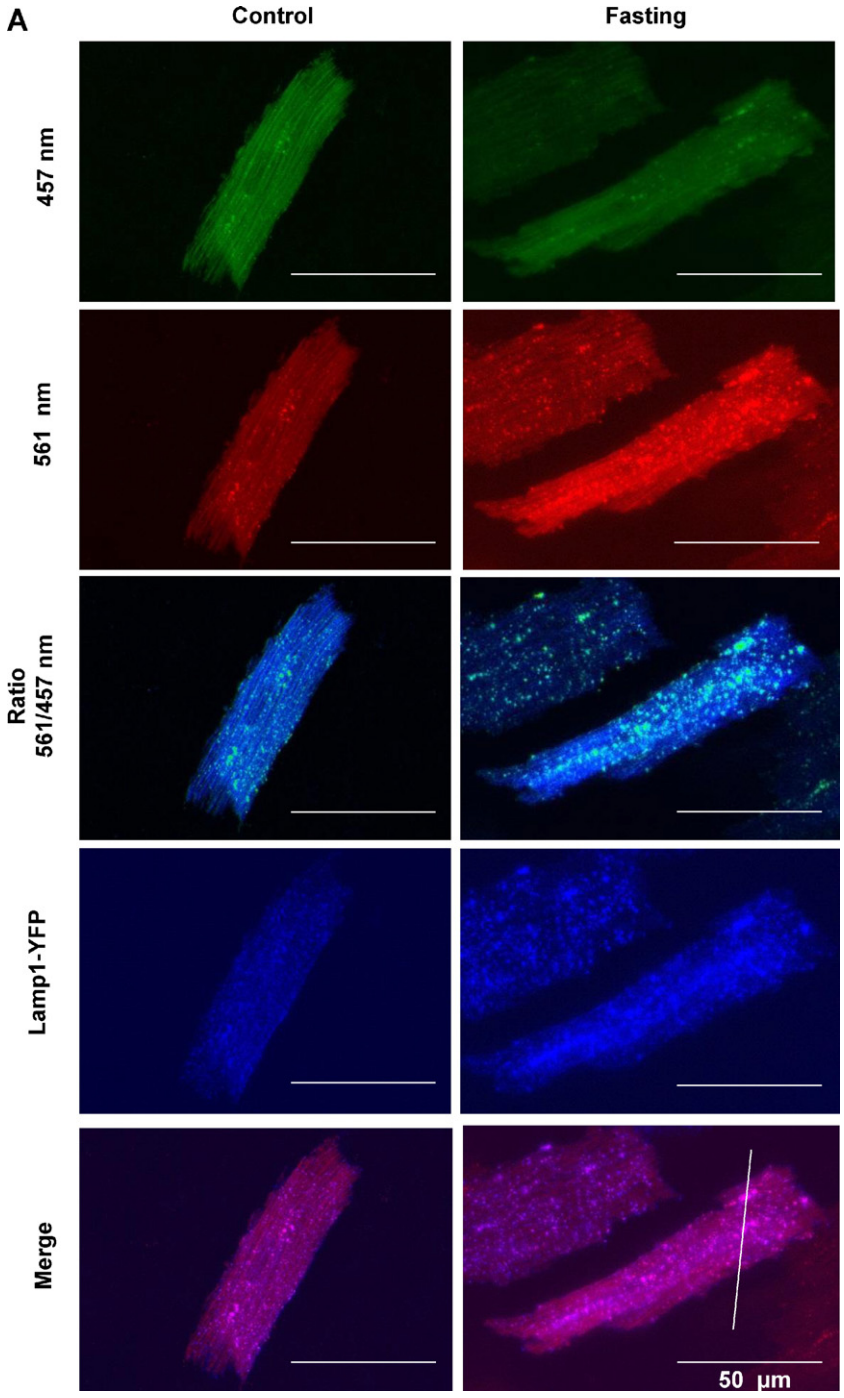

Fig. 1. *Evaluation of mitochondrial autophagy by AAV9-Mito-Keima and AAV9-Lamp1-YFP.* C57BL/6 J mouse hearts were examined in the control group ($n = 3$) or after 48 h of fasting ($n = 4$). Mice were injected with AAV9-Mito-Keima and AAV9-Lamp1-YFP and subjected to either 48 h of fasting or control. Representative images of Mito-Keima green (457 nm), Mito-Keima red (561 nm), a ratiometric image of red to green Mito-Keima (561 nm/457 nm) indicating mitochondrial autophagy, Lamp1-YFP, and the merged image of Mito-Keima red (561 nm) and Lamp1-YFP are shown. A white line in the merged image in the fasting sample indicates a track of the line scan analysis shown in Fig. 2.