Structural and Mechanical Changes in Heart Mitochondria of Rat by AFM

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ABSTRACT

Mitochondria play critical roles in both the life and death of cardiac myocytes. Various factors such as loss of ATP synthesis and increase of ATP hydrolysis, impairment in ionic homeostasis, formation of reactive oxygen species (ROS) and release of proapoptotic proteins are related to the generation of irreversible damage. It has been proposed that the release of cytochrome c is caused by a swelling of the mitochondrial matrix triggered by the apoptotic stimuli. But there is a controversy about whether or not the mitochondria indeed swell during apoptosis. In this study, we observed the morphological and property changes in heart mitochondria which were isolated from rat myocardial infarction model using AFM. The normal mitochondria showed a homogeneous distribution with similar sizes and shapes. The surfaces of normal mitochondria looked smooth and had integrity. However, irregularities in the shapes and sizes of mitochondria were observed in those of ischemic rats. And the surfaces of ischemic mitochondria became rugged. Ultrastructural analysis of mitochondria utilizing AFM demonstrated that myocardial infarction resulted in significant increase in heart mitochondrial size, as compared with that of normal mitochondria. The force-distance curve measurements were performed to investigate the change in the adhesive force of mitochondria affected by myocardial infarction. We inferred that the changes in the adhesion force of mitochondria might be related to destruction of the highly dense mitochondrial outer membrane by swelling.

Keywords: heart mitochondria, myocardial infarction, AFM, swelling, force-distance curve measurements

1 INTRODUCTION

Atomic force microscopy (AFM) has become an important medical and biological tool for the non-invasive imaging of cells and materials since its invention by Binnig et al.[1]. The major advantages of AFM over conventional optical and electron microscopy for bioimaging include the lack of a requirement for special coatings or vacuum and the ability to perform imaging in any environment including air, vacuum or aqueous conditions. Although AFM was originally used to obtain surface topography, it can also precisely measure interactions between its probe tip and the sample surface, and can be used for force-distance curve measurements, which are a fundamental tool in surface chemistry, biochemistry, and material science [2,3]. The slope of an extended half in a force-distance curve is used to determine the stiffness of materials [4-6], and the retracted half in a force-distance curve is used to determine the adhesion force [7].

Changes in mitochondrial morphology, such as swelling and condensation have been associated with a wide range of important biological functions and pathologies [8]. In particular, it has been widely known that mitochondrial swelling is one of the most important indicators of the opening of the mitochondrial permeability transition (MPT) pore. The opening of channels or swelling sufficient to rupture the outer mitochondrial membrane may cause the release of cytochrome c, which, in turn, leads to necrotic or apoptotic cell death [9]. However, the supporting evidence for the mitochondrial swelling theory is based mainly on indirect observations using specific inhibitors of the PT pore or PT pore opening agent [10-13]. In addition, there is a controversy about whether or not the mitochondria indeed swell during apoptosis.

In this study, we investigated quantitatively the morphological changes in rat heart mitochondria that were induced by ischemic stimuli utilizing AFM. We also simultaneously examined the nano-mechanical changes in rat heart mitochondria by myocardial infarction using force-distance curve measurements.

2 MATERIALS AND METHODS

2.1 Animals

Six Male Sprague-Dawley rats (200-300 g) were used for the experiment after one week of acclimation under standard laboratory conditions at 22 ± 2 °C, constant humidity, and photoperiod (12 hour light-dark cycle). Commercial rat chow and water were provided ad libitum. The experiment was performed in accordance with the "Guide for the Care and Use of Laboratory Animals," prepared by the Institute of Laboratory Animal Resources and with prior approval by the Animal Experimentation Committee of the Kyung Hee University School of Medicine.

2.2 Induction of Myocardial Infarction

Rats were anesthetized by an intraperitoneal injection (0.9 ml/100 g body weight) of a mixture of ketamine (50 mg/ml) and xylene (20 mg/ml) at the ratio of 6.25:1. Following tracheotomy, rats were connected to a ventilator, and the respiratory rate was adjusted to obtain an arterial pH of 7.35–7.45.

Rats were randomly divided into two groups: control group (n=3) and myocardial infarction group (n=3). Myocardial infarction was induced by the permanent occlusion of the left anterior descending (LAD) coronary artery, as described previously [14]. The LAD was ligated at 2 mm from the origin, using a 5-0 proline suture. In sham-operated control rats, the same procedure was followed, but the ligation suture was not placed in the heart. Studies commenced three days after permanent myocardial infarction [15].

2.3 Mitochondria Isolation

Subcellular fractions of nuclei or mitochondria were isolated by differential centrifugation from normal and ischemic rat hearts, as described previously [16]. Highly enriched mitochondria were obtained by additional ultra-centrifugation using 30 ~ 50 % (1.1 and 1.6 g/ml) OptiprepTM density gradient media (Sigma-Aldrich, St. Louis, MO, USA). The purity of the mitochondria was confirmed by Western blot analysis using anti-HDAC (Abcam Inc., Cambridge, MA), anti-poly (ADP-ribose) polymerase (PARP) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-Hsp60 (Santa Cruz Biotechnology), anti-mtTFA (Santa Cruz Biotechnology), anti-SOD1 (Santa Cruz Biotechnology, CA, USA), and anti-beta-tubulin (Abcam Inc., Cambridge, MA, USA) antibodies, which are markers for nuclei, mitochondria, and cytoplasm, respectively. Proteins (30 μg) were separated by 12 % SDS-PAGE and transferred onto nitrocellulose membrane (Schleicher & Schuell BioScience, Inc., Keene, NH, USA). The membrane was incubated with primary antibody overnight at 4 °C. HRP-conjugated secondary antibodies (Cell Signaling Technology, Beverly, MA, USA) followed by ECL (Amersham Biosciences Inc. Piscataway, NJ, USA) were used for detection.

2.4 AFM Measurements

The mitochondrial solution was diluted with adsorption buffer (10 mM Tris-HCl (pH 7.2), 150 mM KCl, 25 mM $MgCl_2$) and dropped onto a fresh mica surface. The prepared samples were briefly air-dried at room temperature and immediately imaged by AFM. Imaging was performed using the non-contact mode of NANOS N8 NEOS (Bruker, Herzogenrath, Germany) equipped with a 42.5 × 42.5 × 4 μm^3 XYZ scanner and two Zeiss optical microscopes (Epiplan 200× and 500×). External noise was eliminated by placing the AFM on an active vibration isolation table (Table Stable Ltd., Surface Imaging Systems, Herzogenrath, Germany) inside a passive vibration isolation table (Pucotech, Seoul, Republic of Korea). The mitochondria on mica were scanned at a resolution of 512 × 512 pixels, with a scan rate of 0.8 line/sec.

Force-distance curve measurements were performed by the reflex-coated silicon cantilevers for the contact mode (PR-CO, Surface Imaging Systems, Germany) which had a spring constant of 0.2 N/m. The mitochondrial force data were obtained at locations with similar heights to avoid edge effects.

The shape parameters of the mitochondria, including their areas, perimeters, lengths, breadths, and aspect ratios, were measured from the topographic images using the Scanning Probe Imaging Processor (SPIP^{TM}, Image Metrology, Hørsholm, Denmark). Fifty mitochondria were selected and their shape parameters measured in each specimen, and thirty sites of mitochondria were selected for the force-distance measurements. The adhesion force was calculated using the SPIP^{TM} software from the retraction process of the force-distance curve, and the stiffness was obtained from the slope of the linear region of the extension curve [7].

2.5 Statistics

The results of the shape parameters and adhesion forces were expressed as the means ± standard deviations (SD). A statistical analysis was performed to compare the ultrastructural and nano-mechanical changes between the normal and ischemic heart mitochondria using a two-tailed Student's t-test. P-values less than 0.05 were regarded as statistically significant

3 RESULTS

We observed AFM topographical images of 1 μm × 1 μm to get an initial impression of the morphological differences between normal and infracted mitochondria. The purity of mitochondria was confirmed by Western blot analysis using markers for nuclei, mitochondria, and cytoplasm, respectively. As shown in Fig. 1, the surfaces of normal mitochondria looked smooth and had integrity. However, the surfaces of ischemic mitochondria became rugged. Some debris could be observed around the apical ends of the mitochondrial membranes and the outer membranes had collapsed.

To characterize the morphological changes in mitochondria by myocardial infarction, the particle analysis module was used for the detection and quantification of mitochondria. Normal mitochondria appeared ellipsoidal (mean axial ratio of 1.32 ± 0.16) with a mean area of 3685 ± 923 nm^2 (determined from 30 mitochondria from three different preparations). AFM height and width measurements revealed mitochondria to be unexpectedly flattened, presumably caused by adsorption to the mica surface [17].

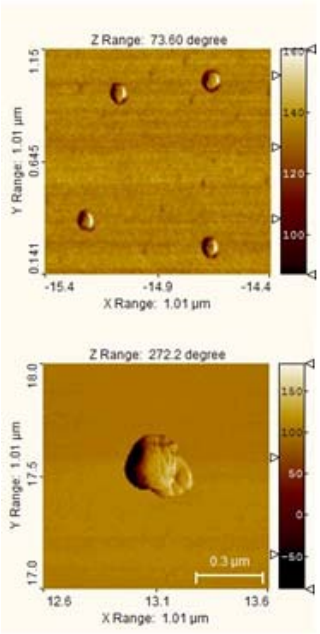

Figure 1 : Representative AFM phase images (1 µm × 1 µm) of mitochondria isolated from normal (left) and ischemic (right) hearts.

As compared to the corresponding normal values, ischemic mitochondria showed a significant increase in all parameters. The mean axial ratio and area were 1.14 ± 0.10 and 29559 ± 21433 nm^2 (n=30, $p < 0.0001$ vs. control, respectively). Ultrastructural analysis of mitochondria utilizing AFM demonstrated that myocardial infarction resulted in significant increase in heart mitochondrial size, as compared with that of normal mitochondria.

The force-distance curve measurements were performed to investigate the change in the adhesive force of mitochondria affected by myocardial infarction. Figure 2 presents representative force-distance curves of mitochondria isolated from normal and ischemic hearts in rats.

As shown in Fig. 2, the adhesion force of ischemic heart mitochondria significantly decreased to 19.56 ± 1.08 nN (n=30, $p < 0.0001$), as compared to normal ones with an adhesion force of 27.64 ± 0.88 nN. Also, the stiffnesses calculated from the slopes of the approach curves of normal and ischemic heart mitochondria were 0.205 ± 0.007 and 0.211 ± 0.011 nN/nm ($p < 0.05$), respectively. It seemed that the ischemic mitochondria were rather stiffer than normal ones.

4 DISCUSSION

It is well known that permanent ischemia causes a loss of matrix density, and this is associated with mitochondrial swelling [18]. It has been proposed that the release of cytochrome c is caused by a swelling of the mitochondrial matrix triggered by the apoptotic stimuli [19]. The most widely recognized biochemical features of apoptosis during ischemic stimuli are the release of cytochrome c from the mitochondria and the activation of a class of cysteine proteases, called caspase, including caspase-3, caspase-8 and caspase-9 [20]. However, there is some controversy about whether or not the mitochondria, indeed, swell during apoptosis. Additionally, there are few reports on the shape changes and swelling of mitochondria isolated from the heart after myocardial infarction utilizing optical measurements, let alone AFM.

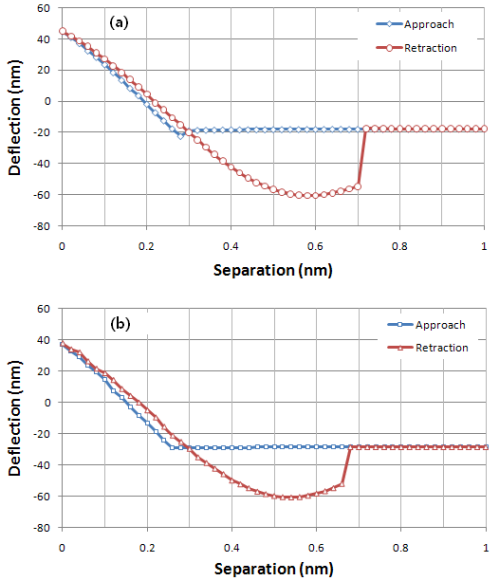

Figure 2 : Representative results of force–distance measurements of mitochondria isolated from normal (a) and ischemic (b) hearts

The morphological differences in the mitochondria between normal and ischemic hearts were quantitatively evaluated using particle shape analysis on AFM topographic images. As a result, it seemed that myocardial infarction resulted in mitochondrial swelling.

Force-distance curve measurements were used to investigate the changes in adhesion forces of swollen mitochondria affected by myocardial infarction. The mitochondrial outer membrane consists of a relatively simple phospholipid bilayer containing proteins such as porins. Therefore, we inferred that the changes in the adhesion force of mitochondria might be related to destruction of the highly dense mitochondrial outer membrane by swelling. Additionally, it seemed that ischemic stimuli might induce the stiffening of mitochondria.

5 CONCLUSIONS

This is the first study using AFM to investigate the morphological and nano-mechanical changes in the isolated rat heart mitochondria after myocardial infarction. From the particle analysis of AFM topographic images, we quantitatively evaluated structural changes in heart mitochondria induced by myocardial infarction. Furthermore, the viscoelastic change in ischemic heart mitochondria was revealed by force-distance analysis. Therefore, we suggest that myocardial infarction might be the cause of mitochondrial swelling and the changes in the adhesion force and stiffness of mitochondria

ACKNOWLEDGEMENTS

This study was supported by Bio R&D program through the national research foundation of Korea funded by the Ministry of Education, Science and Technology (2010-0019917).

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