Morphological Dynamics of Mitochondria in Bovine Aortic Endothelial Cell under Cyclic Stretch

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Abstract Mitochondria are subcellular organelles that synthesize ATP, generate reactive oxygen species (ROS), and control cellular fates such as apoptosis and aging. Mitochondria generate different amounts of ROS in association with their morphologies. Cyclic stretch is a mechanical stimulation exerted on cells due to arterial pulsation, and induces cells to generate mitochondrial ROS. Therefore, one can speculate that morphological changes of mitochondria may play a role in mitochondrial ROS generation in cells under cyclic stretch. However, whether the morphologies of mitochondria are actually altered under cyclic stretch remains unclear. This study attempted to answer this question by time-lapse imaging the morphological dynamics of mitochondria in bovine aortic endothelial cells (BAECs) subjected to two levels of uniaxial cyclic stretch: (1) a physiologic level (5% at 1 Hz) for 1 hour, and (2) a supra-physiologic level (20% at 1 Hz) for 1 hour. Mitochondria were stained with Mito-tracker Orange, and MicroP software and FibrilTool were used for mitochondrial alignment and length analyses. No clear changes in the average length of mitochondria were observed at the physiological level of stretch (5%) compared to no stretch (0%), while the average length was decreased by the supra-physiological level of stretch (20%). In addition, cellular alignment was not different between 0% and 5% stretches, but the cells became perpendicularly aligned in the direction of stretch when 20% stretch was applied. Cellular circularity was not significantly different among the three levels of cyclic stretch. Thus, BAECs exhibited changes in both mitochondrial dynamics and cellular remodeling dynamics under 20% stretch, but showed no changes in both under 5% stretch. The results indicate that changes in morphological dynamics of mitochondria correlate with changes in cellular dynamics, particularly change in cellular alignment.

Keywords: mitochondria, morphology, time-lapse imaging, bovine aortic endothelial cells, cyclic stretch.

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1. Introduction

Cyclic stretch is a mechanical stimulation that is exerted on endothelial cells due to arterial pulsation [1]. Under physiological elongation of 6–10%, cyclic stretch induces anti-apoptotic effects [2, 3]. On the other hand, under supra-physiological elongation of 20%, cyclic stretch causes apoptosis [2, 4]. The difference in response depending on the degree of elongation is thought to involve differences in the amounts of intracellular reactive oxygen species (ROS), superoxide (O_2^•−), and H_2O_2 [5, 6]. Because mitochondria produce ROS in cells under cyclic stretch [7, 8], mitochondria may be involved in the different cellular effects under physiological and supra-physiological cyclic stretches.

Mitochondria are subcellular organelles that synthesize ATP and play important roles in cellular signaling [9]. Mitochondria are dynamic organelles that undergo repeated fusion and fission [10, 11]. When the balance of fusion and fission is disrupted, mitochondrial morphology is altered. In conjunction with these morphological alterations—particularly mitochondrial shortening—ROS production is increased [12]. Thus, morphological alterations of the mitochondria may be involved in mitochondrial ROS production under cyclic stretch.

The morphology of mitochondria depends on the cytoskeleton. Mitochondria are physically anchored to microtubules via adaptor molecules [13]. In that regard, the microtubules function as the backbone of mitochondria [14]. When the microtubules are disrupted, the morphological structures of the mitochondria are also disrupted [15]. In cells subjected to cyclic stretch, the alignment of cytoskeletons, especially actin, is altered so that the cell axis becomes perpendicular to the stretch axis [16]. Recently, alteration of their microtubule alignment has also been demonstrated [17]. Thus, it can be hypothesized that cyclic stretch alters the cytoskeletal alignment, thereby altering mitochondrial morphology and induces ROS production. Because alteration in cytoskeletal alignments has been shown to correlate with the elongation value of cyclic stretch [18], the hypothesis agrees with previous experimental finding that ROS production is enhanced under cyclic stretch with larger elongation values. These findings lead us to ask whether the morphologic alteration of mitochondria under cyclic stretch correlates with the elongation value of cyclic stretch.

In this study, in order to understand whether mitochondrial morphology is altered in cells under cyclic stretch, we time-lapse imaged mitochondria in bovine aortic endothelial cells (BAECs) under cyclic stretch. In addition, in order to understand whether the change in the mitochondrial morphology correlates with the elongation value of cyclic stretch, BAECs were subjected to two types of cyclic stretch, physiologic stretch causing 5% elongation, and supra-physiologic stretch causing 20% elongation.
2. Methods

2.1 Cell culture
BAECs (LONZA Japan Ltd.) were cultured in DMEM (Dulbecco’s Modified Eagle’s Medium; Sigma) supplemented with 10% fetal bovine serum, penicillin/streptomycin and L-glutamine. BAECs were cultured in a 60-mm cell culture dish in a 5% CO₂ incubator at 37°C until they became confluent. For subculture, BAECs were detached with 0.025% trypsin solution. BAECs in the 3rd to 7th passage were used in experiments.

2.2 Stretch chamber
Base and curing agents of polydimethylsiloxane (PDMS) (Sylgard 184; Dow Corning Toray) were mixed in a ratio of 25:2 and poured into a handmade mold. For molding, PDMS was baked at 80°C for 1 h (Fig. 1A). The molded stretch chamber was connected to stretching motors by grippers (Fig. 1B). Before inoculating the cells in a stretch chamber, PDMS was treated with oxygen plasma (PDC-32G; Harrick Plasma) and coated with fibronectin by incubating with fibronectin solution (20 μg/ml in PBS) at room temperature for 1 h. The BAECs were then inoculated into the stretch chamber to 80% confluence, and further cultured overnight before the experiments.

2.3 Cyclic stretch and fluorescence imaging
To visualize the mitochondria, BAECs were stained with MitoTracker Orange (1 μM in serum-free DMEM; Molecular Probes) in a 5% CO₂ incubator for 20 min. After washing with fresh DMEM, the stretch chamber was connected to a stepper motor (SGSP-20-35; Sigma Koki). The BAECs were subjected to cyclic stretch to effect 5% or 20% elongation at 1 Hz frequency. Cyclic stretch was paused for 2 min every 10 min for fluorescence imaging of mitochondria (Fig. 2). During the pauses, fluorescence images were obtained from 6 imaging fields. The positions of the imaging fields were selected prior to cyclic stretch and preset in a PC so that the motorized XY stage on the microscope replicated all 6 positions. Fluorescence images were obtained using a confocal microscope (FV-1000; Olympus). For fluorescence imaging, exposures were made using excitation wavelength of 543 nm and emission wavelength of 576 nm through an objective lens (60×, N.A 1.1; Olympus). During cyclic stretch and fluorescence imaging, BAECs were incubated in a microscope incubator (Tokai Hit) with 5% CO₂ at 37°C. BAECs were incubated in the microscope incubator for 30 min prior to cyclic stretch.

2.4 Image analysis
The fluorescence images obtained were trimmed with ImageJ (freehand selection and clear outside, NIH) to extract the images of a single cell. Brightness was further adjusted with ImageJ (brightness/contrast), and then the image size was standardized to 400 × 400 pixels using Irfan View (freeware) without changing the magnification.

For the evaluation and identification of mitochondrial morphology, the lengths and alignment angles of mitochondria were quantified from fluorescence images. For quantification of the length of mitochondria using MicroP software, a Matlab plugin developed by Peng et al. [19] specifically for the analysis of mitochondrial morphology was used. Although this program is a powerful tool for mitochondrial morphology analysis, it was not suitable for use with the fluorescence images obtained in this study because of low resolution. Thus, the fluorescence images were further analyzed using another software package, FibrilTool. This is a plugin of ImageJ developed by Boudaoud et al. [20] for analyzing the anisotropy and orientation of fibrous images. In this study, the lengths and alignments of mitochondria were estimated using the “anisotropy” and “orientation” analytical functions, respectively, of FibrilTool. For example, when the mitochondria are short, the anisotropy values are low, and vice versa. Since these interpreted values still contain some degree of uncertainty, the correlativity of these values was further evaluated using MicroP and FibrilTool (Fig. S1). The correlativity (R) was 0.31.

The cellular morphology, alignment and circularity were quantified with ImageJ as follows. A single cell was first outlined using the freehand function, and then approximate with ellipse. Finally, the ellipse, angle of the major axis, and circularity were quantified.

2.5 Statistical analysis
All behavioral data are expressed as mean ± SEM. Data were analyzed using one-way analysis of variance (ANOVA), followed by Dunnett’s test for post-hoc analysis to determine whether parameter values under 5% or 20% cyclic stretch were significantly different from those under 0% cyclic stretch (control).

3. Results

3.1 Mitochondrial dynamics
In BAECs under cyclic stretch, the morphology of mitochondria was sequentially imaged with time-lapse fluorescence imaging. Under 0% (Movie S1) and 5% cyclic stretch (Fig. 3A and Movie S2).
S2), the mesh morphology of mitochondria was preserved during the imaging period. On the other hand, under 20% cyclic stretch (Fig. 3B and Movie S3), the mesh morphology was disrupted. The mesh morphology gradually ruptured to exhibit rather sparse condensate. To quantitatively evaluate the morphological differences, the lengths and alignment angles of mitochondria were analyzed with image analysis.

The lengths of mitochondria were measured using two image-analysis tools: MicroP software that uses binary images, and FibrilTool that uses a spatial gradient of fluorescence intensity. Because the former version of MicroP was used to analyze the lengths of mitochondria in a previous study [19], we first used the latest version of MicroP to analyze the lengths of mitochondria in this study. We observed no distinct change in mean mitochondria length after 5% cyclic stretch compared with 0% cyclic stretch, but detected a decrease in the mean length after 20% cyclic stretch (Fig. S2). Although analysis using MicroP showed a trend of change in mitochondrial length for 20% cyclic stretch, the results of the analysis of length alteration remain unclear. Consistent change in the mean length under 20% cyclic stretch was clearly captured using FibrilTool (Fig. 4A). While MicroP uses binary images, which is likely to be influenced significantly by fluorescence blur, the results obtained from FibrilTool may remain robust in the presence of such fluorescence blur because the soft-

**Fig. 3** Morphological dynamics of mitochondria under 5% cyclic stretch (A) and 20% cyclic stretch (B).

**Fig. 4** Alterations in mitochondria morphology: mitochondria length estimated from fluorescence anisotropy (A) and mitochondrial alignment angle estimated from orientation (B). Data are expressed as mean ± SEM (N = 36 for control, N = 28 for 5% cyclic stretch, n = 30, N = 30 for 20% cyclic stretch). *p < 0.05 vs. control, **p < 0.01 vs. control, one way ANOVA followed by Dunnett’s test for post-hoc comparisons.
and mitochondria were altered under 20% cyclic stretch in that mitochondrial length as well as alignment angles of the cells stretch lies between 5% and 20%. In addition, our data showed of stretch that affects mitochondrial morphology under cyclic 5% and 20% elongation. The results suggested that the threshold lapse imaging under two conditions of cyclic stretch resulting in In this study, mitochondrial dynamics were imaged with time-otics, especially changes in cellular alignment, may correlate with remodeling dynamics. These results indicate that cellular dynam-

3.2 Correlation with cellular alignment and circulation
To examine the correlation between mitochondrial dynamics and cellular remodeling dynamics, cellular alignment and cellular circularity were quantified using the fluorescence images. No change in cellular alignment in BAECs was observed under 5% cyclic stretch compared with 0% (control), while they exhibited a consistent increase in alignment angle under 20% cyclic stretch (Fig. 4B). This alteration in mitochondrial alignment was observed between 24 min and 36 min after the start of cyclic stretch. Since the reduction of mitochondrial length started within the first 12 min of cyclic stretch, it appeared that the mito-

ware uses the gradient of the spatial distribution of fluorescence intensities for analysis.

The angle of mitochondrial alignment was also analyzed using FibrilTool. BAECs exhibited no difference in mean alignment angle under 5% cyclic stretch compared with 0% (control), while they exhibited a consistent increase in alignment angle under 20% cyclic stretch (Fig. 4B). This alteration in mitochondrial alignment was observed between 24 min and 36 min after the start of cyclic stretch. Since the reduction of mitochondrial length started within the first 12 min of cyclic stretch, it appeared that the mitochondria were first shortened and then underwent alignment change in BAECs under 20% cyclic stretch.

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Thus, under 5% cyclic stretch, BAECs exhibited no change in mitochondrial dynamics and no change in cellular remodeling dynamics. However, under 20% cyclic stretch, BAECs exhibited changes in both mitochondrial dynamics and cellular remodeling dynamics. These results indicate that cellular dynamics, especially changes in cellular alignment, may correlate with mitochondrial dynamics.

4. Discussion
In this study, mitochondrial dynamics were imaged with time-lapse imaging under two conditions of cyclic stretch resulting in 5% and 20% elongation. The results suggested that the threshold of stretch that affects mitochondrial morphology under cyclic stretch lies between 5% and 20%. In addition, our data showed that mitochondrial length as well as alignment angles of the cells and mitochondria were altered under 20% cyclic stretch in BAECs, but not under 5% cyclic stretch (Fig. 4, Fig. 5A). On the other hand, cellular circularity was not altered under both levels of cyclic stretch (Fig. 5B). Thus we concluded that in BAECs, mitochondrial morphology was altered under 20% cyclic stretch, in association with change in cellular alignment.

We speculate that shortening of mitochondria in BAECs under 20% cyclic stretch may involve the following two mechanisms.

First, fission of mitochondria may be enhanced by application of excessive cyclic stretch. Mitochondrial morphology is altered by repeated fission and fusion [10, 11]. Our observations suggest that this balance is disrupted under excessive cyclic stretch, and fission overwhelms fusion to induce shortening of mitochondria. Although the biochemical mechanism in response to cyclic stretch involved in the overwhelming of fission is unknown, our observation supports the postulation that fission-enhancing proteins such as Drp1 could be activated. Drp1 is activated by the following mechanism. First, intracellular Ca2+ increases in cells under cyclic stretch [21]. When the level of intracellular Ca2+ is increased excessively, mitochondrial Ca2+ concentration also increases [22]. Increase in mitochondrial Ca2+ enhances the translocation of Drp1 to mitochondria in association with calci-nurin [23] to activate Drp1.

Second, mitochondrial fission may be caused by cytoskeleton remodeling. Cyclic stretch stimulates remodeling of cytoskele-

Fig. 5 Alteration in cellular morphology: cellular angle (A) and cellular circularity (B). Data are expressed as mean ± SEM (N = 36 for control, N = 28 for 5% cyclic stretch, n = 30, N = 30 for 20% cyclic stretch). *p < 0.05 vs. control, **p < 0.01 vs. control, one way ANOVA followed by Dunnett’s test for post-hoc comparisons.
result of release of mitochondria from microtubules [19]. If such twisting occurred in the present experiments, it might have been observed as shortening of mitochondrial length. However, it was difficult to distinguish fission from twisting on the fluorescence images in this study, because the quality of the images was insufficient.

Supra-physiological cyclic stretch has been suggested to induce ROS production [5, 6]. However, whether the morphological dynamics of mitochondria as observed in this study are involved in ROS production remains uncertain. We observed shortening of mitochondria. We speculate that mitochondrial shortening may be associated with mitochondrial fission and mitochondrial release from microtubules, as discussed above. Because mitochondrial fission is known to enhance ROS production [12], by analogy mitochondrial shortening could also affect ROS production. On the other hand, the relationship between mitochondrial release from microtubules and ROS production is uncertain. Thus, it is unclear whether ROS production is enhanced in association with mitochondrial release. Enhancement of ROS production is associated with cellular apoptosis. Because cellular apoptosis induced by supra-physiological cyclic stretch appears to be involved in hypertension [2], understanding whether and how the mitochondrial dynamics observed under cyclic stretch are involved in apoptosis would be significant.

Although we observed both mitochondrial dynamics and cellular dynamics simultaneously in cells under cyclic stretch, it remains unclear whether the two are causally related. As a hypothesized causal relation, phosphorylation of focal adhesion kinase induced by mitochondrial ROS [7] may be involved in cell alignment alteration under cyclic stretch [25]. Further experiments, perhaps using inhibitors of mitochondrial and cellular dynamics, are required to understand whether the two are causally related or both depend on common causative factors. Moreover, this study observed no changes in cellular morphology in BAECs under 5% cyclic stretch. However, a previous study showed that cyclic stretch resulting in such small magnitude of elongation induced cellular dynamics [18]. If changes in cellular morphology can be controlled by varying the elongation value, the relationship between mitochondrial dynamics and cellular dynamics can be discussed more easily.

As discussed above, shortening of mitochondria could be associated with mitochondrial fission and release of mitochondria from microtubules. However, these phenomena could not be distinguished in this study. Various technical improvements are required to distinguish them clearly. First, clearer mitochondrial images should be obtained [19], and use of genetically encoded fluorescent proteins such as mitoYFP would be useful [26]. The signal-to-noise ratio of genetically encoded fluorescence proteins is suggested to be lower than that of chemically synthesized dyes. Second, as a means to further clarify mitochondrial dynamics, previous studies have analyzed the levels of photoactivated fluorescent proteins expressed in mitochondria [27, 28]. Using photoactivation, single mitochondria can be visualized independent of neighboring mitochondria. Through combined use of the above technical improvements, it should be possible to elucidate the morphological dynamics of mitochondria much more precisely.

In the present study, significant changes in mitochondrial morphology over time was observed when the cells were under 20% cyclic stretch, but no change was detected under 5% cyclic stretch. The result implies the presence of a threshold between 5% and 20% stretch for alteration of mitochondrial morphology. Proof and identification of the threshold would help explain the mechanism of altered mitochondrial morphology under cyclic stretch, and facilitate planning of experiments that analyze intracellular signal activation under various magnitudes of cell elongation.

5. Conclusion

In this study, to examine whether mitochondrial morphology changes under cyclic stretch, mitochondria were time-lapse imaged in BAECs subjected to cyclic stretch that caused 5% and 20% elongation. The results showed that the mitochondrial length was shortened and mitochondrial alignment was altered under 20% cyclic stretch. However, those morphological changes were not observed under 5% cyclic stretch. Furthermore, cellular alignment was altered when BAECs was subjected to 20% cyclic stretch, but not 5% cyclic stretch. These results suggest that mitochondrial morphology is altered in association with change in cellular alignment, and cyclic stretch of 20% affects mitochondrial dynamics different from cyclic stretch of 5%.

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Conflict of interest

We have no conflicts of interest relationship with any companies or commercial organizations based on the definition of Japanese Society of Medical and Biological Engineering.

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