Mitochondrial Remodeling in Endothelial Cells under Cyclic Stretch is Independent of Drp1 Activation

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Abstract: Mitochondria in endothelial cells remodel morphologically when supraphysiological cyclic stretch is exerted on the cells. During remodeling, mitochondria become shorter, but how they do so remains elusive. Drp1 is a regulator of mitochondrial morphologies. It shortens mitochondria by shifting the balance from mitochondrial fusion to fission. In this study, we hypothesized that Drp1 activation is involved in mitochondrial remodeling under supraphysiological cyclic stretch. To verify the involvement of Drp1, its activation was first quantified with Western blotting, but Drp1 was not significantly activated in endothelial cells under supraphysiological cyclic stretch. Next, Drp1 activation was inhibited with Mdivi-1, but this did not inhibit mitochondrial remodeling. Intracellular Ca^{2+} increase activates Drp1 through calcineurin. First, we inhibited the intracellular Ca^{2+} increase with Gd^{3+} and thapsigargin, but this did not inhibit mitochondrial remodeling. Next, we inhibited calcineurin with cyclosporin A, but this also did not inhibit mitochondrial remodeling. These results indicate that mitochondrial remodeling under supraphysiological cyclic stretch is independent of Drp1 activation. In endothelial cells under supraphysiological cyclic stretch, reactive oxygen species (ROS) are generated. Mitochondrial morphologies are remodeled by ROS generation. When ROS was eliminated with *N*-acetyl-L-cysteine, mitochondrial remodeling was inhibited. Furthermore, when the polymerization of the actin cytoskeleton was inhibited with cytochalasin D, mitochondrial remodeling was also inhibited. These results suggest that ROS and actin cytoskeleton are rather involved in mitochondrial remodeling. In conclusion, the present results suggest that mitochondrial remodeling in endothelial cells under supraphysiological cyclic stretch is induced by ROS in association with actin cytoskeleton rather than through Drp1 activation.

Keywords: Endothelial cells, cyclic stretch, mitochondria, morphology, Drp1.

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1 Introduction

Endothelial cells line the lumen of the vessel wall and are continuously exposed to cyclic stretch due to pulsatile blood pressure. Cyclic stretch with elongation of 5-10% is categorized as physiological stretch [Jufri, Mohamedali, Avolio et al. (2015)]. Physiological stretching maintains vascular homeostasis through the proliferation and sustenance of apoptosis of endothelial cells [Fujiwara (2003); Liu, Ensenat, Wang et al. (2003)]. On the other hand, cyclic stretch with elongation of 20% or more is considered supraphysiological stretch [Jufri, Mohamedali, Avolio et al. (2015)]. Supraphysiological stretch causes ROS generation and apoptosis of endothelial cells [Ali, Mungai and Schumacker (2006); Birukov, Jacobson, Flores et al. (2003)].

Mitochondria are dynamic organelles that undergo fusion, fission, and movement [El-Hattab, Suleiman, Almannai et al. (2018)]. Mitochondrial morphologies regulate their roles in metabolism and cell death [Xie, Shi, Tan et al. (2018); Yu and Pekkurnaz (2018)]. Under cyclic stretch with physiological elongation, mitochondria in endothelial cells maintain their morphologies. On the other hand, under cyclic stretch with supraphysiological elongation, mitochondria remodel from tubular to globular [Shinmura, Tsukamoto, Hamada et al. (2015)]. It remains elusive, however, how mitochondria in endothelial cells remodel their morphologies under supraphysiological cyclic stretch.

Drp1 is a regulator of mitochondrial dynamics [Hoppins (2014); Jayashankar and Rafelski (2014); Longo and Archer (2013)]. When Drp1 is phosphorylated at Ser616, mitochondria are squeezed and divided. Mitochondria shorten as a consequence of Drp1 phosphorylation. Mitochondrial shortening coincides with mitochondrial remodeling in endothelial cells under supraphysiological cyclic stretch. Moreover, in smooth muscle cells, mitochondria remodel morphologically through Drp1 activation in hypertension [Longo and Archer (2013); Marsboom, Toth, Ryan et al. (2012)]. Thus, Drp1 was hypothesized to be involved in mitochondrial remodeling under cyclic stretch in endothelial cells.

Cyclic stretch evokes an intracellular Ca^{2+} increase in endothelial cells [Murase, Naruse, Kimura et al. (2001); Naruse, Yamada and Sokabe (1998); Qiu, Zheng, Hu et al. (2014)]. The intracellular Ca^{2+} increase activates Drp1 through calcineurin [Cereghetti, Costa and Scorrano (2010); Han, Lu, Li et al. (2008); Longo and Archer (2013)]. Thus, it is probable that cyclic stretch activates Drp1 through calcineurin following an intracellular Ca^{2+} increase. In this study, we examined whether intracellular Ca^{2+} increase and Drp1 activation are involved in mitochondrial morphological remodeling in endothelial cells under supraphysiological cyclic stretch.

2 Materials and methods

2.1 Cell culture

Human aortic endothelial cells (HAECs; Lonza) were cultured in Medium 200 (Gibco) supplemented with 2% Low Serum Growth Supplement (Gibco) and 1% penicillin/streptomycin (Gibco). HAECs were cultured in 60 mm-cell culture dishes in a 5% CO_2 incubator at 37°C until reaching 70-100% confluence. For subculture, HAECs were dethatched with 0.025% trypsin solution. HAECs in passages 4-7 were used for experiments.

2.2. Stretch chamber

Stretch chambers were prepared as described previously [Shinmura, Tsukamoto, Hamada et al. (2015)] with modifications. Base and curing agents of PDMS (Sylgard 184, Dow Corning Toray) were mixed at a ratio of 10:1. After being poured into a handmade mold, PDMS was solidified at 80°C for 1 h. The molded stretch chambers were treated with oxygen plasma (PDC-32G, Harrick Plasma) and then incubated with (3-aminopropyl) trimethoxysilane solution (Sigma, 1% in ddH$_2$O) for 5 min. The stretch chambers were washed with ddH$_2$O 3 times and heated at 80°C for 1 h. The stretch chambers were then activated with glutaraldehyde solution (Wako, 0.5% in PBS) for 10 min and washed with PBS 3 times. Finally, the stretch chambers were incubated with collagen solution (Koken, 10 µg/ml in PBS) at room temperature for 20 min and washed with PBS 3 times. On the stretch chambers, HAECs were cultured until reaching 70-100% confluence before experiments.

2.3 Cyclic stretch and fluorescence imaging

HAECs were stained with MitoTracker Orange (Molecular Probes, 1 µM in serum-free Medium 200) for 30 min or with MitoTracker Green (Molecular Probes, 0.2 µM in serum-free Medium 200) for 15 min at 37°C and washed with fresh serum-free Medium 200 3 times. After HAECs were stained, a stretch chamber was connected to a stepper motor (SGSP-20-35, Sigma Koki). Before loading the cyclic stretch, the stretch chamber was stabilized in a microscope incubator (Tokai Hit) under the conditions of 5% CO$_2$ at 37°C for 30 min. The HAECs were subjected to cyclic stretch with an elongation of 5% or 20% and a frequency of 1 Hz. The cyclic stretch was paused for 2 min every 10 min in order to obtain fluorescence images. Fluorescence images of mitochondria were obtained in 6 imaging fields with a XY-stage-adapted confocal microscope (FV-1000, Olympus) through an objective lens (60×, N.A 1.1, Olympus) as described previously [Shinmura, Tsukamoto, Hamada et al. (2015)]. The wavelengths of excitation light and emission light for MitoTracker Orange were 516 nm and 576 nm, respectively. Those for MitoTracker Green were 490 nm and 543 nm, respectively.

2.4 Image analysis

The mitochondrial lengths were measured using fluorescence images as described previously [Shinmura, Tsukamoto, Hamada et al. (2015)]. In brief, fluorescence images were trimmed and their brightness was adjusted with ImageJ (NIH). The image size was standardized with Irfan View (freeware). Mitochondrial morphology was evaluated with FibrilTool [Boudaoud, Burian, Borowska-Wykręt et al. (2014)]. The mitochondrial morphologies were evaluated using the "anisotropy" analytical function of FibrilTool. The mitochondrial lengths were estimated using anisotropy [Shinmura, Tsukamoto, Hamada et al. (2015)]. For example, when the anisotropy values were low, the mitochondria were estimated to be short, and vice versa.

2.5 Western blotting for Drp1 activation

HAECs were subjected to cyclic stretch for 12 min and then were lysed with RIPA buffer. Protein concentrations were determined by a BCA protein assay kit (Pierce). Equal

amounts of proteins were subjected to SDS-polyacrylamide gel electrophoresis. Proteins were transferred to PVDF membranes, which were blocked with 5% skim milk in TBS-T. The membranes were incubated with primary antibodies followed by HRP-conjugated secondary antibodies. Signals were obtained by Clarity/Clarity Max ECL Western blot substrate kits (Bio-Rad). The primary antibodies used in this study were anti-total Drp1 (BD, 611112), anti-phospo-Drp1 (Ser616) (Cell Signaling Technology, #4494), and anti-actin (Millipore, MAB1501).

2.6 Inhibitors

Drp1 was inhibited by loading Mdivi-1 (Sigma, 20 µM) on HAECs for 15 min before the experiments. Ca^{2+} influx through the Ca^{2+} channel was inhibited by loading Gd^{3+} (Sigma, 10 µM) for 5 min before the experiments. Ca^{2+} release from the intracellular Ca^{2+} store was inhibited by loading thapsigargin (Sigma, 1 µM) for 30 min before the experiments. Calcineurin was inhibited by loading cyclosporin A (Sigma, 10 µM) for 15 min before the experiments. Actin polymerization was inhibited by loading cytochalasin D (Sigma, 1 µM) for 30 min before the experiments. All the inhibitors were diluted in Medium 200 and loaded on HAECs under the conditions of 5% CO_2 at 37°C. When cells were loaded with Gd^{3+} or thapsigargin, they were used in experiments without being washed. When loaded with other inhibitors, cells were washed 3 times before the experiments.

2.7 Statistical analysis

All 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. Statistical significance is shown by the symbol ''**'' when $p<0.01$ and ''*'' when $p<0.05$. Otherwise, the symbol ''NS'' is shown for nonsignificant cases as needed.

3 Results

3.1 Mitochondria remodeled in HAECs under supraphysiological cyclic stretch

Mitochondria in endothelial cells remodel their morphologies under supraphysiological 20% cyclic stretch [Shinmura, Tsukamoto, Hamada et al. (2015)]. In a previous study [Shinmura, Tsukamoto, Hamada et al. (2015)], bovine aortic endothelial cells (BAECs) were used as a model of endothelial cells. There was a scarcity of antibodies required for the detection of Drp1 activation in bovine cells. In BAECs, it was thus difficult to detect Drp1 activation with its antibodies. On the other hand, Drp1 antibodies for human cells were readily available. Drp1 activation could be easily detected in human cells. In this study, the model of endothelial cells was changed from BAECs to HAECs.

Under physiological 5% cyclic stretch, mitochondria failed to remodel their morphologies in HAECs (Fig. 1(A)). Under supraphysiological 20% cyclic stretch, mitochondria remodeled their morphologies (Fig. 1(B)). The remodeling in HAECs was remarkable within the first 12 min of the stretch (Figs. 1(C), 1(D)). This remodeling under supraphysiological 20% cyclic stretch is analogous to that in BAECs [Shinmura, Tsukamoto, Hamada et al. (2015)]. These results validated that HAECs are available as a model of endothelial cells in which mitochondria remodel their morphologies under

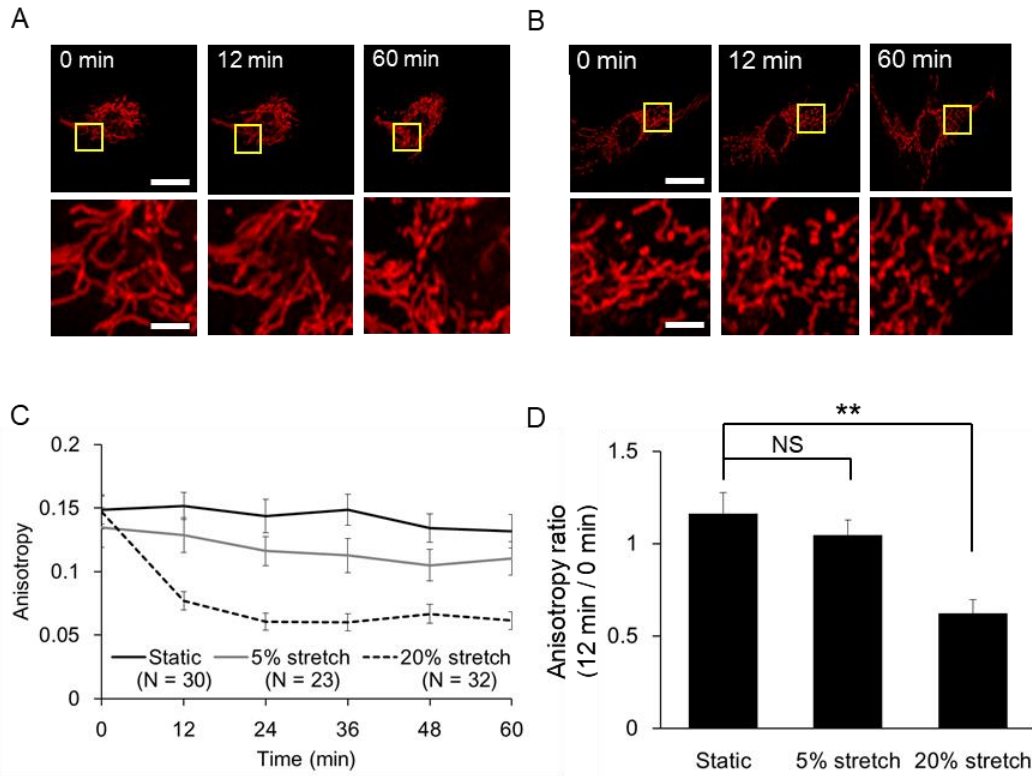cyclic stretch with supraphysiological 20% elongation.

Figure 1: Mitochondria in HAECs remodeled their morphologies. Fluorescence images of mitochondrial morphologies under cyclic stretch with physiological 5% elongation (A) and supraphysiological 20% elongation (B). Bars: 10 μm (top row), 2 μm (bottom row). (C) Alteration of mitochondrial anisotropy, which corresponds with mitochondrial length, under cyclic stretch. (D) Mitochondrial anisotropy after cyclic stretch for 12 min

3.2 Drp1 was not activated in HAECs under supraphysiological cyclic stretch

In HAECs, mitochondria remodeled their morphologies under supraphysiological 20% cyclic stretch within 12 min (Fig. 1(C)). In HAECs following supraphysiological 20% cyclic stretch, Drp1 was subtly activated: however, there was no significant difference between the 20% stretch conditions and the static condition (p=0.161, Fig. 2(A)). This result indicates that mitochondrial morphological remodeling does not involve Drp1 activation.

To further test the involvement of Drp1 activation in mitochondrial remodeling, Drp1 activity was inhibited by Mdivi-1, which inhibits Drp1 polymerization [Cassidy-Stone, Chipuk, Ingerman et al. (2008)]. Although Drp1 was inhibited in HAECs under supraphysiological 20% cyclic stretch, morphological remodeling was not inhibited (Fig. 2(B)). This result further shows that mitochondrial remodeling under supraphysiological 20% cyclic stretch does not involve Drp1 activation.
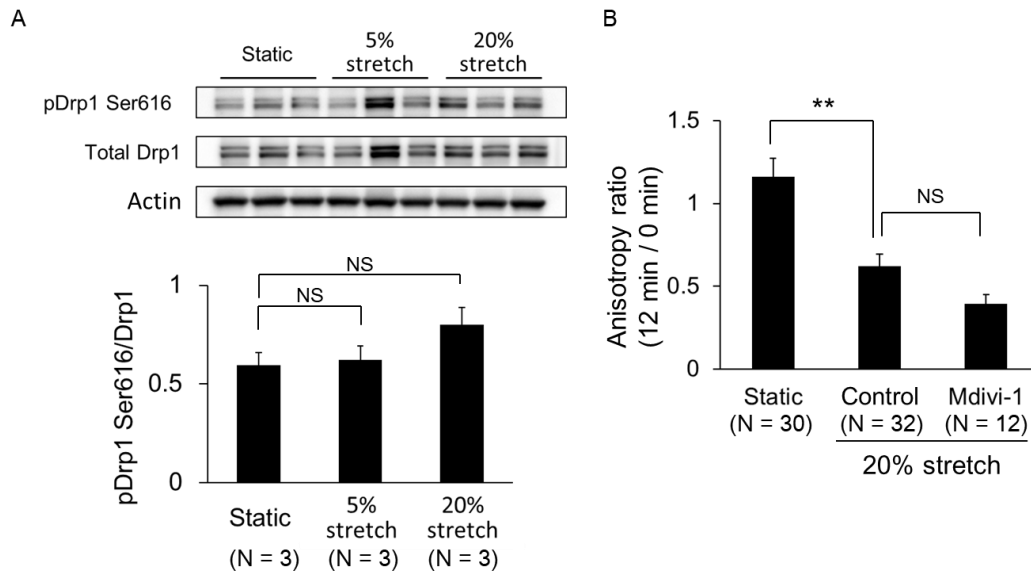
Figure 2: Drp1 phosphorylation at Ser616 under cyclic stretch and its involvement in mitochondrial remodeling. (A) Cyclic stretch failed to phosphorylate Drp1. (B) Inhibition of Drp1 by Mdivi-1 (20 µM) failed to inhibit mitochondrial remodeling under supraphysiological 20% cyclic stretch

3.3 Mitochondrial morphological alterations were independent of intracellular Ca^{2+} increase

Intracellular Ca^{2+} increase activates Drp1 through calcineurin [Cereghetti, Costa and Scorrano (2010); Han, Lu, Li et al. (2008); Longo and Archer (2013)]. Endothelial cells under cyclic stretch increase their intracellular Ca^{2+} [Murase, Naruse, Kimura et al. (2001); Naruse, Yamada and Sokabe (1998); Qiu, Zheng, Hu et al. (2014)]. Thus, Drp1 activation following intracellular Ca^{2+} increase through calcineurin is probable in HAECs under cyclic stretch. Intracellular Ca^{2+} increase has 2 major cascades: one is Ca^{2+} influx through plasma membrane, and the other is Ca^{2+} release from the intracellular Ca^{2+} store. Ca^{2+} influx in endothelial cells under cyclic stretch could be inhibited with Gd^{3+}, an inhibitor for mechanosensitive Ca^{2+} channels [Naruse, Yamada and Sokabe (1998)]. In HAECs under supraphysiological 20% cyclic stretch, Gd^{3+} failed to inhibit remodeling in mitochondrial morphologies (Fig. 3). Thapsigargin inhibits Ca^{2+} release by depleting intracellular Ca^{2+} stores [Murase, Naruse, Kimura et al. (2001)]. Thapsigargin also failed to inhibit remodeling of mitochondrial morphologies (Fig. 3). Cyclosporin A, an inhibitor of calcineurin [Fakata, Elmquist, Swanson et al. (1998)], also failed to inhibit remodeling (Fig. 3). These results are in line with the results that mitochondrial remodeling under supraphysiological 20% cyclic stretch does not involve Drp1 activation.
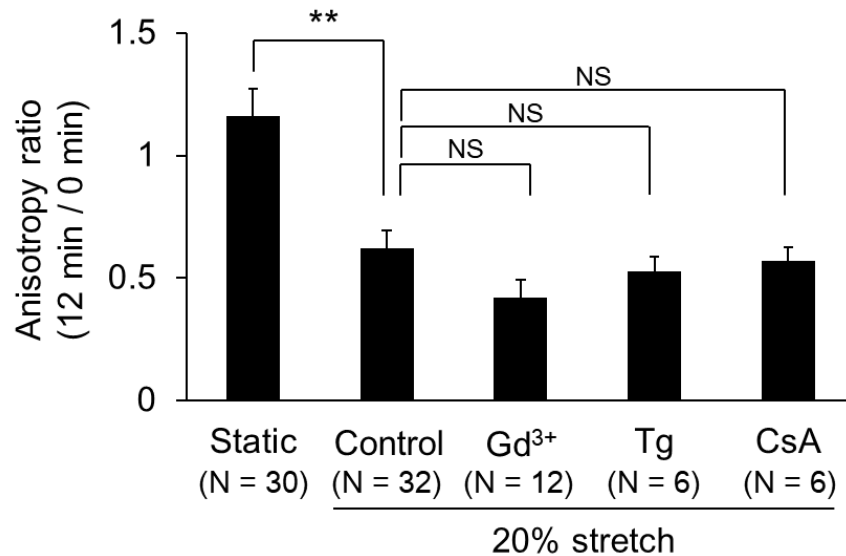
Figure 3: Mitochondrial remodeling does not involve intracellular Ca^{2+} increase or calcineurin. Inhibition of the intracellular Ca^{2+} increase by Gd^{3+} (10 µM) and thapsigargin (Tg, 1 µM), as well as that by calcineurin with cyclosporin A (CsA, 10 µM), all failed to inhibit mitochondrial remodeling under supraphysiological 20% cyclic stretch

3.4 Mitochondrial remodeling under supraphysiological cyclic stretch was dependent on actin cytoskeleton and ROS

Intracellular ROS is another cellular signal involved in mitochondrial remodeling [Deheshi, Dabiri, Fan et al. (2015); Galloway and Yoon (2012); Wu, Zhou, Zhang et al. (2011)]. ROS generation is enhanced when cells are subjected to excessive cyclic stretch [Ali, Mungai and Schumacker (2006); Ali, Pearlstein, Mathieu et al. (2004); Cheng, Chao, Wung et al. (1996); Cheng, Shih, Chen et al. (2001); Sung, Yee, Eskin et al. (2007); Wung, Cheng, Hsieh et al. (1997)]. Thus, intracellular ROS is probably involved in mitochondrial remodeling in HAECs under supraphysiological 20% cyclic stretch. To understand intracellular ROS's involvement, we eliminated it by using *N*-acetyl-L-cysteine. When ROS was eliminated, mitochondrial alterations under supraphysiological stretch were inhibited (Fig. 4).

Actin cytoskeleton remodels its structure under cyclic stretch [Mitsuoka, Tsukamoto, Iwayoshi et al. (2012)]. Actin cytoskeletons regulate mitochondrial physiologies in cells under cyclic stretch [Bartolák-Suki, Imsirovic, Parameswaran et al. (2015)]. To understand the involvement of actin cytoskeleton in mitochondrial remodeling, actin cytoskeleton was depolymerized with cytochalasin D. When actin cytoskeleton was depolymerized, mitochondrial alteration was also inhibited. This and a previous result indicate that ROS and actin cytoskeleton, rather than Drp1 activation, are involved in mitochondrial morphological remodeling under supraphysiological 20% cyclic stretch.
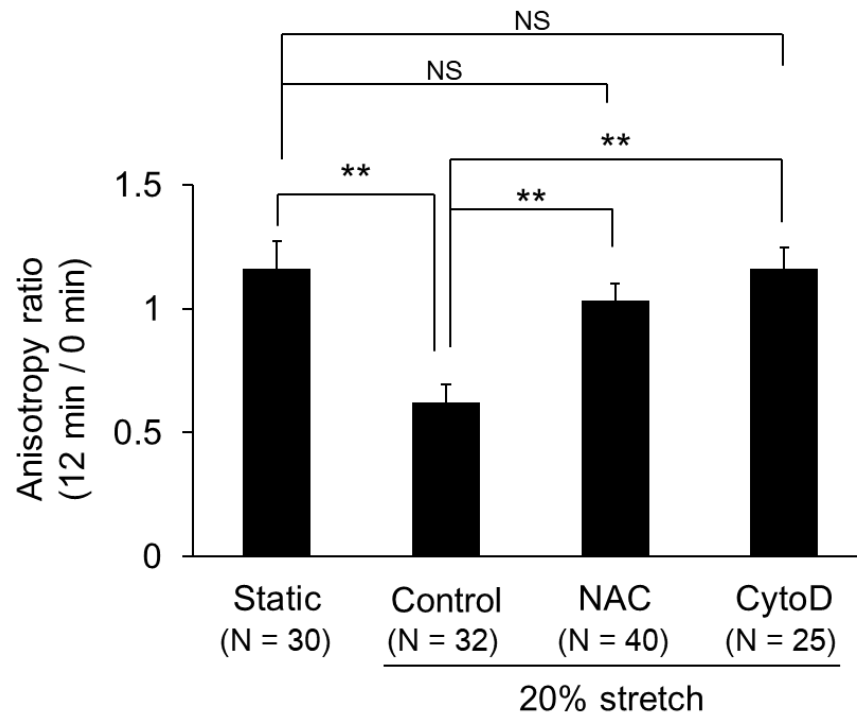
Figure 4: Mitochondrial remodeling involves ROS and actin cytoskeleton. Eliminating ROS with *N*-acetyl-L-cysteine (NAC, 1 mM) and inhibiting actin polymerization with cytochalasin D (CytoD, 1 µM) both inhibited mitochondrial remodeling under supraphysiological 20% cyclic stretch

4 Discussion

In this study, Drp1 was not involved in mitochondrial remodeling in endothelial cells under supraphysiological cyclic stretch. This implies that mitochondrial remodeling is not realized through mitochondrial fission, which is typically induced by Drp1 phosphorylation at Ser616. However, mitochondrial fission independent of Drp1 can occur, e.g., as observed in cells infected by bacteria [Stavru, Palmer, Wang et al. (2013)]. Actin cytoskeleton is involved in mitochondrial fission induced by bacterial infection. This involvement of actin cytoskeleton in mitochondrial remodeling coincides with its involvement in mitochondrial remodeling in endothelial cells under supraphysiological cyclic stretch (Fig. 4). Thus, it is also possible that mitochondrial remodeling under supraphysiological cyclic stretch is realized through mitochondrial fission.

Mitochondrial remodeling is not limited to fission and fusion [Peng, Lin, Chen et al. (2011)]. Other mitochondrial dynamics, including globules, twisted tubules, and loop fragmentation, can be involved in mitochondrial remodeling under cyclic stretch. Those dynamics can apparently shorten mitochondrial. Fluorescence images were obtained with single slices under a confocal microscope in this study. To understand mitochondrial distributions in the Z-axis direction, i.e., in a direction parallel to fluorescence excitation

light, 3D stack images are required. To understand whether those mitochondrial dynamics including lobules, twisted tubules, and loop fragmentation are involved in mitochondrial remodeling in endothelial cells under supraphysiological cyclic stretch, detailed observation and morphological analyses are required.

Mitochondrial remodeling in endothelial cells under supraphysiological cyclic stretch was dependent on ROS (Fig. 4). This result follows previous findings that ROS generation is enhanced in endothelial cells under cyclic stretch [Ali, Mungai and Schumacker (2006); Ali, Pearlstein, Mathieu et al. (2004); Muliyil and Narasimha (2014); Sung, Yee, Eskin et al. (2007)]. Opa1 regulates mitochondrial morphology by enhancing mitochondrial fusion. When ROS is generated, Opa1 is cleaved and mitochondria are fragmented [Garcia, Innis-Whitehouse, Lopez et al. (2018)]. Thus, it is also possible that ROS generated by cyclic stretch released Opa1 from mitochondria and in turn enhanced mitochondrial fragmentation.

Mitochondria anchor to the cytoskeleton via cross-linkers. Mitochondrial morphology, mobility, and positioning are physically supported by cytoskeletons [Jufri, Mohamedali, Avolio et al. (2015); Tang, Luo, Chen et al. (2014)]. Microtubules support mitochondrial long-range movements via microtubule motors. Actin cytoskeletons support those short-range movements and dynamics [Boldogh and Pon (2006); Hoppins (2014); Jayashankar and Rafelski (2014)]. When the cells are subject to high-magnitude stretch of 20% or above, actin is reoriented perpendicular to the stretch direction [Kaunas, Nguyen, Usami et al. (2005); Shinmura, Tsukamoto, Hamada et al. (2015)]. Under the reorientation, actin cytoskeletons remodel. Due to this remodeling, mitochondria could remodel as well.

Although we show that cyclic stretch with large elongation remodel mitochondrial morphologies independently of Drp1, it is not clarified whether this remodeling involves in pathologies including hypertension. Increased ROS generation might trigger endothelial cell dysfunction, possibly contributing to the development of hypertension [Tang, Luo, Chen et al. (2014)]. Although the precise mechanisms by which ROS triggers endothelial cell dysfunction remain elusive, mitochondrial remodeling induced by cyclic stretch can be involved. Further studies are required to understand whether and how mitochondrial remodeling is involved in the development of hypertension.

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