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Isolation of mitochondria by gentle cell membrane disruption, and their subsequent characterization

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ABSTRACT

Mitochondria play a key role in several physiological processes as in integrating signals in the cell. However, understanding of the mechanism by which mitochondria sense and respond to signals has been limited due to the lack of an appropriate model system. In this study, we developed a method to isolate and characterize mitochondria without cell homogenization. By gently pipetting cells treated with streptolysin-O, a pore-forming membrane protein, we disrupted the cell membrane and were able to isolate both elongated and spherical mitochondria. Fluorescence imaging combined with super resolution microscopy showed that both the outer and inner membranes of the elongated mitochondria isolated using the newly developed method were intact. In addition, a FRET-based ATP sensor expressed in the mitochondrial matrix demonstrated that ATP generation by F_oF_1-ATPase in the isolated elongated mitochondria was as high as that in intracellular mitochondria. On the other hand, some of the spherical mitochondria isolated with this method had the outer membrane that no longer encapsulated the inner membrane. In addition, all mitochondria isolated using conventional procedures involving homogenization were spherical, many of them had damaged membranes, and low levels of ATP generation. Our results suggest that elongated mitochondria isolated from cells through gentle cell membrane disruption using a pore-forming protein tend to be more similar to intracellular mitochondria, having an intact membrane system and higher activity than spherical mitochondria.

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1. Introduction

Mitochondria play a key role in energy metabolism, but are also responsible for integrating cell signals related to nutrient sensing, energy usage, oxidative stress, and cell death. In these actions, mitochondria work as information processing organelles, sensing molecular or ionic signals, and responding with an output signal. However, understanding the mechanism by which mitochondria sense and respond to signals has been limited due to the lack of an appropriate model system.

Isolated mitochondria serve as a more easily controllable model system than intracellular mitochondria for studying mitochondrial signaling, because it is possible to control the concentrations of molecules and ions directly acting on isolated mitochondria and easily detect the molecules and ions being released. Mitochondria are typically isolated by cell homogenization, sedimentation of the mitochondrial fraction by centrifugation, and resuspension of the mitochondria. However, such treatments are potentially damaging to mitochondria, as evidenced by the observation that mitochondria isolated using grinding to homogenize cells have a spherical morphology [1,2], while intracellular mitochondria in normal cells have tubular structures [3,4].

Uneven distributions of proteins in intracellular mitochondria have been observed with optical microscopy and the physiological significance has been discussed [5,6]. Although mitochondria isolated with the conventional procedures are much smaller than intracellular mitochondria, if it is possible to isolate the intact mitochondria similar in size to intracellular mitochondria, the lateral movements or redistributions of proteins could be observed

Abbreviations: AM, acetoxymethyl ester; Cell-SLO, mitochondria in cells permeabilized with streptolysin-O; EL-SLO, elongated mitochondria isolated by treatment with streptolysin-O; FRET, Förster resonance energy transfer; GFP, green florescent protein; IM, inner membrane; OFP, orange fluorescent protein; OM, outer membrane; SIM, structured illumination microscopy; SLO, streptolysin-O; SP-CON, spherical mitochondria isolated by conventional procedures; SP-SLO, spherical mitochondria isolated by treatment with streptolysin-O.

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in a single mitochondrion by stimulating the mitochondria. These observations will enhance our understanding of mitochondrial responses to signals. Further, by measuring single intact mitochondria with optical microscopy, we could examine the mechanism by which mitochondria transport the molecules and ions across the membranes with a high degree of accuracy, because the signals from mitochondria with damaged membranes are not included.

Streptolysin-O (SLO) is a membrane-damaging protein that is produced by beta-hemolytic group A streptococci [7]. Since SLO forms a pore in the plasma membranes of cells, SLO has been used to permeabilize plasma membranes [8,9]. However, cells can survive after pores are resealed [10]; therefore, the damage to organelles by SLO is probably negligible. Mitochondria in cells permeabilized with SLO were reported to be intact and to have maintained the ability to generate high levels of ATP [9]. Therefore, in the present study, we isolated mitochondria using SLO, while avoiding cell grinding, sedimentation, and resuspension of mitochondria. By using this new method, we successfully isolated both elongated and spherical mitochondria similar in size to intracellular mitochondria and characterized them structurally and biochemically.

2. Materials & methods

2.1. Reagents

C6 cells were obtained from the Institute of Fermentation, Osaka (Osaka, Japan). The vector pAcGFP1-Mito was purchased from Clontech Laboratories (Mountain View, CA, USA). The vector for mitoGO-ATeam2 [11] was supplied by Dr. H. Imamura (Kyoto University). Calcein acetoxymethyl ester (Calcein AM) was purchased from Dojindo Laboratories (Kumamoto, Japan). Anti-VDAC1 (N-18) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Tetramethylrhodamine ethyl ester (TMRE), MitoTracker Red FM and donkey anti-goat IgG (H + L) antibody conjugated with Alexa fuor® 488 were purchased from Life Technologies (Carlsbad, CA, USA). All other chemicals were of the highest purity available.

2.2. Cell cultures and the generation of stable cell lines

C6 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere of 5% CO_2. C6 cell lines expressing GFP or GO-ATeam2 in the mitochondria were obtained by plasmid transfection using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). For expression of GFP, after 48 h of transfection, the cells were transferred to media containing 800 μg/ml of geneticin. Surviving cells were grown for 2–3 weeks, and colonies were picked. For expression of GO-ATeam2, after 48 h of transfection, the cells were transferred to DMEM without geneticin. After 1–2 weeks, colonies were picked. GFP or GO-ATeam2 protein expression in mitochondria was confirmed by fluorescence microscopy. These cells were cultured on glass bottom culture dishes coated with polyethyleneimine for 2–3 days before microscopic observation.

2.3. Isolation of mitochondria

Mitochondria were isolated from C6 cells by two different procedures. For the conventional procedure of mitochondrial isolation, cells grown in a polystyrene dish were collected with a cell scraper and homogenized with a Teflon homogenizer in Tris-sucrose buffer (250 mM sucrose, 0.5 mM EGTA, and 10 mM Tris, pH 7.4). The homogenate was centrifuged at 1000 × g for 10 min and the resulting supernatant was centrifuged at 8000 × g for 10 min. The resultant pellet contained mitochondria and was resuspended in Tris-sucrose buffer. All procedures were performed at 4 °C. Alternatively, we isolated mitochondria without homogenization. For this procedure, C6 cells grown in a polystyrene dish were incubated in HEPES-CH_3COOK buffer (115 mM CH_3COOK, 2.5 mM $MgCl_2$, 1 mM DTT, and 25 mM HEPES, pH 7.4) supplemented with 1 μg/ml SLO at room temperature for 1–5 min and then transferred to ice. Ten minutes after the transfer, cells were washed with Tris-sucrose buffer at 4 °C to remove free SLO. Cells were then warmed to 37 °C for 10 min in Tris-sucrose buffer. The plasma membranes were permeabilized with gentle agitation by pipetting. The cell suspension was collected and centrifuged at 126 × g for 10 min at 4 °C, and the supernatant containing the mitochondria was then collected.

For microscopic observation of isolated mitochondria, the mitochondrial suspensions were incubated on a cover slip at 4 °C for 120 min so that the mitochondria become adsorbed onto the cover slip. Mitochondria still within cells permeabilized with SLO were also observed. In this case, cells were treated with SLO as described above, but the cells were incubated in HEPES-CH_3COOK buffer at 37 °C after washing away free SLO, without disrupting the plasma membranes of the cells with pipetting.

2.4. Fluorescence staining

The membrane potential of isolated mitochondria was measured using fluorescence microscopy; mitochondria were stained with tetramethylrhodamine ethyl ester (TMRE), a potentiometric fluorescent dye [12]. Before the measurements, mitochondria adsorbed onto a glass bottom dish were incubated with 50 nM TMRE for 10 min at room temperature. To assay the permeability of isolated mitochondria, mitochondria on cover slips were incubated with 3 μM calcein AM for 30 min at room temperature. To observe the membrane structure of isolated mitochondria, mitochondria adsorbed onto a glass bottom dish were incubated with anti-VDAC1 antibody (1:50 dilution) for 30 min at room temperature and then subjected to incubation with 1 μM MitoTracker Red FM for 30 min at 37 °C in the dark. After being washed, the mitochondria were incubated with anti-goat IgG secondary antibody conjugated with Alexa 488 for 1 h at room temperature in the dark. All procedures for fluorescence staining of isolated mitochondria were performed in Tris-sucrose buffer. For staining of mitochondria in living cells with MitoTracker Red FM, cells were incubated with 200 nM MitoTracker Red FM in DMEM with 10% FBS for 20 min at 37 °C. A confirmation of plasma membrane permeability was performed by adding propidium iodide (PI) to cells at 1 μM.

2.5. Fluorescence imaging

The super resolution images of mitochondria were obtained using a structured illumination microscope (N-SIM; Nikon, Tokyo, Japan) combined with a confocal microscope (A1; Nikon). The objective lens was a 100 × oil-immersion lens (Apo TIRF 100 × Oil, NA = 1.49; Nikon). Laser lines at 488 nm for Alexa 488, and 561 nm for MitoTracker Red FM, were used for excitation. Structured illumination microscopy (SIM) images (15 images with five different phases for 3 different angular orientations of illumination for each SIM image) were acquired with an EMCCD camera (iXon3 897, Andor Technology Ltd., Belfast, UK) and processed with NIS-Elements (Nikon) software.

TMRE, PI, calcein, GFP, and OFP fluorescence were observed with an inverted epifluorescence microscope (IX-70; Olympus Corporation, Tokyo, Japan). The magnification of the objective lens was 40 × (Uapo340, NA = 0.9; Olympus Corporation). The excitation light was from a 75-W xenon lamp. The fluorescence signals were

acquired with a cooled CCD camera (Sensicam QE, PCO AG; Kelheim, Germany). For TMRE and PI fluorescence, excitation at 535 nm and emission >580 nm were collected. For calcein and GFP fluorescence, excitation at 480 nm and emission at 532 nm were collected. GO-ATeam2 is a Förster resonance energy transfer (FRET)-based fluorescent ATP probe in which GFP and OFP are connected by the ε subunit of *Bacillus subtilis* F_oF_1-ATP synthase [11]. For the FRET signal, GFP was excited at 480 nm and the fluorescence from OFP was measured at > 580 nm. In all experiments, the readout was digitized to 12 bits and analyzed with image-processing software (MetaMorph; Universal Imaging; Downingtown, PA). All procedures described above were performed at room temperature. For the analysis of individual mitochondria, the fluorescence intensity was averaged over an area of 0.6 μm^2 of the mitochondrion. For analysis of mitochondria in cells, the fluorescence intensity of whole cells was measured.

2.6. Statistical analysis

Data are obtained from at least five independent experiments. The results are expressed as means ± standard error of the means (SEMs), and statistical significance was determined by ANOVA followed by a Student-Newman-Keuls test. Differences with *P* values of less than 0.05 were considered statistically significant.

3. Results and discussion

3.1. The morphology of mitochondria isolated with SLO treatment

To observe the morphology of mitochondria isolated with SLO treatment, we obtained transmission images of mitochondria acquired using optical microscopy. As shown in Fig. S1, we observed elongated mitochondria (EL-SLO) as well as spherical mitochondria (SP-SLO). In contrast, the conventional procedure for the isolation of mitochondria yielded only spherical mitochondria (SP-CON).

The transmission images provided only low-resolution data on mitochondrial morphology. To examine the morphology of the isolated mitochondria more closely, we obtained super-resolution images using SIM (Fig. 1). The major and minor axes of EL-SLO were approximately 2 and 1 μm, respectively. All of the EL-SLO observed had complete outer and inner membranes (Fig. 1A). In addition, EL-SLO were often observed as serially concatenated

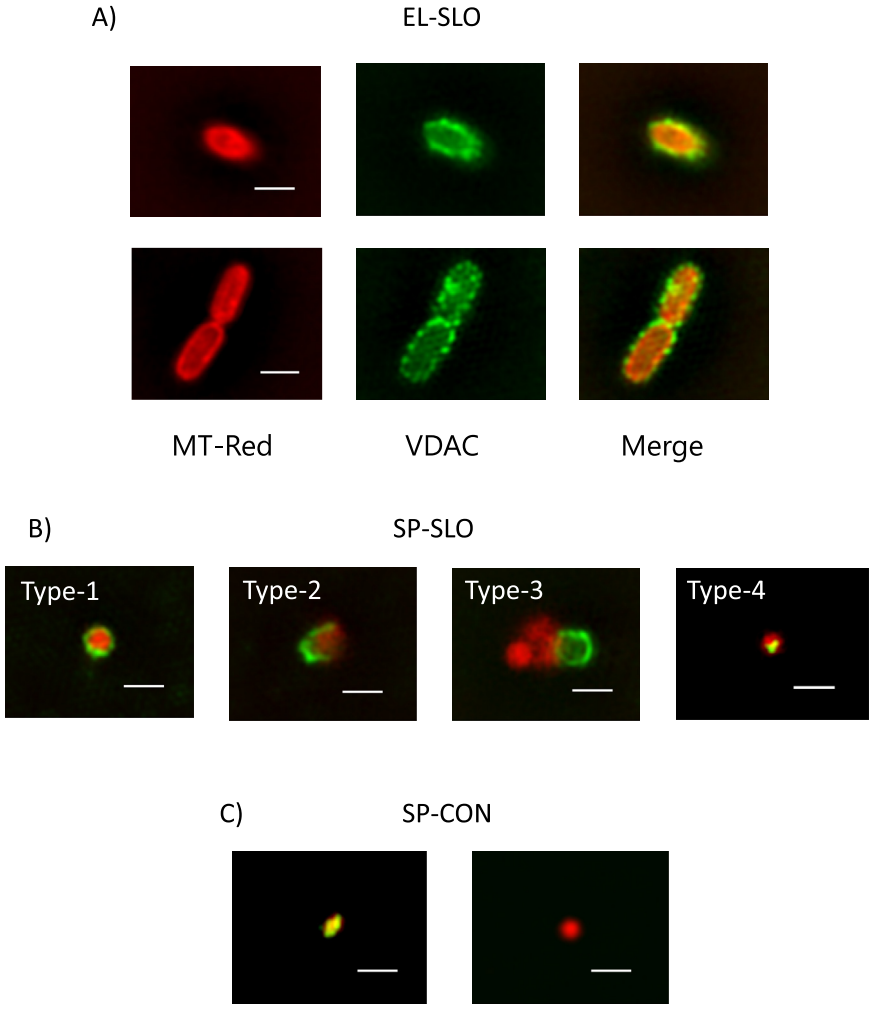

Fig. 1. Super resolution images of isolated mitochondria. Inner membranes were labeled with MitoTracker Red FM (MT-Red). The outer membranes were labeled with anti-VDAC antibody and were visualized with a secondary antibody conjugated to Alexa Fluor 488. Bar, 1 μm, A) Elongated mitochondria isolated with SLO (EL-SLO). Upper: single mitochondrion, Lower: serially concatenated mitochondria. B, C) Red and green show the inner and outer membranes, respectively. In all images, green and red are merged. B) Spherical mitochondria isolated with SLO (SP-SLO): Type-1, mitochondrion with the IM surrounded by a complete OM; Type-2, mitochondrion with IM partially connected to OM; Type-3, mitochondria without OMs; Type-4, small mitochondrion. C) Mitochondrion isolated using the conventional method (SP–CON). Left: mitochondrion with a OM. Right: mitochondrion without a OM. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of this article.)

mitochondria (Fig. 1A, lower row). Immunofluorescence with anti-VDAC antibody produced some bright spots in EL-SLO, indicating that VDAC1 was localized in distinct domains in the outer membranes (OMs). The uneven distributions of VDAC1 in the OMs were consistent with previous observations by Neumann et al. [5]. The diameters of SP-SLO were less than 1 μm. SP-SLO were classified according to four types: 1) mitochondria with the inner membranes (IMs) surrounded by OMs (Type-1, Fig. 1B); 2) mitochondria with IMs partially connected to OMs (Type-2, Fig. 1B); 3) mitochondria without OMs (Type-3, Fig. 1B); and 4) small mitochondria (Type-4, Fig. 1B). The diameters of SP-CON were similar to that of type 4 of SP-SLO. SP-CON were classified according to two types: 1) mitochondria with OMs and 2) mitochondria without OMs (Fig. 1C).

In contrast to EL-SLO, some SP-SLO were partially (Fig. 1B, Type2) or largely (Fig. 1B, Type3) lacking OMs. In these mitochondria, IMs seemed to be partially or completely decapsulated from OMs. These results suggest that a significant part of the IM might be exposed to the cytosol after the OMs become damaged.

3.2. Effects of cell permeabilization on mitochondrial morphology

Before the isolation of mitochondria from cells, the cells were transferred from the cell culture medium to a buffer suitable for isolating mitochondria, and then the cells were permeabilized with SLO. To examine the effects of cell permeabilization with SLO on mitochondrial morphology, we evaluated mitochondria morphology in cells using SIM.

In intact cells bathed in the physiological medium, mitochondria were thin and long with a minor axis of approximately 0.5 μm (Fig. 2, left). When the cells were permeabilized with SLO, the morphology of most mitochondria changed. The major axis became shorter and the minor axis became larger (Fig. 2, right). In these cells, we observed mitochondria similar to EL-SLO and SP-SLO. These results suggest that the morphological changes in mitochondria that occur with cell permeabilization are due to a mitochondrial response to changes in the intracellular environment and are not due to mechanical damage that occurs during isolation. Some mitochondria in cells permeabilized with SLO were longer and thinner than EL-SLO. However, these mitochondria were not observed after isolation, although they were often observed in physiologically normal cells. On the other hand, only a small fraction of mitochondria was small and spherical in cells, although most of mitochondria isolated with the conventional procedure were small and spherical. These results suggest that the small spherical mitochondria might correspond to mitochondria that are fragmented during the isolation procedure.

3.3. Intactness of IMs of isolated mitochondria

To examine whether the IMs of isolated mitochondria are intact, we used the fluorescent molecule calcein as a biosensor of IM integrity. Calcein AM was added to isolated mitochondria adsorbed onto a cover slip; calcein AM is converted to fluorescent calcein by a mitochondrial esterase [13]. Since calcein should be retained in mitochondria with intact IMs due to its hydrophilic nature, it is possible to identify mitochondria with intact IMs by observing the increase in calcein fluorescence after addition of calcein AM. To examine the intactness of the IMs, we observed isolated mitochondria expressing GFP (Fig. S2). First, we identified mitochondria with GFP fluorescence and then measured the increase in fluorescence in each mitochondrion after the addition of calcein AM. The percentages of mitochondria with intact IMs were 81%, 69%, and 60% for EL-SLO, SP-SLO, and SP-CON, respectively (Fig. 3A).

To further confirm the intactness of IMs of EL-SLO, SP-SLO, and SP-CON, we examined the percentage of polarized mitochondria. For this purpose, we isolated mitochondria expressing GFP. After identifying mitochondria with GFP fluorescence, we stained the mitochondria with TMRE and then measured TMRE fluorescence.

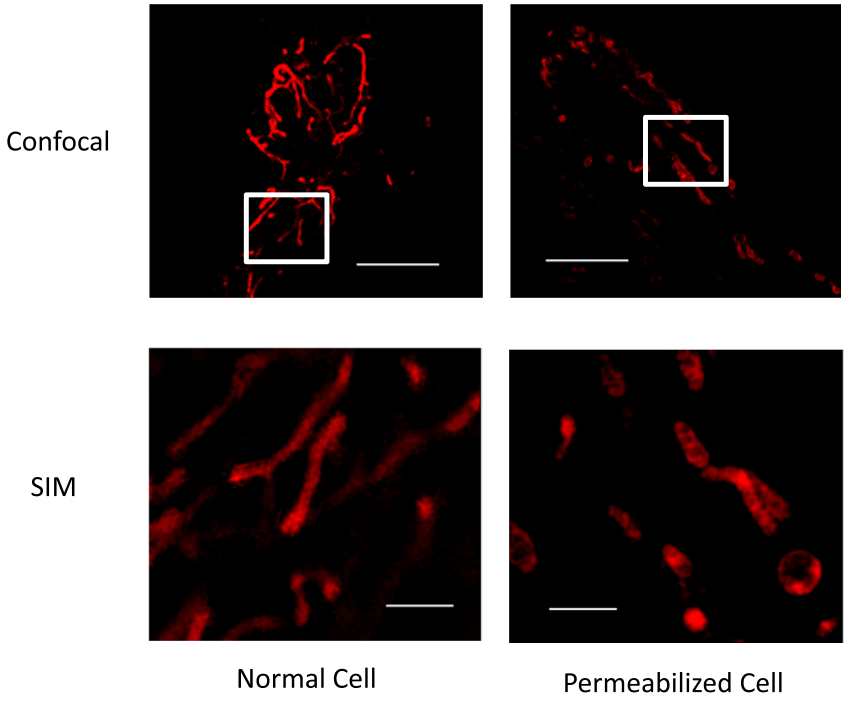

Fig. 2. Mitochondria morphology in cells. Inner membranes were stained with MitoTracker Red FM. The wide field images (upper row, bar, 10 μm) were obtained using confocal microscopy. The field in the square in the upper column was examined using SIM (lower row, bar, 2 μm). Left column: mitochondria in a normal cell in physiological saline (HBS; 120 mM NaCl, 4 mM KCl, 0.5 mM $MgSO_4$, 1 mM NaH_2PO_4, 4 mM $NaHCO_3$, 1.2 mM $CaCl_2$, 25 mM glucose, 0.1% bovine serum albumin, 10 mM HEPES, pH 7.4). Right column: mitochondria in a cell permeabilized with SLO. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of this article.)

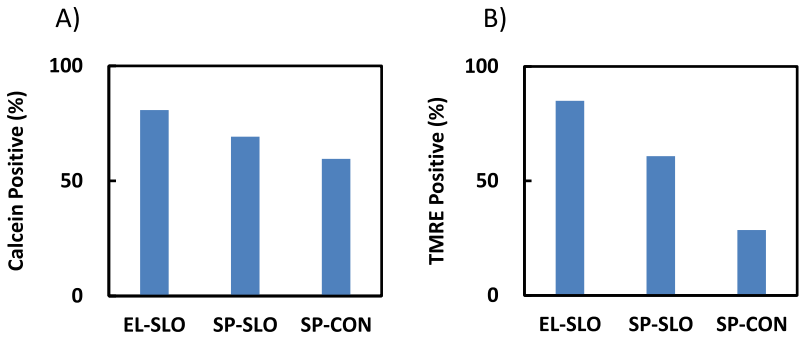

Fig. 3. Intactness of IMs in isolated mitochondria. Isolated mitochondria were identified with GFP fluorescence. A) Percentages of calcein-positive mitochondria. The numbers of mitochondria observed was 26, 13, and 52 for EL-SLO, SP-SLO, and SP-CON, respectively. B) Percentages of TMRE-positive mitochondria. The numbers of mitochondria observed was 20, 51, and 182 for EL-SLO, SP-SLO, and SP-CON, respectively.

To compare TMRE fluorescence among different preparations, the ratio of TMRE fluorescence in individual mitochondria to the fluorescence in the medium [14] was calculated. For identifying polarized mitochondria, we determined the ratios for depolarized mitochondria. When mitochondria were depolarized in the presence of 10 μM carbonyl cyanide m-chlorophenyl hydrazine, the ratios were less than 1.06 for EL-SLO and SP-SLO, and less than 1.10 for SP-CON. Therefore, we considered EL-SLO and SP-SLO with ratios > 1.06 as polarized. For SP-CON, we used 1.10 as the threshold. The percentages of polarized mitochondria were 85% for EL-SLO, 61% for SP-SLO, and 29% for SP-CON (Fig. 3B). Taken together, these results indicate that most of EL-SLO possessed intact IMs. However, significant percentages of SP-SLO and SP-CON had damaged IMs.

3.4. ATP generation in EL-SLO and Cell-SLO

The OMs and IMs of EL-SLO were intact. To further characterize EL-SLO, we compared ATP generation in EL-SLO to that in mitochondria in cells permeabilized with SLO (Cell-SLO). Previous reports suggest that Cell-SLO generate ATP at high levels [9]. Mitochondria expressing GO-ATeam2 were used to detect changes in ATP concentration in the matrix (Fig. S3). The changes were induced by addition of ADP, followed by the addition of a solution containing phosphate, malate, and glutamate. In GO-ATeam2, the FRET efficiency from GFP to OFP, which is defined as the ratio of OFP fluorescence to GFP fluorescence upon excitation of GFP, increases as the ATP concentration rises [11]. When we added ADP to EL-SLO and Cell-SLO, an increase in GFP fluorescence and a decrease in OFP

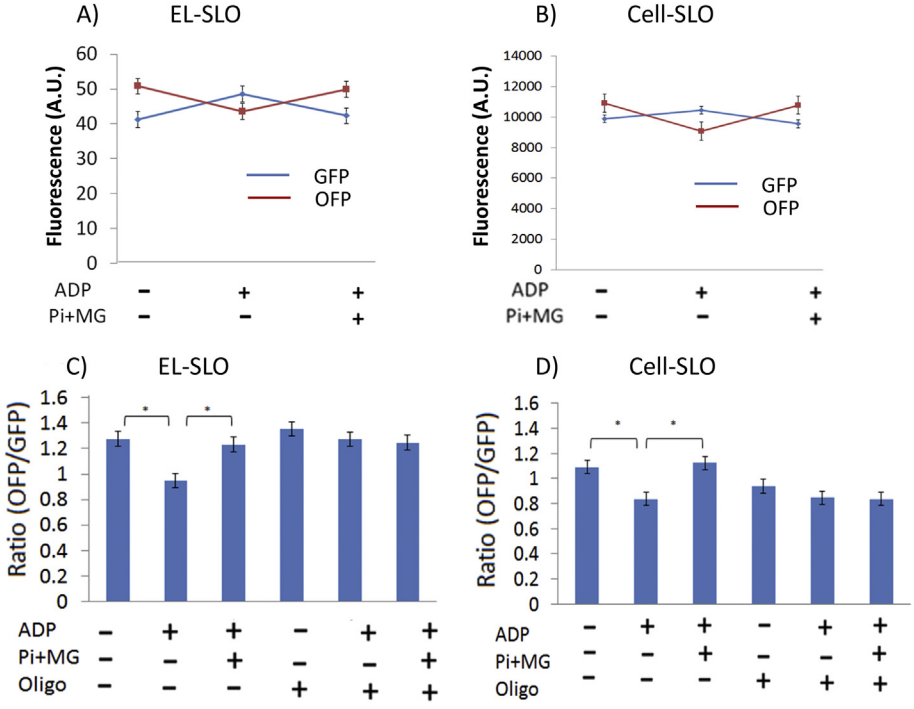

Fig. 4. Changes in ATP concentration in the mitochondrial matrix. For EL-SLO, fluorescence was measured at the single mitochondrion level. For Cell-SLO, fluorescence was measured at the single cell level. ADP and phosphate (P_i) were added at 2 mM (final concentration). The substrates malate and glutamate (MG) were added to a final concentration of 5 mM. When oligomycin was present, cells were incubated with 1 μM oligomycin prior to the addition of ADP. A) Changes in fluorescence of GFP and OFP upon excitation of GFP, measured in single EL-SLO. B) Changes in fluorescence of GFP and OFP upon excitation of GFP, measured in Cell-SLO. C, D) For each sample, N = 5. An * indicates that P < 0.05. C) The ratio of OFP to GFP fluorescence in single EL-SLO. D) The ratio of OFP to GFP fluorescence in Cell-SLO.

fluorescence were observed (Fig. 4A and B), indicating that the ATP concentration decreased in the matrix. This decrease is probably due to exchange of ADP and ATP via the adenine nucleotide translocator. When we added ATP instead of ADP, no significant changes in GFP and OFP fluorescence were observed. Further addition of phosphate, malate, and glutamate following the addition of ADP increased the FRET efficiency, but this effect was completely suppressed by oligomycin, an inhibitor of F_oF_1-ATPase. In addition, the FRET efficiencies in EL-SLO were similar to those in Cell-SLO (Fig. 4C and D). These results indicate that EL-SLO are highly capable of generating ATP and that EL-SLO is a suitable model system for the study of ATP generation at the single mitochondrion level. On the other hand, the responses of SP-SLO and SP-CON to addition of ADP followed by the addition of phosphate, malate, and glutamate were significantly dependent on the mitochondrion (data not shown), so SP-SLO and SP-CON are unreliable model systems for studying ATP generation at the single mitochondrion level.

4. Conclusions

Isolated mitochondria serve as a more easily controllable model system for studying mitochondrial signaling. However, the intactness of isolated mitochondria has been questioned, because there is evidence that mitochondria can be damaged during isolation. In the present study, we successfully isolated intact elongated mitochondria from cells without homogenizing cells, but rather, through permeabilizing the cell membrane using a pore-forming protein. These mitochondria had intact outer and inner membranes, and produced high levels of ATP. However, the fraction of elongated mitochondria was relatively small and the preparation contained many spherical mitochondria damaged during isolation. Although the elongated mitochondria are suitable for studying mitochondrial function at the single mitochondrion level, further improvement is necessary for the large-scale preparation of elongated mitochondria.

Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.05.095.

Transparency document

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