Electron Microscopic Analysis of a Spherical Mitochondrial Structure

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Running title: Structural determination of mitochondrial spheroids

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Background: Mitochondria are dynamic organelles with variable morphological features under different functional status.

Results: Mitochondria treated with an uncoupler presented a spherical structure with an internal lumen containing cytosolic materials as defined by serial sections and electron tomography.

Conclusions: Mitochondria were able to undergo a three-dimensional structural transformation under oxidative stress.

Significance: Mitochondrial spheroid formation represents a novel mitochondrial dynamics.

SUMMARY

Mitochondria undergo dynamic structural alterations to meet changing needs and to maintain homeostasis. We report here a novel mitochondrial structure. Conventional transmission electron microscopic examination of murine embryonic fibroblasts treated with carbonyl cyanide m-chlorophenylhydrazone, a mitochondrial uncoupler, found that more than half of the mitochondria presented a ring-shaped or C-shaped morphology. Many of these mitochondria seemed to have engulfed various cytosolic components. Serial sections through individual mitochondria indicated that they formed a ball-like structure with an internal lumen surrounded by the membranes and containing cytosolic materials. Notably, the lumen was connected to the external cytoplasm through a small opening. Electron tomographic reconstruction of the mitochondrial spheroids demonstrated the membrane topology and confirmed the vesicular configuration of this mitochondrial structure. The outside periphery and the lumen were defined by the outer membranes, which were lined with the inner membranes. Matrix and cristae were retained but distributed unevenly with less being kept near the luminal opening. Mitochondrial spheroids seem to form in response to oxidative mitochondrial damage. The structural features of the mitochondrial spheroids thus represent a novel mitochondrial dynamics.

Mitochondria are dynamic organelles, exhibiting changes in size, morphology, mobility, localization and turnover, which are intimately associated with adaptive and functional alterations, quality control and homeostasis (1-3).
Mitochondria participate in key cellular functions and in turn affect aging, tumorigenesis, neurodegeneration and oxidative tissue injury (2-7). These conditions can also cause mitochondria damage, which in turn further exacerbates disease progression via the increased production of reactive oxygen species.

Mitochondria are delimited by double membranes, enclosing the matrix compartment that contains the mitochondrial DNA. The topology of the inner membrane is dynamically controlled, hence the greater variation in the morphology of the cristae. The outer membrane does not present such variations but set the boundary for the organelle. Another morphological aspect of the mitochondrial dynamics involves the change in size through the fission and fusion process. Abnormality in this process can lead to the formation of giant or fragmented mitochondria, respectively (2,3).

Carbonyl cyanide m-chlorophenylhydrazone (CCCP) is a reversible mitochondria uncoupler. CCCP rapidly causes mitochondrial depolarization and fragmentation, which can in turn broadly affect mitochondrial functions and trigger multiple responses (3,8). Here we report that CCCP can trigger a unique form of mitochondrial dynamic process, which is characterized by a distinct structural transformation. This allows the formation of a spherical mitochondrial spheroid, here called a mitochondrial spheroid, which enwraps cytoplasm and other organelles. Electron serial sectioning and tomographic studies indicate that these structures were nearly completely delimited, with only a small orifice present that connects the lumen to the cytosol. The unique structure represents a novel mitochondrial dynamics.

**EXPERIMENTAL PROCEDURES**

*Chemicals and antibodies-* CCCP was obtained from Sigma-Aldrich. Anti-Tom20 was from Santa Cruz Biotech (Santa Cruz, CA). The secondary antibody for immuno-EM was conjugated with 5 nm gold particles (GE Healthcare).

*Cell lines and cell culture-* Murine embryonic fibroblasts (MEFs) were maintained in DMEM with 10% fetal bovine serum (GIBCO) supplemented with L-glutamine, and penicillin/streptomycin. Cells were treated with CCCP (30 µM) for 6 hours unless otherwise indicated in the figure legend.

*Conventional electron microscopy-* Cells were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), followed by 1% OsO₄. Sections were examined with a JEM 1011CX electron microscope (JEOL, Peabody, MA). Images were acquired digitally.

*Immuno-electron microscopy-* Cells were fixed in 2% paraformaldehyde /0.01% glutaraldehyde in PBS. Cells were pelleted in 3% gelatine in PBS and solidified on ice. Small blocks (~0.5 mm) of the cell pellet were prepared and infiltrated with 2.3 M sucrose at 4°C overnight. Blocks were mounted on aluminum stubs, frozen and sectioned. The sections (60 nm) were picked up in drops of 2.3M sucrose and collected on Formvar-coated mesh grids. After blocking in 1% BSA in PBS the sections were incubated with a primary rabbit anti-Tom20 antibody and subsequently incubated with a secondary antibody conjugated with 5 nm gold particles. The sections were fixed in 1% glutaraldehyde and stained with ice cold 0.4% uranyl acetate/1 % methyl cellulose (pH 4) and dried. The samples were viewed in a FEI Tecnai transmission electron microscope (FEI, Hillsboro, OR).

*Serial section EM.* Serial sections (50 nm thick) were collected onto Cu slot grids and post stained in 2% uranyl acetate and lead citrate. Images were acquired using a Tecnai 12 transmission electron microscope (Philips Electron Optics, Holland) operating at 80 kV. Mitochondrial membranes were traced by hand and Amira software (Visage Imaging Inc.) was used to create 3D models of the mitochondrial structures.

*Electron tomography-* 250-300 nm sections were picked up on 2x1mm Cu slot grids with a 50nm thick LUXFilm™ support film (Ted Pella, Inc.). The grids were stained with 3% uranyl acetate (aq.) followed by Reynolds lead citrate prior to electron microscopic examination. Images were acquired using a JEM 3200FS electron microscope (JEOL) and the HTR (high tilt) specimen holder (JEOL). All tilt series were recorded over the tilt range of approximately ±60° using the automated tilt series collection program serialEM (9). The
thickness of each tomography slice is estimated to be 2-3 nm. Tomograms were generated using IMOD (10). For the work presented here, we used either 15 nm colloidal gold fiducials or local patch tracking to align the images prior to three-dimensional (3D) reconstruction. Membrane boundaries were manually marked and rendered in 3D using IMOD.

RESULTS

**CCCP induces mitochondrial spheroids** - CCCP has been widely used to study a Parkin-dependent mitophagy process (11-17). CCCP does not induce any significant levels of mitophagy in MEFs because the level of Parkin was below the detection in these cells (13,17). Unexpectedly, electron microscopy could readily detect ring-shaped structures (Fig. 1A). They were not autophagosomes but were identified with typical mitochondrial features, including the double membranes and the cristae. It seemed that a portion of the mitochondrion was compressed and the loss of the cristae and the matrix at that part allowed the membrane to bend around cytosolic constituents to form a spherical structure (Fig. 1B). These structures could be detected after only one hour of treatment, reaching the plateau between 6-16 hours and declining at 24 hours (27).

Immuno-EM analysis with an antibody against a mitochondrial outer membrane protein, Tom20, found the signals present in both the outside and inside of the vesicular structure (Fig. 1C), further confirming their mitochondrial origin and the topological evolution.

Various cytoplasmic components, including the cytosol, membranes, vesicles and even another mitochondrion, could be found in the engulfing mitochondria (Fig. 1B), suggesting that they could be non-selectively enclosed by the extended mitochondrial membranes during the formation of the phagosomal structure. As such, these structures were operationally named as mitochondrial spheroids although it is not clear whether the enclosed materials were actively or passively engulfed by the mitochondria.

Ring-shaped mitochondrial structures had been reported in early studies based on either fluorescence microscopy (18-21) or electron microscopy (22-26). It was possible that the mitochondrial structure described here could simply reflect a fusion between two ends of a mitochondrion, as suggested by many of these studies. To determine whether this was the case, we performed serial sections for EM examination.

Through the analysis of a total of twenty-two ring-shaped mitochondria with serial sections, we found that they actually represented spherical structures that were mostly delimited (Fig. 2, Fig. S1A-B). Thus the structure was not simply formed by a simple fusion between two parts of a mitochondrion, resulting in a “donut”, but rather involved a three-dimensional transformation of the entire mitochondrion, forming a clearly defined internal space surrounded by the mitochondrial membranes. Interestingly, in all of such structures analyzed, there was a small orifice present in the spherical structure. This seemed to provide a pathway connecting the internal space, or the lumen, to the cytoplasm. No similar configurations had been found in a regular mitochondrion (Fig. S1C).

**Electron tomography reveals the membrane topology of the mitochondrial spheroids** - To further understand the topological relationship of the membranes that form the mitochondrial spheroids, we conducted electron tomography. Shown in Figure 3A and Video 1 were three examples of mitochondrial spheroids, among which two were subjected to reconstruction (Fig. 3B-C, Videos 2-3). The reconstruction indicated that the vesicular lumen was formed by the original outer membranes, now termed as the inside outer membranes (IOM). The peripheral boundary of the mitochondrial spheroids was still defined by the outer membranes, now termed as the outside outer membranes (OOM). OOM and IOM were connecting to each other due to the presence of the orifice, which was surrounded by these membranes. As such, the luminal contents could have exchanges with the cytoplasm.

Not depicted in Figure 3B-C, but in Figure 4 and Videos 4-5 were the original inner membranes, which were lined against the inside and outside outer membranes, and could thus also be categorized as inside inner membranes (IIM) and outside inner membranes (OIM) (Fig. 4). Between the membranes the matrix and the cristae were redistributed non-symmetrically with certain parts left with membranes only (Fig. 3-4).

Mitochondrial matrix could be defined in a portion
of the mitochondrial spheroid between IIM and OIM, but not in other part of the structure, which was boarded by only four layers of membranes (IOM, IIM, OIM and OOM) (Fig. 1B). Cristae were readily identified in the matrix space (Fig. 4). They remained the lamellar form and were contiguous to the inner membranes. Overall, the electron tomography gave rise to a clear depiction of the spherical nature and membrane topology of the mitochondrial spheroid.

**DISCUSSION**

Mitochondrial spheroids can manifest as ring-shaped mitochondria on conventional transmission EM (Fig. 1). Many studies, using EM [(18,22-26) and references within] or, more recently, fluorescence microscopy (18-21) have described this type of mitochondria in very diverse cellular contexts. However, the actual morphology can vary significantly and it is not clear whether the same subcellular phenomenon had been investigated in these cases. This is particularly true when the description of the mitochondrial morphology is solely based on fluorescence microscopy. Hence different mechanisms could account for the similar but not necessarily exact morphological changes of the mitochondria under these scenarios. While the ring-shaped mitochondria could be found in apparently normal tissues (22,24,26), they are also frequently indentified in stress conditions(18-21,23). Our studies presented in this and a companion paper (27) represent the latter condition, which suggests that mitochondria can undergo active conversion between the normal morphology and the ring-shaped morphology.

This conversion may be due to end-to-end or end-to-side fusion of elongated mitochondria at two-dimensional level, forming donut-shaped mitochondria as suggested by some of the studies (18,21,26). Alternatively, this conversion can represent a vesicle-like structure with a lumen surrounded by the mitochondrial membranes, as supported by the present study. We have also structurally identified the presence of a pore, which would allow the cytoplasm trapped in the lumen to be confluent with the external cytoplasm. However, it is not clear whether this represents an incomplete fusion of the membrane or an integrated part of the structural feature. The significance of such a pore is thus unknown at this time. Our model is supported by both serial sections and tomographic reconstruction. It would be also supported by two earlier studies where serial sections were performed (24,26) and a similar pore was observed (24).

Electron tomography further reveals the membrane topology that may explain how the spherical structure is formed. Mitochondrial spheroid formation would require the extension of the mitochondrial membranes to bend around the cytoplasm to form a new spherical structure with an internal lumen, in which cytosolic materials are likely trapped nonspecifically (Figs. 1-2). The revelation of these three-dimensional features provides the rationale to categorize this mitochondrial structure in its own group. The morphology of the mitochondrial spheroid looks almost like a phagosome in the sense that the spherical structure enwraps the cytosolic contents in the internal space. However, whether this transformation may involve any molecular events similar to the membrane dynamics responsible for phagocytosis (as in heterophagosome or autophagosome formation) is not known.

We have investigated several molecular mechanisms that could be involved in the formation of the spherical mitochondria following CCCP treatment (27). The autophagy machinery does not seem to be required but ROS and mitofusins are required. In addition, Parkin serves as a negative regulator by promoting mitofusin degradation. Mitofusins are known to function in mitochondrial tethering and fusion. Thus mitofusins can be equally important in the self-fusion of the mitochondrial membrane during the formation of mitochondrial spheroid. However, there would be likely other molecular events to affect the membrane curvature for such a process to initiate and to complete. In particular, mechanisms for curvature and branching of filamentous mitochondria are little understood and they could be conceivably involved in the formation of mitochondrial spheroids.

The significance of the mitochondrial spheroids has yet to be fully determined. Common autophagy inducers, such as starvation and endoplasmic reticulum stress, did not induce mitochondrial phagocytosis. CCCP can induce oxidative mitochondrial damage. Another inducer
of mitochondrial spheroids, sodium azide, is also a mitochondrial toxin (27). An earlier study (23) found this type of mitochondria in the livers of rat given long-term alcohol diet. Furthermore, we had found that acetaminophen at a dose that induces significant oxidative mitochondrial damage, also induced mitochondrial phagocytosis in hepatocytes in vivo (27). In both CCCP and acetaminophen cases, antioxidants could prevent mitochondrial spheroid formation. Finally, the number of these spherical mitochondria increased with age in retinal pigment epithelium in birds, which may be related to long-term light stimulation (26). It is thus tempting to suggest that this type of mitochondrial dynamics could be involved in pathological conditions that involve mitochondrial oxidative injury.

In a separate study (27) we reported that CCCP-induced mitochondrial spheroids formation can be a reversible process as other CCCP-induced phenomena, such as mitochondrial depolarization, mitochondrial fragmentation and autophagy induction since all these changes will disappear when CCCP is removed from the medium. These findings could suggest that once an adverse pathological condition is remedied, this mitochondrial alteration could be reversed.

In conclusion, mitochondrial spheroid formation represents a novel mitochondrial dynamic process that is distinctive from other commonly known dynamics. The formation of such a spherical structure poses challenging questions regarding the membrane dynamics, molecular details and pathophysiological consequences, which need to be resolved in the future.

REFERENCES

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FOOTNOTES

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1The abbreviations used are: CCCP, Carbonyl cyanide m-chlorophenylhydrazone; EM, electron microscopy; IIM, inside inner membranes; IOM, inside outer membranes; MEFs: murine embryonic fibroblasts; OIM, outside inner membranes; OOM, outside outer membranes.
FIGURE LEGENDS

Figure 1. CCCP induces mitochondrial spheroid formation. (A) Control or MEFs treated with CCCP for 6 hours were fixed and subjected to EM examination. CCCP induced a significant level of ring-shaped mitochondria in MEFs. (B) Features of ring-shaped mitochondria induced by CCCP included the compression of a portion of the mitochondria to form a quadruple-membrane segment (arrowheads), which seemed to allow the bending of the membrane to circle around cytoplasmic components. These subcellular materials included the cytosol, vesicles (arrows) or a mitochondrion (double arrows). (C) Wild type MEFs treated with CCCP for 6 hours were processed for immuno-EM with anti-Tom 20. Panel a: normal mitochondria; Panel b-c: mitochondrial spheroids. The gold particles indicated the Tom20 signals and were present in both the inside and outside of the ring-shaped mitochondria (b-c), suggesting the present of mitochondrial outer membranes at these locations.

Figure 2. Serial sections of a mitochondrial spheroid. MEFs treated with CCCP for 6 hours were fixed for EM examination. When areas enriched for the mitochondrial spheroids were identified, serial sections were performed. Shown in panel a-h were a serial of sections separated by 50 nm in distance. Note the lumen (asterisk) that contains the cytosol and the orifice (arrow) that connects the lumen to the cytoplasm. The serial sections allowed the construction of a 3D model, and the representative views of the model were shown in panels i-k.

Figure 3. Electron tomography of mitochondrial spheroids. MEFs treated with CCCP for 6 hours were fixed and subjected to electron tomography. The topographic slices (A) illustrated three mitochondrial spheroids (i, ii, iii), from which the 3D models of spheroids i (B) and iii (C) were reconstructed. Images were extracted from Videos 1-3. Only the outer membrane was traced and modeled. The numbers in A represent that of slices along the z-axis of the original volume. The lumen of the spheroid (indicated by an asterisk) was delimited by the inside outer membranes (IOM, green) and opened to the cytoplasm through an orifice (red arrows). The outside outer membrane (OOM, cyan) was the same OM that extended to the IOM and the relationship was indicated by the golden-colored strip. IOM and OOM were linked by a membranous stem that embedded the orifice (C). Between IOM and OOM were the inner membranes (not depicted in the model) and the matrix space (indicated, B-C). The model in B also showed that the spheroid and its internal lumen were completely delimited at one end that was preserved in this volume. Removal of the OOM at this end allowed the visualization of the delimited lumen from the matrix side (B-d). The model in C also illustrated the connection of the lumen to the cytoplasm by the orifice, which was embedded in a membranous stem. Part of the OOM was removed for a clearer view (C-d to C-f).

Figure 4. The topological relationship of inner and outer membranes in a mitochondrial spheroid. (a). A tomographic slice showed the inner membrane (IM), the outer membrane (OM) and the matrix enclosed by the IM, in which two of the five cristae were also indicated. (b-f). The 3D model of the IM (magenta), the OM (blue) and the five cristae (in various colors) were superimposed on a tomographic slice and viewed from the top (b) and bottom (c). Note the connection of the cristae to the IM (white circles). Panels d-f showed the model with all the membrane components (d), the IM and OM only (e) and the cristae only (f). Note the lamellar form of the cristae in f. Images a and b-f were extracted from Video 4 and Video 5, respectively.
Figure 4

(a) Lumen
(b) Cristae
(c) Matrix

IOM/OOM
IIM/OIM

50 nm
100 nm
50 nm
50 nm

IIM
OOM
IOM

OIM
IOM

50 nm
50 nm
50 nm
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