Localization of MTT formazan in lipid droplets. An alternative hypothesis about the nature of formazan granules and aggregates

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MTT (3-(4, 5-dimethyl-2-thiazolyl)-2, 5-dihphenyltetrazolium bromide) assay is a widely used method to assess cell viability and proliferation. MTT is readily taken up by cells and enzymatically reduced to formazan, a dark compound which accumulates in cytoplasmic granules. Formazan is later eliminated by the cell by a mechanisms often indicated as exocytosis, that produces characteristic needle-like aggregates on the cell surface. The shape of formazan aggregates and the rate of exocytosis change in the presence of bioactive amyloid β peptides (Aβ) and cholesterol. Though the cellular mechanisms involved in MTT reduction have been extensively investigated, the exact nature of formazan granules and the process of exocytosis are still obscure. Using Nile Red, which stains differentially neutral and polar lipids, and a fluorescent analog of cholesterol (NBD-cholesterol), we found that formazan localized in lipid droplets, consistent with the lipophilic nature of formazan. However, formazan granules and aggregates were also found to form after killing cells with paraformaldehyde fixation. Moreover, formazan aggregates were also obtained in cell-free media, using ascorbic acid to reduce MTT. The density and shape of formazan aggregates obtained in cell-free media was sensitive to cholesterol and Aβ. In cells, electron microscopy failed to detect the presence of secretory vesicles, but revealed unusual fibers of 50 nm of diameter extending throughout the cytoplasm. Taken together, these findings suggest that formazan efflux is driven by physico-chemical interactions at molecular level without involving higher cytological mechanisms.

Key words: MTT, formazan, lipid droplets, Nile Red, NBD-cholesterol, cholesterol, amyloid, peptides.

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MTT (3-(4, 5-dimethyl-2-thiazolyl)-2, 5-dihphenyltetrazolium bromide) is readily taken up by cells and enzymatically reduced to formazan, an intensely purple substance which accumulates in cytoplasmic granules (Altman 1976). Formazan staining correlates to the number of viable cells and may be used to evaluate changes in the population density due to cell death or proliferation (Mosmann 1983). Formazan is later eliminated by the cell, with the appearance of characteristic needle-like aggregates on the cell surface and the parallel clearance of cytoplasmic granules (Nikkhah *et al.* 1992; Shearman *et al.* 1995). This process is enhanced by cholesterol and cytotoxic amyloidβ peptides (Aβ) (Liu and Schubert 1997; Abe and Saito 1998; Liu *et al.* 1998). In particular it has been shown that a fast formazan elimination, rather than a defective MTT reduction, was responsible for the weak staining of cells treated with Aβ (Liu and Schubert 1997; Abe and Saito 1998; Abe and Saito 1999). In addition, bioactive Aβ induce a peculiar morphology of formazan aggregates. Thus, MTT provides also a sensitive method for the detection of bioactive Aβ in tissue (Liu and Piasecki 2001; Liu *et al.* 2004). The appearance of formazan aggregates on the cell surface was attributed to a mechanism of exocytosis by Liu and coworkers in 1997 (Liu *et al.* 1997). Such hypothesis was suggested by the apparent vesicle-like morphology of formazan granules and the fact that formazan colocalized in part with acridine orange, a marker of acidic vesicles. However the hypothesis of exocytosis has not been substantiated by ultrastructural data, and no conclusive data have been provided about the nature of formazan granules. Using a different approach, based on the use of fluorescent probes, we found that formazan localized in lipid droplets, while electron microscopy failed to reveal the presence of secretory vesicles. In addition, formazan aggregates also developed in cells fixed with paraphormaldehyde (dead cells) and in cell-free systems. The latter were sensitive to the presence of cholesterol and Aβ.

Materials and Methods

Cells

Mouse Swiss 3T3 fibroblasts (ATCC collection) were grown in Dulbecco's modified Eagle's medium with high glucose, supplemented with 10% fetal bovine serum, in a 5% CO_2 incubator at 37°C. Cells were plated in glass-bottomed dishes (MatTek, Ashland, MA) to allow immersion objectives to be used with an inverted fluorescence microscope. Experiments were performed when cells were subconfluent. Before treatments the complete medium was replaced with serum-free medium.

Treatments and probes

Cells were supravitally stained with the following probes (for fluorescent probes, excitation and emission values are indicated in parentheses): 300 nM Nile Red incubated for 15 min (Ex 460±25, Em 535±20 for neutral lipids; Ex 540±12, Em 590 LP for polar lipids); 5 µM 22-[N-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-23,24-bisnor-5-cholen-3-ol or NBD-cholesterol, a cholesterol analog where the terminal segment of the alkyl tail is replaced by the NBD fluorophore, for 1h (Ex 460±25, Em 535±20); 450 µM 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-dihphenyltetrazolium bromide or MTT, for 5 to 30 min; 10 µM calcein for 20 min (Ex 480±20, Em 520±20); 650 nM Hoechst 33258, for 20 min (Ex 360±20, Em 460±25); 100 nm DAPI for 20 min (Ex 360±20, Em 460±25). Vehicles were: Me_2SO for Nile Red; chloroform for NBD-cholesterol; water for Hoechst 33258, calcein, DAPI and MTT. Stock solutions were 1000-fold concentrated, to not exceed the 0.1% of vehicles in the medium. Hoechst 33258, MTT, cholesterol, Aβ fragments $A\beta_{25-35}$ and $A\beta_{12-28}$ were from Sigma (St. Louis, MO, USA); calcein and Nile Red from Fluka (Buchs, SG, Switzerland); DAPI and NBD-cholesterol from Molecular Probes (Eugene, OR, USA).

Quenching of Nile Red and NBD-cholesterol fluorescence by MTT was assessed in cell-free experiments using mixed solutions of Nile Red and MTT or NBD-cholesterol and MTT, in the same proportions applied to cells, but at concentrations 50-fold higher to mimic the higher content in the cytoplasm due to the uptake of probes. These solutions were placed in a glass chamber and observed with the same fluorescence filters used for cells. Both Nile Red and NBD-cholesterol fluorescences were completely quenched by MTT.

The involvement of exocytosis in the generation of formazan aggregates was tested observing formazan changes after cell death. For this purpose, cells were first incubated with MTT for 15 min, photographed, and then fixed with phosphate-buffered 4% paraformaldehyde. Cells were photographed again after fixation, at different time intervals. The generation of formazan aggregates was also tested in cell-free experiments in the presence or the absence of cholesterol and Aβ. In these experiments, MTT reduction was induced by ascorbic acid.

Light microscopy

Observations were made using an Olympus IX 71 inverted microscope (Olympus, Tokyo, Japan) with a 60x planapochromatic oil immersion objective (UPlanSApo series) with an efficient chromatic and spherical correction that minimized the color shift of different emission wavelengths. The use of the inverted microscope excluded any risk of dye contamination, as there was no contact between the immersion objective and the medium. Images were taken with a 12-bit cooled CCD camera (Sensicam PCO, Kelheim, Germany), coupled to a mechanical shutter interposed between the 100W Hg lamp and the microscope, to limit illumination of cells to the time strictly required for acquisition of images. Excitation light was attenuated with a 1.5 or 6% transmittance neutral density filter. The nominal resolution of images was 0.1 µm/pixel. For each microscopic field, at least three images at focus steps of 1 µm were taken and composed into a single picture with extended focus, using the minimum or maximum intensity method (for brightfield or fluorescence images, respectively). Image analysis and measurements were performed with the ImagePro Plus package (Media Cybernetics, Silver Springs, MD, USA).

Electron microscopy

For EM cells were grown on sterilized Thermanox coverslips (Nalge Nunc Intl., Rochester, NY, USA) placed in Petri dishes, and fixed for 2 hr in 1% paraformaldehyde/1.25% glutaraldehyde in 0.15 M sodium cacodylate buffer, pH 7.2. After fixation, cells were rinsed in the same buffer, postfixed in osmium tetroxide, stained in aqueous uranyl acetate 0.25%, dehydrated in ascending concentrations of acetone and infiltrated with Epon resin. Thin sections were post-stained with uranyl acetate and bismuth subnitrate and examined with a Jeol 200 CX transmission electron microscope.

Results

Formazan accumulates into lipid droplets

Nile Red is a fluorescent probe that stains differentially neutral and polar lipids. In particular, lipid droplets are easily identified as yellow spots while cytoplasmic membranes are stained in red (Figures 1A,B). Surprisingly, when Nile Red staining was followed by MTT treatment, all formazan granules were localized in lipid droplets previously stained in yellow by Nile Red (Figure 1C). Unfortunately, Nile Red fluorescence was quenched by MTT, thus the lapse of time between the observation of Nile Red and the appearance of formazan granules resulted in a small displacement of formazan granules with respect to the original position of lipid droplets. To validate the identification of formazan granules with lipid droplets, cells were fixed with paraformaldehyde, washed accurately with PBS to remove the residual MTT and stained again with Nile Red. In this way, colocalization of formazan granules and lipid droplets was shown at pixel level (Figures 1C,D). In parallel experiments, Nile Red was replaced with NBD-cholesterol, a cholesterol analog that accumulates into lipid droplets (Frolov *et al.* 2000). Also in this case, formazan granules colocalized with NBD-cholesterol-stained lipid droplets (Figure 2).

Formazan extrusion is not attributable to exocytosis

The accumulation of formazan into cytoplasmic granules was followed by the extrusion of formazan in the typical form of needle-like aggregates. However, lipid droplets that contained formazan were found in the same position after formazan extrusion (Figures 1B,D). At this stage, the plasma membrane was still intact, as indicated by the exclusion of calcein. Also nuclear stains Hoechst 33258 and DAPI failed to detect chromatin changes attributable to plasma membrane damage (*data not shown*).

The identification of formazan granules with lipid droplets, and the conservation of lipid droplets after formazan extrusion, per se exclude the possibility that formazan granules represent vesicles undergoing exocytosis. The vesicle-like morphology of formazan granules, which is apparent when the microscope contrast is high, is in fact a possible artifact of phase interference, enhanced by the small size of granules. This point was confirmed by electron microscopy, which failed to find any presence of secretory vesicles in MTT-treated cells. On the other hand, electron microscopy revealed the presence of unusual fibers of 50 nm of diameter, extending throughout the cytoplasm and often ragged at

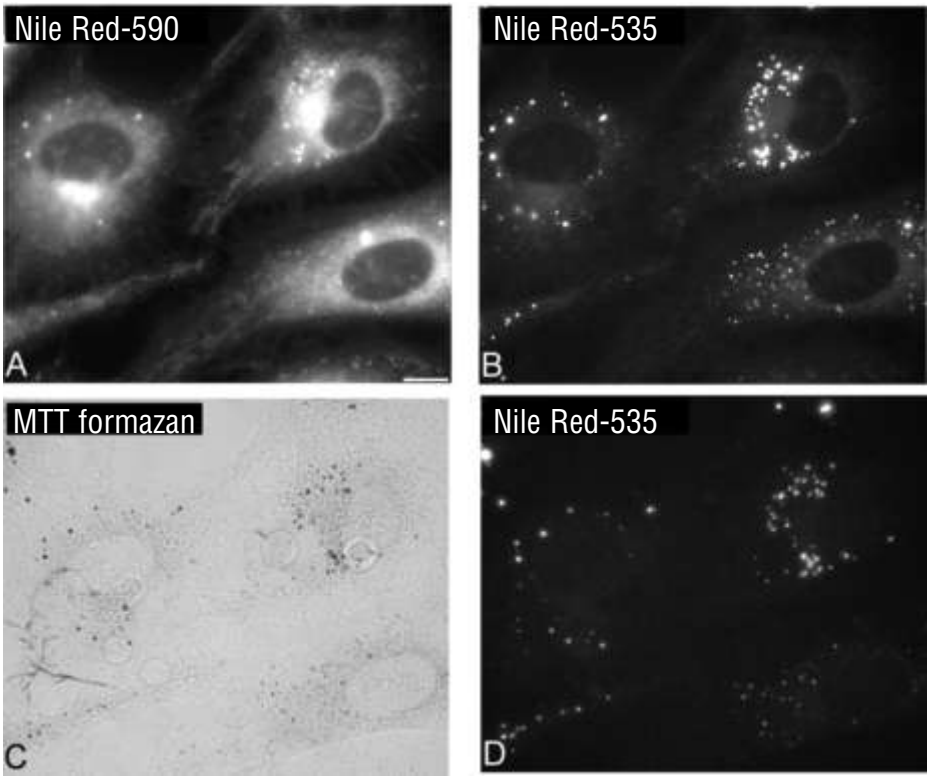

Figure 1. Colocalization of formazan granules and lipid droplets. (A) Overall lipid distribution detected by Nile Red 590 nm long pass emission. Note the intense fluorescence near the nucleus and the Golgi area, where intracellular membranes are most concentrated. (B) Lipid droplets detected by Nile Red 535±20 nm band pass emission. (C) Formazan granules after 10 min of incubation with MTT. The small displacement of formazan granules with respect to lipid droplets is due to the lapse of time between observations *in vivo*, as Nile Red fluorescence was completely quenched by MTT. (D) Nile Red re-staining after fixation of cells with paraformaldehyde and several washes in PBS to remove MTT. A complete correspondence is found between lipid droplets and formazan granules. Bar 10 µm.

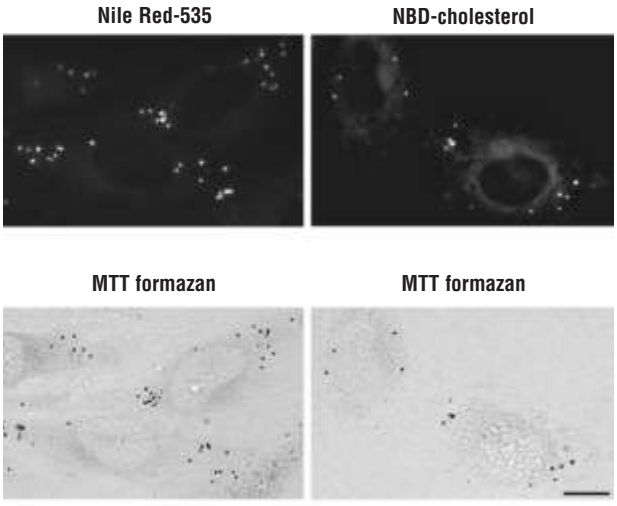

Figure 2. Colocalization of formazan granules and lipid droplets stained with Nile Red or NBD-cholesterol. As in Figure 1, a small displacement between formazan granules and lipid droplets may be found, due to the lapse of time between observations *in vivo*. Bar 10 µm.

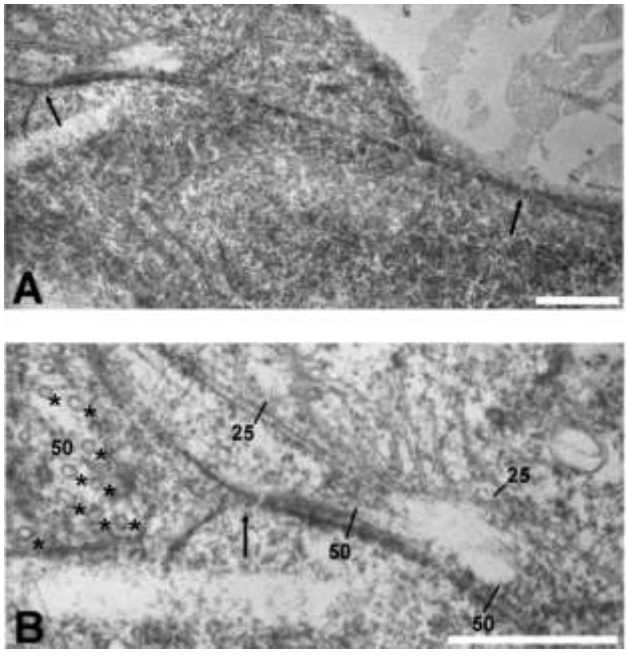

Figure 3. Electron microscopy of cells incubated with MTT for 20 min. (A) The cytoplasm is crossed by a long fiber with an average diameter of 50 nm. Both ends of the fiber (arrows) are ragged, suggesting a process of aggregation of finer components. (B) Higher magnification showing one end of the 50 nm fiber and several round and elliptical profiles (asterisks). The minor axis of these profiles is invariably 50 nm. This means that they are sections of continuous structures (i.e., fibers or tubules) and not vesicles. Typical microtubules (25 nm) are also shown. Bar 0.5 µm.

ends, as being formed by a process of aggregation of finer filaments (Figure 3). Normal cytoskeletal elements, such as microtubules and microfilaments, were also found.

Formazan aggregates are self-assembled structures

Generation of formazan aggregates was also found in cells killed by fixation with paraformaldehyde after 15 min of MTT incubation (Figure 4A). The morphology of aggregates was in all similar to that observed in living cells. Cells that contained small formazan granules at the time of fixation, after fixation also exhibited an enlargement of granules followed by the generation of aggregates (Figure 4B). The growth of formazan aggregates was accompanied by the clearance of granules, in fixed cells as in living cells.

To verify the effect of cholesterol and Aβ on the generation of formazan aggregates, cell-free experiments were made in 14 mm dishes uniformly coated with 0.15 µmoL of cholesterol dissolved in ethanol, which evaporated. Dishes were then filled with 250 µL of water containing 150 nmol of MTT and 10 nmol of Aβ peptide fragments $A\beta_{25\text{-}35}$ (bioactive cytotoxic) or $A\beta_{12\text{-}28}$ (nontoxic). MTT reduction was induced 20 hrs later with the addition of ascorbic acid. Data are shown in Figure 5. Long, needle-like formazan aggregates, similar to those observed in cells, were found only in the presence of cholesterol. However, regardless of the shape, the number of aggregates was higher with

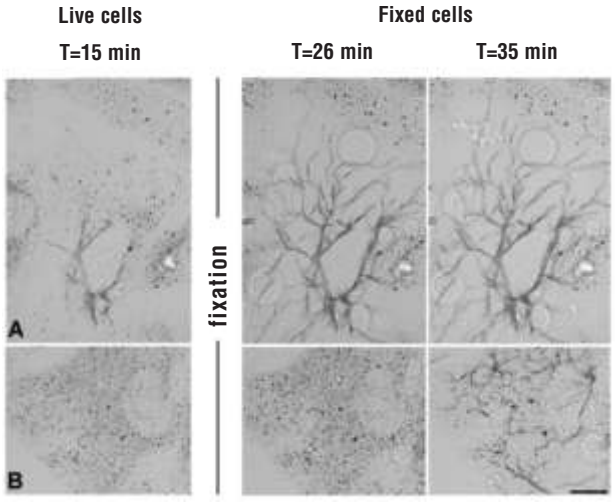

Figure 4. Formazan changes in fixed (dead) cells. Cells were incubated with MTT for 15 min and then killed by fixation with phosphate-buffered 4% paraformaldehyde. Images were taken immediately before fixation (live cells, left column) and after fixation (right columns). T is the duration of MTT incubation. After fixation, a prominent extension of pre-existing formazan aggregates is observed (A). In some cells, formazan granules exhibit an increase of size followed by the original generation of formazan aggregates (B). In all cells, the production of aggregates is accompanied by the clearance of granules. Bar 10 µm.

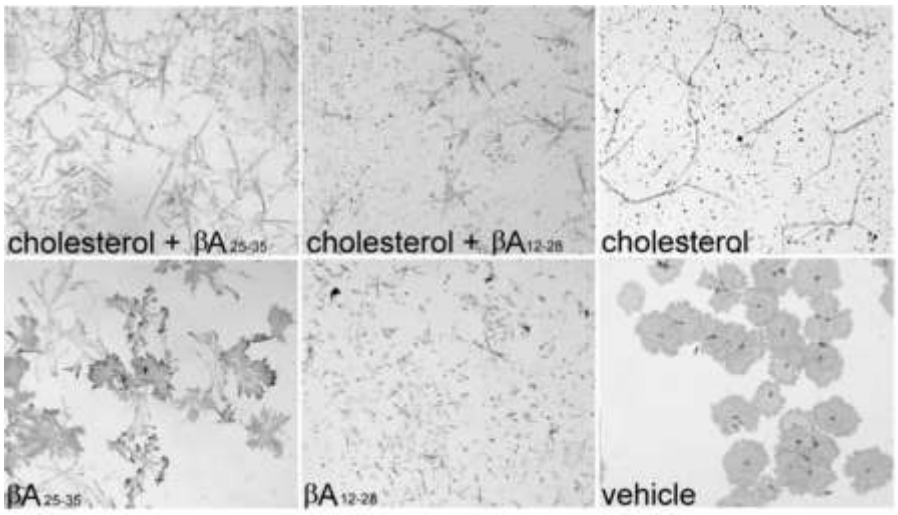

Figure 5. Self-assembled formazan aggregates in cell-free media with or without cholesterol and with or without Aβ fragments $A\beta_{25\text{-}35}$ or $A\beta_{12\text{-}28}$ (method given in detail in the Result section). Long, needle-like formazan aggregates, comparable to those observed in living cells, are seen only in the presence of cholesterol. Aggregation is strongly enhanced by the co-presence of the bioactive Aβ fragment $A\beta_{25\text{-}35}$. In the absence of both cholesterol and Aβ, formazan produce only isotropic aggregates.

cholesterol + $A\beta_{25\text{-}35}$, less with cholesterol + $A\beta_{12\text{-}28}$ and low with cholesterol alone. In the absence of cholesterol, formazan aggregates were short and compact. Isotropic aggregates were found in pure water.

Discussion

MTT assay can be subdivided into four steps regulated by distinct cytological mechanisms which can be separately recognized by optical microscopy: (1) MTT uptake, detected by the quenching of pre-loaded fluorochromes; (2) MTT reduction to formazan, detected by the appearance of a spread gray staining of cytoplasm; (3) formazan compartmentalization, detected by the appearance of dark granules in the cytoplasm and the clearance of the spread staining; (4) formazan extrusion, detected by the appearance of needle-like aggregates on the cell surface and the clearance of cytoplasmic granules. In this paper we describe new findings that contribute to understand the nature of formazan granules and the mechanism of formazan extrusion (steps 3 and 4).

Formazan granules are usually regarded as cytoplasmic vesicles that deliver formazan to the cell surface by a mechanism of the exocytosis (Liu *et al.* 1997; Abe and Saito 1998; Isobe *et al.* 1999; Molinari *et al.* 2005). This hypothesis was suggested by the vesicle-like morphology of formazan granules and the fact that formazan colocalized in part with acridine orange, a marker of acidic vesicles (Liu *et al.* 1997). In contrast with this hypothesis, we found a complete colocalization of MTT formazan granules with lipid droplets. Lipid droplets are dynamic structures potentially present in all eukaryotic cells that develop from discrete regions of the endoplasmic reticulum and accumulate neutral lipids, mostly triacylglycerols and cholesteryl esters. These substances are used for metabolism, membrane synthesis (phospholipids and cholesterol) and steroid synthesis. Lipid droplets are also associated to proteins (perilipin, ADRP and TIP47-related and Rab proteins) which control the mechanisms of lipid droplets' lipolysis, fusion, motility and the link to cytoskeletal components and other organelles (reviewed in Martin and Parton 2006). The presence of formazan in lipid droplets is consistent with the strongly lipophilic nature of formazan. This feature has been often considered in relation to the problem of densitometric measurements, as formazan does not dissolve in aqueous media. On the other hand, to our knowledge, it has been scarcely considered in relation to the distribution of formazan in the cytoplasm.

A second, unexpected finding was the clearance of formazan granules and generation of formazan aggregates in cells killed by paraformaldehyde fixation. This finding indicates that the generation of formazan aggregates is not attributable to cytological processes, such as vesicular transport and exocytosis, which occur only in living cells, but depend on physico-chemical mechanisms such as electrostatic forces, hydrogen bonds and hydrophobic interactions at molecular level. As a consequence of their small size, compared to the range of optical wavelengths, molecular aggregates often exhibit a marked change of the absorption spectrum (Jelley 1936). These structural properties are in agree-

ment with the changes in the rate of formation (enhanced *exocytosis*), shape and color of formazan aggregates induced by cholesterol and Aβ in living cells (Liu and Schubert 1997, Abe and Saito 1998; Liu *et al.* 1998). Our data show that cholesterol- and Aβ-sensitive formazan aggregates can be also induced in cell-free media.

In experiments with live cells, the membrane-impermeant probe calcein was excluded from the cytoplasm for several minutes from the first appearance of formazan aggregates. Likewise, Hoechst 33258 staining, which is very sensitive to plasma membrane alterations, was not affected by the presence of formazan aggregates. This condition, for some aspects, is similar to that observed in mitochondria stained with the potential-sensitive mitochondrial probe 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolyl-carbocyanine iodide, or JC-1. JC-1 accumulates in the mitochondrial matrix where it forms needle-like aggregates in the presence of a sustained transmembrane potential. With time, JC-1 aggregates continue to extend linearly up to distort remarkably the normal mitochondrial structure (Diaz *et al.* 2001). On the other hand, the presence of JC-1 aggregates proves the preservation of the transmembrane potential, indicating that they do not cause rupture or permeabilization of the mitochondrial membrane.

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