Mitochondrial Aberrations in Mucolipidosis Type IV*

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Mucolipidosis type IV is a genetic lysosomal storage disease associated with degenerative processes in the brain, eye, and other tissues. Mucolipidosis type IV results from mutations in the gene MCOLN1, which codes for the TRP family ion channel, mucolipin 1. The connection between lysosomal dysfunction and degenerative processes in mucolipidosis type IV is unclear. Here we report that mucolipidosis type IV and several unrelated lysosomal storage diseases are associated with significant mitochondrial fragmentation and decreased mitochondrial Ca2+ buffering efficiency. The mitochondrial alterations observed in these lysosomal storage diseases are reproduced in control cells by treatment with lysosomal inhibitors and with the autophagy inhibitor 3-methyladenine. This suggests that inefficient autophagolysosomal recycling of mitochondria generates fragmented, effete mitochondria in mucolipidosis. Mitochondria accumulate that cannot properly buffer calcium fluxes in the cell. A decrease in mitochondrial Ca2+ buffering capacity in cells affected by these lysosomal storage diseases is associated with increased sensitivity to apoptosis induced by Ca2+-mobilizing agonists and executed via a caspase-8-dependent pathway. Deficient Ca2+ homeostasis may represent a common mechanism of degenerative cell death in several lysosomal storage diseases.

Mucolipidosis (ML)3 is the collective name for a group of autosomal recessive diseases characterized by accumulation of membranous lipid inclusions in patients’ cells (1, 2). There are four types of ML, three of which (MLII, MLIIIa, and MLIIIc) involve incorrect intracellular targeting of lysosomal lipid hydrolases, resulting in inefficient processing of endocytosed lipids (3–5). Although the cellular phenotype of type IV (MLIV) is similar to other MLs, MLIV is linked to mutations in the ion channel, mucolipin 1 (TRP-ML1), a member of the TRP family of ion channels (6–8). The current consensus assigns TRP-ML1 a role in some aspect of lysosomal/endosomal function (4, 9, 10).

The pathological manifestations of MLIV are retinal degeneration, corneal cloudiness, and neuromotor retardation (11, 12). Brain MRI studies of autopsied cases are consistent with demyelination, neuronal death, and neuronal loss in the cerebral cortex, basal ganglia, and deep cerebellar and brainstem nuclei (13, 14). The degenerative processes in MLIV and in most other lysosomal storage diseases (LSDs) are not limited to brain but also involve skeletal and cardiac muscle, skin, parietal cell, and other tissues (15–17).

The range of pathological manifestations in many LSDs is similar and suggests that a generalized mechanism of degeneration exists. One such mechanism may involve the lysosomal-mitochondrial axis, as proposed for aged cardiac myocytes (18, 19). This model suggests that accumulation of lipofuscin in lysosomes of post-mitotic cardiac myocytes impairs lysosomal function. Because lysosomes are major players in autophagic recycling of mitochondria (20–22), lysosomal inactivation by lipofuscin deposits results in accumulation of fragmented, dysfunctional mitochondria (18, 23). Normally, mitochondrial fragmentation is the result of activity of a dynamin-related protein, DRP1 (24, 25), and other proteins (26–30) that cleave mitochondrial membranes and lead to mitochondrial fission. Mutations in the gene DRP1 cause disease (27, 31), and this parallels the disease in autosomal recessive optic atrophy (MCOA). Our results demonstrate that LSDs also affect mitochondrial homeostasis.

Normal mitochondria are frequently arranged in extended linear networks, allowing intermitochondrial communication to facilitate crucial energetic and calcium buffering functions (reviewed in Refs. 31 and 32). Our results demonstrate significant changes in both the appearance and function of mitochondria in skin fibroblast cultures from patients with MLIV and other LSDs. This is pathogenically significant for mucolipidoses because post-mitotic neurons and cardiomyocytes are especially sensitive to alterations in mitochondrial function (18, 24, 33). We hypothesize that impaired lysosomal function in MLIV affects autophagy of mitochondria, which leads to accumulation of effete mitochondria that cannot effectively buffer cyto-
plasmic Ca\(^{2+}\). As a result, cells affected by MLIV are more sensitive to pro-apoptotic effects of Ca\(^{2+}\)-mobilizing agonists than cells with normal mitochondria.

At present, little is known about the pathways leading to cell death in LSDs. A recent paper by Schaheen et al. (34) reports that mutations in CUP-5, a *Caenorhabditis elegans* orthologue of TRP-ML1, lead to increased apoptosis, but it did not explain why a cell death pathway is activated in the CUP-5-deficient cells. We believe that our results provide a mechanistic explanation that links suppression of lysosomal function in MLIV, and perhaps other LSDs, with cell death.

**EXPERIMENTAL PROCEDURES**

**Materials and Solutions**—FCCP, 123-rhodamine, DISC3 (5), Mitotracker Red, fluorescein isothiocyanate-conjugated annexin V, DAPI, Rhod2, BAPTA-AM, and the TUNEL apoptosis kit were from Molecular Probes (Eugene, OR); bradykinin, bafilomycin, nigericin, ionomycin, and cell-permeable caspase inhibitors (caspase-3/7, -8, and -9) were from Calbiochem; 3-methyladenine (3-MA), monodansylcadaverine (MDC), and ammonium chloride were from Sigma. Fura-2AM was from TefLabs (Austin, TX).

**Cells**—Skin fibroblasts from MLIV, MLII, and MLIII patients (clones WG 0909, WG 0229, and WG 0109) and corresponding heterozygous nondiseased parent skin fibroblasts (clones WG 0987 and WG 0140) were obtained from the Repository for Mutant Human Cell Strains of Montreal Children’s Hospital (Montreal, Canada). MLIV fibroblasts clone GM02525 and CLN2 fibroblasts clone GM09404 were from Coriell Cell Repositories (Boston, MA). Fibroblasts were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 1% streptomycin (all from Atlanta Biologicals, Atlanta, GA).

**Confocal Immunocytochemistry**—For experiments with fixed cells, the cells grown on glass coverslips were fixed and permeabilized by 10 min of incubation at –20 °C with 100% methanol or were fixed by a 5 min incubation with 3.7% formaldehyde and permeabilized by incubation with 0.01% Triton X-100 at 4 °C for 5 min. After fixation, nonspecific sites were blocked by incubation in 5% goat serum. Subsequently the cells were incubated with the primary antibodies in blocking solution. Following wash-out of the primary antibodies with PBS, the cells were stained with fluorescent secondary antibodies and analyzed as above. Primary antibodies used for immunocytochemistry for LC3 were clone H-50 and clone 6H2 for cytochrome c. They were from Santa Cruz Biotechnology (Santa Cruz, CA). The primary cytochrome c antibodies used in Western blotting were clone 6H2.B4 from Zymed Laboratories Inc. (San Francisco, CA). Primary antibodies for caspase-8 (clone 1C12) were from Cell Signaling (Danvers, MA).

For experiments with live cells, the cells on coverslips were bathed in the standard bath solution, which contained 140 mm NaCl, 5 mm KCl, 1 mm CaCl\(_2\), 1 mm MgCl\(_2\), 10 mm Hepes, pH 7.4. Following loading with dyes and three washes, the coverslips were mounted into a perfusion chamber containing standard solution. The chamber was placed on the stage of a Bio-Rad 1024 confocal microscope, and fluorescence was recorded and analyzed off-line using ImageJ software.

**Measurement of \([Ca^{2+}]_i\)**—For standard fluorescence recording experiments, the cells grown on coverslips were loaded with 5 μM Fura-2AM in the standard solution for 30 min at 37 °C. Following three washes, the coverslips were placed in the perfusion chamber that allows rapid exchange of bath solutions. The solutions were applied by bath perfusion with ten volumes of the perfusion chamber. Fura was excited by alternating 340/380-nm light, and the resulting 510-nm fluorescence was recorded through a CCD-cooled camera and analyzed using Metafluor software (Universal Images Corporation).

To record mitochondrial Ca\(^{2+}\), the cells were loaded with Rhod2 and analyzed essentially as above. To record MDC fluorescence, the coverslips were washed three times with 1× PBS and incubated with 0.05 mM MDC diluted in 1× PBS for 10 min at 37 °C. The coverslips were washed three times with 1× PBS to remove excess and placed in the perfusion chamber. MDC fluorescence was excited by 340-nm light and recorded at 510 nm.

**Apoptosis Assays**—TUNEL and annexin V-based assays were performed according to the manufacturer’s instructions ( Molecular Probes, Eugene, OR). Confluent coverslips, either untreated or treated with an apoptosis-inducing agonist, were washed three times with 1× PBS. The cells were fixed with –20 °C methanol for 10 min and washed three times with 1× PBS. The coverslips were incubated in 300 nm DAPI (Molecular Probes, Eugene, OR) diluted in 1× PBS for 5 min, washed three times with 1× PBS, and mounted on slides.

**Caspase-8 Fluorogenic Substrates**—The fluorogenic Z-IETD-AFC substrate (Calbiochem, San Diego, CA) was used to measure the caspase-8 activity in this study (35). Lysate (10 μg of protein) was prepared in 200 μl of reaction buffer (0.1% CHAPS buffer containing 20 mM PIPES, 10 mM NaCl, 10 mM dithiothreitol, 1 mM EDTA, and 10% sucrose) and mixed with 20 μM Z-IETD-AFC. The fluorescence of the cleavage product was measured over time at 37 °C in a microplate spectrofluorometer (Molecular Devices; excitation wavelength, 400 nm; emission, 505 nm). Proteolytic activity was expressed as relative fluorescence units normalized to control cultures.

**RESULTS**

**Mitochondrial Appearance in MLIV**—Live skin fibroblasts from a heterozygous control relative and a MLIV patient, homozygous for the TRP-ML1 mutation (clones WG0987 and WG0909, henceforth identified as MLIV-control and MLIV fibroblasts, respectively) were stained with 1 μM Mitotracker Red or 1 μM 123-rhodamine. At these concentrations, both dyes showed nearly perfect overlap, and from this point on, the description and statistical analysis of mitochondrial appearance in the two cell types were performed on 123-rhodamine-stained cells. Confocal analysis revealed significant differences in mitochondrial appearance between MLIV-control and MLIV cells. Mitochondrial chain length was estimated by manually tracing unbranched mitochondria on confocal images of the cells and measuring their length using ImageJ software. In MLIV-control fibroblasts, mitochondria are organized as extended threads with average lengths of 73.8 ± 4.5 μm (n = 25 cells and all results are the means ± S.E.) (Fig. 1, A and B). In comparison, MLIV cells contain mitochondria that appear sig-
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Mitochondrial fragmentation in MLIV fibroblasts cannot be explained by differences between proliferation rates of control and MLIV fibroblasts because our own observations do not indicate such a difference. TRP-ML1 did not colocalize with mitochondria (data not shown) and is therefore unlikely to directly regulate mitochondrial length and/or function.

Mitochondrial fragmentation was previously reported in post-mitotic myocytes with lipofuscin deposits in lysosomes (18, 19, 23, 36), and because MLIV is associated with suppressed lipid degradation in the lysosomal pathway, we hypothesized that depressed lysosomal function contributed to the mitochondrial fragmentation observed in MLIV fibroblasts. Furthermore, a prediction can be made that mitochondrial fragmentation similar to that observed in MLIV fibroblasts and aged cardiac myocytes should also be observed in fibroblasts with other LSDs or in cells whose lysosomal function is suppressed by pharmacological means.

Mitochondrial Alterations in Other Mucolipidoses—Fig. 1 (A and B) shows that mitochondrial fragmentation is also observed in fibroblasts from an MLIII patient but not from a matching heterozygous control relative (clones WG0109 and WG0140 will be identified as MLIII and MLIII-control, respectively). Average mitochondrial numbers were 34.8 ± 3.5 (n = 11) in MLIII-control fibroblasts and 123.6 ± 13.7 in MLIII fibroblasts (n = 10, p < 0.05) (Fig. 1, A and B). Fibroblasts from an MLII patient (clone WG0229) showed a similar trend (39.5 ± 8.1 μm (n = 25) and 91.6 ± 6.1 average mitochondrial number (n = 10, p < 0.05)) (Fig. 1, A and B). Fibroblasts from an unrelated storage disease, neuronal ceroid lipofuscinosis 2 (CLN2, clone GM09404), which is a result of mutations in a gene coding for tripeptidyl peptidase I, also contained numerous, fragmented mitochondria (average mitochondrial length was 19.1 ± 1.2 μm (n = 25), and the average mitochondrial number was 105.8 ± 7.2 (Fig. 1, A and B).

Lysosomal Function, Autophagy, and Mitochondrial Fragmentation—MLII, MLIII, MLIV, and CLN2 are caused by lysosomal dysfunctions linked to inefficient breakdown of lipids or proteins (37, 38). The fact that these LSDs result from mutations in completely different proteins yet cause same mitochondrial phenotype indicates a common cause of mitochondrial fragmentation in these diseases, such as lysosomal dysfunction. We used a series of lysosomal degradation inhibitors to determine whether impaired completion of autophagy/lysosomal degradation could explain the observed alterations in mitochondrial organization in the mutant cells. Lysosomal function was suppressed using bafilomycin, which blocks the lysosomal V-type H+ pump, nigericin, the H+ ionophore, and ammonium chloride, which disrupt the acidic environment essential for lysosomal hydrolase activities (39). Fig. 2 shows that treatment of MLIV-control and MLIII-control cells with bafilomycin (0.1–100 nm) for 3 days induced mitochondrial fragmentation similar to that observed in the LSD fibroblasts (Fig. 2A). Average mitochondrial lengths in bafilomycin-treated mitochondria were 21.0 ± 0.8 and 10.7 ± 0.5 μm, respectively (n = 20 and 20, p < 0.05 for both sets). Short treatment with bafilomycin did not induce significant mitochondrial fragmentation (Fig. 2, A and B). Therefore, these changes do not reflect rapid induction of mitochondrial fission by lysosomal or autophagy inhibitors. Counting individual mitochondria in control and treated cells confirmed that cells treated with lysosomal inhibitors contained more discrete mitochondrial fragments than untreated cells (Fig. 2C). Similar results were obtained with nigericin and ammonium chloride used as lysosomal inhibitors (data not shown).

The primary pathway through which mitochondria recycle and renew is autophagy, a process in which lysosomes are key participants (33, 40, 41). Upstream signals that stimulate autophagy include Vps34, a class III PI3K, and inhibition of Vps34...
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FIGURE 2. Mitochondrial fragmentation in control cells after conditions of inhibited lysosomal function or autophagocytosis. A and B, confocal images of 1 μM 123-rhodamine-stained mitochondria in MLIV-control and MLIII-control cells under control conditions and following long (A) and short (B) treatment with lysosomal and autophagy inhibitors. 3-MA was used at 5 mM, and bafilomycin (Baf) was used at 0.01 μM. C, left panel, quantitative analysis of the average lengths of mitochondria in MLIV and MLIII human fibroblasts after corresponding cultures. Right panel, average number of individual mitochondria in various fibroblast clones. Statistical details are given in the text.*p < 0.05 within group.

by 3-MA effectively inhibits starvation-induced autophagy (42). To see whether disrupted autophagy could result in mitochondrial changes observed in MLIV and MLIII fibroblasts, we used a pharmacologic inhibitor of autophagy. Fig. 2A shows that treating MLIV-control and MLIII-control fibroblasts with 5 mM 3-MA for 3–4 days induced mitochondrial fragmentation similar to that observed in MLIV and MLIII fibroblasts. Average mitochondrial lengths of 3-MA-treated MLIV-control and MLIII-control fibroblasts were 32.3 ± 1.7 and 34.7 ± 2.4 μm, respectively (n = 20 and 20, p < 0.05). Short, 1-h treatment with 3-MA did not induce noticeable mitochondrial fragmentation (Fig. 2, B and C), indicating that 3-MA does not directly induce mitochondrial fission. Thus, inhibition of autophagocytosis results in the loss of the extended, filamentous mitochondrial network and accumulation of individual, isolated mitochondria as observed in MLIV and MLIII fibroblasts. The number of individual mitochondria per cell was also higher in 3-MA-treated control fibroblasts than in untreated controls (Fig. 2C).

Based on these three series of experiments, we conclude that suppression of lysosomal function in LSDs results in the accumulation of isolated, fragmented mitochondria. By analogy with lipofuscin-induced mitochondrial fragmentation reported by Terman et al. (18), we hypothesize that mitochondrial fragmentation in LSDs is a result of suppressed autolysosomal degradation of mitochondria.

Mitochondrial Ca2+ Buffering in MLIV—Mitochondria are involved in Ca2+ homeostasis because they work as Ca2+ sinks buffering cytoplasmic Ca2+ and preventing large Ca2+ transients that might otherwise be damaging for cells (43–45). Mitochondria are normally organized as extended cellular networks, and we hypothesized that disruption of this network would significantly impair mitochondrial Ca2+ buffering.

To test [Ca2+]i buffering by mitochondria, the cells were stimulated with a Ca2+-mobilizing agonist, bradykinin (Bk), and the amount of Ca2+ accumulated in mitochondria after completion of the Ca2+ flux was compared. Bk activates phospholipase Cβ, generates inositol (1, 4, 5) trisphosphate, and induces both Ca2+ release for endoplasmic reticulum and associated Ca2+ influx (46). MLIV-control and MLIV fibroblasts showed normal Ca2+ release and influx (Fig. 3A). The endoplasmic reticulum Ca2+ load in MLIV-control and MLIV fibroblasts was previously analyzed using ionomycin and was found to be very similar in the two cell types (8). Therefore, the differences in mitochondrial Ca2+ load described below can be directly attributed to variations in mitochondrial Ca2+ uptake in control and LSD cells.

During the [Ca2+]i spike, some of the Ca2+ is accumulated by mitochondria; this amount can be estimated by releasing mitochondrial Ca2+ with the mitochondrial uncoupler FCCP, which collapses mitochondrial membrane potential. When applied during Ca2+ response, 0.1 μM FCCP induced an increase in [Ca2+]i in 9 of 14 of MLIV-control cells (Fig. 3A). The increase was never seen in MLIV fibroblasts (25 cells tested). Similar results were obtained with GM402525 MLIV fibroblasts. We conclude that the mitochondrial Ca2+ buffering capacity is markedly reduced in MLIV fibroblasts as compared with MLIV-control fibroblasts. To determine whether suppressed mitochondrial Ca2+ accumulation could be a general feature of LSDs, we used MLIII-control and MLIII fibroblasts. Fig. 3A shows that much like MLIV fibroblasts, MLIII fibroblasts did not release Ca2+ in response to FCCP application during Bk-induced Ca2+ response, whereas MLIII-control SF did. These data represent 11 of 11 MLIII-control and 12 of 12 MLIII fibroblasts. No mitochondrial Ca2+ accumulation was detected in 20 of 20 MLII or 10 of 10 CLN2 fibroblasts (data not shown).

Because FCCP is a H+ ionophore, a possibility exists that the FCCP-induced Ca2+ release originates, at least partially, from lysosomes. To remove this uncertainty, we depleted lysosomal Ca2+ by bursting lysosomes using 100 μM glycyrrhetinic acid (47, 48). We found that bursting lysosomes with glycyrrhetinic acid...
Alanine-naphthylamide released lysosomal Ca^{2+} into the cytoplasm but did not affect FCCP-induced Ca^{2+} release (data not shown). Therefore, FCCP-induced Ca^{2+} release originates largely or entirely from mitochondria. FCCP has equal access to mitochondria in MLIV-control and MLIV fibroblasts because it was equally effective in dissipating 123-rhodamine mitochondrial stain (Fig. 3B).

The FCCP-based assay was validated by directly measuring mitochondrial Ca^{2+} using mitochondrial Ca^{2+} indicator Rhod2. Fig. 3C shows that stimulation with Bk significantly increases mitochondrial Ca^{2+} in MLIV-control, but not MLIV cells. Although Rhod2 may pick up cytoplasmic Ca^{2+}, and cytoplasmic Ca^{2+} signals may bias mitochondrial Ca^{2+} measurements, the amplitudes of Ca^{2+} signals induced by Bk in MLIV-control and MLIV cells were essentially the same (Fig. 3A; see also Ref. 8). Therefore, the difference between Ca^{2+} signals detected with Rhod2 in MLIV and MLIV-control cells is a direct reflection of different Ca^{2+} uptake by mitochondria in these cells. Two independent approaches demonstrate the loss of mitochondrial Ca^{2+} uptake in MLIV fibroblasts.

The link between lysosomal dysfunction and mitochondrial Ca^{2+} buffering was further probed using MLIV-control and MLIII-control cells in which autophagocytosis or lysosomal function was inhibited. Fig. 4A shows that incubating the cells with 5 mM 3-MA or 0.01 μM bafilomycin for 3 days abolished FCCP-induced Ca^{2+} release. Short (1 h) exposure to either drug did not decrease the mitochondrial ability to release Ca^{2+} (Fig. 4B). Similar results were obtained with nigericin or ammonium chloride used as lysosomal inhibitors (data not shown). These data demonstrate that inhibiting lysosomal function and/or autophagocytosis impairs mitochondrial Ca^{2+} accumulation. Therefore, abnormal lysosomal function in MLIV and MLIII leads to accumulation of dysfunctional mitochondria, which are unable to efficiently scavenge cytoplasmic Ca^{2+}. Although a prolonged treatment of the cells with these compounds may have nonspecific effects, it should be noted that such treatments did not have a noticeable effect on Bk-induced Ca^{2+} signal but resulted in a specific mitochondrial phenotype that recapitulates mitochondrial abnormalities detected in MLIV and MLIII.
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**FIGURE 5.** Apoptosis in control, MLIV, and MLIII fibroblasts induced by Ca\(^{2+}\)-mobilizing agonists. A, apoptosis induced by 1 \(\mu\)M Bk was measured in MLIV-control (Ctrl) and MLIV cells using three different assays: DAPI, annexin V, and TUNEL. Statistical details are given in the text. B, apoptosis in MLIII-control and MLIII cells after stimulation with 1 \(\mu\)M Bk was analyzed using DAPI, C, calcium release induced by 3 \(\mu\)M thapsigargin (Tg) was measured by Fura-2AM (left panel) and associated apoptosis rates (right panel) were assessed after 1 h of thapsigargin treatment using DAPI staining in MLIV and MLIV-control cells. Apoptosis percentages show that cell death occurs to a greater extent in patient cells treated with Tg than in MLIV-control cells. The results are representative of three independent experiments. D, left panel, Fura-2AM experiments demonstrating BAPTA-AM-induced blockade of Ca\(^{2+}\) spike induced by 1 \(\mu\)M Bk. The results are representative of six experiments in MLIV-control and MLIV cells. Right panel, quantitative analysis of apoptosis induced by a 3-h treatment with 1 \(\mu\)M Bk in MLIV fibroblasts with and without 30 \(\mu\)M BAPTA-AM. Statistical details are given in the text. *, \(p < 0.05\) within group.

Apoptosis—Mitochondria are major players in apoptosis because they release cytochrome c (CytC) that triggers the caspase-9-dependent branch of apoptosis and because their Ca\(^{2+}\)-buffering function regulates apoptotic events triggered by Ca\(^{2+}\) (49–51). Bearing this in mind, we tested whether decreased mitochondrial Ca\(^{2+}\) buffering function in MLIV translates into increased cell death rates for cells stimulated with Ca\(^{2+}\)-mobilizing agonists. Apoptosis rates were estimated using three different apoptosis markers: nuclear morphology visualized using DAPI stain, phosphatidylserine translocation to the outer layer of the plasma membrane visualized using fluorescein isothiocyanate-conjugated annexin V, and DNA fragmentation visualized using TUNEL assays. The stained cells were analyzed using either a fluorescence imaging system or a confocal microscope; the percentage of apoptotic cells was calculated as the ratio of cells displaying apoptotic characteristics to the total number of cells in the field of view. Five to seven images were taken in each trial with 5–25 cells each.

In the first set of assays, MLIV-control and MLIV fibroblasts were stimulated with 1 \(\mu\)M Bk for 1–16 h and assayed for apoptosis rates. There was some degree of variability to the exact values yielded by these assays, which has been previously observed (52, 53). Nonetheless, the three assays demonstrated the same trend: under control conditions, the percentage of MLIV cells undergoing apoptosis was not statistically different from MLIV-control fibroblasts (Fig. 5A). However, when stimulated with 1 \(\mu\)M Bk for 1–3 h, MLIV fibroblasts showed dramatically increased apoptosis rates as compared with control cells (Fig. 5A). Specifically, in annexin V assays, 2–4% of untreated MLIV-control and MLIV fibroblasts displayed apoptotic phenotype. After 3 h of stimulation with Bk, the percentage of annexin V-positive cells was 4.6 ± 1.9% in MLIV-control fibroblasts and 18.4 ± 3.9% (\(n = 10\) and 10, \(p < 0.05\)) in MLIV fibroblasts, a statistically significant difference (Fig. 5A). A similar response was observed using the DAPI and TUNEL assays (Fig. 5A). These data indicate increased sensitivity of MLIV fibroblasts to apoptotic stimuli brought about by Ca\(^{2+}\)-mobilizing agonists. MLIII fibroblasts were also more sensitive to Bk-induced apoptosis than were MLIII-control cells (Fig. 5B). It was only after a 16-h exposure to Bk that apoptosis rates in control cells approached the levels detected in LSDs.

Two lines of experiments confirmed that Bk-induced apoptosis in LSD-affected cells is mediated by Ca\(^{2+}\). First, thapsigargin, a plant alkaloid that blocks endoplasmic reticulum Ca\(^{2+}\) pump and thus releases the Ca\(^{2+}\) stored in the endoplasmic reticulum without activating phospholipase C (54), also induced apoptosis in MLIV, but not in MLIV-control fibroblasts (Fig. 5C). Second, Bk-induced apoptosis was abolished by incubating the cells with a cell-permeable form of the Ca\(^{2+}\) chelator BAPTA, BAPTA-AM (Fig. 5D). At a 30 \(\mu\)M concentration, BAPTA-AM inhibited Bk-induced Ca\(^{2+}\) transients and Bk-induced apoptosis in MLIV fibroblasts. In two separate experiments, stimulation of MLIV fibroblasts with Bk for 1 h increased apoptosis from 5.5 ± 1.6% (\(n = 10\)) to 18.0 ± 6.6% (\(n = 10\), \(p < 0.05\)) in the absence of BAPTA-AM, whereas in the presence of BAPTA-AM, apoptosis increased from 3.2 ± 1.8% (\(n = 10\)) only to 4.5 ± 2.0% (\(n = 10\), not significant) as determined by annexin V assay.

Staurosporin (Sta), is known to enhance the rate of Ca\(^{2+}\)-induced apoptosis by inhibiting protein kinase C, a regulator of Ca\(^{2+}\) signals (55, 56). We found that similar to Bk-stimulated cells, apoptotic rates are higher in MLIV fibroblasts treated with 0.1 \(\mu\)M Sta than in Sta-treated MLIV-control fibroblasts (Fig. 7A) as determined by DAPI staining.

To test whether the cell stress induced by Ca\(^{2+}\) stimulation involves liberation of CytC from mitochondria and apoptosis by a caspase-9-dependent scenario, we stained untreated and stimulated cells with antibodies specific to the native form of CytC (clone 6H2) and analyzed their cellular localization in control and treated cells. The cells were incubated with the drugs for 1–3 h, and CytC translocation was surveyed using confocal immunocytochemistry. Neither Bk nor 0.1 \(\mu\)M Sta caused a noticeable change in the CytC localization pattern (Fig. 6A). At the same time, 0.25 mM H\(_2\)O\(_2\), which is used to induce apoptosis by liberating CytC, changed CytC appearance from the highly compartmentalized pattern detected in
unreated cells to a smooth stain pattern associated with cytoplasmic localization of CytC (Fig. 6A). High concentrations of Sta also changed the CytC localization pattern (not shown).

Similar results were obtained using Western blotting analysis of cytoplasmic and mitochondrial CytC. Untreated and stimulated MLIV-control and MLIV fibroblasts were homogenized, and unbroken cells and nuclei were separated by centrifugation at 700 × g in the presence of protease inhibitors. The mitochondrial fraction was then isolated by centrifugation at 5,000 × g, and mitochondrial and cytoplasmic fractions were analyzed using SDS-PAGE and Western blotting with CytC antibodies (clone 6H2). Fig. 6B shows that H2O2 increased the levels of CytC in cytoplasm of MLIV cells, whereas Bk-induced CytC translocation was less pronounced.

It is important to note that H2O2 is equally efficient in inducing apoptosis in MLIV-control, MLIII-control, MLIV, and MLIII fibroblasts (Fig. 6C). Therefore, oxidative stress- and Ca2+-induced cell death likely involves different pathways in our system, and the consequences of altered mitochondrial morphology specifically impact survival-death mechanisms involving Ca2+ buffering.

The mechanism of cell death was further probed using caspase-3/7, -8, and -9 inhibitors and fluorogenic substrates. Our assumption was that if the cell death pathway triggered by Bk and Sta is, indeed, apoptosis, it should be sensitive to the inhibitors of the downstream executioner caspase-3. This idea proved to be correct. Pretreatment of cells with cell-permeable caspase-3 inhibitor abolished Bk- and Sta-induced apoptosis in both MLIV-control and MLIV fibroblasts (Fig. 7A). Importantly, both Bk- and 0.1 μM Sta-induced apoptotic responses were also abolished by inhibition of caspase-8, suggesting that a caspase-8-dependent mechanism mediates Ca2+-induced cell death in MLIV fibroblasts.

To further confirm the involvement of caspase-8 in apoptosis induced by Ca2+ stimulation of MLIV fibroblasts, we used a fluorogenic substrate of caspase-8, Z-IETD-AFC. Its fluorescent group, AFC, is quenched until the polypeptide is cleaved by caspase-8 (35). After the cleavage, the fluorescent group can be excited by 400-nm light to emit at 505 nm. The net fluorescence can be used to estimate caspase-8 activity in a given sample. To compare caspase-8 activity using this assay, untreated and Bk-stimulated MLIV-control and MLIV fibroblasts were homogenized, and the protein loads were normalized between the samples. Fluorescence readings were taken before and 30 min after the addition of the fluorogenic substrate. Four independent experiments showed an increase in fluorescence in Bk-treated MLIV cells, which was significantly higher than that detected in Bk-treated MLIV-control cells (Fig. 7B). Therefore, we conclude that Ca2+ stimulation activates caspase-8 in MLIV fibroblasts.

Although the 0.1 μM Sta elicited caspase-8-dependent apoptosis in these cells, higher concentrations of Sta (1 μM) are typically used to induce apoptosis by a caspase-9-dependent pathway. At this higher dose, Sta-induced cell death rates were essentially the same in MLIV-control and MLIV fibroblasts (65.0 ± 6.1% and 69.1 ± 5.4%, respectively, n = 3 independent experiments). Thus, the enhanced sensitivity of MLIV fibroblasts is specifically manifested through Ca2+-induced, caspase-8-dependent pathways rather than via oxidative stress- or caspase-9-dependent pathways.

DISCUSSION

Mechanisms of cell death in LSDs are not well understood, and the lack of a MLIV mouse model impedes elucidation of the pathways that lead to cellular death in this disease. Although psychomotor retardation is a major consequence of MLIV, TRP-ML1 is ubiquitously expressed, and the degenerative processes are not limited to the brain but are also documented in cornea, retina, skeletal muscles, pituitary gland, and other tissues (11, 16, 57). Indeed corneal opacification and retinal degeneration are often the presenting symptoms. Given the similarity between fibroblasts and corneal keratinocytes and the availability of fibroblasts isolated from patients with mucolipidoses, we selected these cells as an initial model system to dissect mechanisms of cell death in MLIV.

We found that mitochondria in MLIV fibroblasts are fragmented and hampered in Ca2+ buffering capacity. Because MLIV is a lysosomal storage disease, we suggested that these aberrations were due to inefficient autophagocytosis and cycling of effete mitochondria (19, 23). Our idea is supported by the fact that every aspect of the mitochondrial phenotype observed in MLIV fibroblasts has been observed in three unrelated lysosomal storage disease systems (MLIII, MLII, and
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Furthermore, control fibroblasts express similar mitochondrial dysmorphology and corrupted function when autophagy or lysosomal function is inhibited. Thus, we established a lysosomal-mitochondrial connection in MLIV, and possibly, in other lysosomal storage diseases: depressed lysosomal function inhibits autophagic turnover of aged and/or damaged mitochondria, which in turn results in accumulation of dysfunctional mitochondria and increased susceptibility to Ca^{2+} toxicity.

It is possible that accumulation of short, fragmented mitochondria could result from the failure of mitochondria to fuse (24), potentially related to lipid deficiencies in cells affected by MLIV. On the other hand, the similarity between our system and the aged post-mitotic cells with lipofuscin accumulation as well as the fact that mitochondrial fragmentation can be reproduced in control cells by lysosomal or autophagy inhibitors support our interpretation that mitochondrial abnormalities detected in our system are the result of inhibited autophagocytosis of effete mitochondria.

Additional data further implicate abnormalities in autophagy in MLIV fibroblasts. Autophagy can be monitored by the redistribution of microtubule-associated protein light chain 3 (LC3, also known as autophagy gene 8, Atg8) from a perinuclear position to the nascent autophagosome membrane (58–60). We used immunofluorescence to study the dynamics of LC3 translocation in untreated, starved, and refed cells. In our experiments, LC3-positive immunofluorescence was primarily located in the perinuclear area of untreated MLIV and MLIV-control cells (supplemental Fig. S1, left column). A 1-h starvation induced translocation of LC3-positive stain into cytoplasm, where it was associated with numerous puncta, presumably autophagosomes (supplemental Fig. S1, middle column). Refeeding control fibroblasts for 3 h restored perinuclear LC3 localization. At the same time, clumps of LC3 stain were retained in the cytoplasm of refed MLIV fibroblasts (supplemental Fig. S1, right column). These results suggest that although autophagic sequestration commences normally in both MLIV and MLIV-control fibroblasts, completion of the degradation process is impaired/delayed in MLIV fibroblasts.

Another autophagy marker, MDC (61) confirmed this trend. Nearly all MLIV-control cells and a majority of untreated MLIV cells displayed smooth cytoplasmic MDC signals (supplemental Fig. S2, left column). However, a large fraction of MLIV fibroblasts showed punctate MDC localization similar to that shown in the middle column in supplemental Fig. S2. Both starvation and mitochondrial damage induce the transformation of MDC stain into a punctate morphology shown to be associated with autophagosomes (61, 62). In our system starvation elicits punctate MDC staining (supplemental Fig. S2, middle column). The punctate MDC stain was completely abolished by refeeding of MLIV-control fibroblasts but was largely retained in refed MLIV fibroblasts (supplemental Fig. S2, right column). Again, generation of autophagosomes seems to commence at similar rates after starvation in both MLIV and MLIV-control cells, but completion of autophagy seems to be delayed in MLIV cells. These data further support the model that abnormal autophagy in MLIV contributes to accumulation of dysfunctional mitochondria.

Mitochondria are well known to regulate apoptosis by buffering Ca^{2+} and by releasing CytC. One of the better known connections between mitochondria, apoptosis, and Ca^{2+} has been documented in neurons, where it has been convincingly shown that mitochondrial Ca^{2+} overload during prolonged Ca^{2+} stimulation destabilizes mitochondrial membranes and releases CytC (reviewed in Ref. 63). In fact, inhibition of mitochondrial Ca^{2+} uptake by FCCP has been shown to have a neuroprotective effect (64). Our results prompt a complimentary model, in which pro-apoptotic effects of Ca^{2+} may also manifest under conditions of suppressed Ca^{2+} uptake by mitochondria. Interestingly, although the cell death induced by mitochondrial Ca^{2+} overload involves the “intrinsic,” caspase-9-dependent path-

FIGURE 7. Mechanisms of apoptosis in control and MLIV fibroblasts.

A. Bk- or Sta-stimulated cells

- **Control**
- **3 hr Bk**
- **3 hr Bk + Cas3 Inh**
- **3 hr Bk + Cas8 Inh**
- **3 hr Bk + Cas9 Inh**

B. Bk-stimulated cells

- **MLIV Ctrl**
- **MLIV**

**FIGURE 7.** Analysis of 1 μM Bk- and 0.1 μM Sta-induced apoptosis under control conditions or under conditions of caspase-3/7, -8, or -9 inhibition. The cells were pretreated with caspase-3/7 (30 μM), caspase-8 (20 μM), or caspase-9 (20 μM) cell-permeable inhibitors at 37 °C for 30 min before treatment with apoptosis stimulus. It is evident that inhibition of caspase-9 has no effect on the rate of apoptosis when cells are stimulated with Ca^{2+} -releasing agonists and can be excluded as the caspase that mediates Ca^{2+} -induced apoptosis in MLIV cells. The results are representative of five experiments; *, p < 0.05 within group.

**Conclusion:**

- CLN2
- Aged mitoClus and lipofuscin accumulation
- Autophagy and mitochondrial function
- Caspase-dependent vs. independent pathways
- Mitochondrial Ca^{2+} and CytC release
- Neuroprotective effects
- Complementary model for Ca^{2+} and apoptosis
way (63), we found that the caspase-8-dependent pathway executes Ca\textsuperscript{2+}-dependent apoptosis in the fibroblasts of MLIV patients. Based on the fact that mitochondria in cells affected by LSDs are deficient in Ca\textsuperscript{2+} uptake function, we hypothesize that in this situation, Ca\textsuperscript{2+} exerts its pro-apoptotic effects in the cytoplasm, perhaps in the immediate proximity of mitochondria.

The perimitochondrial Ca\textsuperscript{2+}-sensitive elements that activate caspase-8 in our system remain to be identified. However, this is not an unprecedented suggestion; recently, it has been shown that the Ca\textsuperscript{2+}-sensitive protease calpain-10 localizes to mitochondria (65). Notably, calpain-cleaved forms of caspase-8 are active, whereas calpain-cleaved caspase-9 is inactive (66), which concurs with our findings that apoptosis induced in MLIV and MLIII fibroblasts by Bk involves caspase-8, not caspase-9.

Autophagosome-rich forms of cell death elicited by other stimuli have also been reported to occur through caspase-8 rather than caspase-9-dependent pathways (67, 68). Although the term autophagic cell death or type II programmed cell death rather than caspase-9-dependent pathways (67, 68). Although the term autophagic cell death or type II programmed cell death is not an unprecedented suggestion; recently, it has been shown that in this situation, Ca\textsuperscript{2+} uptake function, we hypothesize whether the increased autophagy contributes to cell death or represents a failed compensatory reaction (20–22, 69, 70).

To summarize, we observed a common mitochondrial phenotype in MLIV, MLIII, and other LSDs, namely accumulation of fragmented mitochondria with impaired calcium buffering capacity. These changes could be recapitulated in control fibroblasts treated with inhibitors of autophagy in MLIV, MLIII, and other LSDs, namely accumulation of fragmented mitochondria with impaired calcium buffering capacity. These changes could be recapitulated in control fibroblasts treated with inhibitors of autolysosomal degradation, implicating a role for impaired recycling of effete mitochondria.

Moreover, accumulation of abnormally fragmented mitochondria was associated with ineffective mitochondrial Ca\textsuperscript{2+} homeostasis and increased susceptibility to apoptosis induced by Ca\textsuperscript{2+} mobilizing agents. In particular, a pathway of caspase-8-dependent apoptosis is implicated in degenerative cell death associated with MLIV.

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