We have investigated here the mechanism of dephosphorylation and activation of death-associated protein kinase (DAPK) and the role of lysosome in neuroblastoma cells (SH-SY5Y) treated with mitochondrial toxins, such as MPP⁺ and rotenone. Mitochondrial respiratory chain inhibitors and uncouplers decreased mitochondrial membrane potential leading to DAPK dephosphorylation and activation. The class III phosphoinositide 3-kinase inhibitors attenuated DAPK dephosphorylation induced by mitochondrial toxins. Complex I inhibition by mitochondrial toxins (e.g. MPP⁺) resulted in mitochondrial swelling and lysosome reduction. Inhibition of class III phosphoinositide 3-kinase attenuated MPP⁺-induced lysosome reduction and cell death. The role of DAPK as a sensor of mitochondrial membrane potential in mitochondrial diseases was addressed.

Many neurodegenerative diseases are associated with mitochondrial dysfunction. Defects in complexes I, II, and IV of the mitochondrial respiratory chain have been detected in several neurodegenerative diseases (e.g. Alzheimer, Parkinson, Huntington, and amyotrophic lateral sclerosis) (1–4). Experimental models of these diseases typically involve inhibition of complex I, II, and IV enzymes in the mitochondrial respiratory chain (5–7). Thus, it is of interest to fully understand the signaling consequences of inhibiting the mitochondrial respiratory chain.

Drugs inhibiting mitochondrial function decrease mitochondrial potential, depolarize mitochondria, and inhibit ATP production (8, 9). Mitochondrial dysfunction has been causally linked to a variety of human disorders, including neurodegenerative diseases, diabetes, and cancer (9–11). Understanding the molecular mechanisms of the mitochondrial stress-signaling pathway is, therefore, critical for elucidating the mechanism(s) of neurodegenerative and cardiovascular disease processes (12).

Mitochondria are one of the main sources of reactive oxygen species (ROS), and how they transduce oxidative stress and energy-related signaling is an active area of research (13, 14). In addition, mitochondria act as a dynamic receiver and integrator of numerous proteins in cellular stress signals to coordinate the intrinsic apoptotic pathway (15). Mitochondria regulate intracellular Ca²⁺ and membrane phospholipid signaling as well (16, 17). Mitochondria, as stress-integration machinery, play a pivotal role in the determination of the life and death of cells in neurodegenerative diseases and cancer (12). Identification of mitochondrial signaling pathways and the key lipid or protein affecting the cell death could provide new therapeutic targets for neurodegeneration and cancer.

In this study, we sought to investigate the role of death-associated protein kinase (DAPK) and lysosomes in autophagy and apoptosis induced by mitochondrial electron transport chain inhibitors. DAPK is a cytosolic multidomain protein containing a catalytic domain, death domain, Ca²⁺/calmodulin domain, and ankyrin repeats that are involved in protein-protein interactions (18). The pro-cell death mechanisms and the regulatory signaling pathways of DAPK are not fully understood. The present results indicate that DAPK is an effective sensor of the mitochondrial membrane potential (Δψ⁰) loss in cells treated with respiratory chain inhibitors and mitochondrial uncoupling agents. The relevance of DAPK as a sensor of Δψ⁰ loss in cells treated with neurotoxic agents has been addressed.

**EXPERIMENTAL PROCEDURES**

**Materials**—1-Methyl-4-phenylpyridinium (MPP⁺), 3-(4,5-dimethylylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), rotenone, antimycin A, glucose oxidase, tert-butyldihydroperoxide, 3-nitropropionic acid, thienyltrifluoroacetone, carbonyl cyanide m-chlorophenyl hydrazone (CCCP), dinitrophenol, cyclosporin A, okadaic acid, rapamycin, 3-methyladenine, bafilomycin A, ammonium chloride, phosphatase inhibitor mixture 1, and mouse anti-phosphorylated DAPK antibody were purchased from Sigma. BAPTA and JC-1 were from Molecular Probes, Inc. (Eugene, Oregon). Mouse anti-DAPK protein antibody was from BD Transduction Laboratories. Mouse anti-actin antibody was from Chemicon International, Inc. (Temecula, California).

**Synthesis of Mito Vit-E**—The method of Murphy and co-workers was used with some modifications (19, 20). 6-Hydroxy-2-methoxy-2, 5,7,8-tetramethylchroman was synthesized from trimethylhydroquinone, trimethyl ortho-formate, and methyl vinyl ketone. This chroman was converted to the corresponding nitrile, which upon hydrolysis with aqueous ethylene glycol in potassium hydroxide, gave the corresponding acid. The acid was reduced to the alcohol that was converted to the bromo derivative by using carbon tetrabromide and triphenylphosphine in dimethyl formamide. The bromo derivative was heated with tert-butyl hydroperoxide, 3-nitropropionic acid, thienyltrifluoroacetone, carbonyl cyanide m-chlorophenyl hydrazone (CCCP), dinitrophenol, cyclosporin A, okadaic acid, rapamycin, 3-methyladenine, bafilomycin A, ammonium chloride, phosphatase inhibitor mixture 1, and mouse anti-phosphorylated DAPK were purchased from Sigma. BAPTA and JC-1 were from Molecular Probes, Inc. (Eugene, Oregon). Mouse anti-DAPK protein antibody was from BD Transduction Laboratories. Mouse anti-actin antibody was from Chemicon International, Inc. (Temecula, California).
ple with 2,3-dimethoxy-5-methyl-1,4-benzoquinone to yield the 6-(10-bromodecyl) ubiquinone. The quinone was reduced to the quinol using sodium borohydride and heated with triphenylphosphine in dioxane for 4 days. The oily product separated from the reaction medium was purified and analyzed by mass spectroscopy.

**Culturing of Human Neuroblastoma SH-SY5Y Cells**

Human neuroblastoma cells (SH-SY5Y) obtained from the American Type Cell Collection were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, L-glutamine (4 mmol/liter), penicillin (100 units/ml), and streptomycin (100 μg/ml) and incubated at 37 °C in a humidified atmosphere of 5% CO2 and 95% air. For the experiments, the cells were seeded in 6-well plates and grown to a confluence of 70–80%.

**Subcellular Fractionation and Aconitase Measurements**

After treatment, cells were washed three times and scraped in 1-ml isolation buffer (320 mM sucrose, 1 mM potassium EDTA, 10 mM Tris-HCl, 20 mM fluorocitrate, 0.0035% digitonin, pH 7.4) (22). Cell suspension was homogenized in a tight-fitting glass/teflon homogenizer using 20 up-and-down strokes. The homogenate was centrifuged at 1,500 g for 10 min at 4 °C. The supernatant was centrifuged at 17,000 g for 10 min at 4 °C. The final pellet was resuspended in a 100 μl of isolation buffer. The activity of aconitase in the cytosol and mitochondrial fractions were measured in a 100-μl Tris-HCl (100 mM, pH 8.0) containing 20 mM D,L-trisodium isocitrate buffer. The rate of change of absorbance was followed for 3 min at 240 nm in a Shimadzu UV-visible spectrophotometer at room temperature. An extinction coefficient for cis-aconitase of 3.6 mm⁻¹ cm⁻¹ at 240 nm was used to calculate the enzyme activity.

**Determination of DAPK Protein Levels**

SH-SY5Y cells were washed with an ice-cold phosphate-buffered saline buffer and resuspended in 100 μl of radioimmune precipitation assay buffer (20 mM Tris-HCl, pH 7.4, 2.5 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 1% SDS, 100 mM NaCl, and 100 mM sodium fluoride). To a 10-ml solution of the above were added 1% phosphatase inhibitors mixture 1 and 1 tablet of protease inhibitor mixture (Roche Applied Science). The cells were homogenized, and the lysate was centrifuged for 10 min at 12,000 g at 4 °C. Proteins were resolved on 8% SDS-polyacrylamide gels and blotted onto nitrocellulose membranes. The membranes were probed with a mouse anti-phosphorylated DAPK monoclonal antibody. After washing, the membranes were incubated with horseradish peroxidase-conjugated, rabbit anti-mouse IgG. Protein bands were detected using the ECL method (Amersham Biosciences). Subsequently, the blots were stripped twice and reprobed with an antibody against DAPK and actin, respectively.

**Electron Microscopy**

SH-SY5Y cells were cultured in 100-mm Petri dishes. After treatment with MPP⁺ (1 mM) for 24 h, media were removed as much as possible. The cells were washed once with prewarmed 1/16 phosphate-buffered saline, and then 5 ml of cold freshly made fixative (2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4) were poured into dishes to cover the cells. The cells were then placed on ice. Routine electron microscopy was performed after a 30-min fixation.

**Mitochondrial Membrane Potential Measurement**

Mitochondrial potential was assessed using the fluorescent potentiometric dye JC-1.
as described previously (23). Briefly, cells were incubated with JC-1 (5 μg/ml) in serum-free Dulbecco’s modified Eagle’s medium for 15 min at 37 °C. After staining, the cells were collected at room temperature and washed three times with pre-warmed 1X phosphate-buffered saline. The cell pellet was then resuspended in 1 ml of phosphate-buffered saline. JC-1 fluorescence was quantitated using a Shimadzu spectrofluorophotometer. The fluorescence of the JC-1 monomer was measured at 485 nm of excitation/530 nm of emission wavelength. The fluorescence of the JC-1 aggregate was measured at 535 nm of excitation/590 nm of emission wavelength. For each experiment, the aggregate monomer ratios were normalized to untreated controls (100%).

MTT Assay—After treatment, the culture media were removed and cells were washed with Dulbecco’s modified Eagle’s medium and were incubated in Dulbecco’s modified Eagle’s medium containing 0.25 mg/ml MTT for 2 h at 37 °C. Media were removed and cells were solubilized and mixed thoroughly in isopropyl alcohol/0.08 N HCl (1:1). The absorption was measured at 562 nm with reference at 630 nm.

Data Analysis—Statistical significance was obtained using a Student’s t test employing the Sigmasat software.

RESULTS

Mitochondrial Respiratory Chain Inhibitors (Complexes I–IV) Induce DAPK Dephosphorylation and Cell Death—Treatment of SH-SY5Y cells with MPP⁺ (1 mM) for 36 h increased cell toxicity, as measured by the cell viability assay (Fig. 1A). To investigate the mechanism of cell death, we monitored DAPK, which is involved in autophagic cell death. DAPK is activated by dephosphorylation via an unknown phosphatase. The dephosphorylation of DAPK was measured by Western blot using an antibody against phosphorylated DAPK. MPP⁺ treatment induced DAPK dephosphorylation (Fig. 1B, top row, and C).

Next we examined the effects of rotenone, another mitochondrial complex I inhibitor, and antimycin A, the inhibitor of mitochondrial complex III. Rotenone (0.5 μM) treatment decreased the cell viability with time (Fig. 1D) and induced DAPK dephosphorylation (Fig. 1, E and F).

The complex III site is another major site of ROS formation in the mitochondrial respiratory chain (24). Antimycin A (4 μg/ml) treatment decreased the cell viability in a time-dependent manner (Fig. 1G). It induced DAPK dephosphorylation as well (Fig. 1H and I).

Additional information concerning the mechanism of DAPK dephosphorylation was obtained using the inhibitors of mitochondrial complexes II and IV. Complex IV is not a major site of ROS production in mitochondria. Mitochondrial complex II inhibitors, 3-nitropropionic acid, thienyltrifluoroacetone, and thenoyltrifluoroacetone time-dependently decreased the cell viability (Fig. 2, A and D) and induced DAPK dephosphorylation (Fig. 2, B and E). Treatment with sodium azide (2 mM), a mitochondrial complex IV inhibitor, also decreased the cell viability with time (Fig. 2G) and induced DAPK dephosphorylation (Fig. 2, H and I).
Role of ROS in Mitochondrial Toxin-induced DAPK Dephosphorylation—Mitochondrial complexes I and III are major sites of ROS formation (24–25). To investigate whether exogenously added oxidants induce DAPK dephosphorylation, we examined the effects of glucose/glucose oxidase (glucose/GO) and tert-butyl hydroperoxide (tert-BuOOH). Hydrogen peroxide (H$_2$O$_2$) was generated in the media using the glucose/glucose oxidase (glucose/GO). Treatment with glucose/GO (10 milliunits/ml) or tert-BuOOH (200 μM) decreased the cell viability with time (Fig. 3, A and E). Aconitase serves an important sensor of intracellular oxidant formation (26). Treatment with glucose/GO (10 milliunits/ml) or tert-BuOOH (200 μM) only decreased the cytosolic and not the mitochondrial aconitase activity (Fig. 3, B and F), suggesting a high level of cytosolic ROS compared with that in mitochondria. However, glucose/GO or tert-BuOOH treatment did not correspondingly induce DAPK dephosphorylation (Fig. 3, C and G), coinciding with increased cell death. These results suggest that cytosolic ROS may not be involved in DAPK dephosphorylation and activation.

Mitochondrial respiratory chain inhibitor-induced ROS formation in the cytosol and mitochondria was examined by monitoring the corresponding aconitase activity. Treatment with MPP$^+$ or antimycin A for 12 h decreased both the cytosolic and mitochondrial aconitase activities, indicating the formation of ROS in both compartments (Fig. 4, A and C). Treatment with rotenone for 12 h only decreased the cytosolic aconitase activity, not the mitochondrial aconitase activity, suggesting only cytosolic ROS formation at this time point (Fig. 4B). However, with prolonged incubation time (e.g. 18–36 h), rotenone treatment caused a decrease in the mitochondrial aconitase activity as well, suggesting ROS formation both in cytosol and mitochondria (data not shown).

To investigate whether mitochondrial ROS are involved in DAPK dephosphorylation, the effects of mitochondrial targeted antioxidants, Mito-Q and Mito Vit-E, were determined. Mito-Q and Mito Vit-E are derivatives of ubiquinone and vitamin E, respectively. Ubiquinol has been reported to function as an antioxidant by donat-
ing a hydrogen atom from one of its hydroxyl groups to a lipid per-oxyl radical, thereby decreasing lipid peroxidation within the inner membrane (19). The ubisemiquinone radical formed during this process disproportionates into ubiquinone and ubiquinol. The respiratory chain subsequently recycles ubiquinone back to ubiquinol, restoring its antioxidant function (19). The tocopheroxyl radical formed from the one-electron oxidation of vitamin E was proposed to regenerate vitamin E by reacting with ubiquinol (19). Neither Mito-Q nor Mito Vit-E prevented MPP⁺–induced DAPK dephosphorylation (Fig. 4, D and F). The effects of thiol antioxidants glutathione and N-acetylcysteine were investigated. Neither glutathione nor N-acetylcysteine prevented MPP⁺–induced DAPK dephosphorylation (Fig. 4, H and J). These results suggest that mitochondrial ROS may not be involved in DAPK dephosphorylation and activation. However, additional studies with mitochondrial superoxide dismutase or catalase overexpressing cells are needed to further corroborate this conclusion.

FIGURE 4. Mitochondrially targeted antioxidant did not prevent MPP⁺–induced DAPK dephosphorylation in SH-SYSY cells. SH-SYSY cells were treated with MPP⁺ (1 mM) (A), rotenone (0.5 μM) (B), or antimycin A (4 μg/ml) (C) for 12 h, and cytosolic and mitochondrial aconitase activities were measured. SH-SYSY cells were treated with MPP⁺ in the presence (+) or absence (−) of different concentrations of Mito-Q (D), Mito Vit-E (F), glutathione (H), or N-acetylcysteine (J) as indicated. DAPK dephosphorylation levels and total protein levels were determined by Western blotting using antibodies against phosphorylated DAPK and total DAPK, respectively. E, G, I, and K show the densitometric analyses of D, F, H, and J. Gels are representative of three independent experiments. Values are means ± S.D. of three separate experiments. *, p < 0.05 compared with control.
Mitochondrial Membrane Potential (Δψm) Loss Induces DAPK Dephosphorylation—As most mitochondrial respiratory inhibitors could effectively decrease Δψm, the effects of mitochondrial respiratory chain inhibitors on Δψm were determined using the JC-1 staining technique. As shown in Fig. 5A, all mitochondrial respiratory inhibitors decreased Δψm, as did the mitochondrial uncouplers CCCP and DNP. In contrast, glucose/GO and tert-BuOOH, which had no effect on DAPK dephosphorylation, did not decrease the Δψm. To confirm that

FIGURE 5. Mitochondrial respiratory chain inhibitors and uncouplers induced changes in Δψm and DAPK dephosphorylation in SH-SY5Y cells. A, SH-SY5Y cells were treated with MPP⁺ (1 mM, 12 h), 3-nitropropionic acid (10 mM, 24 h), antimycin A (4 μg/ml, 12 h), sodium azide (2 mM, 12 h), DNP (2 mM, 12 h), CCCP (25 μM, 2 h), tert-BuOOH (200 μM, 1 h), or glucose/GO (10 milliunits/ml, 4 h), and then cells were collected and Δψm was measured by JC-1 staining and expressed as percent of control. B, SH-SY5Y cells were treated with CCCP (25 μM, 2 h) or DNP (2 mM, 12 h). The cells were then collected, and DAPK dephosphorylation levels and total protein levels were determined by Western blotting using antibodies against phosphorylated DAPK and total DAPK, respectively. C shows the densitometric analyses of B. Gels are representative of three independent experiments. Values are means ± S.D. of three separate experiments. *, p < 0.05 compared with control; **, p < 0.01 compared with control.

FIGURE 6. PP1 and PP2A were not involved in MPP⁺-induced DAPK dephosphorylation in SH-SY5Y cells. SH-SY5Y cells were treated with 1 mM MPP⁺ for 12 h in the presence or absence of 10 nM okadaic acid (A), 10, 20, or 50 mM glucose (C), 25 μM BAPTA or 50, 100, and 250 nM cyclosporin A (E). DAPK dephosphorylation levels and total protein levels were determined by Western blotting using antibodies against phosphorylated DAPK and total DAPK, respectively. B, D, and F show the densitometric analyses of A, C, and E. Gels are representative of three independent experiments. Values are means ± S.D. of three separate experiments.
**DAPK and Lysosome in Mitochondrial Toxicity**

$\Delta \psi_m$ reduction induces DAPK dephosphorylation, we investigated the effect of CCCP and DNP. Both CCCP and DNP induced DAPK dephosphorylation and activation (Fig. 5, B and C), indicating that DAPK is a sensor of $\Delta \psi_m$.

**DAPK Dephosphorylation Is Mediated by a Class III PI 3-Kinase-dependent Phosphatase and Not by the mTOR Pathway**—Next we defined the phosphatase that is responsible for DAPK dephosphorylation. PP1 and PP2A, which are inhibited by okadaic acid, were studied. Okadaic acid did not prevent the MPP$^+$-induced DAPK dephosphorylation (Fig. 6, A and B). ATP depletion was reported to activate PP2A (14). Therefore, glucose (10, 20, or 50 mM) was added to the medium to increase the intracellular ATP levels. Previous reports indicated that the addition of glucose attenuated MPP$^+$ toxicity in neuroblastoma cells via enhanced ATP synthesis (27, 28). Glucose treatment, however, did not prevent MPP$^+$-induced DAPK dephosphorylation (Fig. 6, C and D). These results suggest that PP1 and PP2A are unlikely to trigger DAPK dephosphorylation.

The calcium-dependent phosphatase (PP2B), which is inhibited by cyclosporin A, was examined. Cyclosporin A treatment did not prevent MPP$^+$-induced DAPK dephosphorylation (Fig. 6, E and F). As the activity of PP2B is calcium-dependent, the effect of the calcium chelator, BAPTA, was examined. BAPTA treatment did not prevent MPP$^+$-induced DAPK dephosphorylation (Fig. 6, E and F). These results suggest that PP2B is not involved in DAPK dephosphorylation.

The class I PI 3-kinase inhibits autophagy through the mTOR pathway, whereas inhibition of mTOR by rapamycin stimulates autophagy (29). mTOR is also a sensor of mitochondrial dysfunction. It was reported that ATP depletion and $\Delta \psi_m$ loss inhibit mTOR activity, resulting in the activation of PP2A and an undetermined phosphatase (14, 23). Rapamycin treatment did not prevent MPP$^+$-induced DAPK dephosphorylation (Fig. 7, A and B). Interestingly, rapamycin treatment had a synergistic effect on MPP$^+$-induced cell toxicity, as measured by the cell viability assay (Fig. 7C). MPP$^+$ treatment for 36 h decreased cell viability to 65% of control; the cell viability significantly decreased to nearly 39% of control in the presence of rapamycin.

The class III PI 3-kinase stimulates autophagy and is inhibited by 3-methyladenine (3-MA) (29). 3-MA treatment attenuated MPP$^+$-induced DAPK dephosphorylation (Fig. 7, D and E). 3-MA also increased the cell viability as compared with MPP$^+$ alone (Fig. 7F). DAPK dephosphorylation and cell death induced by antimycin A (complex III inhibitor) were attenuated by 3-MA (Fig. 7, G–J). These results suggest that a class III PI 3-kinase-dependent phosphatase is involved in DAPK dephosphorylation.

**MPP$^+$ Induces Lysosome Diminution and Not Autophagy in SH-SY5Y Cells**—Unexpectedly, we observed a significant diminution of the lysosomes in MPP$^+$-treated (24 h) SH-SY5Y cells as revealed by electron microscopy (Fig. 8). In MPP$^+$-treated cells, there was mitochondrial...
swelling. Under these conditions, there was no nuclear condensation, suggesting a non-apoptotic mechanism of cell death. In the presence of 3-MA, there were more normal mitochondria and lysosomes compared with MPP⁺ alone. The double membranes of mitochondria were maintained in MPP⁺-treated cells, even under severely swollen conditions. The Western blotting technique was used to examine whether cytochrome c was released under these conditions. The majority of the cytochrome c was retained in the mitochondria, confirming that there was no intrinsic apoptotic cell death under these conditions (data not shown). It should, however, be noted that high concentrations of MPP⁺ induced apoptotic cell death in SH-SY5Y cells (31).

**DISCUSSION**

Here we report that treatment of SH-SYSY cells with mitochondrial respiratory chain inhibitors decreased mitochondrial membrane potential. This was accompanied by an increase in DAPK dephosphorylation through a class III PI 3-kinase-dependent pathway. The complex I inhibition by MPP⁺ and other mitochondrial toxins inhibited lysosomes. Inhibition of class III PI 3-kinase attenuated MPP⁺-induced lysosome reduction and cell death. Loss in Δψₘ induced DAPK dephosphorylation and activation leading to cell death. We propose that DAPK is an important sensor of Δψₘ (Scheme 1).

We propose that depolarized mitochondria release a factor that leads to their specific removal. Previous results demonstrated that the direction of mitochondrial transport is modulated through a pathway that involves phosphatidylinositol (Scheme 1, Ptdins) (32). Our findings connecting DAPK dephosphorylation and activation to a class III PI 3-kinase-dependent pathway also suggest that mitochondrial membrane-derived lipid second messengers are possibly involved in Δψₘ-induced mitochondrial turnover. Membrane lipid second messengers are generated when plasma membrane is depolarized (33). Thus, similar messenger molecules are also generated during mitochondrial membrane depolarization (Scheme 1). Release of peptides from mitochondrial proteins presumably triggers the mitochondrial stress signaling pathway (34).

It is of interest to note that a phosphatase and tensin homolog deleted from chromosome 10 (PTEN), which is an antagonist of the PI 3-kinase signaling cascade, was reported to be sensitive to mitochondrial redox status (17). PTEN antagonizes the class I PI 3-kinase/Akt pathway and positively regulates autophagy (29). Collectively, these results suggest that the mitochondrial membrane lipid second messenger is closely coupled to the mitochondrial function and is involved in the signaling cascade induced by mitochondrial dysfunction.

Δψₘ-mediated DAPK Signaling in Mitochondrial Turnover—Mitochondria undergo a continuous turnover in cells (35). Depolarized or aged mitochondria are selectively eliminated by autophagy (36). In neurons, mitochondria are synthesized in the cell bodies and are transported down
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Our findings that mitochondrial respiratory chain inhibitors result in Δψm diminution and DAPK activation suggest that DAPK may play a role as a sensor in mitochondrial diseases. DAPK is highly expressed in brain, especially in the hippocampus (30). DAPK exists as an inactive form under physiological conditions, so the active form (dephosphorylated form) is a likely target for drug development in the treatment of neurodegenerative diseases (e.g. Parkinson disease) and cancer.

**REFERENCES**


**SCHEME 1.** A proposed mechanism for DAPK as a sensor of Δψm in mitochondrial toxin-induced cell death in SH-SYSY cells. Mitochondrial respiratory chain inhibitors decreased Δψm, and caused depolarization leading to formation of mitochondrial membrane lipid second messengers. This resulted in the activation of a phosphatase and DAPK dephosphorylation. DAPK activation led to lysosome dysfunction and cell death.