Mechanism of Cell Death Caused by Complex I Defects in a Rat Dopaminergic Cell Line*\S

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Defects in the proton-translocating NADH-quinone oxidoreductase (complex I) of mammalian mitochondria are linked to neurodegenerative disorders. The mechanism leading to cell death elicited by complex I deficiency remains elusive. We have shown that expression of a rotenone-insensitive yeast NADH-quinone oxidoreductase (Ndi1) can rescue mammalian cells from complex I dysfunction. By using the Ndi1 enzyme, we have investigated the key events in the process of cell death using a rat dopaminergic cell line, PC12. We found that complex I inhibition provokes the following events: 1) activation of specific kinase pathways; 2) release of mitochondrial proapoptotic factors, apoptosis inducing factor, and endonuclease G. AS601245, a kinase inhibitor, exhibited significant protection against these apoptotic events. The traditional caspase pathway does not seem to be involved because caspase 3 activation was not observed. Our data suggest that overproduction of reactive oxygen species (ROS) caused by complex I inhibition is responsible for triggering the kinase activation, for the release of the proapoptotic factors, and then for cell death. Nearly perfect prevention of apoptotic cell death by Ndi1 agrees with our earlier observation that the presence of Ndi1 diminishes rotenone-induced ROS generation from complex I. In fact, this study demonstrated that Ndi1 keeps the redox potential high even in the presence of rotenone. Under these conditions, ROS formation by complex I is known to be minimal. Possible use of our cellular model is discussed with regard to development of therapeutic strategies for neurodegenerative diseases caused by complex I defects.

The proton-translocating NADH-quinone oxidoreductase (complex I)\A is the first of four enzyme complexes in the mammalian mitochondrial respiratory chain. A dysfunction in complex I is known to be involved in a large number of human diseases such as myopathies (1), stroke-like syndrome (2), and Leber’s hereditary optic neuropathy (3). More recent developments revealed that diminution of the complex I activity is also linked to neurodegenerative disorders, most notably Parkinson disease (PD). In PD, a systemic reduction of complex I activity was found in the brain of patients (4). Additional evidence for complex I impairment in PD comes from the finding that 1-methyl-4-phenylpyridinium ion, the active metabolite of the Parkinsonism toxin MPTP, acts as a complex I inhibitor (5). In fact, animal models developed for PD involve administration of complex I inhibitor, MPTP, or rotenone (6).

In the rotenone rat PD model, many characteristics of PD were demonstrated, including selective nigrostriatal dopaminergic degeneration, formation of Lewy bodies, and motor deficits (7, 8). Since the doses of rotenone used did not alter mitochondrial oxygen consumption in isolated brain mitochondria (9), it did not appear that a bioenergetic defect could account for rotenone-induced neurodegeneration. Instead, it was suggested that rotenone toxicity resulted from oxidative stress (10). In the case of MPTP mouse PD models, it has been reported that apoptosis is involved in the neurodegeneration. For example, ablation of proapoptotic protein Bax was shown to prevent dopaminergic neurodegeneration (11). Also, neuronal apoptosis in the substantia nigra was observed in the early stage of chronic degeneration (12).

Because complex I inhibitors trigger characteristic features of PD, their use would allow us to understand the mechanisms of cell death during the development of the disease. The role of the mitochondrial respiratory chain and particularly of complex I in the process of cell death is still elusive. It is known that complex I is involved in formation of ROS (13) and that compounds stimulating ROS formation can trigger apoptosis (14). Complex I is known to generate ROS in at least two modes as follows: one is through inhibition of forward electron transfer, and the other is associated with reverse electron transfer (15, 16). The ROS production in the latter mode is suppressed by rotenone. Recently, Kussmaul and Hirst (15) reported that ROS production caused by complex I inhibition involves FMN and occurs only when the NADH/NAD+ ratio is significantly high. We recently dem-

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**Abbreviations used:**
- complex I, proton-translocating NADH-quinone oxidoreductase; Ndi1, internal rotenone-insensitive NADH-quinone oxidoreductase from *S. cerevisiae*; PD, Parkinson disease; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; ROS, reactive oxygen species; AIF, apoptosis-inducing factor; EndoG, endonuclease G; DMEM, Dulbecco’s modified Eagle’s medium; 8-oxo-dG, 7,8-dihydro-8-oxo-deoxyguanine; NAC, N-acetylcysteine; TBS, Tris-buffered saline; FBS, fetal bovine serum; PBS, phosphate-buffered saline; cyt c, cytochrome c; JNK, c-Jun N-terminal kinase; FITC, fluorescein isothiocyanate; DAPI, 4’,6-diamidino-2-phenylindole.

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onstrated that the yeast Ndi1 enzyme, when introduced in mammalian respiratory chain, is capable of diminishing ROS formation caused by complex I inhibition (17). Therefore, we can utilize the Ndi1 enzyme as a versatile tool with which to investigate participation of complex I in cellular events relating to ROS (for reviews see Refs. 18–20).

We sought to clarify the mechanism of cell death in a dopaminergic cell line PC12 caused by complex I inhibitors and the mechanism of protective effects of Ndi1 expression on ROS generation by complex I defects. Here we showed that complex I inhibitors induce apoptosis not by the traditional caspase pathway but mainly by the release of AIF and EndoG. We also demonstrated that the Ndi1 enzyme could prevent the activation of all apoptotic cellular pathways triggered by the production of ROS following complex I inhibition. This event occurs when the Ndi1 expression suppressed NADH/NAD+ ratios in the matrix to the level where complex I cannot produce ROS. This suggests that the very first step in complex I defect cell death is the elevation of ROS production.

**EXPERIMENTAL PROCEDURES**

**Materials**—Antibodies against AIF and EndoG were purchased from Chemicon International (Temecula, CA). JNK inhibitor SP600125, antibodies against cyt c, caspase 3, caspase 7, caspase 9, p42/44 mitogen-activated protein kinase, p38 kinase, and JNK were from Cell Signaling Technology (Beverly, MA). MitoSOX, JC-1, anti-COX antibody, anti-F0F1-ATPase antibody, and secondary antibodies labeled with FITC or rhodamine red-X were from Invitrogen. Antibody against 8-oxo-dG was from R & D Systems (Minneapolis, MN). Secondary antibodies used for immunoblotting were purchased from Pierce. Complete mixture of protease inhibitors tablets were from Roche Applied Science. JNK inhibitor AS601245 was from Pierce. Complete mixture of protease inhibitors. Cells were plated on a microcentrifuge tube with 50 μl of pre-washed glass beads (250 μm size; Sigma). The tubes were vortexed at maximum speed for 1 min and centrifuged at 500 × g for 5 min at 4 °C to eliminate nuclei and unbroken cells. The supernatant was transferred to a new tube and centrifuged at 10,000 × g for 30 min at 4 °C. The pellet obtained was enriched in heavy membrane organelles and used as the mitochondrial fraction. The resulting supernatant was stored as the cytoplasmic fraction. Protein concentration was determined by the micro-Bradford assay at 595 nm. The cytoplasmic fraction was treated with 5% trichloroacetic acid for 30 min on ice, and the precipitated protein was dissolved in sample buffer (65 mM Tris-Cl, pH 6.8, containing 5% SDS, 3% urea, 5% beta-mercaptoethanol, 10% glycerol and 0.05% bromphenol blue) by sonication, heated at 65 °C for 15 min, and loaded on a 15% SDS-polyacrylamide gel.

**Determination of Mitochondrial Membrane Potential**—PC12 and PC12-NDI1 cells were cultivated in 384-well plates (black well, clear bottom, 1 × 104 cells/well) (Greiner bio-one, Monroe, NC) for 24 h in DMEM with 10% serum. Medium was changed to DMEM supplemented with 1% serum and 1g/liter glucose 15 h before the start of the experiment. After addition of an inhibitor to test, cells were incubated for a designated period of time and washed twice with warm PBS, JC-1 (2 μM in DMEM with 1% serum) was added, and the cells were incubated for 20 min at 37 °C under 5% CO2 atmosphere.

**Cell Culture**—PC12 and PC12-NDI1 cells (21) were cultured in DMEM (Irving Scientific) containing 4.5 g of glucose/liter and supplemented with penicillin/streptomycin (50 units/ml), glutamate (2 mM), and 10% fetal bovine serum. Cells were grown in humified chamber at 37 °C under a 5% CO2 atmosphere.

**Cell Viability Assay**—PC12 and PC12-NDI1 (5 × 104 cells) were seeded in 96-well plates. Eighteen hours before the start of the experiment, cells were placed in DMEM with 1% serum. Then rotenone was added at a final concentration of 1 μM. At the end of incubation with rotenone, cells were washed with warm DMEM with 10% serum. Cell viability was performed with the CellTiter 96® AQueous nonradioactive cell proliferation assay (Promega) by following the manufacturer’s recommendation. Briefly, 80 μl of warm culture medium was mixed with 20 μl of reagent and incubated for 1 h. Then the absorbance was monitored at 490 nm on a plate reader (SpectraMax M2, Molecular Devices) every 15 min for 4 h.

**DNA Laddering Assay**—PC12 and PC12-NDI1 cells were grown in 6-well plates in DMEM with 1% serum. Cells were incubated with 1 μM rotenone for 24 h, washed with cold PBS, and lysed in 1.5 ml of 10 mM Tris-Cl, pH 8, 5 mM EDTA, 0.5% SDS for 30 min on ice. The cell lysate (600 μl) was incubated with RNase A (200 μg/ml) for 30 min at 37 °C and with proteinase K (150 μg/ml) for 4 h at 54 °C. The incubation was then continued at 65 °C for 1 h. After phenol/chloroform extraction and isopropyl alcohol precipitation, DNA samples were loaded on a 1.5% agarose gel. Electrophoresis was carried out for 2 h at 100 V in a cold room.

**Immunoblotting**—Caspase activation was measured by Western blotting. Cells were grown in DMEM with 10% serum and then in DMEM with 1% serum 18 h before the start of the experiment. Rotenone was added at a final concentration of 1 μM. After incubation, cells were lysed for 10 min on ice with 150 μl of lysis buffer (50 mM Tris-Cl, pH 7.4 containing 150 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate, and 5 mM EDTA) and centrifuged at 500 × g for 5 min. Protein was precipitated with 2.5 volumes of methanol. Samples (80 μg of total protein) were loaded on a 15% SDS-polyacrylamide gel. Activated forms of caspases 9, 3, and 7 were visualized, respectively,
with anti-caspase 9 (1/1000), anti-caspase 3 (1/1000), and anti-caspase 7 (1/1000) antibodies after transfer onto a nitrocellulose membrane. The incubation was carried out in TBST (40 mM Tris-Cl, pH 7.8, 140 mM NaCl, and 0.1% Tween 20) with 5% nonfat milk overnight at 4 °C.

For kinase phosphorylation analysis, PC12 and PC12-NDI1 cells were grown in 12-well plates, and the medium was replaced with DMEM with 1% serum for 18 h before the start of the experiment. Cells were incubated with 1 μM rotenone in Krebs medium (140 mM NaCl, 4.7 mM KCl, 1.2 mM KH2PO4, 1.25 mM MgSO4, 1 mM CaCl2, 15 mM glucose, 10 mM HEPES, pH 7.4, and 0.1% bovine serum albumin). Cells were lysed in lysis buffer supplemented with 1 mM Na2VO4, 10 mM NaF, and a complete mixture of protease inhibitors. Forty μg of total protein were loaded per lane on a 15% SDS-polyacrylamide gel and electroblotted onto a nitrocellulose membrane (0.2 μm). The membrane was incubated with appropriate antibodies (1/1000) overnight at 4 °C in TBST with 5% milk. Blots were revealed using an enhanced chemiluminescence system (Pierce) and exposed on blue X-rays film. To ensure equal loading of proteins in each lane, the same blot was reprobed with the anti-total kinase antibody.

Immunoblotting analysis for AIF and EndoG relocalization was performed by loading the mitochondrial fraction (2 μg of protein) and the cytoplasmic fractions (60 μg of protein) (see “Subcellular Fractionation”). After blotting onto a nitrocellulose membrane AIF and EndoG were revealed by anti-AIF antibody (1/1000) and anti-EndoG antibody (1/500).

**Fluorescence Microscopy Analysis**—Ndi1 staining was performed on PC12-NDI1 cells as described previously (22). Briefly, cells were grown on 12-mm coverslips and fixed in 4% paraformaldehyde for 10 min on ice. After washing with TBS the cells were blocked for 45 min with image-iT FX signal enhancer (Invitrogen). Antibody against Ndi1 (1/250) was applied on the coverslips for 18 h at 4 °C in TBS with 10% FBS and 0.1% Triton X-100. Ndi1 staining was revealed by incubation with a rabbit secondary antibody labeled with FITC for 5 h at room temperature. After washing with TBS (three times for 5 min), cells were mounted with Mowiol and observed under an inverted fluorescence microscope (Zeiss).

AIF relocalization was monitored on PC12 and PC12-NDI1 cells after 48 h of rotenone (1 μM) exposure. Non-specific staining was blocked by incubation of coverslips with TBS containing 10% FBS and 0.1% Triton X-100 for 45 min at ambient temperature. Antibodies against AIF (1/300) and COX (1/300) were applied overnight at 4 °C. Secondary antibodies, anti-mouse FITC (1/100), and anti-rabbit rhodamine red-X (1/500) were used to show AIF and COX staining, respectively. Coverslips were mounted on glass slides with Mowiol and examined under a fluorescence microscope. For assessment of cyt c relocalization, cells were fixed and stained with antibodies against F3F1-ATPase (1/300) and cyt c (1/200) in TBS with 10% FBS and 0.1% Triton X-100 overnight at 4 °C. Staining of the α-subunit of F3F1-ATPase and cyt c was done with the anti-mouse rhodamine red-X and anti-rabbit FITC, respectively.

8-Oxo-dG staining was performed on the cells grown on glass coverslips. After rotenone incubation, cells were washed for 10 min with TBS and fixed with 70% ethanol pre-cooled at −20 °C for 10 min on ice. The coverslips were washed with a large amount of TBS and were soaked in 37 °C using TBS supplemented with 100 μg/ml of DNase-free RNase A for 1 h. To increase the 8-oxo-dG recognition, cells were incubated with 4 M HCl for 7 min. The pH was raised by two 2-min washes with 500 mM Tris-Cl, pH 7.4. Cells were blocked with TBS containing 5% FBS, 5% horse serum, and 0.05% Triton X-100 for 2 h.

Primary mouse antibody anti-8-oxo-dG (1/300) was added to the cells in TBS with 2.5% FBS, 2.5% horse serum, and 0.05% Triton X-100 overnight at 4 °C. Anti-mouse FITC (1/100) was used in the same incubation medium for 5 h at room temperature. The mitochondrial network was counterstained using 50 mM of red Mito-Tracker CMXRs (Molecular Probes, Invitrogen) on live cells for 30 min immediately before the fixation step.

ROS formation in PC12 and PC12-NDI1 cells was visualized as follows. Cells were incubated with 1 μM rotenone in DMEM with 1% serum for up to 6 h and then washed twice with warm medium. MitoSOX (Invitrogen) was diluted at a final concentration of 1.5 μM in warm DMEM with 1% serum and added to cells. After incubation with MitoSOX for 10 min, cells were fixed with 4% paraformaldehyde for 10 min, mounted onto glass slides with Mowiol, and observed under a fluorescence microscope. All images were taken under identical exposure conditions in order to evaluate the intensity of the MitoSOX fluorescence accurately (23).

The NADH concentration in mitochondria was measured by using mitochondria isolated from PC12 and PC12-NDI1 cells as before (17). The mitochondria (16.4 mg) were suspended in 220 μl of the isotonic mitochondrial buffer containing 210 mM mannitol, 70 mM sucrose, 1 mM EDTA, and 10 mM HEPES, pH 7.5, and the fluorescence was monitored with excitation and emission wavelengths set to 351 and 466 nm, respectively, by using a 455 nm cutoff filter.

**Other Analytical Procedures**—Protein concentration was determined by a Coomassie protein assay kit from Pierce. SDS-PAGE was performed by a modified method of Laemmli (24). Any variations from the procedures and other details are described in the figure legends.

**RESULTS**

**Expression of the Ndi1 Enzyme in PC12 Cells and Its Protection against Toxic Effects Caused by Complex I Inhibitors**—In order to evaluate the protection of PC12 cells provided by expression of the Ndi1 enzyme, we compared the effects of complex I inhibition on cell survival and morphological changes between control PC12 cells and the PC12 cells infected with recombinant adeno-associated virus carrying the NDI1 gene (PC12-NDI1) (21). The expressed Ndi1 enzyme was predominantly located to mitochondria as evidenced by its colocalization with cytochrome c oxidase in the immunostaining images of the cells (supplemental Fig. 1). PC12 and PC12-NDI1 cells were incubated with increasing concentrations of rotenone for 4 days, and the extent of cell death was assessed by staining the cells with trypan blue (Fig. 1). The control cells exhibited 25% of cell death at 0.25 μM rotenone. Under the same conditions, no cell death was observed in the PC12-NDI1 cells. At the highest rotenone concentration used (1 μM), 75% of...
the control cells died, whereas the PC12-NDI1 cells retained a survival rate of 75%. It should be noted that rotenone tends to bind to serum in the incubation medium; therefore, the serum concentration was reduced to 1%. In fact, the inhibition efficiency of rotenone was varied with the amount of serum present in the medium (data not shown).

One of the characteristic features of apoptosis in cells is condensation and fragmentation of the nucleus. These features could be seen on cells treated with rotenone. In Fig. 2A, PC12 and PC12-NDI1 cells were exposed to 1 μM rotenone for 24 h, after which nuclei were stained with DAPI. The apoptotic nuclei, marked with arrowheads in Fig. 2A, are clearly visible in the rotenone-treated PC12 cells. Fragmentation of nuclei was significantly less with the PC12-NDI1 cells. Another typical feature of apoptosis is DNA fragmentation. As seen in Fig. 2B, PC12 cells incubated with rotenone for 24 h exhibited the characteristic pattern of DNA laddering. Again, the cells expressing the Ndi1 enzyme did not show discernible DNA fragmentation.

We have reported earlier that inhibition of complex I causes ROS formation which oxidatively damage mtDNA to a significantly greater extent than nDNA and that the Ndi1 enzyme exerts protective effect against this DNA modification (17). These experiments were done with a relatively short (6 h) incubation of the cells. When PC12 cells were exposed to rotenone for 24 h, nDNA was also extensively oxidized as seen in immunostaining of cells for 8-oxo-dG, an indicator of damaged DNA (Fig. 3A). Even under these severe conditions, PC12-NDI1 cells showed little or no oxidative damage in either mtDNA or nDNA, which is in good agreement with the nDNA fragmentation data presented above. To examine the ROS production more directly, we used MitoSOX which is reported to work as a probe for mitochondrial formation of \( \text{O}_2^\cdot \) (23, 25). PC12 and PC12-NDI1 cells were incubated with rotenone for 4 h and then loaded with MitoSOX for 10 min. Positive staining with MitoSOX was observed with the control cells but not with the PC12-NDI1 cells (Fig. 3B). We then tested other complex I inhibitors, fenpyroximate and pyridaben, as well as a complex III inhibitor antimycin A. They all caused \( \text{O}_2^\cdot \) generation in control cells. As expected, the presence of the Ndi1 enzyme suppressed \( \text{O}_2^\cdot \) formation by complex I inhibitors but not by complex III inhibitor. MitoSOX staining was seen as early as 2 h of incubation with the inhibitors (data not shown). An image at a higher magnification clearly showed that MitoSOX mainly stained the mitochondrial network (Fig. 3B).

**Activation of Caspase 9 but Not Caspase 3 by Complex I Inhibitors**—Knowing that the Ndi1 enzyme can be used to prevent ROS generation and apoptosis induced by complex I inhibitors, we investigated apoptotic events in more detail by comparing the control PC12 cells and the PC12-NDI1 cells. First, among many caspases known to date, we focused on caspase 9 and caspase 3 because they both act as a key factor in mitochondria-driven apoptosis (26). Caspase activation occurs through a cleavage of the pro-form of the protein that can be assessed by Western blotting using specific antibodies. The
cells were grown to 80% confluence and then incubated with 1 μM rotenone for 0–6 h (Fig. 4A). In control cells, the cleaved active form of caspase 9 began to appear within 1 h of rotenone exposure. The activation was also observed with the other complex I inhibitors, fenpyroximate and pyridaben (Fig. 4B). On the contrary, the PC12-NDI1 cells did not show any cleaved form of caspase 9 within 6 h of incubation. Antimycin A, which is reported to exert apoptotic effects on mammalian cells (27), induced activation of caspase 9 in both control and PC12-NDI1 cells (Fig. 4B). It is generally believed that caspase 9 activation is mainly elicited by cyt c released from the mitochondria. Therefore, we examined relocalization of cyt c into the cytoplasm in the presence and absence of rotenone by analyzing fluorescence images obtained by immunostaining of the cells with anti-cyt c antibody (supplemental Fig. 2). Fig. 4D summarizes the result of such analysis. The release of cyt c becomes apparent at 4 h of rotenone incubation in PC12 cells, whereas no cyt c was detected in the cytoplasm of PC12-NDI1 cells.

In a typical caspase pathway, caspase 3 activation follows caspase 9 activation. We assayed for pro-caspase 3 activation by immunoblotting analysis. Surprisingly, we could not detect the active form of caspase 3 in the PC12 or PC12-NDI1 cells after 6 h of rotenone exposure (Fig. 4C). Similarly, no activation of caspase 7 was observed even after 18 h of incubation with rotenone (Fig. 4E). To ensure that activation of these caspases could occur in PC12 or PC12-NDI1 cells, we incubated the cells with staurosporine, a well-known chemical that promotes apoptosis in cells (28). As seen in Fig. 4, C and E, staurosporine was able to activate both caspase 3 and caspase 7, indicating that the machinery or cellular components required for the activation of either caspase are available in these cells.

Release of AIF and Endonuclease G from Mitochondria in PC12 Cells—Failure of complex I inhibitors to induce the complete cascade activation of the caspase pathway led us to investigate other mechanisms that would cause nuclear fragmentation and DNA laddering associated with complex I inhibition. Mitochondria have been reported to contain proapoptotic factors that are translocated to the nucleus and induce apoptosis (29, 30). We examined relocalization of proapoptotic factors such as AIF and EndoG in PC12 and PC12-NDI1 cells. Cells were incubated for 0, 24, or 48 h with 1 μM rotenone and immunostained with antibodies against AIF and a subunit of cytochrome oxidase. Nuclei were visualized using DAPI. Fluorescence images of cells without rotenone treatment showed colocalization of AIF with cytochrome oxidase, indicating that all AIF is present in the mitochondria (data not shown). At 24 h of incubation of PC12 cells with 1 μM rotenone, a small but significant fraction of AIF was found in the nuclei (Fig. 5A). A greater amount of redistribution of AIF in the nuclei was observed after 48 h of rotenone incubation. In contrast, no relocalization of AIF occurred in PC12-NDI1 cells. We were not able to monitor EndoG in this experiment because commercially available antibody for this protein worked only in immunoblotting studies but not in immunohistochemical assays (see below). To further confirm the release of the proapoptotic factors from mitochondria, we prepared the mitochondrial and the cytoplasmic fractions of the cells and assayed for the presence of AIF and EndoG using immunoblotting (Fig. 5B). In agreement with the result from fluorescence images above, AIF was detected in the cytoplasm fraction of the PC12 cells after 48 h of rotenone incubation. The size of the released AIF was smaller because of a cleavage of this protein during the process of the release from the mitochondrial membrane (31). Under the same conditions, EndoG also seems to be released into the cytoplasm. These results clearly indicate the involvement of mitochondrial proapoptotic effectors in the cell death by rotenone inhibition of complex I.
The release of proapoptotic factors is known to be closely related to the status of the mitochondrial permeability transition pore. Opening of the pore results in a decrease of mitochondrial membrane potential. We evaluated the status of the membrane potential associated with rotenone exposure using a fluorescent probe, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1). JC-1 is accumulated in mitochondria and shifts its fluorescence from green to red. The decrease in the ratio of red to green fluorescence is used as a measure of depolarization in the mitochondria. Fig. 5C presents such measurements during rotenone exposure of PC12 and PC12-ND11 cells. A partial decrease in the membrane potential was apparent at 6 h of rotenone exposure with PC12 cells, which may coincide with the cyt c release. Loss of membrane potential became greater with a prolonged incubation. Relocalization of AIF, on the other hand, seems to be delayed compared with that of cyt c. A similar delay for AIF relocation has been reported (32) and is most likely because cleaving is necessary for this molecule to be released from the mitochondrial membrane (see above). The membrane potential of PC12-ND11 cells remained unchanged under the same conditions tested. The value obtained after addition of an uncoupler, SF6847, represents a total collapse of membrane potential.

**Involvement of Apoptotic Kinase Phosphorylation**—The release of proapoptotic factors from the mitochondria is believed to be initiated by activation of kinases, especially p38 kinase and the c-Jun-N-terminal kinase (JNK) (33). We have therefore examined the phosphorylation of these kinases in our cell model. As shown in Fig. 6A, with increasing time of incubation with rotenone, PC12 cells showed a marked enhancement in phosphorylation of p38 kinase. The level of p38 phosphorylation of PC12-ND11 cells remained unchanged under the same conditions. Basically the same result was obtained with JNK; rotenone exposure caused phosphorylation of JNK in PC12 but not in PC12-ND11 (Fig. 6B). It was confirmed that other complex I inhibitors, fenpyroximate and pyridaben, also induced the phosphorylation only in PC12. Antimycin A, on the other hand, could trigger the kinase activation in both PC12 and PC12-ND11.

Because p38 kinase and JNK represent two different pathways, we used the specific inhibitors to determine which pathway was mainly involved in rotenone-induced cell death. AS601245 and SP600125 specifically inhibit JNK, and sodium selenite selectively decreases the phosphorylated form of p38 kinase (34). PC12 cells were preincubated with each kinase inhibitor for 2 h prior to rotenone exposure. As shown in Fig. 7A, the JNK inhibitor AS601245 showed 80% survival rate at 48 h of rotenone exposure. The other JNK inhibitor SP600125 exhibited similar protection of cells to AS601245 at 36 h of rotenone incubation. In contrast, sodium selenite failed to show the same extent of protection until 48 h of rotenone exposure. We also investigated effects of these kinase inhibitors on AIF release from mitochondria (Fig. 7B). JNK inhibitors AS601245 and SP600125 almost completely prevented the AIF release, whereas sodium selenite revealed partial protection. It seems likely that JNK is more involved in this apoptotic pathway than p38 kinase.

**Partial Protection by Antioxidants against Cell Death Caused by Complex I Inhibition**—As described above, ROS is likely to be involved in the apoptotic process caused by inhibition of complex I. One of the earliest events associated with oxidative stress may be activation of the JNK/p38 kinase pathway (33). We have investigated the effect of antioxidants on the activation of JNK using PC12. In Fig. 8A, PC12 cells were preincubated with α-tocopherol, NAC, or Trolox before they were exposed to rotenone or pyridaben, and we then assessed the amount of phosphorylated JNK. All antioxidants lowered the level of JNK phosphorylation regardless of the complex I inhibitor used. At the concentration tested, NAC and Trolox showed a slightly higher protection than α-tocopherol. We obtained
were incubated with 1 μM JC-1, and the fluorescence was recorded on a plate reader at 529 and 590 nm. The ratio between 590 and 529 nm. SF6847 was used to achieve complete depolarization. *, hyde-3-phosphate dehydrogenase (Purity of the cytoplasmic and the mitochondrial fractions were evaluated using antibodies against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and F1β, β-ATPase, respectively. C, cells were cultivated in a 384-well plate and exposed to 1 μM rotenone for the period of time indicated. The cells were stained with 2 μM of JC-1, and the fluorescence was recorded on a plate reader at 529 and 590 nm. The histogram represents the ratio between 590 and 529 nm. SF6847 was used to achieve complete depolarization. *, p < 0.05; **, p < 0.01 (Student’s t test, against 0 h control).

Mechanism of Protection by NDI1 Expression against ROS Overproduction by Complex I Inhibition—One of the key factors by which Ndi1 prevents apoptotic cell death by rotenone is the suppression of ROS. In an earlier report, we showed that introduction of Ndi1 into the mammalian respiratory chain indeed diminishes generation of ROS from complex I (17). However, it was unclear how the presence of this alternative NADH dehydrogenase could possibly influence existing complex I, which would otherwise produce ROS under the inhibited conditions. One plausible explanation comes from reports regarding the dependence of complex I-derived ROS formation on the redox environment in the matrix. Kushnareva et al. (35) reported that the ROS production from complex I is critically dependent upon a highly reduced state of the mitochondrial matrix NADH pool and is achieved upon nearly complete inhibition of the respiratory chain. As reported in our previous paper (21), NADH oxidase activity of PC12-NDI1 cells was two times as high as that of PC12 cells and was only partially sensitive to rotenone. Furthermore, as shown in Fig. 5C, the PC12-NDI1 mitochondria exhibited the membrane potential similar to the PC12 mitochondria. Therefore, we speculated that the NADH level in the matrix of mitochondria from PC12-NDI1 cells might never be elevated high enough to realize ROS formation even in the presence of rotenone. This, in fact, turned out to be the case as shown in Fig. 9. In mitochondria from control PC12 cells, addition of NADH-linked substrates increased the NADH content, and ~100% reduction of NADH pool was observed by rotenone as expected. In quite contrast, in the case of mitochondria from PC12-NDI1, addition of rotenone caused only a slight increase in the NADH content, which is still lower than that of control mitochondria in the absence of rotenone. In other words, under these conditions, the presence of Ndi1 keeps the redox potential high enough, so complex I is unable to generate ROS regardless of whether it is inhibited by rotenone or not.

DISCUSSION

In addition to their function as the major energy source, mitochondria play a central role in cellular processes such as apoptosis (36, 37). In this study we probed the molecular pathway triggered by complex I inhibition leading to the death of a dopaminergic cell line and the mechanism of protection by the Ndi1 enzyme against ROS production by complex I defects. As reported in a series of our papers, the Ndi1 enzyme can be functionally expressed in mammalian cells and act as a member of the respiratory chain in the host mitochondria (21, 22). Because Ndi1 is insensitive to typical complex I inhibitors, its presence provides a complete protection against complex I inhibition. In addition, we have shown that the expressed Ndi1 suppressed ROS generation caused by complex I inhibition...
These characteristics of Ndi1 allowed us to study the process of cell death caused by complex I inhibitors. When PC12 cells were treated with rotenone or other complex I inhibitors, we observed morphological hallmarks of apoptosis such as condensation of nuclei and DNA fragmentation. Another key observation in apoptosis is formation of ROS. This is particularly important because inhibition of complex I is known to stimulate ROS generation in a variety of cell lines (38–40). In dopaminergic cells such as PC12, it is suspected that the presence of dopamine may potentiate the inhibitory effect by enhancing the amount of intracellular ROS (41). In fact, ROS turned out to play an essential role in our model system as indicated by the observation of oxidative damage to mtDNA and nDNA and, more directly, by probing O2. In these cases, inclusion of Ndi1 in the mitochondria counteracted the effect of complex I inhibition as expected, indicating that elimination of functional complex I was the sole cause of the observed deleterious events. The mechanism of ROS overproduction by complex I inhibition is associated with the extent of NADH/NAD+ in the mitochondrial matrix as described under “Results.”

On the molecular level, it is generally agreed that typical apoptosis in mammalian cells is associated with a series of caspase activations (36). Participation of mitochondria in the overall process of apoptosis is not fully understood, but one of the major events is the release of cyt c. Once outside the mitochondria, cyt c becomes part of the apoptosome, which induces the activation of caspase 9, and apoptosome formation leads to the sequential activation of pro-caspase 3 (42). In our cell model using complex I inhibitors, we clearly detected the release of cyt c and the cleavage of pro-caspase 9 into the active caspase 9 form. However, under the same conditions, activation of caspase 3 or caspase 7 did not take place. The failure to detect activation of caspase 3 led us to investigate proapoptotic factors in mitochondria such as AIF and EndoG, which can induce DNA breakdown in the nucleus (29, 30). In PC12, AIF and EndoG were relocalized from the mitochondria to the nucleus when complex I was inhibited. The relocalization was accompanied by the depolarization of the mitochondrial membrane, illustrating the release of the proapoptotic factors.

In our cellular model for complex I defects, the ROS production and phosphorylation of the JNK/p38 kinase was observed prior to the release of AIF/EndoG. Our results also indicate that complex I inhibition induces phosphorylation of both p38 kinase and JNK in PC12 and that antioxidants suppress, at least partially, the activation of these kinases. It has been reported that endogenous or exogenous ROS are responsible for the acti-
vation of JNK (for review see Ref. 43). One of the actions of the activated form of JNK is considered to phosphorylate the Bcl-2 family members, such as BAX, which regulate the mitochondrial apoptotic machinery (44, 45). In addition, our data indicated that the JNK inhibitors (SP60125 and AS601245) have protective effects against the AIF release from mitochondria and cell death. Therefore, we speculate that the release of AIF/EndoG is dependent on activation of JNK/p38 kinase. This hypothesis would need further scrutiny.

In this study, we showed that apoptotic cell death caused by complex I inhibition does not appear to involve the classic caspase pathway but is rather closely related to the relocalization of the AIF/EndoG into the nucleus (Fig. 10). Newhouse et al. (46) reported that rotenone caused phosphorylation of JNK/p38 kinase in human dopaminergic SH-SY5Y cells and suggested that the caspase-dependent pathway participates in rotenone-induced apoptosis. Because they did not examine the release of AIF/EndoG, it is unclear whether the difference is due to the cell line used or other factors. In a recent paper using a cybrid model for Leber’s hereditary optic neuropathy, Zanna et al. (47) observed the relocation of AIF and EndoG into the cytosol and concluded that complex I defects resulted in caspase-independent cell death. The results are consistent with our data obtained using PC12 cells, supporting the notion that the caspase-dependent pathway may not be essential.

Clarification of the molecular basis for cell death initiated by complex I inhibition may help us develop therapeutic strategies for neurodegenerative diseases such as Parkinson’s disease. In fact, proteins or genes involved in the apoptosis machinery have been the target of many studies aimed at treatment of the diseases. For example, knocking out the Bax gene reportedly rescued dopaminergic neurons in mouse substantia nigra from neuronal apoptosis through mitochondrial oxidative damage in vivo (48). Use of kinase inhibitors such as AS601245 suppressing apoptotic cell deaths is one approach. But kinase inhibitors or Bax deletion is not effective for damages directly caused by the ROS (e.g. modification and mutation of mtDNA and nDNA). Use of antioxidants is another method to diminish harmful ROS. In our study, protection by antioxidants was observed but only partial. This is most likely because arresting

**Figure 8.** Antioxidants prevent kinase activation in PC12 cells and improve the survival rate of cells. A, PC12 cells were preincubated with antioxidant, α-tocopherol (α-toc), NAC, or Trolox for 30 min and treated with the complex I inhibitor in Krebs medium for 6 h. Phosphorylation of JNK was monitored with the specific antibody. B, PC12 cells were treated with antioxidant as in A and incubated with 1 μM rotenone (roten) for up to 48 h. The survival rate of cells was measured as described under “Experimental Procedures.” **, p < 0.01 (Student’s t test, against no inhibitor control).

**Figure 9.** Effects of respiratory substrates and inhibitors on the NADH concentration in the matrix of mitochondria isolated from PC12 and PC12-NDI1 cells. A, fluorescence of NADH of a mitochondrial suspension was recorded every 5 s. Where indicated, 5 mM glutamate plus 5 mM malate (glut/mal), 1 μM rotenone, and 2 mM KCN were added. B, NADH fluorescence was normalized using the average value obtained after KCN as 100%. The assays were carried out in triplicate at the ambient temperature.
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