The mitochondrial oxidative phosphorylation system consists of five multimeric enzymes (complexes I–V). NADH dehydrogenase or complex I (CI) is affected in most of the mitochondrial diseases and in some neurodegenerative disorders. We have studied the physiological consequences of a partial CI inhibition at the cellular level. We used a genetic model (40% CI-inhibited human-ape xenomitochondrial cybrids) and a drug-induced model (0–100% CI-inhibited cells using different concentrations of rotenone). We observed a quantitative correlation between the level of CI impairment and cell respiration, cell growth, free radical production, lipid peroxidation, mitochondrial membrane potential, and apoptosis. We showed that cell death was quantitatively associated with free radical production rather than with a decrease in respiratory chain function. The results obtained with human xenomitochondrial cybrid cells were compatible with those observed in rotenone-induced 40% CI-inhibited cells. At high concentrations (5–6-fold higher than the concentration necessary for 100% CI inhibition), rotenone showed a second toxic effect at the level of microtubule assembly, which also led to apoptosis. The correlation found among all the parameters studied helped clarify the physiological consequences of partial CI inhibitions at the cellular level.

Electron transport and oxidative phosphorylation are mediated by five multimeric complexes (complexes I–V) that are embedded in the mitochondrial inner membrane. Mammalian complex I (CI) or NADH:ubiquinone oxidoreductase has at least 40 subunits (1), seven of which are coded by the mitochondrial DNA (mtDNA; Ref. 2), and catalyzes electron transport from NADH to ubiquinone, which is coupled to vectorial proton movements.

A CI deficiency is characteristic of mitochondrial diseases, as an isolated defect or, in most of the cases, as a part of multiple respiratory chain deficiencies. Isolated CI deficiency has been associated with a wide spectrum of symptomatic phenotypes, varying from fatal infantile lactic acidosis to some cases of Leigh's disease, adult onset exercise intolerance, and some neurodegenerative diseases as Leber's hereditary optic neuropathy (LHON), focal dystonia, and Parkinson's disease (3–6).

Some specific complex I inhibitors have been classically used to model CI deficiencies. Rotenone is an inhibitor of the NADH dehydrogenase, which shuts off the supply of electrons to the quinol (QH2)-cytochrome c oxidoreductase (14). 1-Methyl-4-phenylpyridinium, the bioactivated product of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, binds to the rotenone-binding site, thereby limiting CI activity (15). Inhibition of CI by rotenone or 1-methyl-4-phenylpyridinium not only leads to a decline in mitochondrial ATP production but also enhances the generation of free radical by the mitochondrial respiratory chain and initiates lipid peroxidation reaction in isolated bovine heart mitochondria or submitochondrial particles (16–19). Because 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine causes Parkinson's disease in humans (20) and rats (21), these data suggested a role for CI deficiency and increased oxidative stress in Parkinson's disease substantia nigra and in the dopaminergic cell death in this disorder (22).

The underlying physiological consequences of a partial complex I inhibition at the cellular level remain mostly unknown. Here we report the effect of different levels of CI impairment on the cell physiology. We used a genetic model (40% CI inhibited HXC lines; Ref. 13), and a drug-induced model (partial CI inhibition in the human osteosarcoma-derived cell line 143B) with rotenone to quantitatively correlate CI inhibition and its
effect on cell respiration, cell growth, free radical production, lipid peroxidation, mitochondrial membrane potential, and apoptosis.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Culture Conditions**—Human xenomitocondrial cybrids (HXC) (human-chimpanzee clone HC1, human-gorilla HG13, and human-pigmy chimpanzee clone HP4) were produced and published previously (12). The human osteosarcoma-derived cell line 143B (RK1) and its mtDNA-less derivative, 143B/206 rho−, were cultured as described (23). Culture conditions were modified as described below under “Growth Curves.” SUB2B cell line (transmitochondrial cybrid clone containing wild type mtDNA) was previously characterized (24) and used in some experiments as a human control cell line.

**Geneic and Experimentally Drug-induced Models of Complex I Inhibition**—We modeled a partial CI inhibition by treating the osteosarcoma cell line 143B with different concentrations of the CI substrates rotenone. To define the model and to assess how the CI (NADH de-cytochrome c reductase) inhibition affects the KCN-sensitive endogenous cell respiration, 143B cells were treated for 24 h with 0–1 μM rotenone. CI was measured spectrophotometrically in isolated mitochondria, and cell respiration was measured polarographically in whole cells as described previously (13). In all the experiments, the culture medium was changed 2 h before harvesting the cells. To validate the results obtained, a genetic model of CI deficiency was used. The model consisted in HXC lines harboring mtDNA from common chimpanzee (Pan troglodytes), pigmy chimpanzee (Pan paniscus), and gorilla (Gorilla gorilla; Ref. 12). HXC cells showed an approximately 20% decrease in the cell respiration and a 40% decrease in the CI activity (13).

**Immunoblotting**—To determine whether the CI inhibition induced adaptive variations on the expression of the complex I and II subunits, immunoblotting was performed as described previously. 40 μg of mitochondrial proteins from 143B cells treated with rotenone during 24 h were used, as well as human succinate dehydrogenase flavoprotein subunit (SDH (Fp)) mononal and human ND1 polyclonal antibodies. After scanning the autoradiograms, band signals were quantified using NIH Image 1.62b7 software. The ratio between the different bands (ND1/SDH ratio) was considered as the division of the arbitrary densitometric values of the signals using each antibody. These ratios were used to compare untreated and rotenone-treated 143B cells.

**Growth Curves**—Growth measurements were made by plating 5 × 10⁴ cells on 100-mm² dishes in 10 ml of the appropriate medium. Cells were grown in Dulbecco’s modified Eagle’s medium lacking glucose supplemented with 5 mM glucose or Dulbecco’s modified Eagle’s medium lacking glucose supplemented with 5 mM galactose, both supplemented with 1 mM pyruvate and 10% dialyzed fetal calf serum. Cells were incubated at 37 °C, and cell counts were performed at daily intervals. For 143B and 143B/206 rho− cells, medium was supplemented with different concentrations (0–1 μM) of rotenone. HXC lines (13) were grown in media with glucose or galactose without rotenone supplementation.

**Oxidative Stress Analyses**—For each assay, three independent samples from each cell type (human control lines SUB2B and 143B and the HXC lines HP4, HG13, and HC1), either intact cells (independently collected) or isolated mitochondria (independently isolated), were analyzed, and at least three measurements of each sample were obtained. To reproduce different degrees of CI inhibition, various concentrations of rotenone (0–600 nM) were added to 143B cells at approximately 80% confluence. After 4, 24, 48, and 72 h the oxidative stress determinations were conducted. Experiments were performed in triplicate.

**Free Radical Production**—To monitor oxidative activity, the cell-permeant probe 2′,7′-dichlorofluorescin diacetate (H₂DCFDA) was used. H₂DCFDA passively diffuse into cells where intracellular esterases cleave the acetates, and the oxidation of H₂DCF by hydrogen peroxide produces a fluorescent response (25). Cells were collected by trypsinization. 50 μg of cellular protein were resuspended in 500 μl of phosphate-buffered saline (PBS) medium and labeled with H₂DCFDA (2 μM) (Molecular Probes, Eugene, OR) for 10–60 min in the dark at 37 °C. Fluorometric analyses at 507 nm excitation and 530 nm emission were performed using a MFP-66 fluorescence spectrophotometer (Perkin-Elmer Corp.). For all the measurements, the signal fluorescent (time 0, t₀) was subtracted. The increase in the fluorescence was used to measure the chemical process of hydrogen peroxide production.

**Lipid Peroxidation Assays**—Peroxidation of cellular lipids was measured using 50 μg of cellular protein resuspended in 500 μl of PBS and labeled with cis-parinaric acid (5 mM) (Molecular Probes, Eugene, OR) (26) for 10–60 min in the dark at 37 °C. Cells were washed by pelleting to remove excess probe, and fluorescence at 318 nm excitation and 410 nm emission was detected as described (27). Loss of parinaric acid fluorescence was used to measure the chemical process of lipid peroxidation. To measure peroxidation of mitochondrial membranes lipids, 50 μg of fresh mitochondrial protein were resuspended in 500 μl of medium A. The experimental conditions were the same as described above.

**Mitochondrial Membrane Potential (Δψₒ) was estimated using 5,5′,6,6′-tetramethylrhodamine, 3,3′,2-tetraethylrhodamine isothiocyanate dye (JC-1).** JC-1 is a fluorescent compound (excitation maximum, 490 nm) that exists as a monomer at low concentrations. At higher concentrations, JC-1 forms aggregates. Fluorescence of the monomer is green (emission, 527 nm), whereas that of the J-aggregate is red (emission, 590 nm). Mitochondria with intact membrane potential (higher than 100 μM) aggregate JC-1 to form a green fluorescent probe whereas de-energized mitochondria cannot concentrate JC-1 and fluoresce green (28). Changes in plasma membrane potential do not affect the JC-1 status. JC-1 was chosen because has been described as a reliable probe for analyzing Δψₒ, changes in intact cells, whereas other different probes capable of binding mitochondria show a lower sensitivity (rhodamine 123) or a noncoherent behavior due to a high sensitivity to changes in plasma membrane potential (3,3′-dihexyloxacarbocyanine iodide) (29). Cells were grown in 25-cm² dishes until 90% confluence and incubated for 45 min with 6.5 μM JC-1. Cells were collected by trypsinization, washed in PBS, and resuspended in 500 μl of PBS, and the samples were measured on a MFP-66 fluorescence spectrophotometer (Perkin-Elmer Corp.). The ratio of the reading at 590 nm to the reading at 527 nm (590/527 ratio) was considered as a relative Δψₒ value.

**Cell Death Studies**—The apoptotic rotenone-induced cell death was assessed using a cell death detection enzyme-linked immunosorbent assay kit (Roche Molecular Biochemicals). Approximately 10⁴ cells were used for the photometric enzyme immunosassay of cytoplasmic histone-associated DNA fragments after rotenone-induced cell death. 143B and 143B/206 rho− were treated with different concentrations of rotenone and harvested at 24–72 h. To study the role of the mitochondrial permeability transition (MPT) induced by the rotenone-potentiated MPT, cells were pretreated for 30 min with 5 μM of the MPT inhibitor cyclosporin A (CyA) and incubated for 48 h, changing the medium every 8.5 h. As CyA is only able to prevent MPT in short term experiments, the assay was repeated with 15- and 30-h incubation periods and using 5 μg CyA and 50 μM of the phospholipase A₂ inhibitor arachidonic acid (AraA) (Sigma), which enhances and prolongs the effect of CyA (30, 31). The cell culture medium was changed after 15 h when necessary, and the cells were processed after 30 h.

**Immunocytochemical Staining**—143B and 143B/206 rho− cells were seeded on glass coverslips and treated with different concentrations of rotenone for 24 h. Cells were fixed with 4% parafomaldehyde in PBS, permeabilized with 0.1% saponin, and incubated for 4 h with monoclonal antibody anti-a-tubulin (Sigma). A Texas Red-conjugated Anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was used as secondary antibody. Coverslips were incubated for 30 min with 10 μg/ml bis-benzimide Hoechst 33422 (Sigma) to reveal the nuclei and mounted onto glass slides with ProLong™ Antifade kit (Molecular Probes). Fluorescence was inspected with a Wild Leitz microscope (Fluovert, Heerbrugg, Switzerland).

**Statistical Analysis**—The data were analyzed using the SPSS software. Results are expressed as the means ± S.D. Comparisons between two groups of measurements were carried out using Student’s t test (for equality of means) and the Student’s t test (for equality of variances) and the Student’s t test (for independence) for independent data. When a potential relationship between variables was of interest, a linear regression analysis was performed. Values with p < 0.050 were considered statistically significant.

**RESULTS**

**Defining the Complex I-inhibited Models**

** modeling Complex I Partial Impairment by Incubating 143B Cells with Rotenone**—The rotenone-inhibited model was created by titrating the effect of different concentrations of the drug on the CI activity, measured as NADH decytochrome c reductase. Exposure of 143B cells to increasing concentrations of rotenone produced a progressive inhibition of CI activity in isolated mitochondria (Fig. 1). A 100% inhibition was achieved with 100 nM rotenone. The endogenous cell respiration was also inhibited in a dose-dependent manner but showed different inhibi-
ion kinetics (Fig. 1). CI inhibition followed a classical hyperbolic shape, whereas cell respiration inhibition showed an S-shaped curve. Only when CI was inhibited by 35–40% (< 5 nM rotenone), cell respiration began to decrease. Between 40 and 60% of CI inhibition (5–10 nM rotenone), cell respiration decreased linearly until 30% of the normal rate. Increasing concentrations of rotenone produced further but slower decrease in CI activity and cell respiration.

**Effect of Rotenone Treatment on the Steady-state Levels of mtDNA and Mitochondrial Respiratory Chain Complexes**

To determine whether the inhibition of CI induced adaptative variations on the expression of the complex I and II subunits, immunoblotting was performed. Using mitochondria isolated from cells incubated with different concentrations of rotenone for 24 h, no significant changes in the ND1/SDH(Fp) ratio were observed (Fig. 2). A rotenone-induced CI deficiency did not produce an adaptive response in the form of an increased expression of the complex II (CII) in a period of 24 h.

**Effect of CI Inhibition on Cell Growth**

Respiratory competent 143B cells grew exponentially in medium containing glucose as carbon source, and they showed a substantial, although slightly reduced, growth rate in galactose-containing medium (Fig. 3A). When 143B/206 ρ0 cells were cultured in a medium in which glucose is substituted for galactose, these cells died and detached from the culture plate in less than 12 h (Fig. 3B, r°). Cells grown in galactose medium in the presence of 5 nM rotenone (concentration inducing 54% of CI inhibition, Fig. 1) but not in the presence of 7.5 nM (72% of CI inhibition, Fig. 1). HXC cells grew in galactose medium if supplemented with less than 1 nM rotenone (concentration that produced approximately 19% of CI inhibition, Fig. 1). The rotenone-induced inhibition was additive to the genetically produced 40% deficiency in these cells. Fig. 3C compares growth parameters of the cell lines studied in the different culture conditions. The growth of the 143B/206 ρ0 in glucose medium was affected by rotenone at concentrations ≥500 nM (the growth rate with 500 nM rotenone was 30% slower than untreated cells), and cells did not grow at a concentration of 600 nM. These data indicated that rotenone kills cells in a CI inhibition-independent way, when present at concentrations substantially higher than that producing complete CI inhibition in 143B cells.

**Effects of CI Inhibition on Oxidative Stress**

**Studies in HXC Lines**—We studied whether a 40% deficiency in CI activity resulted in greater reactive oxygen species (ROS) production in HXC lines. ROS production was estimated using H2DCF, a dye that is converted to a fluorescent product upon oxidation by hydrogen peroxide. Fig. 4A shows ROS production as a function of time. The slope of the regression lines was significantly higher in the HC (5.64 ± 0.25; p < 0.002), HP (5.07 ± 0.31; p < 0.016), and HG (5.09 ± 0.57; p < 0.050) xenomitochondrial cell lines compared with the set of values obtained for the 143B (4.22 ± 0.40) and SUB21 (4.67 ± 0.32) control cell lines. The electron transport in HXC may be inefficiently coupled to ATP synthesis, producing an increase in the rate of ROS formation. Lipid peroxidation is a deleterious consequence of oxidative stress. To determine whether the observed increase in the rate of ROS production in the HXC cell lines produced a higher lipid peroxidation of the membranes in these cells, the loss of cis-parinaric acid fluorescence was used to measure the chemical process of lipid peroxidation (26). In a whole cell assay, the time course of the fluorescence decrease showed no significant differences among cell lines (Fig. 4B). However, when using isolated mitochondria, an increase in the membrane lipid peroxidation was observed in HXC cells (Fig. 4C). The fluorescence value at the start of the reaction (t0) subtracted by the value measured after 30 min (t30), was 1.36-fold higher in HC (p = 0.044), and 1.51-fold higher in HG (p = 0.036), compared with the mean values obtained for the control human cell lines.

**Studies in Rotenone-treated 143B Cells**—ROS production and mitochondrial membrane lipid peroxidation were enhanced by incubation of cells with rotenone (Fig. 5, A and B). Both parameters increased roughly linearly when concentra-
tions of 0–100 nM rotenone were used (concentration range to produce CI inhibition) but did not increase significantly at higher concentrations. At the same concentration of rotenone, both parameters increased with longer treatment (between 4 and 72 h).

**Effect of CI Inhibition on the Mitochondrial Membrane Potential**

A CI deficiency leads to a reduction in proton extrusion to the intermembrane space, partially depleting the cells from ATP. These factors, together with the associated increase in oxidative stress, could produce a significant drop of the mitochondrial membrane potential ($\Delta \psi_{m}$). The dye JC-1 was used to monitor $\Delta \psi_{m}$, estimated as the 590/527 nm emission ratio. Used as low $\Delta \psi_{m}$ controls, 143B/206 $\rho^{0}$ value was 30–32% of 143B, and 143B cells incubated for 45 min with 10 $\mu$M of the uncoupler carbonyl-cyanide m-chlorophenylhydrazone showed an estimated $\Delta \psi_{m}$ corresponding to 45–48% of the 143B value (Fig. 5C). HXC lines exhibited a $\Delta \psi_{m}$ that was 83–87% of the 143B level. Rotenone-treated 143B cells showed a mild decrease in their $\Delta \psi_{m}$ (Fig. 5C). Low concentrations of rotenone (between 1 and 10 nM), produced a consistent hyperpolarization of the mitochondrial membrane (a reproducible 15–18% increase compared with the control level) when cells were incubated with the drug for 4 h. At those concentrations, the $\Delta \psi_{m}$ was maintained above 80% of the normal value. Only in cells that were incubated for 3 days with concentrations of rotenone between 100 and 600 nM did their $\Delta \psi_{m}$ decrease to 60–65% of the untreated 143B cell value. For each concentration of rotenone used, $\Delta \psi_{m}$ decreased slightly with the time of incubation.

**Fig. 3. Effect of complex I inhibition on cell growth.** A, human xenomitochondrial cybrid cell growth. Cells grew in Dulbecco’s modified Eagle’s medium lacking glucose supplemented with 5 mM glucose (□) or Dulbecco’s modified Eagle’s medium lacking glucose supplemented with 5 mM galactose (●), both supplemented with 1 mM pyruvate and 10% dialyzed fetal calf serum. HXC lines (HC, human-common chimpanzee; HP, human-pigmny chimpanzee; HG, human-gorilla), which exhibited a 40% complex I deficiency, showed a reduced growth rate in both glucose and galactose medium compared with 143B cells. B, rotenone effects on cell growth. Concentrations from 0 to 500-nM rotenone produced progressively greater growth impairment of 143B in glucose medium. 143B/206 $\rho^{0}$ cells growth was affected only with high concentrations of the drug. In galactose medium, 5 nM rotenone (producing ~54% complex I inhibition) was enough to block 143B cell growth, whereas 1.5 nM rotenone (~20% of complex I inhibition) blocked cell growth in the 40% complex I defective HC (human-common chimpanzee xenomitochondrial cybrid) line. C, comparison of growth properties of rotenone-treated 143B cells and human-primate xenomitochondrial cybrids. The doubling time of different cell lines in medium containing glucose or galactose as the only carbon source, and the galactose/glucose medium ratio (number of cells in galactose medium at 72 h after plating divided by the number of cells in galactose medium at 72 h) were determined from the growth curves. Error bars represent the standard deviation of at least three independent measurements.
Effect of CI Inhibition on Cell Death

143B and 143B/206 $\rho^-$ were treated with different concentrations of rotenone and harvested at 24, 48, and 72 h. In 143B cells, cell death was increased with all concentrations of rotenone up to 10–13 times the level of untreated cells after 3 days of incubation with 100 nM rotenone (Fig. 6A). Higher concentrations increased cell death exponentially. Rotenone did not significantly kill 143B/206 $\rho^-$ cells until present at concentrations higher than 100 nM. Using 600 nM rotenone, both cell lines died at indistinguishable rates (Fig. 6A).

Experiments were carried out to test whether cell death in the presence of rotenone requires MPT. Fig. 6B shows the ability of 5 $\mu$M CyA and 50 $\mu$M ArA to prevent cell death from taking place in the presence of different concentrations of rotenone during 15 and 30 h. CyA inhibits the opening of the MPT pore (32), and phospholipase A2 inhibitors, such as ArA, enhance and prolong this effect (30, 31). Because CyA is not able to maintain mitochondrial function, it did not prevent rotenone-induced apoptosis after long periods of rotenone treatment. In cells treated with rotenone for 48 h, CyA essentially did not inhibit apoptosis (15–30% inhibition), even with changing the medium and adding fresh CyA every 8.5 h (data not shown). These data indicate that additional CyA-resistant mechanisms can have a role in the rotenone-induced apoptosis. ArA prolongs the effect of CyA for at least 16 h (31). We incubated the cells for 15 and 30 h with different concentrations of rotenone in the presence of CyA and ArA with a change of the medium at the latter at 15 h. When CyA and ArA were used in cells treated with < 500 nM rotenone, the cell death prevention was almost complete in the 15-h incubation experiments. However, cell death was prevented by 55–75% in the 30-h incubation experiments. In experiments using 600 nM of rotenone, cell death prevention dropped to under 50%. At these concentrations of rotenone, the mtDNA-less 143B/206 $\rho^-$ cells also were killed, suggesting a second mechanism for rotenone toxicity.

Exploring the Secondary Effects of Rotenone and Checking the Validity of the CI Inhibition Model

Rotenone seems to have at least two modes of action, because the mtDNA-less cell line was also killed by relatively high doses of the drug. To distinguish between these two effects and to define the effective range of rotenone concentrations that induce only or mostly a partial mitochondrial defect without major secondary effects, we studied the effect of rotenone in the microtubular cytoskeleton by immunocytochemical staining. 143B (data not shown) and 143B/206 $\rho^-$ cells (Fig. 7) were treated with different concentrations of rotenone for 24 h and stained with a monoclonal antibody against tubulin. The nuclei were revealed by the specific dye Hoechst 3342. When the cells were incubated with 0–100 nM rotenone (Fig. 7), a normal microtubular pattern and normal nuclear morphology were observed in all cells (Fig. 7). Concentrations of 500 nM rotenone produced a disorganization of the microtubular structure and condensation-fragmentation of the nucleus, which are typical apoptotic signs (Fig. 7).
Correlations between CI Impairment and Physiological Consequences

After 72 h of rotenone treatment, the percentage of CI inhibition correlated positively, giving an exponential curve, with ROS production ($R^2 = 0.973$), decay in $\Delta_{m}$ ($R^2 = 0.985$), mitochondrial membrane lipid peroxidation ($R^2 = 0.964$), and apoptotic cell death ($R^2 = 0.924$) (Fig. 8b). Because the effect of rotenone on CI inhibition and cell respiration was not exactly the same (Fig. 1), all the curves were modified accordingly, showing hyperbolic shapes, when cell respiration was plotted against all the parameters studied. In all cases, $R^2$ was $> 0.93$ (Fig. 8a). Fig. 1 shows that incubation of 143B cells in medium supplemented with 1 nM rotenone exhibited a 19% of CI inhibition but maintained 98% of the cell respiration rate. Percentages of CI inhibition lower than 20% produced mild changes in all parameters, whereas any change in cell respiration produced significant increases in all of them (Fig. 8, A and B). A summary of our findings for four putative levels of CI deficiency is depicted in Fig. 8c.

**DISCUSSION**

In the last few years, many studies focused in the causal relationship between physiological or pathological decreases in mitochondrial polarization, ROS generation, the MPT, and subsequent apoptotic cell death (33–35). The main goal of the present work was to define quantitative correlations between partial mitochondrial CI inhibition and the physiological consequences at the cellular level by using a drug-induced model (rotenone dose-dependent CI inhibition in a human osteosarcoma-derived cell line) and a genetic model (40% CI inhibited HXC lines; Ref. 13).

**Effect of CI Inhibition on Cell Respiration and Growth—Exposure of 143B cells containing normal levels of mtDNA ($\rho^+$ cells) to increasing concentrations of rotenone produced a progressive inhibition of CI activity and, subsequently, of cell respiration. We observed a hyperbolic titration curve of CI inhibition, whereas the cell respiration inhibition showed a sigmoidal curve. Our results suggest that there is an excess CI of activity relative to the maximum rate of electron flow through the respiratory chain. Consistent with the respiratory control theory (36), a reduction in CI activity up to 40% may be able to limit the flux rate of the electron transport chain only modestly. In the case of HXC, a 40% CI inhibition produced a 20% cell respiration reduction, which is in good agreement with the pharmacological model. Cultured cells, even the ones defective in oxidative phosphorylation, can grow at essentially normal rates in medium containing glucose, a sugar that can be well metabolized glycolytically. Using a medium in which galactose replaced glucose, cells were forced to derive much of their ATP from oxidative metabolism. The decrease in respiratory efficiency of the HXC and in the rotenone-treated cells reduced their rate of ATP production by oxidative phosphorylation to an extent sufficient to affect their growth capacity in galactose medium. A 65% CI inhibition, producing approximately a 35% inhibition in cell respiration, seems to be the threshold in our pharmacological model that allows cells to grow in conditions where the respiratory chain is essentially the only source of ATP. The rotenone-induced CI inhibition was additive to the genetically determined 40% CI deficiency in HXC cells (13). HXC cells could grow in galactose medium, although their doubling time was very high (~80 h). HXC cells were still able to grow in galactose medium for an additional ~20% CI inhibition (~5% cell respiration decline), showing that also in this model, the threshold seems to be ~60% CI inhibition (corresponding to ~35% cell respiration decay). This threshold may change with the cell type, depending on the...
control coefficient of their CI. Changes in the threshold can be the basis for understanding the cell type specificity of CI deficiencies in some neurodegenerative disorders such as Parkinson's disease. Our results suggest that if cells are maintained in glucose medium, a partial CI deficiency is more deleterious than a complete respiratory chain deficiency for some cellular functions. In glucose-containing medium, \( r^1 \) cells experienced a rotenone dose-dependent (from 0 to 500 nM) reduction in their growth rate. \( r^0 \) cells did not experience any major change in their growth rate in glucose medium (10%) at concentrations lower than 200 nM rotenone and decreased by only 35% with 500 nM rotenone, a concentration that seems to be a threshold for killing these cells. It has been suggested that inhibition of respiratory chain activity by drugs such as rotenone directly interferes, as an acute insult, with the normal mitochondrial energy metabolism, triggering an apoptotic response. The generation of \( r^0 \) cell lines, on the other hand, undergoes a progressive mtDNA depletion by treatment with ethidium bromide during at least 30 days, a period of time that could allow the cells to adapt metabolically (23). Nevertheless, because cells with complete rotenone-induced CI inhibition still maintained a cell respiration rate of approximately 20% (because of the electron flow through the CII) and because of the gradual decline observed on the growth rate of \( r^1 \) cells with partial CI inhibition in nonselective glucose medium, it is clear that factor(s) in addition to the oxidative impairment are implicated in the CI impairment.

**Effect of CI Inhibition on Oxidative Damage—** In both genetic and pharmacological models, CI impairment was accompanied by an enhancement of ROS production and lipid peroxidation. Under aerobic conditions, the respiratory chain is a potent source of free radical (33, 37, 38). A CI impairment leads to enhanced formation of ROS, as has been shown in submitochondrial particles (16, 18) and in different cell systems (39, 40). In the case of HXCI, defects in CI may predispose the respiratory chain to produce excess superoxide because of structural or stoichiometric alterations in the subunits. This alteration may lead to increased interaction of oxygen with an electron carrier, as it has been proposed for patients with genetically determined CI deficiency (39). In 143B cells treated with rotenone, ROS production increased in a dose-dependent manner at concentrations partially affecting CI activity in 143B cells and at higher concentrations in 143B and 143B/206 \( r^0 \) cells. B, CyA plus ArA prevention of rotenone-induced cell death. Incubation of cells with 5 \( \mu M \) CyA plus 50 \( \mu M \) ArA (see "Experimental Procedures") partially prevented killing of 143B cells after 15 or 30 h of chronic treatment with rotenone. The cell death prevention was higher at low concentrations of rotenone (see insets).
At 100% CI inhibition, the rate of ROS generation increased by a maximum of 20–25% in short term incubations (4 h). Similar results were reported on a human skin fibroblasts treated with 1 \( \mu \)M rotenone (39). This pattern is compatible with a scenario where ROS production may result directly from CI inhibition, being enhanced during the subsequent apoptotic process (see below). Activation of apoptosis has been associated with generation of ROS, and it has been shown that superoxide is produced by mitochondria isolated from apoptotic cells due to a switch from the normal four-electron reduction of \( O_2 \) to a one-electron reduction when cytochrome \( c \) is released from mitochondria (41). It is still unclear whether the site of superoxide production in CI inhibited mitochondria is CI itself (16–19) or not (42). In any case, a partial inhibition of CI by rotenone should favor the transfer of electrons by CII and, as a consequence, an increase in the superoxide anion production. Electrons channeled via CII produce greater than four times more mitochondrial superoxide than electrons channeled via CI (43), and in studies in isolated cerebral mitochondria using succinate as substrate, ROS production was increased almost 9-fold (44). This problem will be of greater magnitude if cells try to compensate the CI impairment by overexpressing CII subunits. Although the question of whether CI inhibition up-regulates the mitochondrial CII activity was not directly addressed in this study, the unchanged ND1/SDH ratio observed in our experiments suggest that the compensatory mechanism of CII overexpression, if any, cannot be observed in a period up to 24 h. The possible adaptive mechanisms in the form of modulation of enzymic activities of distal respiratory chain complexes, increase in mitochondrial content of the cell, or other effects in the mitochondrial biogenesis remain unknown and are currently under investigation.

It has been shown that CI inhibition induces increase in the MnSOD levels (39, 45). Because oxidative stress is considered an imbalance between oxidants being metabolized or scavenged by antioxidants, our work cannot discern whether the ROS generation was underestimated by the action of intramitochondrial scavenging activity. To evaluate the consequences of increased level of ROS under the circumstance of a partial CI impairment, we examined the degree of lipid peroxidation. Lipid peroxidation increased linearly until approximately 75% CI inhibition, remaining basically constant until total CI blockade. The increased membrane sensitivity to oxidants could be due to changes in membrane lipid composition (46) and may

**FIG. 8.** Correlation between the percentage of complex I inhibition and the percentage of residual cell respiration with the physiological parameters studied. 

A, CI inhibition correlated exponentially with mitochondrial membrane potential decay (\( \Delta \psi_m \)), ROS production, mitochondrial membrane lipid peroxidation, and cell death. 

B, percentage of cell respiration inhibition correlated with all the parameters giving a hyperbolic curve. The different shapes of the curves in A and B are explained because percentages of CI inhibition lower than 20% produced mild changes in all the parameters, whereas any change in cell respiration produced significant increases in all of them. 

C depicts a model whereby cells with a partial complex I deficiency (exemplified as 25, 50, 75, and 100% impairment) suffer different levels of physiological consequences. When mitochondrial electron transport is inhibited at CI, cells are depleted of ATP and ROS production is enhanced, damaging mitochondrial membranes, resulting in small membrane depolarization and permeability transition. These mitochondrial alterations are the starting point of a cascade of events promoting apoptosis. In response to mitochondrial impairment, a calcium efflux from mitochondria probably occurs, which could contribute to perpetuate a vicious cycle of MPT and ROS production, resulting in further complex I inhibition, disruption of calcium homeostasis, and cell death.
contribute to a decreased fluidity (47), affecting in turn parameters such as $\Delta \psi_m$ and cell death. Certainly, ROS have damaging effects on cellular components triggering defensive responses by the cells and even apoptosis (48, 49).

**Effect of CI Inhibition on $\Delta \psi_m$ and Apoptosis**—Although the regulation of apoptosis is complex and not fully understood, it is known that mitochondria play an important role in the process. A reduction in $\Delta \psi_m$ is an early event in many cells undergoing apoptosis (50–53); isolated mitochondria induced to undergo MPT can induce nuclear apoptosis (53), CyA inhibits MPT pore opening, inhibiting apoptosis in some cases, and the subsequent loss of $\Delta \psi_m$ (31, 53, 54). It is interesting to consider whether or not a fall in $\Delta \psi_m$ is a signal for the CI impairment-associated cell death. According to this and previous studies (55), the failure to generate ATP oxidatively, rotenone only slightly depolarized mitochondria, maintaining more than 75% of control $\Delta \psi_m$ even when CI was completely inhibited for long incubation periods (3 days). Glycolysis provides the cells with sufficient ATP, which can be used via the mitochondrial ADP/ATP translocator to create a $\Delta \psi_m$ across the mitochondrial inner membrane (56). Such a fall in $\Delta \psi_m$ may be insufficient to trigger apoptosis when cells grow in glucose medium, and additional biochemical insults (e.g., oxidative stress) may finally lead to cell death. It is interesting that by using concentrations of rotenone lower than 10 nM ($\approx$75% CI inhibition), mitochondria actually became hyperpolarized, especially under incubation times shorter than 48 h. Similar $\Delta \psi_m$ increases have been shown before in Jurkat cells early during the apoptotic response to different stimuli (57). As a result of a decreased electron transport, the rate of $^+H$ ion delivery to the intermembrane space should be decreased. However, alterations in ionic homeostasis may produce an increase in that rate or a decrease in the removal of the $^+H$ ions from the intermembrane space, inducing the observed hyperpolarization. When cells lose ion homeostasis, this scenario could be reversed, producing a fall in the $\Delta \psi_m$. The $^+H$ ion cells used in this study maintained approximately 30% of the $\Delta \psi_m$ exhibited by the $^+H$ ion cells (20% in previous studies using the dye R123; Ref. 56), enough to support a growth rate in glucose medium comparable with that from $^+H$ ion cells. In the light of these results, HXC reduced growth rate in glucose medium, and additional biochemical insults (such as the starting point of a cascade of events promoting apoptosis. In response to respiratory chain impairment, a calcium efflux from mitochondria probably occurs, which could contribute to perpetuate a vicious cycle.

Our results show that the cytotoxicity of rotenone at high concentrations does not correlate to its inhibitory effect on CI because: (i) $^+H$ ion cells underwent apoptosis in the presence of high doses of rotenone; the levels of cell killing by concentrations of the drug higher than 600 nM were indistinguishable from those corresponding to $^+H$ ion cells and (ii) $^+H$ ion cells did not survive, even in high glucose medium, at concentrations of rotenone five times higher of that producing a total CI inhibition. It was important to address whether or not side effects of the drug would affect its use in creating pharmacological models of CI inhibition. Rotenone, at $\mu M$ concentrations, is able to arrest mammalian cells in metaphase (61), not by inhibiting a specific energy-requiring step (62) but by binding directly to tubulin and preventing microtubule assembly (61, 63), similarly to colchicine or other “c-mitotic agents” (64). Our immunocytochemical studies demonstrated that by using rotenone concentrations that produce 0–100% of CI inhibition, the microtubule assembly was not affected, and no nuclear condensation was observed. Therefore, it is evident that the two modes of action of rotenone occur at nonoverlapping ranges of drug concentration, validating the rotenone-induced model of CI impairment.

**Conclusions**—The goal of this study was to provide a comprehensive analysis of the cellular consequences of a partial complex I deficiency. Our chief conclusions were as follows: (i) We demonstrated a toxic effect of CI inhibition that is different from the one caused by a decrease in ATP production. This effect is likely to be mediated by increased free radical production, which triggers an apoptotic program. This cell death, however, could be prevented by blocking the mitochondrial permeability transition pore. (ii) Our studies validated rotenone inhibition as a good model for studying complex I, provided that the concentrations used do not exceed the ones sufficient to cause a complete inhibition of the enzyme, thus avoiding secondary toxicity. (iii) Finally, the quantitative correlation found among all the parameters studied provided a “guide” for the several physiological consequences of a partial CI inhibition at the cellular level and underscored the important deleterious effects of even a mild, chronic impairment in CI activity.

**Acknowledgments**—We thank Dr. Ashok Verma and Dr. Runu Dey for critically reading the manuscript and all the members of the Mito-Club (University of Miami) for constructive criticism. We are indebted to Dr. Michael P. King (Thomas Jefferson University, Philadelphia, PA) for the cell line 143B/206 $^+H$ ion, Dr. R. A. Capaldi (Institute of Molecular Biology, University of Oregon, Eugene, OR) for SDH (Fp) monoclonal antibody, and Dr. A. Lomberes (Groupe Hospitalier Pitié-Salpétrière, INSERM U-153, Paris, France) for the human NDI polyclonal antibody.

**REFERENCES**

1. Walker, J. E. (1992) Q. Rev. Biophys. 25, 253–324
2. Chomyn, A., Cleeter, M. W., Ragan, C. I., Riley, M., Doolittle, R. F., and Attardi, G. (1986) Science 234, 614–618
3. Robinson, B. H., McKay, N., Goodyer, P., and Lancaster, G. (1985) Am. J. Hum. Genet. 37, 938–946
4. Wallace, D. C., Singh, G., Lott, M. T., Hodge, J. A., Schurr, T. G., Lezza, A. M., Elsay, L. J., III, and Nikoskelainen, E. K. (1988) Science 242, 1427–1430
5. Schapira, A. H., Cooper, J. M., Dexter, D., Jenner, P., Clark, J. B., and Marsden, C. D. (1989) Lancet 1, 1269
