Inhibition of complex I of the electron transport chain causes $O_2^-$-mediated mitochondrial outgrowth


Inhibition of complex I of the electron transport chain causes $O_2^-$-mediated mitochondrial outgrowth. *Am J Physiol Cell Physiol* 288: C1440–C1450, 2005. First published January 12, 2005; doi:10.1152/ajpcell.00607.2004.—Recent evidence indicates that oxidative stress is central to the pathogenesis of a wide variety of degenerative diseases, aging, and cancer. Oxidative stress occurs when the delicate balance between production and detoxification of reactive oxygen species is disturbed. Mammalian cells respond to this condition in several ways, among which is a change in mitochondrial morphology. In the present study, we have used rotenone, an inhibitor of complex I of the respiratory chain, which is thought to increase mitochondrial reactive oxygen species is disturbed. Mammalian cells respond to this condition in several ways, among which is a change in mitochondrial morphology. In the present study, we have used rotenone, an inhibitor of complex I of the respiratory chain, which is thought to increase mitochondrial reactive oxygen species.

Mitochondria generate ATP through oxidative phosphorylation (OXPHOS), and defects in this system lead to decreased energy production, increased formation of $O_2^-$, and derived reactive oxygen species such as hydrogen peroxide and $\cdot$OH, and the release of death-promoting factors (44, 47, 56). Defects occur in a wide variety of degenerative diseases, aging, and cancer and primarily affect tissues that have high energy requirements and are unable to adapt to conditions of reduced mitochondrial energy supply. Cells that can survive under such conditions, such as cancer cells, show a variety of adaptations, including upregulation of glucose transport, glycolysis, and lactate formation (38). At the same time, these cells show a marked reduction in mitochondrial content (10) and OXPHOS capacity (46), whereas the mitochondrial reticulum is largely perinuclear (49). Cancer cells that are forced to grow on galactose and glutamine readily switch from anaerobic to aerobic energy production (43). This adaptation is accompanied by an increase in OXPHOS protein, a decrease in mitochondrial matrix pH, a more oxidized matrix redox state, an increase in the amount of cristae but no increase in mitochondrial morphology, and a more extended mitochondrial network. Similar observations were initially reached in budding yeast, in which a change of substrate induced a threefold increase in mitochondrial volume (16). Qualitative and/or quantitative changes in the mitochondrial reticulum are also observed under pathological conditions that are caused by inherited mutations in mitochondrial DNA or in nuclear OXPHOS genes (8, 20) and suggest a tight relationship between mitochondrial structure and function (11).

Recent insights suggest that $O_2^-$ anions, formed as a byproduct of the OXPHOS process, may activate specific redox-sensitive signaling pathways (13). Evidence has been provided that these pathways control uncoupling of protein-mediated proton conductance (7). In addition, these pathways are implicated in mitochondrial biogenesis (29, 32, 33) and regulation of cellular antioxidant capacity (40). Failure to make the appropriate changes is thought to lead to increased $O_2^-$ production, which, if not properly balanced by the cell’s antioxidant mechanisms, may cause structural and functional damage to the cellular mitochondrial network are observed during differentiation, cellular senescence, and apoptosis, whereas subtle rearrangements occur during cellular growth and division (56).

Marked changes in the structure of the cellular mitochondrial network are observed during differentiation, cellular senescence, and apoptosis, whereas subtle rearrangements occur during cellular growth and division (56).
polyunsaturated fatty acids in membrane lipids, proteins, and DNA. There is good evidence that increased oxygen radical formation is the cause of atherosclerosis and possibly also of the major neurodegenerative and chronic inflammatory diseases (21). Moreover, increased radical formation has been implicated in aging (17) and apoptosis (31). However, in the majority of diseases in which tissue damage occurs, increased radical formation is regarded as a consequence rather than a cause (21). Human mitochondrial complex I (NADH:ubiquinone oxido-reductase; EC: 1.6.5.3) is the largest multisubunit assembly of the OXPHOS system, comprising 39 nuclear encoded and 7 mitochondrially encoded subunits (23). Malfunction of this complex is associated with a wide variety of clinical syndromes (47). To enhance the understanding of the pathophysiology of these diseases, with the final aim of developing new treatment strategies to stabilize or even cure them, we have studied genetically characterized human complex I-deficient fibroblast cell lines as a model for OXPHOS system disease with the knowledge that these cells are glycolytic (41).

In doing so, we recently showed that agonist-induced mitochondrial Ca$^{2+}$ accumulation and ensuing ATP production are significantly decreased in skin fibroblasts derived from patients with an isolated complex I deficiency caused by mutations in nuclear encoded structural subunits of the complex (53).

Pham et al. (39) reported that mitochondrial morphology and dynamics are altered in skin fibroblasts from patients with mitochondrial complex I deficiency. Similar observations were made with regard to control fibroblasts treated for 5 min with the complex I inhibitor rotenone (40 µM). Studies with mitochondrial membranes isolated from patient fibroblasts showed that NADH-stimulated mitochondrial O$_{2}^{-}$ formation is increased in human complex I deficiency and that 10 µM rotenone readily increases formation of this radical in control membranes (40). Together with observations that exogenous application of hydrogen peroxide increases mitochondrial mass in human lung fibroblasts (29), these findings suggest a causal relationship between increased mitochondrial O$_{2}^{-}$ formation and alterations in mitochondrial reticulum and dynamics in complex I deficiency. However, no definitive proof has yet been offered.

In the present report, we show that a sustained increase in mitochondrial O$_{2}^{-}$ production brought about by chronic inhibition of complex I of the electron transport chain (100 nM rotenone, 72 h) causes a marked increase in mitochondrial length and branching.

**MATERIALS AND METHODS**

**Cell culturing, NADH:ubiquinone oxido-reductase measurements, and cell cycle analysis.** Fibroblasts were obtained from a skin biopsy of a healthy individual and cultured in medium 199 with Earle’s balanced salt solution (Invitrogen, Carlsbad, CA) in a humidified atmosphere of 95% air-5% CO$_{2}$ at 37°C. This sample was collected after the person provided informed consent. The medium contained 5 mg/l Tween 20 and was supplemented with 10% (vol/vol) fetal calf serum, 100 IU/ml penicillin, and 100 µg/ml streptomycin (Invitrogen). The activities of NADH:ubiquinone oxido-reductase (complex I), cytochrome c oxidase (complex IV), and citrate synthase (CS) were measured in a mitochondria-enriched fraction as described previously (48). For fluorescence microscopy, fibroblasts were seeded on glass coverslips (22 mm diameter) and cultured to ∼70% confluence. For cell cycle assessment, cells were trypsinized and stored on ice. After being stained with propidium iodide, cell suspensions were analyzed using flow cytometry (52).

**Quantitative analysis of mitochondrial morphology by video-rate laser-scanning confocal microscopy.** Stock solutions of the lipophilic cation rhodamine 123 (R123), Mitotracker Green FM (MG), and Mitotracker Red CMXRos (MR; all from Molecular Probes, Leiden, The Netherlands) were freshly prepared in dimethyl sulfoxide (DMSO) before each measurement. Fibroblasts were incubated in culture medium containing 200 µM R123 or 5 µM MR or MG for 40 s (R123), 3 min (MR), or 20 min (MG) at 37°C. After being loaded, cells were thoroughly washed with HEPES-Tris medium containing (in mM) 132 NaCl, 4.2 KCl, 1 CaCl$_{2}$, 1 MgCl$_{2}$, 5.5 d-glucose, and 10 HEPES, pH 7.4. For confocal imaging, coverslips were mounted in an incubation chamber and placed on the stage of an inverted microscope (Nikon Diaphot, Tokyo, Japan) attached to an Oz confocal microscope (Noran Instruments, Middleton, WI). Measurements were performed at 20°C. The light from an argon ion laser (488 nm; Omnichrome, Chino, CA) was delivered to the cells using a ×40 oil-immersion planapochromat lens objective (numerical aperture (NA), 1.4; Nikon). For all dyes, fluorescence emission light was directed through a 500-nm LP barrier filter (Chroma Technology, Brattleboro, VT) and quantified using a photomultiplier tube (Hamamatsu Photonics, Bridgewater, NJ). Given the flat morphology of the fibroblasts (<3 µm in the axial direction), slit settings were chosen such that axially each cell was entirely present within the confocal volume (27). This prevented exclusion of mitochondrial structures from the image and guaranteed an optimal fluorescence signal at minimal laser intensity. Hardware and image acquisition were controlled using Intervi-sion software (version 1.5; Noran Instruments) run under IRIX 6.2 on an Indy workstation (Silicon Graphics, Mountain View, CA, equipped with 512 Mb of memory. Before image acquisition, brightness and contrast settings were optimized using a custom-made lookup table that colored the upper and lower 10 gray levels red and blue, respectively. Images (512 × 480 pixels) were collected at 30 Hz with a pixel dwell time of 100 ns. To reduce random noise, images were averaged in real time using the running average algorithm of the Intervision Acquisition software with a window size of 32. This acquisition protocol, in combination with the low mitochondrial mobility at 20°C, effectively prevented distortion of the image by mitochondrial movement. Images were recorded from a cross-shaped area transecting the center of the coverslip and converted to tagged image file format using a Silicon Graphics O2 workstation running IRIX 6.5. Quantitative analysis of mitochondrial morphology was performed using Image Pro Plus 4.5 (Media Cybernetics, Silver Spring, MD) as described in RESULTS.

**Quantitative analysis of mitochondrial O$_{2}^{-}$ production using digital imaging microscopy.** Fibroblasts were incubated in HEPES-Tris medium containing 10 µM hydroethidine (HEt; Molecular Probes) for 10 min at 37°C. HEt is an uncharged compound that readily enters the cell, whereas positively charged product ethidium (Et) (15). The reaction was stopped by thoroughly washing the cells with PBS to remove excess HEt. For quantitative analysis of Et emission signals, coverslips were mounted in an incubation chamber placed on the stage of an inverted microscope (Axiovert 200 M; Carl Zeiss, Jena, Germany) equipped with a Zeiss ×40/1.3 NA fluor lens objective. Et was excited at 490 nm using a monochromator (Polychrom IV; TILL Photonics, Gräfelfing, Germany). Fluorescence emission was directed using a 525DRLP dichroic mirror (Omega Optical, Brattleboro, VT) through a 565ALP emission filter (Omega Optical) onto a CoolSNAP HQ monochrome charge-coupled device camera (Roper Scientific, Vienna, The Netherlands). The image-capturing time was 100 ms. Routinely, 10 fields of view per coverslip were analyzed. Hardware was controlled using Mettlerflow 6.0 software (Universal Imaging, Downingtown, PA). Quantitative image analysis was performed using Metamorph 6.0 software (Universal Imaging) as described in RESULTS.

**Quantitative analysis of the extent of lipid peroxidation by video-rate laser-scanning confocal microscopy.** The extent of lipid peroxida- tion was quantified using the fluorescent ratio probe C11-BODIPY™581/591 (Molecular Probes). Upon oxidation, the red emitting form of the dye (595 nm) is converted into a green emitting form (520 nm).
Fig. 1. Visualization of mitochondria in living human skin fibroblasts. To determine which mitochondria-specific dye was best suited for image analysis, cells were stained with rhodamine 123 (R123) (A), Mitotracker Red CMXRos (MR) (B), or Mitotracker Green FM (MG) (C). Image segmentation into three discrete gray-level ranges (second column, 0–50; third column, 51–150; and fourth column, 151–255) revealed that only in the case of R123 mitochondrial structures were present within a discrete gray-level interval required for computerized image analysis (A, third image).

RESULTS

Visualization of mitochondria in living skin fibroblasts. Successful quantitative analysis of mitochondrial morphology requires a visualization protocol that is compatible with off-line computer-assisted image analysis. The latter requirement implies that fluorescence originating from mitochondria can easily be separated from background signals. In the ideal case, this means that the recorded images contain a discrete range of gray levels associated solely with these organelles. To find the mitochondrial dye most compatible with this requirement, we stained fibroblasts with three widely used mitochondria-specific fluorophores: rhodamine 123 (R123) (n = 34 cells), Mitotracker Red CMXRos (MR; n = 37), and Mitotracker Green FM (MG; n = 42). To reduce the illumination time of the fluorophores, prevent artifacts associated with mitochondrial movement, and increase the signal-to-noise ratio, 32 images were captured during 1 s and averaged in real time.

All three dyes revealed extensive mitochondrial reticula as well as individual mitochondrial filaments (Fig. 1, A–C, left). Next, images were segmented using three gray-level intervals (0–50, 51–150, and 151–255; see columns below intensity scale in Fig. 1). For only R123, a discrete range of gray levels was associated exclusively with mitochondrial structures (Fig. IA, third image from left). When R123-stained cells were treated with 1 μM concentration of the protonophore carbonyl cyanide-p-trifluoromethoxy-phenylhydrazone (FCCP), the mitochondrial staining pattern was acutely lost, thus confirming the mitochondrial localization of the dye (data not shown).

Quantitative analysis of mitochondrial structure. To identify mitochondrial structures in images of R123-stained fibroblasts (Fig. 2A; solid line indicates cell boundary), an image-processing algorithm was developed that consisted of three steps. First, the contrast in the image was optimized by reassigning the gray values of the pixels to cover the entire available range from 0 to 255 (Fig. 2B; intensity histograms before and after optimization are displayed in Fig. 2E). Next, a “top-hat” spatial filter was applied (Fig. 2C). This filter is particularly suited to isolating bright features from a dark background and consists of two steps. First, the pixel values in a certain region of the image are multiplied by a matrix h (or kernel) of integer-filtering coefficients given by

\[
h = \begin{bmatrix}
0 & 0 & -1 & -1 & -1 & 0 & 0 \\
0 & -1 & -1 & -1 & -1 & -1 & 0 \\
-1 & -1 & +3 & +3 & +3 & -1 & -1 \\
-1 & -1 & +3 & +3 & +3 & -1 & -1 \\
0 & -1 & -1 & -1 & -1 & -1 & 0 \\
0 & 0 & -1 & -1 & -1 & 0 & 0
\end{bmatrix}
\]

Second, the newly assigned pixel values are summed and used...
to replace the original intensity of the central pixel. This process is repeated until each pixel in the image is processed. Of note, the filter always uses the original intensity values of the neighborhood pixels as an input. We chose a dimension of 7 × 7 pixels for the top-hat filter because it is compatible with the size of the mitochondrial objects. The result in Fig. 2C was obtained by applying the top-hat filter three times using a filter strength of 30%. The latter value indicates that each time the filter is applied, only 30% of the difference between the pixel value after and before filtering is added. Finally, a threshold operation was performed to remove the distinct spherical objects of intermediate intensity introduced by the top-hat filter (Fig. 2D). The effects of the various steps of the image-processing protocol are shown in Fig. 2, F–H, depicting the intensity profiles across a horizontal line transecting the cell (dotted line in Fig. 2A–D). The clear distinction between background (black) and mitochondrial (white) signals in the binary image (Fig. 2D and H) allows automated shape analysis of mitochondrial structures.

For morphological analysis, two parameters (descriptors) were calculated for each mitochondrial object: the form factor \( F = \text{perimeter}^2/4\pi \cdot \text{area} \) and the aspect ratio \( AR \) (ratio between...
the major and minor axes of the ellipse equivalent to the object). Moreover, the total number of mitochondria per cell (Nc) was determined. Both F and AR are independent of image magnification and have a minimal value of 1 (corresponding to a perfect circle). F was used previously to analyze cell shape (28). Figure 3 illustrates the morphological meaning of F and AR. Images of R123-stained fibroblasts were contrast optimized (Fig. 3A), and mitochondrial structures were isolated using image processing (Fig. 3B). Plotting AR as a function of F for all 392 mitochondria depicted in Fig. 3C revealed that AR is a measure of mitochondrial length, whereas F is a measure of both length and degree of branching (representative mitochondrial structures are indicated by filled circles). For statistical analysis, we routinely calculated the average values of F and AR for each recorded field of view.

**Chronic rotenone treatment inhibits complex I activity and induces mitochondrial outgrowth in human skin fibroblasts.** To assess a possible connection between mitochondrial stress and mitochondrial morphology, cells were cultured in the presence of rotenone, an archetypal inhibitor of complex I of the respiratory chain. Enzyme activity measurements in mitochondria-enriched fractions of cells chronically treated with this drug for 72 h revealed a dose-dependent decrease in complex I activity (Fig. 4A, filled circles). In contrast, the activities of complex IV (open circles) and the Krebs cycle enzyme citrate synthase (squares) were not altered.

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Visual inspection of R123-stained cells suggested an increase in mitochondrial length and/or branching after rotenone treatment (Fig. 4B). Subsequent quantitative analysis revealed that rotenone did not alter AR or Nc but caused a dose-dependent increase in F, which was significant at a concentration of 100 nM (Fig. 4C; *P < 0.01*). Importantly, rotenone did
not change the cell cycle phase. For vehicle-treated cells and cells treated with 2.5 and 100 nM rotenone, values of G0/G1, G2/M, and S were, respectively, 86%, 4%, and 10% (n = 3); 91%, 5%, and 5% (n = 2); and 92%, 4%, and 4% (n = 2). The drug did not detectably alter the mitochondrial membrane potential as indicated by the lack of effect on the R123 fluorescence emission intensity (Fig. 4C). Of note, the latter was determined in unprocessed images recorded at identical hardware settings using the binary image obtained after image processing as a mask.

Chronic treatment with rotenone increases $\text{O}_2^\cdot$ production in human skin fibroblasts. Measurement of the effect of rotenone on $\text{O}_2^\cdot$ formation in living cells has yielded conflicting results. Increases were observed in the human osteosarcoma-derived cell line 143B (4), mesencephalic neurons (37), and HL-60 cells (31), whereas decreases occurred in hepatocytes (55), cultured mouse hippocampal neurons (45), and monocytes and macrophages (30). In this study, we used hydroethidine (HEt), a redox-sensitive probe that is widely used to measure mitochondrial $\text{O}_2^\cdot$ production in living cells (5, 6, 15, 57). HEt is a cell-permeant compound that is oxidized by $\text{O}_2^\cdot$ to its positively charged product Et. Digital imaging microscopy of fibroblasts loaded with 10 $\mu$M HEt for 10 min and thoroughly washed to remove nonoxidized HEt, revealed the presence of Et in both nucleoli and a widespread network of tubular structures present in the cytosolic compartment (Fig. 5A). The

Fig. 5. Rotenone increases mitochondrial $\text{O}_2^\cdot$ production. A: human skin fibroblasts incubated in the presence of HEt (10 $\mu$M) for 10 min and thoroughly washed to remove nonoxidized HEt revealed the presence of Et in both nucleoli and a widespread network of tubular structures present in the cytosolic compartment. B: staining of the cytosolic tubular structures was lost immediately after addition of the mitochondrial uncoupler carbonylcyanide-\(p\)-trifluoromethoxy-phenylhydrazone (FCCP; 1 $\mu$M). C and D: stimulatory effect of rotenone (100 nM, 1 h) on Et accumulation in nucleoli (n) and mitochondrial structures (m) during 10-min incubation with 10 $\mu$M HEt. Vehicle-treated cells were incubated with 0.001% (vol/vol) ethanol. For quantification of the rotenone effect, the average fluorescence intensity was determined in the nucleoplasm and a cytosolic region of equal size containing a high density of mitochondrial structures (C, ovals). E: linear relationship (slope = 0.51 ± 0.03; P < 0.001) between the average fluorescence intensities in nucleoplasm and cytosolic region for fibroblasts exposed to vehicle (n = 35 cells), 100 nM rotenone for 1 h (n = 34), 0.1 $\mu$M rotenone for 1 h (n = 30), or 100 nM rotenone for 72 h (n = 28). $^a$P < 0.01, significantly different from vehicle-treated cells. $^b$P < 0.01, significantly different from fibroblasts treated with 100 nM rotenone for 1 h. $^c$P < 0.01, significantly different from fibroblasts treated with 100 nM rotenone for 72 h.
intensity of fluorescence emission did not change during 10 min of illumination at 0.2 Hz as demonstrated by a linear fit with a slope of $4.4 \times 10^{-5} \pm 2.3 \times 10^{-6}$ ($P < 0.001$). This indicates that excess HEt was effectively removed, Et did not leak out of the cell, and photobleaching and/or photoactivation did not occur during at least 10 min of image acquisition. The mitochondrial nature of the tubular structures was demonstrated by the immediate loss of Et fluorescence upon addition of the mitochondrial uncoupler FCCP (1 μM; Fig. 5B). This confirms that mitochondrial Et accumulation depends on mitochondrial membrane potential (5, 6, 15).

Fibroblasts treated with 100 nM rotenone for 1 h before the 10-min incubation period with HEt displayed a marked increase in Et fluorescence in both nucleoli and mitochondria (Fig. 5, C and D). For quantification of the effect of rotenone, we determined the average fluorescence intensity in the nucleoplasm and a cytosolic region of equal size containing a high density of mitochondrial structures (Fig. 5C, circles n and m, respectively). Figure 5E shows that the average fluorescence intensities in both regions, determined at different degrees of rotenone inhibition, are linearly correlated ($R = 0.99$; slope $= 0.51 \pm 0.03$; $P < 0.05$). This demonstrates that the nucleolar Et is of mitochondrial origin. Figure 5 also shows that the average fluorescence intensity in cells treated with 100 nM rotenone for 1 h ($n = 35$ cells) was twofold higher than in vehicle-treated cells ($n = 37$). A further increase to threefold the control value was reached with 1 μM rotenone ($n = 31$). Cells chronically treated with 100 nM rotenone for 72 h still displayed a twofold increase in average fluorescence intensity ($n = 32$). Finally, addition of H$_2$O$_2$ (100 μM) did not increase Et fluorescence, demonstrating that HEt is not oxidized by this O$_2^-$ derivative. Taken together, these findings show that chronic rotenone treatment causes a rapid and persistent increase in the rate of mitochondrial O$_2^-$ production in cultured human skin fibroblasts.

**MitoQ prevents rotenone-induced mitochondrial outgrowth without affecting rotenone-induced O$_2^-$ formation.** To investigate the mechanism underlying rotenone-induced mitochondrial outgrowth, we applied the antioxidant mitoquinone (MitoQ) (25). MitoQ is a ubiquinone derivative that is mitochondria-targeted by covalent attachment to a lipophilic triphenylphosphonium cation through an aliphatic carbon chain. Chronic treatment of human fibroblasts with MitoQ for 72 h did not alter mitochondrial morphology (Fig. 6A) or number (not shown). At a concentration of 10 nM, however, MitoQ abolished the effect of chronic rotenone treatment on mitochondrial outgrowth. Figure 6B shows that MitoQ alone had no effect on mitochondrial O$_2^-$ production. In addition, Fig. 6 shows that MitoQ did not inhibit rotenone-induced O$_2^-$ formation. These findings demonstrate that MitoQ acts downstream of O$_2^-$ to inhibit rotenone-induced mitochondrial outgrowth.

**MitoQ prevents the rotenone-induced increase in lipid peroxidation.** To address the question of the specificity of MitoQ, we used C11-BODIPY$_{581/591}$, a lipid peroxidation reporter molecule that is insensitive to O$_2^-$, NO, and hydroperoxides (14), to assess the effect of rotenone, alone and in combination with MitoQ, on lipid peroxidation. Cells chronically treated with 100 nM rotenone for 72 h and subsequently incubated in the presence of 4 μM C11-BODIPY$_{581/591}$ for 30 min showed a twofold increase in the ratio between the oxidized and nonoxidized forms of the reporter molecule (Fig. 6C). This effect of rotenone was fully absent in cells cotreated with 10 nM MitoQ. MitoQ alone did not alter the relative amount of oxidized C11-BODIPY$_{581/591}$. These data show that lipid peroxidation is markedly increased in cells chronically treated with 100 nM rotenone and that this effect of rotenone is completely blocked by MitoQ.

**DISCUSSION**

Human mitochondrial complex I (NADH:ubiquinone oxidoreductase) is the largest multisubunit assembly of the OXPHOS system. Its malfunction is associated with a wide variety of clinical syndromes ranging from often early lethal disorders, of which Leigh disease, a progressive encephalopathy, is the most frequent, to neurodegenerative disorders in adulthood, including Leber’s hereditary optic neuropathy and Parkinson’s disease. In recent years, all human nuclear structural complex I genes have been characterized, which allowed us to elucidate the genetic defect in 40% of a cohort of complex I-deficient patients in whom the enzyme defect was present in at least skeletal muscle and cultured skin fibroblasts (47). To enhance the understanding of the pathophysiological consequences of complex I deficiency, we studied fibroblasts from genetically characterized patients (53). Pilot experiments using the protocol for quantitative analysis of mitochondrial morphology described in the present report revealed significant differences between control and patient fibroblasts (Koopman WJH, Visch HJ, Verkaart S, Smeltink JAM, van den Heuvel LWPJ, and Willems PHGM, unpublished data). This prompted us to investigate the relationship between complex I activity and mitochondrial morphology in control human skin fibroblasts. To mimic the pathological condition as closely as possible, control fibroblasts were chronically treated with rotenone concentrations that decreased the activity of complex I to values similar to those measured in patient fibroblasts.

The data presented show that chronic treatment of fibroblasts with 100 nM rotenone for 72 h decreased complex I activity by 80% and caused significant mitochondrial outgrowth. Importantly, the alterations in mitochondrial shape were not the result of changes in cell cycle phase. This finding is in agreement with the findings of previous studies showing that 100 nM rotenone did not affect cell growth and viability of human B lymphoma cells (1). The morphological parameters analyzed were aspect ratio ($AR$), which is a measure of mitochondrial length, and form factor ($F$), which is a combined measure of mitochondrial length and degree of branching. Both parameters are independent of objective or zoom factor and therefore are most suited for comparison between different microscopes and cell types. To minimize the effects of phototoxicity and mitochondrial movement, images were acquired at video speed (30 Hz) and averaged in real time. For statistical evaluation, we calculated the average values of $AR$, $F$, and $Ne$ (the number of mitochondria per cell) for each field of view. These averages were not influenced by the small number of partially imaged mitochondria, because typically 80–500 mitochondria per field of view were present. Day-to-day variations in $AR$, $F$, and $Ne$ were effectively corrected by expressing each value as a percentage of the corresponding control value recorded on the same day. Because our R123 staining procedure was very short (40 s) and because image analysis can be automated allow for
future application of this protocol in the rapid screening of many mitochondrial structures.

Chronic treatment with 100 nM rotenone for 72 h caused a marked increase in form factor but did not significantly alter the aspect ratio or the number of mitochondrial structures. These findings show that human skin fibroblasts respond to chronic complex I inhibition with the formation of a more complex mitochondrial reticulum. Chronic treatment with 15 nM rotenone tended to increase both AR and F, suggesting that the effect of rotenone is dose dependent. When the concentration of rotenone was increased to 100 nM, however, the effect of chronic rotenone treatment on AR decreased rather than increased, whereas the effect on F increased further to reach statistical significance. Pham et al. (39) recently reported that human skin fibroblasts displayed increased amounts of mitochondria in the swollen filamentous forms, nodal filaments, and ovoid forms upon acute (5 min) treatment with 40 μM rotenone. We did not observe such aberrations in mitochondrial morphology in our study, indicating that mitochondria respond completely differently depending on the concentration of the inhibitor and the duration of treatment. Chronic treatment of fibroblasts with 5 μM rotenone caused massive cell death (data not shown), demonstrating the inability of these cells to cope with relatively high inhibitor concentrations. It should be noted, however, that the relatively low concentration of 100 nM rotenone used in the present study decreased the activity of complex I by 80%. Moreover, acute addition of 100 nM rotenone caused an immediate increase in the rate of O$_2^·•$ production (data not shown), indicating that the inhibitor acts instantaneously. These observations suggest that the cytotoxicity of the higher rotenone concentrations is not directly related to its inhibitory effect on complex I activity and that therefore the results obtained with these concentrations should be treated with caution.

Several studies have shown that high concentrations of rotenone or exogenously added H$_2$O$_2$ can induce apoptosis (1, 12, 26, 31, 51). In general, this induction is accompanied by permeabilization of the mitochondrial inner membrane, opening of the permeability transition pore, and dissipation of the mitochondrial membrane potential (18). It has been demonstrated that chronic (36 h) rotenone treatment induces apoptosis by enhancing mitochondrial reactive oxygen production in HL-60 cells (31). However, the lowest concentration of rotenone that produced a significant increase in the percentage of apoptotic cells was 200 nM. In agreement with this finding, the present study did not show any adverse effects of chronic rotenone or exogenously added H$_2$O$_2$.

Fig. 6. MitoQ inhibits rotenone-induced mitochondrial outgrowth and lipid peroxidation but not O$_2^·•$ generation. Human skin fibroblasts were incubated in the presence of ethanol (0.001% vol/vol; vehicle), rotenone (100 nM), MitoQ (1 nM, 10 nM), or the combination of both drugs for 72 h before analysis of mitochondrial morphology, rate of mitochondrial O$_2^·•$ production, and extent of lipid peroxidation. A: effect on form factor. Experiments were performed on 2 days. On each day, the average value obtained with vehicle was set at 100%, to which the other values were related. The values presented are the average of n = 56 cells (vehicle; V), n = 71 cells (100 nM rotenone), n = 84 cells (1 nM MitoQ), n = 102 cells (10 nM MitoQ), n = 34 cells (100 nM rotenone + 1 nM MitoQ), and n = 87 cells (100 nM rotenone + 10 nM MitoQ). B: effect on Et accumulation in the nucleoplasm during 10 min incubation with 10 μM HEt as a measure of the rate of mitochondrial O$_2^·•$ production. Experiments were performed on 2 days. On each day, the average fluorescence intensity in vehicle-treated cells was set at 100%, to which the other values were related. The values presented are the average of n = 60 cells (V), n = 64 cells (100 nM rotenone), n = 61 cells (1 nM MitoQ), n = 63 cells (10 nM MitoQ), n = 77 cells (100 nM rotenone + 1 nM MitoQ), and n = 30 cells (100 nM rotenone + 10 nM MitoQ). C: effect on the emission ratio of C11-BODIPY581/591 as a measure of the extent of lipid peroxidation. Data are from two independent measurements. The values presented are the average of n = 55 cells (V), n = 44 cells (100 nM rotenone), n = 62 cells (10 nM MitoQ), and n = 53 cells (10 nM MitoQ + 100 nM rotenone). **P < 0.01, significantly different from vehicle-treated cells.
treatment with 100 nM rotenone on cell growth and viability. To the contrary, rotenone-treated fibroblasts displayed an increase in complexity of the mitochondrial network, suggesting the induction of an adaptive response. A similar increase was observed in cancer cells that were forced to grow on galactose and glutamine (43). Importantly, the latter study showed that the increase in complexity of the mitochondrial reticulum was accompanied by an increase in OXPHOS protein, indicating the adaptive nature of this response. These findings support the existence of a tight relationship between mitochondrial structure and function (11).

The rotenone-induced increase in mitochondrial outgrowth was completely prevented by cotreatment with MitoQ, a mitochondria-targeted derivative of coenzyme Q10 (25, 44). Given its very large hydrophobicity, MitoQ is preferentially adsorbed to the matrix-facing leaflet of the inner mitochondrial membrane, with the triphenylphosphonium moiety at the membrane surface at the level of the fatty acid carbonyls and the alkyl chain and ubiquinol moiety inserted into the hydrophobic core of the lipid bilayer (2). The inhibitory effect of MitoQ on rotenone-induced mitochondrial outgrowth suggests that an increase in mitochondrial oxidative stress is the primary cause of this cellular response.

MitoQ completely blocked rotenone-induced outgrowth and lipid peroxidation but had no effect on the rotenone-induced increase in mitochondrial O$_2^\cdot$ formation. The latter finding shows that MitoQ exerts its effect downstream of this O$_2^\cdot$. It has been demonstrated that O$_2^\cdot$ produced upon complex I inhibition are released into the mitochondrial matrix (9, 50). The rotenone-induced increase in O$_2^\cdot$ production found in the present study might very well be the basis of the rotenone-induced increase in H$_2$O$_2$ production observed in previous studies (4, 31, 51). The observation that exogenous application of H$_2$O$_2$ increases mitochondrial mass in human lung fibroblasts (29) indeed suggests the involvement of matrix manganese superoxide dismutase in the mechanism of action of rotenone. Recent work concerning the mechanism by which O$_2^\cdot$ activates mitochondrial uncoupling proteins has suggested that O$_2^\cdot$ releases ferrous iron from iron-sulfur center-containing enzymes, which reacts with hydrogen peroxide, produced by the action of manganese superoxide dismutase, to form the •OH. This radical then extracts a hydrogen atom from an unsaturated fatty acyl chain of a phospholipid to generate carbon-centered radicals that initiate lipid peroxidation, yielding breakdown products that activate the uncoupling proteins (34). Because MitoQ reacts mainly with lipid peroxidation products (Murphy M, unpublished observations), the present findings may suggest that rotenone acts through these products to increase mitochondrial outgrowth. However, further research is required to define the exact site of action of MitoQ. The present findings support recent insights that intracellular oxidants may act as specific signaling molecules under both physiological and pathological conditions (17).

The present study shows that 100 nM rotenone causes a twofold increase in the rate of mitochondrial O$_2^\cdot$ production in intact human skin fibroblasts. In agreement with this finding, NADH-stimulated mitochondrial O$_2^\cdot$ formation was found to be increased in mitochondrial membranes isolated from complex I-deficient human skin fibroblasts (40, 42). These findings support the existence of a site of electron leakage upstream from the rotenone binding site (19). A considerably higher concentration of 10 μM rotenone was used to evoke a significant increase in NADH-stimulated mitochondrial O$_2^\cdot$ formation in mitochondrial membranes isolated from control human skin fibroblasts (40). Therefore, the present method of determining the accumulation of Et in intact cells incubated for a short period in the presence of HEt is highly sensitive and most suitable for quantification of the rate of mitochondrial O$_2^\cdot$ production in patient fibroblasts.

In conclusion, the data presented herein are compatible with the existence of a O$_2^\cdot$-induced mechanism of mitochondrial outgrowth that is activated at subapoptotic levels of complex I inhibition and leads to a possibly adaptive increase in the complexity of the mitochondrial reticulum. Importantly, we have shown that this mechanism is activated at pathological levels of complex I inhibition. Detailed analysis of mitochondrial morphology in patient fibroblasts will reveal whether this mechanism is activated or whether activation of this mechanism is impaired in human complex I deficiency.

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REFERENCES


