The mechanism of tumor necrosis factor-α (TNFα)-induced cytotoxicity in metabolically inhibited cells is unclear, although some studies have suggested that mitochondrial dysfunction and generation of reactive oxygen species may be involved. Here we studied the effect of TNFα on the redox state of mitochondrial cytochromes and its involvement in the generation of reactive oxygen species in metabolically inhibited L929 cells. Treatment with TNFα and cycloheximide (TNFα/CHX) induced mitochondrial cytochrome c release, increased the steady-state reduction of cytochrome b, and decreased the steady-state reduction of cytochromes c1 and a2. TNFα/CHX treatment also induced lipid peroxidation, intracellular generation of reactive oxygen species, and cell death. Furthermore, as the cells died mitochondrial morphology changed from an orthodox to a hyperdense and condensed and finally to a swollen configuration. Antimycin A, a mitochondrial respiratory chain complex III inhibitor that binds to cytochrome b, blocked the formation of reactive oxygen species, suggesting that the free radicals are generated at the level of cytochrome b. Moreover, antimycin A, when added after 3 h of TNFα/CHX treatment, arrested the further release of cytochrome c and the cytotoxic response. We propose that the reduced cytochrome b promotes the formation of reactive oxygen species, lipid peroxidation of the cell membrane, and cell death.

Tumor necrosis factor α (TNFα) is a cytokine that is cytotoxic against certain tumor cells (1), and this effect is enhanced by cycloheximide (CHX) (2). In the presence of CHX not only is less TNFα required, but also cell death occurs in a shorter period of time (3). Although these effects have been described extensively, the molecular mechanisms of action are not well understood (4, 5). The reported ability of antioxidants to protect cells against TNFα-induced cytotoxicity suggests that mitochondrial dysfunction and generation of reactive oxygen species (ROS) in the mitochondria may play an essential role (6–11). Oxidative stress can result in severe metabolic dysfunction, including the peroxidation of lipid membranes (12), an increase in cytosolic Ca2+ (13), induction of the mitochondrial permeability transition (14), and DNA damage (reviewed in Refs. 15 and 16).

Previous investigations have shown contradictory results regarding whether TNFα induces cytotoxicity through necrosis or apoptosis (17). Recent results indicate that TNFα induces necrosis in L929 cells, although apoptosis-like features have also been observed (18, 19). Regardless of the mode of cell death, however, it has been shown that TNFα-induced cytotoxicity of L929 cells is mediated by mitochondrial formation of ROS (20, 21). In intact mitochondria, three components of the respiratory chain have been found to be involved in the generation of ROS (22, 23); one is located in complex I, and the other two are ubisemiquinone (24) and reduced cytochrome b (25, 26), which are both located in complex III. Furthermore, substantial evidence in other systems implicates mitochondria and mitochondrial cytochrome c release in both apoptosis and necrosis (27–29). As part of the mitochondrial electron transport chain, cytochrome c transports electrons from the b-c1 complex (complex III) to cytochrome c oxidase (complex IV). Consequently, cytochrome c release upon a cell death-inducing stimulus may block the normal flow of electrons and may promote the increased steady-state reduction of the respiratory components upstream of cytochrome c, such as the b-c1 complex. Increased steady-state reduction of components of complex III (e.g. cytochrome b) may then promote free radical generation.

The purpose of the present study was to investigate the effect of TNFα in the presence of CHX on cytochrome c release and the redox state of mitochondrial cytochromes and the potential role of the redox state in ROS generation and TNFα-induced cytotoxicity. We show that TNFα treatment promoted mitochondrial cytochrome c release, increased the steady-state reduction of cytochrome b, and generated elevated amounts of ROS. We also demonstrate that blocking cytochrome b with antimycin A abrogated ROS generation and arrested the cytotoxic response. Finally, we show that mitochondria underwent a sequence of morphological changes that preceded cell death.

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**EXPERIMENTAL PROCEDURES**

**Reagents**—Recombinant human TNFα was purchased from Genzyme Co. (Cambridge, MA). RPMI 1640 medium was from Biochrome (Berlin, Germany). Rotenone, CHX, antimycin A, horse heart cytochrome c, oligomycin, myxothiazol, thenoyltrifluoroacetone (TTF), α-tocopherol, amidopyridine iodide (PI), Triton X-100, and trypsin were purchased from Sigma. Dihydrorhodamine 123 and 2,7′-dichlorofluorescein diacetate were obtained from Molecular Probes, Inc. (Eugene, OR). Potassium cyanide (KCN) was supplied by Ferrosa (Barcelona, Spain). L-Glutamine, penicillin, phosphate-buffered saline, and streptomycin were from ICN Biomedicals Inc. (Costa Mesa, CA). Fetal bovine serum was purchased from Sera-Lab (Sussex, United Kingdom).

**Cell Culture**—Cells from the murine fibrosarcoma cell line L929 (American Type Culture Collection, Manassas, VA) were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, penicillin (100 units/ml), and streptomycin (0.1 mg/ml) in a humidified incubator in 5% CO₂ in air at 37 °C.

**Separation of Viable and Dead Cells**—During the process of cell death, L929 cells detach from the flask and float in the medium. In this study, we exploited this phenomenon as an easy way to separate attached/living cells and floating/dead cells. Thus, after various incubation times, the medium containing the nonadherent cells was removed, and the cells were collected. The remaining monolayer of attached cells was harvested by trypsinization. Viability of floating and attached cells was determined by PI staining and microscopic visualization. Only samples of attached cells with >85% of viable cells were used for further studies. The floating cell population was 100% PI-positive.

**Cytotoxicity Assays**—Cytotoxicity was measured using lactate dehydrogenase leakage of damaged cells. LDH was expressed as a percentage of total cellular lactate dehydrogenase activity, as described by Decker and Lohmann-Matthes (30). Lactate dehydrogenase activity was measured using a commercial assay kit (Cromatex, Laboratorios Knickerbocker, S. A. E., Barcelona, Spain).

**DNA Fragmentation Analysis**—DNA fragmentation was measured by quantifying hypoploid nuclei after DNA staining with PI. L929 cells were harvested, fixed with 2% formaldehyde, and ethanol at 4 °C overnight. After centrifugation, the fixed cells were resuspended in 1 ml of PI staining solution (5 μg/ml propidium iodide in 0.1% sodium citrate, 0.1% Triton X-100), followed by incubation for 30 min at 0 °C. Stained nuclei were analyzed on the FACScan (Becton Dickinson Immunocytometry System, San Jose, CA). Hypoploid cells appeared as a sub-G₁ peak.

**Measurement of Intracellular Generation of ROS**—Flow cytometric analysis of intracellular generation of ROS was performed using dihydorhodamine 123 as probe (21). Cells were cultured in six-well plates, and at confluence (1 × 10⁵ cells/well) they were treated with TNFα (25 ng/ml), CHX (0.1 mM/liter), or a combination of TNFα/CHX. After 6 h of incubation, dihydorhodamine 123 (1 μM) was added and the incubation was continued for an additional 4 h. The cells were harvested, washed, centrifuged for 5 min at 1000 rpm, resuspended in RPMI 1640 medium, and analyzed by flow cytometry (excitation, 488 nm; emission, 530 nm). Rhodamine 123 fluorescence was analyzed in viable cells. To measure intracellular hydroperoxide production, we determined the conversion by endogenous esterases of 2,7′-dichlorofluorescein diacetate to membrane-impermeant 2,7′-dichlorofluorescein, which reacts with hydroperoxides to form highly fluorescent 2,7′-dichlorofluorescein (DCF) (31). Cells were cultured as above in the presence of 2,7′-dichlorofluorescein diacetate (1 μM). After 6 h, the cells were washed, resuspended in RPMI medium, and analyzed by flow cytometry (excitation, 488 nm; fluorescent detection between 515 and 565 nm). DCF fluorescence was analyzed in viable cells only.

**Determination of Lipid Peroxydation**—Lipid perxydation was determined by measuring thiobarbituric acid-reacting substances (TBARS) as described by Ohkawa et al. (32). TBARS were measured fluorometrically (excitation, 515 nm; emission, 553 nm) using malondialdehyde and tetramethoxypropane standards.

**Cellular Glutathione**—Cellular glutathione was measured using the Eddy et al. modification (33) of Tietze's assay (34).

**Cytochrome Activity in Whole Cells**—Cells were treated with TNFα, CHX or the combination TNFα/CHX for 8 h. At this time, the attached cells were harvested, centrifuged, and resuspended in respiration buffer (0.25 M sucrose, 0.1% bovine serum albumin, 10 mM MgCl₂, 10 mM K⁺ Heps, 5 mM KH₂PO₄, pH 7.2) at a final concentration of 2 × 10⁷ cells/ml. One-half ml of the suspension was injected into a chamber containing 3.5 ml of air-saturated respiration buffer and 1 mM ADP at 37 °C. The cells were permeabilized with digitonin (final concentration 0.005%), and substrates and inhibitors were added in the following order and final concentrations: antimycin A, 50 mM; ascorbate, 1 mM; tetramethyl-p-phenylenediamine (TMPD), 0.4 mM. Antimycin A was used to inhibit autologous mitochondrial electron transport. TMPD is an electron donor that reduces cytochrome c nonenzymatically. Once TMPD is added, as a substrate, changes in O₂ uptake rates reflect changes in cytochrome c oxidase activity. Ascorbate was used to reduce TMPD. Oxygen consumption was calibrated with air-saturated buffer, assuming 390 ng atoms of oxygen/ml of buffer. Rates of potassium cyanide-sensitive oxygen consumption are expressed as ng atoms of oxygen/min/1 × 10⁶ cells.

**Western Blot Analysis of Cytosolic and Mitochondrial Fractions**—Cytosolic and mitochondrial fractions were prepared as described (36). Potential mitochondrial contamination of the cytosol was monitored by Western blotting for cytochrome c oxidase subunit I. Twenty-five μg of cytosolic proteins were separated on a 15% denaturing SDS-polyacrylamide gel electrophoresis minigel. After protein transfer, the membrane was incubated with various primary antibodies. Anti-cytochrome c monoclonal antibody (clone 7H8.2C12; Molecular Probes, Inc., Eugene, OR) was diluted 1:1000; anti-GAPDH monoclonal antibody (clone 6C5; Research Diagnostic, Inc., Flanders, NJ) was diluted 1:5000; anti-CPP32 polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was diluted 1:1000; and anti-PARP polyclonal antibody (Roche Molecular Biochemicals) was diluted 1:1000. The membrane was incubated with the corresponding secondary antibody coupled to horseradish peroxidase at 1:10,000 dilution. The specific protein complexes were identified using the "Supersignal" substrate chemiluminescence method (Pierce).

**Fluorescent Microscopy Inspection of Nuclear Morphology**—L929 cells were cultured on 1-mm glass coverslips and treated with TNFα/CHX for 8 h. Both attached and floating cells were then stained with Hoechst 33342 (1 μg/ml) and PI (5 μg/ml) and analyzed under a fluorescence microscope (Zeiss).

**Spectrophotometry**—The redox state of mitochondrial cytochromes was studied by absorption spectroscopy as described by Chance (37). Cultures of confluent L929 cells were incubated in RPMI 1640 in the absence (control) or presence of 25 ng/ml TNFα and 0.1 mM/liter CHX for 2, 3, 4, and 8 h. After the indicated times, floating cells were removed, and attached cells were harvested by trypsinization and re-suspended in 2.5 ml of phosphate-buffered saline/albumin (0.1%) at a final concentration of 30 × 10⁶ cells/ml. Control cells were placed into the reference cuvette, and TNFα/CHX-treated cells were placed into the sample cuvette. The difference absorption spectra were recorded on an Aminco DW2000 spectrophotometer (SLM-AMINCO, Urbana, IL) at room temperature and with continuous stirring. The spectra were obtained in a path length of 1 cm with a scan speed of 2 nm/s and a slit width of 2 nm.

**Electron Microscopy**—L929 cells were treated with 25 ng/ml TNFα and 0.1 mM/liter CHX for 0, 3, 6, and 8 h. The growth medium and floating cells were removed by two washes with phosphate-buffered saline and replaced by Karnovsky's reagent. The floating cells were resuspended in Karnovsky's reagent and incubated at 4 °C, with periodic agitation, for 2 hours. After fixation for 30 min at room temperature, attached cells were washed in 0.1 mM cacydolate buffer, rinsed with 0.1 mM cacodylate buffer (pH 7.4), and postfixed in 2% osmium tetroxide for 10 min. Cells were dehydrated with increasing concentrations of ethanol, and, after passage through propylene oxide, the blocks were embedded in Epon 812 according to standard technique (38). Semithin, 1-μm cross-sections were stained with toluidine blue. Representative samples of the cells were chosen, and ultrathin 60–90-nm cross-sections were cut and mounted on bare copper grids. The staining was done with uranyl acetate and lead citrate. The samples were examined in a Hitachi H-12A and in a JOEL-100SX microscope. An independent, trained, unbiased observer evaluated all cells in one section from each sample. The various mitochondrial conformations were classified into four different categories, and the number of cells with each conformation was counted. The four categories were defined according to the following criteria: 1) orthodox configuration, mitochondria with normal matrix density and regularly spaced and oriented cristae; 2) hyperdense configuration, cristae with increased density and normal orientation and increased matrix density; 3) ultracondensed configuration and distortion of the cristae, mitochondria with engrossed cristae that have lost their parallel configuration, increased volume in the outer and intracistriate compartments, decreased matrix volume, and increased matrix density; and 4) lytic configuration, swollen mitochondria with hydropic matrix, loss of cristae and often rupture of the outer membrane.

**Statistical Analysis**—All results are expressed as means ± S.D. unless stated otherwise. The unpaired Student's t test was used to...
evaluate the significance of differences between groups, accepting $p < 0.05$ as the level of significance.

RESULTS

TNFα/CHX Induces an Apoptosis-like Mode of Cell Death—In order to address the modality of cell death induced by TNFα/CHX treatment, several morphological and biochemical tests were performed. An increased number of cells showing the typical apoptotic feature of plasma membrane blebbing was detected by light microscopy (Fig. 1A). Flow cytometric analysis showed a 10-fold increase in the sub-G₁ population in TNFα/CHX-treated cells, suggesting the presence of DNA fragmentation (Fig. 1C), and Western blot analysis of cellular extract showed activation of caspase-3 and PARP cleavage in attached and floating cells. Cells were treated with TNFα/CHX for 8 h. At the indicated times, floating and attached cells were harvested separately and processed for Western blot analysis of caspase 3 and PARP as described under “Experimental Procedures.”

Together, these observations suggested that TNFα/CHX-treated L929 cells underwent a particular form of cell death, which was characterized by events common to both the apoptotic (DNA fragmentation, plasma membrane blebbing, caspase activation) and the necrotic pathways (early plasma membrane disruption). Oxidative Stress Induced by TNFα/CHX—Intracellular generation of ROS, hydroperoxide formation, lipid peroxidation, and glutathione levels. A, cellular generation of ROS was determined by flow cytometry using rhodamine 123 fluorescence. Cells were incubated for 6 h in the absence (C) or presence of 25 ng/ml TNFα (T), 0.1 mM CHX (CHX), or the combination of TNFα and CHX (T/CHX) for 6 h. Rhodamine 123 (R123) fluorescence was also measured in TNFα/CHX-treated cells co-incubated with 50 μM α-tocopherol (Tph). B, intracellular generation of hydroperoxide was measured by flow cytometry using DCF fluorescence. Cells were incubated as described for A. Data represent fluorescence at 6 h minus background due to DCF alone. C, intracellular content of lipid peroxides determined by measuring TBARS. Cells were incubated for 6 h as described for A. D, effect of α-tocopherol (Tph) on TNFα/CHX-induced cytotoxicity. L929 cells were incubated for 0–12 h with 25 ng/ml TNFα and 0.1 mM CHX in the absence (×) or presence of 50 μM α-tocopherol (Tph). Cytotoxicity was quantified as described under “Experimental Procedures.” E, effect of TNFα on the intracellular concentration of glutathione. Total glutathione was measured in cells incubated for 6 h in the absence (C) or presence of 25 ng/ml TNFα (T), 0.1 mM CHX (CHX), or TNFα/CHX (T/CHX). Results are means ± S.D. of three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ between control and experimental cells, a, $p < 0.001$ between TNFα or CHX and TNFα/CHX.
with TNFα in the presence of 0.1 mmol/liter CHX (TNFα/CHX), whereas increased ROS formation by CHX alone was not significant. Increased ROS formation induced by TNFα/CHX treatment was prevented by co-incubation of cells with 50 μM α-tocopherol, a well established antioxidant.

The fluorescent probe DCF was used to measure intracellular hydroperoxide formation. Treatment of L929 cells with 25 ng/ml TNFα/CHX for 6 h led to increased intracellular hydroperoxide formation (Fig. 2D). Again, this increase was significantly higher in cells treated with TNFα/CHX, whereas increased hydroperoxide formation by CHX alone was not significant.

Next, we also assessed whether these treatments induced lipid peroxidation by measuring TBARS. Incubation of cells with TNFα/CHX for 6 h increased the production of TBARS 2.78 ± 0.13-fold (p < 0.001), which was prevented by co-incubation with 50 μM α-tocopherol (Fig. 2C). Treatment of cells with TNFα alone also enhanced TBARS production significantly (p < 0.01), although the increase was less marked than with the combination treatment. CHX alone again did not significantly change the production of TBARS.

To investigate the antioxidant reserves under TNFα/CHX treatment, we analyzed the levels of glutathione. Although incubation of cells with TNFα or CHX alone for 6 h resulted in a significant decrease in total cellular glutathione, this effect was again intensified by treating cells with TNFα/CHX (p < 0.001) (Fig. 2E).

Finally, we showed that both TNFα/CHX-induced cytolysis (Fig. 2D) and DNA fragmentation (Fig. 1C) were prevented by the antioxidant α-tocopherol.

Release of Cytochrome c Shifts the Steady-state Reduction of Mitochondrial Cytochromes—Recently, the release of mitochondrial cytochrome c has been proposed as a critical event in both apoptosis and necrosis. Cytochrome c is the mobile redox protein that shuttles electrons from complex III to complex IV. Therefore, its release and exclusion from the electron transport chain can result in dysfunction of the normal electron flow. As a consequence, electrons are retained upstream of cytochrome c and can alter the steady-state reduction of mitochondrial cytochromes. The accumulation of reducing equivalents in some components of the respiratory chain might promote free radical generation. Therefore, to examine a possible association between mitochondrial cytochrome c release and the steady-state reduction of mitochondrial cytochromes, both events were monitored simultaneously over several hours after TNFα/CHX treatment.

To study cytochrome c release, cytosolic extracts were prepared at various times after the addition of TNFα/CHX under conditions that kept mitochondria intact, and cytosolic and mitochondrial cytochrome c protein levels were measured by immunoblot analysis. Cytochrome c accumulated in the cytosol within 2 h of treatment with TNFα/CHX and reached maximum levels at 8 h (Figs. 3A and 7B). Concomitantly, cyto-
treated with TNF 
substrate, in TNF 
activation of caspase-3 and the cleavage of PARP, a caspase-3 
cytosolic caspases in our model system, we also examined the 
activity. Thus, the loss of cytochrome 
c results in a reduction of cytochrome 
and an interruption of normal electron flow that could divert elec-
crondia, one might expect an inactivation of cytochrome c 
oxidase-dependent oxygen uptake when using ascorbate/TMPD 
as electron donor. As shown in Fig. 3C, TNFα/CHX treatment 
did indeed result in a reduction of cytochrome c oxidase activity. 
In order to study the effect of cytochrome c release on the 
redox state of the mitochondrial cytochromes, difference 
spectra between control cells and cells treated with 25 
ng/ml TNFα and 0.1 mM CHX were performed. Fig. 4 shows 
typical difference spectra between control cells and cells 
treated for different times with TNFα/CHX. After 2 h of incub-
ation, two small peaks located at 428 and 438 nm and two 
valleys at 550 and 605 nm were observed, which became dra-
matically more prominent at 3 h. At this time, the difference 
spectrum showed two large peaks at 428 and 438 nm and two 
deep valleys at 420 and 445 nm of the Soret region. Likewise, a 
pronounced shoulder appeared at 558–560 nm and two valleys 
appeared at 550 and 605 nm of the α region of the spectrum. A 
similar pattern was also observed at 4 and 8 h, although there 
was a gradual disappearance of the peak at 428 nm, probably 
due to the greater predominance of the peak at 438 nm. These 
results indicate that cytochrome b is reduced, while cyto-
chomes cc, and aa, are oxidized.

Together these observations suggest that cytochrome c re-
lease is associated with and possibly precedes the accumulation 
of reducing equivalents at the cytochrome b level.

**Effect of Mitochondrial Respiratory Chain Blockade on 
TNFα/CHX-induced Cytotoxicity and ROS Generation**—Previ-
ously, we have shown that TNFα/CHX treatment induced a 
marked increase in ROS generation, which was abolished or 
strongly reduced by the simultaneous addition of mitochondrial 
inhibitors (39). These results suggested that the mitochondrial 
electron transport chain was the likely source of ROS. In order 
to investigate which component of the mitochondrial respira-
tion chain was responsible for ROS production, we used a 
different strategy in the present study. Various inhibitors of 
the mitochondrial respiratory chain were added to the cells 
only after 3 h of incubation with TNFα/CHX when cytochrome 
b had already become reduced. Under these conditions, only 

| Inhibitors added after 3 h of incubation | Control | TNFα/CHX |
|-----------------------------------------|---------|----------|
| +0                                      | 77 ± 10 | 254 ± 15*|
| +Ro                                     | 76 ± 11 | 215 ± 34*|
| +TTFA                                   | 78 ± 12 | 205 ± 26*|
| +AA                                     | 72 ± 10 | 58 ± 16**|
| +Mx                                     | 77 ± 9  | 210 ± 23*|
| +KCN                                    | 74 ± 6  | 192 ± 28*|
| +Oligo                                  | 76 ± 5  | 227 ± 23*|

**TABLE I**

Effect of mitochondrial inhibitors on the TNFα/CHX-induced 
intracellular generation of ROS

L929 cells were incubated for 6 h with 25 ng/ml TNFα and 0.1 mM 
CHX. After 3 h, one of the following mitochondrial inhibitors was added 
and was present for the last 3 h of incubation: control (+0), 1 mM 
rotenone (+Ro), 400 μM TTFA (+TTFA), 30 μM antimycin A (+AA), 
2 μM myxothiazol (+Mx), 1 mM cyanide (+KCN), or 10 μg/ml oligomycin 
(+Oligo). Generation of ROS was quantified by flow cytometry using 
R123 fluorescence. Results are expressed as means ± S.D. of three 
different experiments. *, p < 0.001 between control and experimental 
cells (TNFα/CHX); **, p < 0.001 between the absence and the presence 
of inhibitors of mitochondrial respiration in TNFα/CHX-treated cells.
antimycin A caused a marked decrease in the ROS generation induced by TNFα/CHX treatment (Table I). Since antimycin A specifically binds to cytochrome b, this observation strongly suggested that this cytochrome may be involved in ROS formation. The effect of antimycin A was also associated with significantly decreased TNFα/CHX-induced cytoxicity (Fig. 5), and the protection by antimycin A was dose-dependent (Fig. 6A). Furthermore, flow cytometric analysis revealed a decrease in the sub-G₁ population in TNFα/CHX-treated cells after the addition of antimycin A (Fig. 1C). In contrast, neither of the other inhibitors of complex b-c₁, particularly myxothiazol, as well as blockers of complex I (rotenone), complex II (TTFA), cytochrome c oxidase (KCN), and the ATPase (oligomycin) affected ROS production or protected cells from the cytidal effect of TNFα/CHX (Table I, Fig. 5). The lack of an effect of these mitochondrial inhibitors was dose-independent (rotenone: 0.24, 0.48, 1, and 2 μM; TTFA: 50, 200, and 400 μM; myxothiazol 0.5, 1, 2, and 10 μM; KCN: 0.1, 0.5, 1, and 10 mM; oligomycin: 0.05, 0.1, 1, 1.2, 10, and 20 μg/ml), since none prevented ROS generation or increased the resistance of cells against the cytidal effect of the treatment (data not shown).

Free Radicals Enhance Mitochondrial Cytochrome c Release—To determine whether preventing free radical generation could arrest cytochrome c release, cytochrome c levels in the cytosol of L929 cells treated with TNFα/CHX followed by the addition after 3 h of antimycin A or α-tocopherol were measured. The results showed that when added after 3 h, at a time when cytochrome b was already reduced, both antimycin A and α-tocopherol prevented further cytochrome c release (Fig. 7, A and B). This observation suggested that free radical production initially triggered by cytochrome c loss could provide a positive feedback mechanism that can amplify cytochrome c release.

Effects of TNFα/CHX on Mitochondrial Ultrastructure—Normal L929 cells showed a relatively electron-dense cytoplasm due to its content rich in free ribosomes and polyribosomes and with the mitochondria in their typical orthodox configuration (40) with regularly spaced and oriented cristae and a homogeneous matrix (Fig. 8A). Incubation in the presence of 25 ng/ml TNFα and 0.1 mM CHX induced significant changes in mitochondrial appearance (Table II). After 3 h of treatment, 44% of cells showed mitochondria with a hyperdense configuration (Fig. 8B). The percentage of cells with this mitochondrial morphology increased to 84 and 66% after 6 and 8 h of incubation, respectively. Moreover, at these time points, 12 and 15% of cells showed mitochondria with ultracondensed and distorted cristae (Fig. 8, C–E), and at 8 h 19% of the cells displayed mitochondria with a lytic pattern (Fig. 8F). All of the floating cells showed mitochondria with a lytic configuration. No myelin-like figures, inclusions, or granules were seen in mitochondria from TNFα/CHX-treated cells. These data clearly indicate that there was a distinct deterioration of mitochondria preceding cell death.

![Figure 6](http://www.jbc.org/)

**Fig. 6.** Dose-response curves of antimycin A-mediated protection from TNFα/CHX-induced cytoxicity. A, cells were preincubated with TNFα/CHX for 3 h and then treated with increasing concentrations of antimycin A: 0 μg/ml (×), 10 μg/ml (■), 20 μg/ml (▲), and 30 μg/ml (●). Cytotoxicity was determined at the indicated times as described under “Experimental Procedures.” B, cytotoxicity of increasing concentrations of antimycin A alone (■, 10 μg/ml; ▲, 20 μg/ml; ●, 30 μg/ml). Results represent percentage of dead cells. Data are the means ± S.D. of three experiments.

![Figure 7](http://www.jbc.org/)

**Fig. 7.** Effect of antimycin A and α-tocopherol on TNFα/CHX-induced mitochondrial cytochrome c release. A, Western blot analysis of cytosolic extracts from cells treated for 8 h with TNFα/CHX, TNFα/CHX with 30 μg/ml antimycin A (AA), or 50 μM α-tocopherol (Tph) added at 3 h. Membranes were probed with antibodies against cytochrome c and GAPDH. The last right-hand lane contained 10 ng of horse heart cytochrome c as a standard. GAPDH protein levels confirmed that the same amount of protein was loaded in each lane. B, quantitation of cytochrome c release. The blots in Figs. 3A and 7A were analyzed by densitometry, and the density of the bands was graphed against the time of treatment. ■, no inhibitor; ●, antimycin A; ●, α-tocopherol. The data are the means ± S.D. of two separate experiments. The arrow indicates the time of antimycin A (AA) or α-tocopherol (Tph) addition.
**FIG. 8. Structural changes in mitochondria induced by TNFα/CHX in L929 cells.** A, normal L929 cells showing a relatively electron-dense cytoplasm and mitochondria with typical, orthodox configuration (magnification, ×11,500). B, mitochondria of cells treated with TNFα/CHX for 3 h showing a hyperdense configuration (magnification, ×11,500). C, ultracondensed mitochondria of cells treated for 6 h with TNFα/CHX (magnification, ×25,000). D, cells treated for 6 h with TNFα/CHX showing mitochondria with twisted, rounded, and condensed cristae (magnification, ×51,000). E, ultracondensed configuration in mitochondria of cells treated for 6 h with TNFα/CHX (magnification, ×25,000). Some mitochondria have lost their external membrane (arrow). F, mitochondria of cells treated with TNFα/CHX for 8 h showing a swollen and rounded appearance with clear matrix, fragmented cristae, and breaks in their external membrane (magnification, ×28,500).

**DISCUSSION**

The mechanism(s) of TNFα cytotoxicity is currently incompletely understood, although mitochondrial function has been suggested to be essential. For example, treatment of a number of transformed cell lines with TNFα caused morphological alterations of the mitochondria (6, 8), inhibition of mitochondrial electron transfer (7, 8), and a decrease in the mitochondrial membrane potential (11, 40). Moreover, manganous superoxide dismutase, a mitochondrial matrix enzyme, is synthesized in response to TNFα in target cells (9, 41–43). This enzyme has been shown to prevent mitochondrial damage and to protect cells from TNFα cytotoxicity (41). Because manganous superoxide dismutase is also involved in the dismutation of superoxide anions into hydrogen peroxide, it has been proposed that superoxide radicals or other ROS might participate in TNFα-induced cytotoxicity (8–10, 20). Our results support this mechanism of action, since TNFα/CHX treatment increased ROS generation, enhanced intracellular hydroperoxide formation and lipid peroxidation, and reduced the intracellular glutathione concentration. Moreover, α-tocopherol, a lipophilic antioxidant, decreased the intracellular concentrations of ROS and lipid peroxides and prevented TNFα/CHX cytotoxicity and DNA fragmentation.

The exact site in the mitochondrial respiratory chain responsible for the formation of ROS has not been defined, although a number of authors have demonstrated a relationship between the redox state of cytochrome b and the formation of ROS (25,26,44). The key role played by reduced cytochrome b in the generation of ROS and the pathogenesis of TNFα/CHX cytotoxicity is supported by our observation that antimycin A, when added to the cells after cytochrome b has been reduced, blocked the formation of ROS and prevented cell death. According to the widely accepted proton motive cycle (45, 46), the cytochrome bc1 complex has two ubiquinone-reactive sites: center P, where ubiquinol is oxidized, and center N, where ubiquinone is reduced (Fig. 9). Center P is inhibited by myxothiazol, and center N is blocked by antimycin A. Antimycin A is a quinone analog that binds to the quinone site of cytochrome b562 and blocks the transfer of electrons from this cytochrome to ubiquinone (47–50). Antimycin A produces a conformational change of the bc1 complex (48), causes a red shift in the α and γ peaks of the reduced cytochrome b562 (48, 49), and prevents the ATP-induced increase in the redox potential of cytochrome b566 (50). Once cytochrome b had been reduced, treatment of cells with myxothiazol, which blocks oxidation of ubiquinol by the Rieske iron-sulfur center of complex III and prevents ubisemiquinone formation, did not inhibit the formation of ROS and did not protect cells from the cytoidal effect of TNFα combined with CHX. Thus, ubisemiquinone does not seem to be the source of electrons for the formation of ROS in this experimental model, a conclusion that is in agreement with results reported by Hennet et al. (10). These authors showed that the chemiluminescence signal generated by superoxide anions in TNFα-treated cells was abolished or strongly inhibited in the presence of antimycin A. In contrast, Schulze-Osthoff et al. (8) suggested that ROS are generated at the ubisemiquinone site. However, the present study does not support this conclusion, since we did not observe any increase in ROS formation when antimycin A was applied to TNFα-treated cells. Likewise, other inhibitors of cellular respiration, added to the cells only after 3 h of treatment with TNFα/CHX, prevented neither ROS generation nor the cytoidal effect of the treatment. Together, these results suggest that TNFα added to metabolically inhibited cells causes electrons to be retained preferentially along the cytochrome b-related pathway, instead of being transferred through the Rieske iron-sulfur center to the cytochrome c oxidase complex. This electronic shift could be a response to the early loss of cytochrome c, which functions to shuttle electrons

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**TABLE II**

**Time course of mitochondrial conformational changes induced by TNFα/CHX in L929 cells**

| Mitochondrial configuration | Treatment with TNFα/CHX | Floating cells |
|----------------------------|-------------------------|---------------|
|                            | 0 h         | 3 h            | 6 h            | 8 h            |               |
|                            | %           | %              | %              | %              | %             |
| Orthodox                   | 99          | 56             | 0              | 0              | 0             |
| Hyperdense                 | 1           | 44             | 84             | 66             | 0             |
| Ultracondensed and         | 0           | 0              | 12             | 15             | 0             |
| Distortion of cristae      |             |                |                |                |               |
| Lytic                      | 0           | 0              | 4              | 19             | 100           |
| Number of cells examined   | 165         | 178            | 169            | 128            | 150           |

Cells were incubated for the indicated times with 25 ng/ml TNFα in the presence of 0.1 mM CHX. At each time point, cells were collected and processed as described under “Experimental Procedures.” Orthodox configuration, mitochondria with regularly spaced and oriented cristae, matrix of normal density. Hyperdense configuration, mitochondria with hyperdense and engrossed cristae and increased matrix density. Ultra-condensed and distortion of cristae, mitochondria with ultracondensed or twisted cristae that have lost their parallel configuration. Lytic configuration, rounded, swollen mitochondria with pale matrix, fragmented cristae, and often rupture of the outer membrane. Data are shown as percentage of cells with the indicated mitochondrial morphology at each time point.
from the Rieske iron-sulfur center to cytochrome c oxidase. Consequently, electrons accumulated in reduced cytochrome b may be transferred to molecular oxygen, leading to the generation of ROS.

Our results suggest a model in which TNFα/CHX treatment initially results in cytochrome c translocation to the cytosol, which compromises mitochondrial electron flow and triggers ROS formation. ROS in turn stimulates further release of cytochrome c, which leads to more ROS formation. According to this model then, one would expect that blocking ROS formation would prevent further cytochrome c release. The data in Fig. 7B support this hypothesis. When antimycin A or α-tocopherol was added to cells 3 h after TNFα/CHX, no further release of cytochrome c occurred. Concomitantly, DNA fragmentation and cell death were significantly delayed (Figs. 1C and 2D).

The molecular mechanism(s) for the release of cytochrome c from mitochondria to the cytosol during apoptosis and necrosis is unknown. However, a number of different models have been proposed to explain cytochrome c translocation. These include pore formation by the translocation to the mitochondrial membrane of proteins such as BAX (51, 52), the induction of the mitochondrial permeability transition (27), the disruption of the outer membrane (53), and the activation of specific caspases (54, 55). Moreover, it has been proposed that Bel-2 family proteins block cytochrome c release by preventing ROS generation (56–59), implying a direct role for ROS. This hypothesis is supported by our data that demonstrated that the blocking of ROS generation with antimycin A or α-tocopherol prevented further cytochrome c release. When we compared the time course of cytochrome c release and cytochrome b reduction (Figs. 4 and 7B), the data suggested that initially cytochrome c release preceded cytochrome b reduction. At 2 h, when there was little cytochrome b reduction, a substantial amount of cytochrome c had already been released. As cytochrome b became reduced, more cytochrome c was released, supporting our model of a positive feedback mechanism.

Assuming that cytotoxicity of TNFα is mediated by ROS and mitochondria are the major source of these free radicals, one might expect that these organelles would develop early structural changes preceding cell death. Consistent with this hypothesis, electron microscopy of L929 cells treated with TNFα/CHX for 3–8 h revealed that most cells exhibited mitochondria with hyperdense or ultracondensed and distorted cristae. Furthermore, the matrix volume was decreased, and its electron density was increased. The cristae became more electron-dense, rounded, protruding, or twisted, or they contained enlarged intra cristae spaces. Some mitochondria also lost their outer membrane. These effects increased gradually as time progressed, until at 8 h, 19% of the cells contained mitochondria with a lytic configuration. They had become large and rounded, with fragmented cristae and a clear matrix, and frequently contained a ruptured outer membrane.

These observations are reminiscent of those by Matthews (60), who in cells treated with TNFα alone found enlarged and translucent mitochondria, and by Schulze-Osthoff et al. (8), who described the appearance of mitochondria with rounded and electron-dense cristae and onion-like structures inside the matrix. The sequence of structural changes observed in the mitochondria of TNFα-treated cells has also been found in many other models of cell injury (61–63). The mechanisms leading to these conformational changes are, however, not well understood. The condensed conformation has been related to the blockade of mitochondrial electron transport (64), the drop of the cellular ATP/ADP ratio (65), and the loss of ions and water (66). Swelling and subsequently rupture of the outer mitochondrial membrane have been proposed as a mechanism for the release of cytochrome c into the cytosol (53), events that are usually associated with the mitochondrial permeability transition (66, 67) and with loss of Δψm (67). However, the predominance of hyperdense mitochondria seen in our system at a time when substantial cytochrome c release has already occurred indicated that swelling of mitochondria is unlikely to be the primary mechanism of cytochrome c release. This observation was also consistent with results reported by Dinsdale et al. (68), which showed that ultracondensed but not swollen mitochondria were involved in apoptotic monocytes.

In conclusion, we found that TNFα in metabolically inhibited L929 cells induced the release of mitochondrial cytochrome c, the reduction of cytochrome b, and the oxidation of cytochromes aa3 and cytochrome c1. We propose that the release of cytochrome c leads to the reduction of cytochrome b, which in turn favors the formation of ROS and the lipid peroxidation of cell membranes, and ultimately leads to plasma permeabilization and cell death.

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Tumor Necrosis Factor-α Increases the Steady-state Reduction of Cytochrome b of the Mitochondrial Respiratory Chain in Metabolically Inhibited L929 Cells

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