Tumor Necrosis Factor-α Increases ATP Content in Metabolically Inhibited L929 Cells Preceding Cell Death

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The effects of tumor necrosis factor-α (TNF) on ATP levels were studied in metabolically inhibited L929 cells. Treatment of these cells with TNF in the presence of actinomycin D or cycloheximide induces cyclic changes in the intracellular ATP content preceding cell death. After 3 h of incubation, the intracellular ATP content increased by 48 ± 6% (p < 0.001), but at 4 h, it decreased to the control level. Two hours later, it increased again by 23 ± 5% over the control level (p < 0.001). Coinciding with cell death, ATP content decreased progressively until almost complete depletion. These changes in ATP content were associated with parallel alterations in the respiratory coupling and with increased generation of reactive oxygen species. The mechanism by which TNF/actinomycin D or TNF/cycloheximide increased cellular ATP seemed to be dependent on the mitochondrial ATP synthesis and related to the cytotoxic effect of TNF, since blockade of mitochondrial electron transport prevented the increase in cellular ATP, the formation of reactive oxygen species, and the apoptotic cell death caused by TNF. We suggest that the TNF/actinomycin D-or TNF/cycloheximide-induced changes in intracellular ATP levels may be involved in the cytotoxic effect of TNF in metabolically inhibited L929 cells.

Experimental Procedures

Materials

Recombinant human TNF was purchased from Genzyme Co. (Cambridge, MA); RPMI 1640 medium was from Biochrom (Berlin, Germany), and Dulbecco’s modified Eagle’s medium was from Bio-Whittaker (Verviers, Belgium). Rotenone, AD, CHX, antimycin A, oligomycin, trypsin, Hepes, Mops, EDTA, proteinase K, Triton X-100, ethidium bromide, propidium iodide, mithoxanthol, thenoyltrifluoroacetone (TTFA), SDS, and pyruvate kinase were purchased from Sigma (Alcobendas, Spain). Potassium cyanide (KCN) was from FEROSA (Barcelona, Spain); t-glutamine, penicillin, streptomycin, and phosphate-buffered saline (PBS) were from ICN Biomedicals Inc. (Costa Mesa, CA); and trichloroacetic acid was from Panreac (Barcelona, Spain). Potassium hydroxide and Tris were from Merck (Barcelona, Spain). Fetal calf serum was from Sera-Lab (Sussex, UK). Dihydroxycholine 123 (DHR-123) was purchased from Molecular Probes (Eugene, OR).

Methods

Cell Cultures—L929 murine fibrosarcoma cell line and HepG2 human hepatoma cell line were obtained from the American Type Culture Collection (Rockville, MD). L929 cells were grown in RPMI 1640 medium, and HepG2 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, penicillin (100 units/ml), and streptomycin (0.1 mg/ml) at 37 °C and 5% CO2. For TNF cytotoxicity, lactate, and ATP/ADP assays, cells were cultured in six-well plates (35-mm diameter well). At
confluence (1 × 10^6 cells/plate), the medium was replaced with fresh RPMI 1640 medium in the absence (control) or presence of 25 ng/ml TNF, 1 μg/ml AD, 0.1 μM CHX or the combined treatment, TNF and AD (TNF/AD) or TNF and CHX (TNF/CHX), for the times indicated under "Results" and figure legends. Mitochondrial inhibitors were added at the indicated concentrations to TNF/CHX. ATP/ADP assay kit (Sigma). ATP was converted into ATP by pyruvate kinase (22). Luminescence was measured in a bioluminometer equipped with injector (Lumat LB 9501, Berthold). Separation of cytosolic and mitochondrial compartments was carried out by digitonin fractionation (23). After the indicated times, L929 cells were harvested by trypsinization and suspended in ice-cold medium containing 0.2% BSA. Flow cytometric analysis (24) was measured in the supernatant to ensure a proper permeabilization (>95%) of L929 cells without mitochondrial contamination. ATP/ADP ratios were calculated from these data.

**DNA Fragmentation Analysis—**For DNA fragmentation analysis, 3 × 10^5 L929 cells were exposed to TNF, AD, CHX, TNF/CHX, or TNF/AD for 8–72 h. Cells were centrifuged and washed with cold phosphate-buffered saline, and the pellet was lysed by the addition of 400 μl of a hypotonic lysis buffer consisting of 10 mM Tris, pH 7.5, 1 mM EDTA and 0.2% Triton X-100. Microfuge tubes were spun at 13,000 rpm for 15 min, and 350 μl of supernatant were incubated with 106 μl of lysis buffer (150 mM NaCl, 10 mM Tris-HCl, pH 8, 40 mM EDTA, 1% SDS, and 0.2 mg/ml proteinase K, final concentrations) for 4 h at 37 °C. DNA was treated with phenol/chloroform/isooamylic acid (25:25:1, v/v/v) and precipitated in ice-cold 100% ethanol and 4 mM NaCl at −20 °C for 12–18 h. After centrifugation for 5 min at 13,000 rpm and 4 °C, the DNA pellet was washed with 500 ml of 70% ethanol and resuspended in 15 μl of 10 mM Tris, 1 mM EDTA, pH 8.5, and 50 μg/ml RNase for 1 h at 37 °C. Loading buffer (2 μl) was added to each sample. Samples were analyzed on 1% agarose gel containing 0.1 μg/ml ethidium bromide. The same amount of DNA, as assessed by spectrophotometric measurement, was loaded in each lane. A mixture of HenIII-digested dX174 DNA and λ HindIII-digested DNA was run as a size marker.

DNA fragmentation was also measured by quantitation of apoptotic cell death on single cell level using a flow cytometric kit (in situ cell death detection kit, fluorescein; Boehringer Mannheim, SA, Barcelona, Spain). This test is based on the detection of single- and double-stranded DNA breaks occurring at early stages in apoptosis. This assay was performed according to the manufacturer’s protocol. Briefly, cells were washed twice in PBS containing 1% bovine serum albumin and fixed in PBS containing 2% paraformaldehyde for 30 min at room temperature. After one wash with PBS, cells were permeabilized for 2 min on ice in 0.1% Triton X-100, 0.1% sodium citrate. After two washes with PBS, cells were resuspended in 50 μl of TUNEL mixture (terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling) reaction and incubated for 60 min at 37 °C. Cells were washed twice before they were analyzed by flow cytometry.

Flow cytometric analysis of apoptosis was also performed after DNA staining with propidium iodide as described by Nicoletti et al. (20). L929 cells were treated without or with TNF, TNF/AD, TNF/CHX, or AD, for 24 h. After the treatment period, cells were harvested, centrifuged (500 rpm for 5 min), washed once with PBS, and resuspended in 1 ml of propidium iodide staining solution (5 μg/ml propidium iodide in 0.1% sodium citrate, 0.1% Triton X-100). Cell suspensions were then incubated for 30 min at 4 °C. Stained nuclei were analyzed with an Elite Flow Cytometer (Coulter Electronics, Hialeah, FL). Hypodiploid apoptotic cells and apoptotic bodies appear as a sub-G0/G1 peak. The intensity of this peak is related to the amount of apoptotic cells.

**Measurement of Oxygen Consumption—**L929 cells were harvested by trypsinization at the indicated times. After centrifugation for 10 min (4 °C, 500 × g), the pellet of packed cells was resuspended in RPMI medium without serum (1 × 10^6 cells/ml) and placed in a polargraphic chamber at 37 °C. Oxygen consumption was measured using a Clark-type electrode (Yellow Springs Instruments Co.) fitted to a 600-μl thermally insulated sample chamber (37 °C) under constant stirring. The oxygen consumption rate of the whole cells was calculated by assuming that dissolved oxygen concentration in 1 ml of media was initially 200 nmol/ml (21). The initial rate of oxygen consumption observed after cells were added to the chamber was termed the basal respiration. To determine the fraction of respiration attributable to coupled oxidative phosphorylation, oligomycin (20 μg/ml), which inhibits the Fo,F1-ATPase, was added during cellular basal respiration. To determine the nonmitochondrial respiration, KCN (1 mM), an inhibitor of the cytochrome c oxidase, was added during cellular basal respiration. Oxygen consumption rates are expressed as nmol of oxygen/min/10^6 cells.

Lactate Assay—L-Lactic acid was determined in the culture media using a commercial kit (Boehringer Mannheim, Germany). Results are expressed in nmol/10^6 cells.

**ATP/ADP Assay—**Cellular, cytosolic, and mitochondrial content of ATP and ADP were measured in trichloroacetic acid (10%, w/v)-precipitated samples using the luciferin/luciferase reaction with an adenosine 5'-triphosphate bioluminescent assay kit (Sigma). ATP was converted into ATP by pyruvate kinase (22). Luminescence was measured in a bioluminometer equipped with injector (Lumat LB 9501, Berthold). Inhibitors—couples oxidative phosphorylation, oligomycin (20 μg/ml), which inhibits the Fo,F1-ATPase, was added during cellular basal respiration. Oxygen consumption rates are expressed as nmol of oxygen/min/10^6 cells.

**RESULTS**

Sensitivity of L929 Cells to TNF Is Enhanced by Metabolic Inhibitors—In this investigation, we studied the cytotoxic activity of TNF toward L929 cells metabolically inhibited with AD or CHX for 24 h. Fig. 1 shows that murine L929 cells are

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susceptible to the cytotoxic action of TNF. However, sensitivity of these cells to this cytokine is enhanced significantly in the presence of CHX or AD. In the presence of 1 μg/ml AD or 0.1 mM CHX, 25 ng/ml TNF increased the cell death from 7 ± 2% to 100% in 24 h (Fig. 1). Treatment with either CHX or AD also yielded cytotoxic effects on L929 cells, but cell death started later (12 ± 1 h) and was to a lesser extent than with combined TNF treatment (Fig. 1).

Apoptosis was identified on the basis of the occurrence of internucleosomal DNA cleavage on agarose gel electrophoresis. The electrophoretic pattern of the DNA extracted from TNF-treated cells showed fragments of molecular weights corresponding to multiples of about 180 base pairs at 48 and 72 h, but not at 24 h, of incubation (Fig. 2A). Similarly, laddering of DNA was induced in L929 cells treated with actinomycin D (1 μg/ml) for 24 h. Finally, DNA fragmentation was observed in cells exposed to TNF/AD for 8 h or more. These results were supported by the quantitative measurement of fragmented DNA using two different methods (TUNEL assay and sub-G0/G1 peak). The time course study of DNA fragmentation in L929 cells incubated with TNF/AD showed that significant DNA cleavage (17 ± 1.3%) was first seen by the TUNEL assay after 8 h of treatment, reaching 61.2 ± 5.8% and 100% after 12 and 24 h, respectively, of incubation (Fig. 2B). Similar results were obtained when apoptosis was quantified by measuring the sub-G0/G1 peak (Fig. 2C). Incubation of cells with TNF or AD alone also elicited DNA fragmentation, but the slope of the time curve was more attenuated than the slope of the curve caused by TNF/AD treatment. DNA fragmentation after 12 and 24 h of incubation with AD was 13.4 ± 1.9 and 22.5 ± 1.8%, respectively. At 24, 48, and 72 h of treatment with TNF, the DNA fragmentation was 8.2 ± 1.1%, 23.1 ± 9.3% and 39.4 ± 0.7%, respectively.

**TNF/AD and TNF/CHX Increase the Intracellular ATP Content**—TNF/AD and TNF/CHX induced a biphasic increase in intracellular content of ATP during the first 6 h of treatment (Fig. 3). After 3 h of incubation with TNF/AD or TNF/CHX, the ATP content in cells increased by 44 and 52%, respectively (p < 0.001, for both cases), over the basal level. At 4 h, ATP content had declined to control levels. However, 2 h later, cellular ATP content was enhanced again by 19 and 27%, respectively (p < 0.001, for both cases). These early increases were followed by a progressive decrease in cellular ATP content until 24 h, at which time cellular ATP was almost undetectable (Fig. 3). This late decrease coincided in time with the release of LDH, which began at 5–6 h of incubation and was steadily enhanced until 24 h. None of these changes were seen in untreated cells. The slight decrease in cellular ATP levels observed in control cells after 24 h of incubation can be ascribed to the depletion of nutrients in the culture medium. Treatment of L929 cells with AD or CHX, but not with TNF, enhanced intracellular ATP levels significantly for the 24 h of observation. However, these elevations were significantly less marked than those obtained after 2 and 3 h of treatment with TNF/AD or TNF/CHX. While these conditions resulted in the death of about 30% of cells at 12 h of incubation, treatment with TNF, AD, or CHX alone did not cause cell death at this time (Fig. 1). Treatment with TNF alone had no effect on cellular ATP levels during the 72 h of incubation, although about 60% of cells have died at this time, and 39% showed apoptotic features (Fig. 2).

Effects of TNF/AD on cytosolic and mitochondrial ATP content were similar to those found in total cellular ATP. The cytosolic ATP level reached its maximum at 2 h, and mitochondrial ATP reached its maximum 1 h later (Table I). At 4 h, both cytosolic and mitochondrial ATP decreased to control level. The ADP content did not change significantly during the 4 h of
treatment with TNF/AD (Table I). Therefore, determination of the ATP:ADP ratio, as a measurement of the energy charge, showed that at 2 h, TNF/AD increased this ratio markedly in the cytosolic compartment. These results obtained by a luminometric method were confirmed by $^{31}$P NMR spectroscopy of cellular extracts. After 2 h of incubation with TNF/AD, the area of the peaks assigned to ATP increased by $68 \pm 11\%$. This elevation was also significantly more marked than those induced by AD alone (AD, $43 \pm 10\%$; TNF/AD, $68 \pm 11\%, p < 0.01$). The intensity of the peaks assigned to AD did not change during treatment with TNF/AD or AD alone (Fig. 4). Peaks exhibited a significant decrease both in TNF/AD- and AD-treated cells.

To determine the relevance of these findings, we tested the ability of TNF to increase ATP levels in the human hepatoma HepG2 cell line. TNF added to these cells did not change cellular ATP significantly and did not induce cell death either. However, sensitivity of these cells to this cytokine increased in the presence of AD. Treatment of HepG2 cells with TNF/AD led to a marked increase in cell death at 24 h and to cyclic changes in the ATP content similar to those induced in L929 cells. Cellular ATP increased after 1 and 4 h of treatment but decreased at 3 and 6 h (Fig. 5A). Both ATP increase and cell death were significantly higher in cells treated with TNF/AD than in those incubated with AD alone (Fig. 5A and B).

Effects of TNF/AD and TNF/CHX Treatments on Lactate Production—Because glycolysis contributes to the formation of ATP, we studied the effects of these treatments on the glycolysis rate by measuring lactate concentration in the culture medium. As Fig. 6 shows, ATP accumulation in L929 cells cannot be ascribed to an increased glycolysis, since lactate levels in culture medium did not change significantly during the initial 2–3 h of incubation. After 4 h, both TNF/AD and TNF/CHX caused a significant accumulation of lactate in the medium, indicating that glycolysis increased after this time (Fig. 6).

Treatment of cells with either AD or CHX also resulted in an increase in the lactate concentration after 4 h of incubation, although it was significantly lower than the increase caused by the combined treatment with TNF. In the absence of metabolic inhibitors, TNF increased lactate moderately through all of the

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**TABLE I**

Effects of TNF and AD on cytosolic and mitochondrial ATP and ADP and the ratios on L929 cells

Cells were incubated with TNF (25 ng/ml) and AD (1 $\mu$g/ml) or TNF/AD for 2, 3 and 4 h. After the indicated incubation period, the cells were harvested and suspended in ice-cold medium containing sucrose, Mops buffer, EDTA, and digitonin. After 1 min, the suspension was centrifuged at 3000 x g, for 1 min. ATP and ADP contents were measured in the mitochondrial pellet and supernatant (cytosolic fraction) as described under "Experimental Procedures." Values shown are means ± S.D. of six independent experiments. ATP values were normalized to the number of surviving cells. *, $p < 0.05$; **, $p < 0.01$; ***$, p < 0.001$ between control and experimental cells.

| ATP | Cytosolic | Mitochondrial | Cytosolic | Mitochondrial | Cytosolic | Mitochondrial |
|-----|-----------|---------------|-----------|---------------|-----------|---------------|
|     | 2 h       |               | 3 h       |               | 4 h       |               |
| ATP | mmoles/10^6 cells | mmoles/10^6 cells | mmoles/10^6 cells | mmoles/10^6 cells | mmoles/10^6 cells | mmoles/10^6 cells |
| Control | 7.60 ± 0.27 | 0.50 ± 0.09 | 8.30 ± 1.00 | 0.44 ± 0.07 | 7.36 ± 0.92 | 0.39 ± 0.05 |
| TNF | 7.72 ± 1.50 | 0.50 ± 0.10 | 7.86 ± 1.50 | 0.53 ± 0.13 | 8.10 ± 0.60 | 0.52 ± 0.10 |
| AD | 10.90 ± 1.80** | 0.49 ± 0.09 | 10.50 ± 3.00* | 0.55 ± 0.23 | 8.93 ± 1.10 | 0.66 ± 0.15 |
| TNF/AD | 13.40 ± 1.70*** | 0.64 ± 0.23 | 9.70 ± 1.80 | 0.73 ± 0.27* | 7.11 ± 1.00 | 0.42 ± 0.06 |

| ADP | mmoles/10^6 cells | mmoles/10^6 cells | mmoles/10^6 cells | mmoles/10^6 cells | mmoles/10^6 cells | mmoles/10^6 cells |
|-----|------------------|------------------|------------------|------------------|------------------|------------------|
|     | 2 h       |               | 3 h       |               | 4 h       |               |
| ADP | mmoles/10^6 cells | mmoles/10^6 cells | mmoles/10^6 cells | mmoles/10^6 cells | mmoles/10^6 cells | mmoles/10^6 cells |
| Control | 4.78 ± 0.97 | 0.49 ± 0.16 | 5.64 ± 0.93 | 0.40 ± 0.08 | 5.39 ± 0.93 | 0.35 ± 0.03 |
| TNF | 5.29 ± 0.84 | 0.43 ± 0.10 | 6.43 ± 0.86 | 0.50 ± 0.16 | 5.28 ± 0.77 | 0.52 ± 0.01 |
| AD | 6.00 ± 0.57 | 0.44 ± 0.10 | 5.80 ± 1.00 | 0.48 ± 0.06 | 6.41 ± 0.59 | 0.58 ± 0.24 |
| TNF/AD | 5.41 ± 0.89 | 0.50 ± 0.17 | 5.44 ± 1.12 | 0.57 ± 0.13 | 6.03 ± 0.47 | 0.46 ± 0.03 |

| ATP/ADP | mmoles/10^6 cells | mmoles/10^6 cells | mmoles/10^6 cells | mmoles/10^6 cells | mmoles/10^6 cells | mmoles/10^6 cells |
|---------|------------------|------------------|------------------|------------------|------------------|------------------|
| ATP/ADP | 2.47 ± 0.50*** | 1.28 ± 0.14 | 1.77 ± 0.21 | 1.28 ± 0.21 | 1.17 ± 0.16** | 0.91 ± 0.20 |

*$p < 0.05$ between TNF/AD and AD.
incubation period (Fig. 6).

**TNF/AD and TNF/CHX Alter the Coupling between Mitochondrial Electron Transport and Oxidative Phosphorylation**—Most ATP synthesis takes place during the mitochondrial respiration. Therefore, we analyzed the effects of TNF, AD, CHX, and TNF/AD or TNF/CHX on the oxygen consumption by L929 cells in RPMI medium in the presence or absence of either oligomycin or KCN. These conditions allow us to determine the basal respiration, the coupled respiration, and the nonmitochondrial respiration.

Through the first 8 h of incubation, the total basal respiration and the nonmitochondrial respiration did not change significantly by any of these treatments. Nevertheless, there were major changes in the oligomycin-sensitive respiration, which provides us information about the coupling degree between the mitochondrial electron flux and the ATP synthesis by the mitochondrial ATPase.

In basal conditions, L929 cells consumed 1.87 ± 0.20 nmol of O2/min/106 cells (Fig. 7A). About 20 ± 2% of the respiration rate was insensitive to cyanide, and it should be attributed to nonmitochondrial reactions. When oligomycin was added to the polarographic chamber, the oxygen consumption decreased by 32 ± 3%, indicating the degree of coupled respiration. The remaining 48 ± 3% represents the uncoupled respiration (Fig. 7A).

Treatment of cells with TNF alone increased the respiratory coupling from 32 ± 3% of the total respiration in untreated cells to 47 ± 3% and 42 ± 2.8% in cells treated with TNF for 2 and 3 h, respectively (p < 0.01) (Fig. 7A). After this time, coupled respiration decreased and remained at the control levels for the next 69 h (Fig. 7B). Treatment either with AD or CHX induced a smooth cyclic change of the coupled respiration (Fig. 7B). At 2 h of incubation, the coupled respiration was only 20 ± 1 and 19 ± 4%, respectively, of the total respiration (p < 0.01 against the control) (Fig. 7A).

Finally, the cytotoxic treatment combining TNF/AD or TNF/CHX induced marked cyclic changes in the coupled respiration. At 2 and 3 h of incubation, the presence of TNF increased the respiratory coupling as compared with AD or CHX alone (Fig. 7B). At 4 h, there was a great decrease of coupling, and at 6 h, this returned to control levels. Again, there was a huge decrease in respiratory coupling after 6 h of incubation (Fig. 7B).

These cyclic changes in the coupled respiration induced by the TNF in metabolically inhibited L929 cells were closely correlated with those induced in the intracellular ATP levels (r = 0.98, p < 0.01 for TNF/CHX-treated cells; r = 0.93, p < 0.05 for TNF/AD-treated cells).

To elucidate the cause of the elevated respiratory coupling through the first 3 h of incubation with TNF, we investigated whether treatment with this cytokine enhances ATP consump-
Fig. 6. Effect of TNF, AD, CHX, TNF/AD, and TNF/CHX on lactate accumulation. L929 cells were cultured in RPMI medium without serum at 37 °C for 8 h in the absence (Control) or presence of 25 ng/ml TNF, 1 μg/ml AD, 0.1 mM CHX, and the combined treatments TNF/AD and TNF/CHX. Lactate was measured as described under “Experimental Procedures.” Values shown are means ± S.D. of three independent experiments. Results are expressed as nmol/10^6 cells. * * * , p < 0.001; * * , p < 0.01 between control and experimental cells; a, p < 0.01 between TNF/AD and AD or TNF/CHX and CHX.

Blockade of Mitochondrial Respiration Prevented the Increase in Intracellular ATP Content at 3 h and Apoptotic Cell Death Induced by TNF/AD—Previous experiments have suggested that the increase in intracellular ATP induced by TNF/AD or TNF/CHX was a result of the mitochondrial respiration. To assess this relationship, we studied the effects of blockade of mitochondrial respiration on the ATP content in L929 cells treated with TNF/AD or TNF/CHX. We added the mitochondrial inhibitors simultaneously with TNF, since we have previously found that the pretreatment of L929 cells with mitochondrial inhibitors resulted in a significant decrease in the binding of human TNF to cell surface receptors (27). As expected, incubation of these cells with any inhibitor of cellular respiration for 3 h resulted in a significant decrease in the intracellular ATP levels. In TNF/AD-treated cells, inhibition of NADH-coenzyme Q reductase with 2 μM rotenone decreased cellular ATP content from 14.3 ± 0.93 to 7.30 ± 1.4 nmol/10^6 cells (p < 0.01). TTFA, an inhibitor of the electron transport at complex II, decreased cellular ATP to 6.6 ± 1.3 nmol/10^6 cells (p < 0.01). Antimycin A (5 μg/ml), an inhibitor of the b-cyt complex, diminished ATP content in cells to 3.45 ± 0.53 nmol/10^6 cells (p < 0.001). Finally, blockade of ATPase with 10 μg/ml oligomycin decreased cellular ATP to 2.90 ± 0.66 nmol/10^6 cells (p < 0.001). These results indicate that accumulation and, thus, whether it is able to modulate the respiratory coupling in a short period without changing significantly the total respiration rate. To approach this issue, we prevented de novo synthesis of mitochondrial ATP by incubating L929 cells with oligomycin and measured the effect of TNF on the cellular ATP content in the absence and presence of AD and CHX. In all of these conditions, we measured the lactate production to assess any change in the glycolysis rate. As Fig. 8 shows, AD increased cellular ATP content over the control level, which suggests that the consumption of ATP was reduced by AD treatment. A similar effect was observed when protein synthesis was inhibited by CHX. On the contrary, TNF decreased significantly intracellular ATP content in comparison with control cells and cells treated with AD or CHX alone. This effect of TNF on ATP levels suggests that this cytokine increases energy consumption by a process independent of the gene transcription or protein synthesis (Fig. 8).

Fig. 7. Panel A, determination of the quantitative importance of the nonmitochondrial and oligomycin-sensitive respiration on TNF-, AD-, TNF/AD-, and TNF/CHX-treated L929 cells. L929 cells were cultured for 72 h in RPMI medium with 25 ng/ml TNF, 1 μg/ml AD, 0.1 mM CHX, and the combined treatments TNF/AD and TNF/CHX. The respiration rate in RPMI medium was measured in the absence and presence of oligomycin (20 μg/ml) to inhibit oxidative phosphorylation and in the presence of KCN (1 mM) to prevent mitochondrial respiration. Results are expressed as the percentage of total respiration (indicated above each bar). Values shown are means ± S.D. of three independent experiments. * , p < 0.01 between control and experimental cells; a, p < 0.01 between TNF/AD and AD or TNF/CHX and CHX. Panel B, coupled respiration in TNF-, AD-, CHX-, TNF/AD-, and TNF/CHX-treated L929 cells. L929 cells were cultured for 72 h in RPMI medium with 25 ng/ml TNF or for 8 h with 1 μg/ml AD, 0.1 mM CHX, or TNF/AD or TNF/CHX. Coupled respiration was determined as the oligomycin-sensitive respiration. Results are expressed as the percentage ± S.D. of coupled respiration in control cells of three independent experiments. *, p < 0.01 between control and experimental cells; a, p < 0.01; aa, p < 0.01 between TNF/AD and AD or TNF/CHX and CHX.

To investigate the implications, if any, of these changes in the mitochondrial function on the cytotoxic effect of TNF/CHX treatment, we measured apoptotic cell death induced by this treatment in the absence or presence of a variety of mitochondrial blockers. Our study showed that these agents reduced the cytotoxicity of TNF/CHX treatment. Thus, TNF/CHX killed 94 ± 4% of cells after 24 h of incubation, while this treatment killed only 9.5% of cells in the presence of 10 μg/ml oligomycin (Fig. 10A). Similar effects were obtained when mitochondrial electron transport was blocked with 10 μg/ml antimycin A. In this case, apoptosis decreased from 94 ± 4 to 15%. This percentage was not much higher than the 9 ± 3% of apoptotic cell death caused by antimycin A alone (Fig. 10B). Rotenone (2 μM), which decreased ATP levels by about 50%, reduced cytotoxicity
cytotoxic activity of TNF toward metabolically inhibited L929 cells has been ascribed to ROS generated in mitochondria, we investigated intracellular production of ROS by L929 cells in response to TNF/AD and TNF/CHX treatments using the DHR-123 probe. As Fig. 12A shows, these treatments induced a marked increase in the ROS generation, which started after 4 h of incubation and was maintained until cell death. Blockade of the mitochondrial respiratory chain with rotenone, antimycin A, mixothiazol, or cyanide or blockade of the ATPase with oligomycin prevented this increase in ROS generation. TTFA and malonate were less effective and decreased ROS generation significantly only in cells treated with TNF/CHX (Fig. 12B). Incubation of cells with TNF alone enhanced ROS generation but only after 72 h of treatment. AD and CHX treatments also increased the formation of ROS, but these changes were less marked than those obtained when these agents were added in combination with TNF (Fig. 12A).

DISCUSSION

This study shows that treatment of L929 cells with a combination of TNF and actinomycin D (TNF/AD) or cycloheximide (TNF/CHX) led to cyclic increases in the intracellular ATP content, both cytosolic and mitochondrial, preceding cell death (Fig. 3). ATP increases were significantly greater than the changes induced by AD or CHX alone. These changes in the intracellular content of ATP were also found in the HepG2 cell line (Fig. 5) and were confirmed by 32P NMR spectroscopy of cellular extracts (Fig. 4). This increase in intracellular ATP content without a parallel increase in ADP levels led to a rise in the ATP-ADP ratio, which, as has been shown, plays a central metabolic role (28, 29).

The increase in ATP levels in cells treated with AD or CHX may be ascribed to a decline in the ATP consumption secondary to the inhibition of the gene transcription and protein synthesis, since these are high energy-consuming processes (30–32). The additional and significant increase in ATP levels induced by TNF in these metabolically inhibited cells may be a consequence of either an enhanced ATP production or a decreased ATP consumption. The rise in ATP production may result from an increased glycolysis or from a more active or efficient mitochondrial respiration. Our study shows that the TNF-induced ATP increase is not a result of an enhanced rate of glycolysis, since the accumulation of lactate did not change significantly during the first 3 h of treatment (Fig. 6). Similarly, treatment of cells with TNF alone or combined with CHX or AD did not change the total rate of mitochondrial respiration (Fig. 7A). These results contrast with those obtained by others (15), who found that these treatments inhibit mitochondrial electron flow. The reason for this discrepancy may lie in the different experimental conditions used in these studies. While we measured oxygen consumption by whole cells incubated in RPMI, other authors used digitonized cells suspended in a respiratory medium containing ADP and a number of substrates to evaluate all complexes of the mitochondrial respiratory chain (15).

Our study shows that although TNF alone or in combination with CHX or AD did not induce any change in the oxygen consumption, these treatments induced deep changes in the efficiency of mitochondrial synthesis of ATP, as assessed by measuring the degree of the respiratory coupling. It is well known that oxidative phosphorylation varies its coupling efficiency depending on demand without requirements for large changes in oxygen consumption rate (33). Short term changes in the coupling between mitochondrial electron flux and oxidative phosphorylation could be advantageous to the cells. They could adapt the cells to short term environmental changes induced by optimizing heat production or the rate of ATP synthesis (34).
Treatment of cells with either AD or CHX alone decreased the respiratory coupling during the first 3 h of treatment (Fig. 7B). This change may be ascribed to the low energy utilization induced by these agents, as was previously suggested (31). The combined treatment of cells with either TNF/CHX or TNF/AD enhanced the respiratory coupling degree over that found in cells treated with just CHX or AD alone (Fig. 7A and B). This enhanced respiratory efficacy may be responsible for the increased ATP content we found in cells after 3 h of combined treatment. As Fig. 7B shows, this energy accumulation was followed by a large respiratory uncoupling and by a fall in the ATP levels at 4 h. These decreases were succeeded by a new respiratory coupling and by a rise in the intracellular ATP levels at 6 h of incubation. Finally, there was a significant decline in the respiratory coupling and intracellular ATP levels, which coincided in time with the start of cell death. ATP content and coupling degree in TNF/AD- and TNF/CHX-treated cells were closely correlated \( r = 0.93 \) and \( r = 0.98 \), respectively, suggesting that between both groups of changes there may exist cause-effect relationships. The uncoupling phase starting at 4 h of incubation may be responsible for the marked increase in lactate production observed in cells treated with TNF/AD and TNF/CHX (Fig. 6).

Treatment with TNF alone resulted in an increase of the respiratory coupling during the first 3 h of treatment (Fig. 7B), without inducing any significant modification in the ATP levels (Fig. 3). These effects may be ascribed to a rise in the energy consumption induced by TNF. Thus, the addition of TNF to L929 cells, in which ATP synthesis had been inhibited with oligomycin, resulted in a significant decrease in the ATP levels (Fig. 8). This increased consumption of ATP does not appear to be related to the protein synthesis or gene transcription, seeing that it also occurred in the presence of CHX or AD (Fig. 8). Our study could not clarify the process in which this enhanced ATP consumption takes place. However, we could speculate that this rise in energy utilization might occur in any intracellular signal transduction process induced by TNF. In these processes participate many reactions of phosphorylation mediated by kinases (8). Thus, an increase in respiratory coupling would compensate for the enhanced energy consumption induced by TNF without causing any significant change in the ATP levels.

Implications of these effects of TNF in metabolically inhibited cells are not well understood. However, we speculate that these changes in the cellular ATP levels might be related to the...
cytotoxic effect of TNF, since inhibition of mitochondrial electron transfer prevented both effects of TNF, the early increase in intracellular ATP content and its cytotoxicity to L929 cells (Figs. 9 and 10). Moreover, cytotoxicity of TNF/AD or TNF/CHX was closely correlated with ATP concentration in L929 cells inhibited with blockers of the mitochondrial respiration ($r = 0.91$; $n = 16$; $p < 0.001$). The discrepancies among our results and the reports of other authors (16, 35) can be ascribed to the different methods used to determine cell cytotoxicity and the time at which cytotoxicity was measured.

Our study shows that 100% of cell death caused by TNF/AD treatment for 24 h was induced by apoptosis (Figs. 2 and 10), and a dose-response curve demonstrated that there was a close negative relationship between oligomycin-induced ATP depletion and apoptosis (Fig. 11B). Over the past few years, many investigators have shown that the functional integrities of mitochondria and intracellular ATP are important factors during the early phases of apoptotic cell death (36–39). Apoptosis involves the activity of hydrolytic enzymes, chromatin condensation, and vesicle formation. Therefore, apoptosis is considered to have a high energy demand. In accord with this concept, Chou et al. (36) demonstrated that reduction of cellular ATP content with antimycin A blocks AD-induced apoptotic cell death. Likewise, Eguchi et al. (38) showed that ATP depletion induced with an inhibitor of mitochondrial ATPase completely blocks Fas/apo-1 stimulated apoptosis. Moreover, Leist et al. (39), not only confirmed that nuclear condensation and DNA fragmentation did not occur in cells depleted of ATP but also demonstrated that ATP replenishment was sufficient to kill cells by apoptosis.

How an early event, like TNF-induced increases in the intracellular ATP level, might result in a late effect (apoptotic cell death) is not known. However, there is ample evidence that apoptosis is accompanied by oxidative stress (40–42) and that antioxidants, including Bcl-2 (41, 42), may prevent apoptosis (43, 44). The role for ROS in TNF-mediated cell death has been postulated by many authors (15–17, 25, 45–49). The present study confirms that treatment of cells with TNF/AD or TNF/CHX for more than 3 h was followed by a significant increase in intracellular ROS production (Fig. 12A). In nonphagocytic cells, mitochondria are the main source of ROS, and the present study shows that blocking mitochondrial respiration with rotenone, TTFA, antimycin A, or oligomycin resulted in both reduced ROS production (Fig. 12B) and TNF-induced cell death (Figs. 10 and 11). These results are in agreement with those presented by others. Thus, Schulze et al. (16) showed that antioxidants, iron chelators, and some inhibitors of mitochondrial electron transport can interfere with TNF-mediated cytotoxicity.

The present study shows that the increase in the intracellular ATP content at 3 h was followed by a large respiratory uncoupling (Fig. 7). It is conceivable that ROS might be formed during these phases of uncoupled respiration, as it has been shown in other models of cell death (50). Accordingly, our study demon-
Fig. 12. Effect of TNF/AD and TNF/CHX on cellular generation of ROS. Panel A, cells were incubated with TNF (25 ng/ml), AD (1 
μg/ml), CHX (0.1 mM), or the combined treatment with TNF/AD or TNF/CHX for the indicated times. a, p < 0.05; b, p = 0.01; c, p < 0.001 
between control and metabolically inhibited cells. *, p < 0.01 between control and TNF-treated cells. Panel B, cells were treated for 6 h with 
25 ng/ml TNF and 1 mM CHX or TNF and 0.1 mM CHX in the presence of one of the following inhibitors: 2 μM rotenone (+Ro.), 5 
μM 2-aminopyridine (±A.A.), 250 μM TTPA (+TTPA), 1 mM cyanide (+KCN), 10 μM oligomycin (±Olig.), 2 μM mitoxantrone (+Mix.), or 10 
mM malonate (±Mal.). Cellular generation of ROS was determined by flow cytometry using rhodamine 123 fluorescence as described under “Experimental Procedures.” Results are expressed as means of three 
different experiments and represent the experimental:control ratio of the DHR-123 fluorescence. a, p < 0.001 between control and metabolically 
inhibited cells (TNF/AD or TNF/CHX). In TNF/AD or TNF/CHX-treated cells, * represents p < 0.05, ** represents p < 0.01, and *** 
represents p < 0.001 between the absence and the presence of mitochondrial respiration.

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