Tumor Necrosis Factor Alpha Inhibits Hepatocyte Mitochondrial Respiration

JOSEF STADLER, M.D., BRANDON G. BENTZ, B.S., BRIAN G. HARBRECHT, M.D., MAURICIO DI SILVIO, M.D., RONALD D. CURRAN, M.D., TIMOTHY R. BILLIAR, M.D., ROSEMARY A. HOFFMAN, B.S., and RICHARD L. SIMMONS, M.D.

From the Department of Surgery, University of Pittsburgh, Pittsburgh, Pennsylvania

Although direct cytotoxicity is a well-established phenomenon of tumor necrosis factor alpha (TNFα)-induced tissue damage, the intracellular events leading to cell death are still poorly understood. To study the cytotoxic effects of TNFα on normal parenchymal cells, rat hepatocytes were purified and incubated with various concentrations of TNFα. Mitochondrial respiration, total protein synthesis, and enzyme release were measured to assess metabolic performance and cell integrity. Treatment with TNFα suppressed mitochondrial respiration in a concentration-dependent fashion, resulting in a reduction of the activity of complex I of the respiratory chain to 67.0 ± 3.5% of that of untreated hepatocytes by 2000 U/mL TNFα. Under these conditions protein synthesis and the release of intracellular enzymes were significantly increased. Both hepatocellular enzyme release and inhibition of mitochondrial respiration appear to be associated with the generation of reactive oxygen intermediates by the hepatocyte itself, because oxygen radical scavengers prevented these adverse effects of TNFα. Inhibition of protein synthesis by cycloheximide as well as addition of cyclic adenosine monophosphate synergistically enhanced the suppression of mitochondrial respiration by TNFα, resulting in complex I activity of 6.9 ± 1.6% and 24.9 ± 2.9% of that of untreated cells. These data indicate that inhibition of mitochondrial respiration is one of the mechanisms by which TNFα induces tissue injury.

TUMOR NECROSIS FACTOR ALPHA (TNFα) was originally described as an agent responsible for hemorrhagic necrosis of murine sarcoma after administration of endotoxin.[1] Since then, multiple metabolic alterations of both malignant and nonmalignant cells have been attributed to the action of TNFα.[2] Depending on the concentration of this cytokine, the response of cells, tissues, or the whole organism is extremely variable. At low levels, TNFα acts mainly as a paracrine mediator, and trophic effects enhancing tissue repair and microbiostasis have been identified.[3] At higher concentrations, TNFα leads to tumor necrosis, but eventually also to damage of normal tissue. Finally, at even higher concentrations when spillover into the systemic circulation occurs, TNFα causes a lethal shock syndrome similar to that seen in the endotoxic shock.[4]

Although specific receptors for TNFα[5] and second messenger pathway that are activated by receptor occupancy have been identified,[2] it still remains to be elucidated how TNFα mediates cell death and organ dysfunction.[6] Important observations have been made using *in vivo* models of hemorrhagic and septic shock. In some of these experiments, significant changes of hepatocyte (HC) mitochondrial morphology and function have been described.[7,8] At very high concentrations, endotoxin appears to directly inhibit HC mitochondrial function *in vitro*.[9] It is unclear, however, whether the *in vivo* findings are also due to direct effects of lipopolysaccharide (LPS) or to induction of secondary factors, such as TNFα, which inhibits mitochondrial respiration in tumor cell lines.[10] Our own preliminary work has shown that TNFα inhibits respiratory activity in normal rat HC, too.[11]

In a long-term effort of studying liver failure in patients with surgical sepsis, our laboratory has demonstrated that Kupffer cell activation appears to play a major role in the development of liver dysfunction in sepsis.[12-14] Because Kupffer cells, the largest population of fixed macrophages in the body, are thought to be a major source of TNFα,[15,16] it seems relevant to study possible influences of TNFα on the parenchymal cells of the liver, the HC. The following

Supported in part by National Institutes of Health grants GM-44100 (to T. R. Billiar) and GM-37753 (to R. L. Simmons). J. Stadler was supported by a fellowship of the Deutsche Forschungsgemeinschaft, Bonn, Germany (Sta 311/1-1).

Address correspondence to Timothy R. Billiar, M.D., Department of Surgery, University of Pittsburgh, 497 Scaife Hall, Pittsburgh, PA 15261.

Reprints will not be available from the author.

Accepted for publication November 5, 1991.

experiments were undertaken to investigate the effects of TNFα on HC mitochondria, in an attempt to clarify the role of TNFα in the induction of hepatocellular dysfunction after hemorrhagic and septic shock.

Materials and Methods

Cell Isolation

Male Sprague-Dawley rats (Harlan Sprague-Dawley, Inc., Indianapolis, IN) weighing 200 to 300 g, were used as a cell source in all experiments. Hepatocytes were harvested with an *in situ* collagenase (Type IV; Sigma Chemical Co., St. Louis, MO) perfusion technique, as previously described.[11,12] The cells were purified by repeated differential sedimentation at 50*g* for 2 minutes. Purity of the HC was greater than 96%, and viability exceeded 85% as determined by trypan blue exclusion.

Cell Culture Technique

Cultures were performed in Williams medium E (Gibco Laboratories, Grand Island, NY) supplemented with 10^{-6} mol/L insulin, 2 mmol/L L-glutamine, 10^5 U/L penicillin, 100 mg/L streptomycin, 10^{-8} mol/L dexamethasone, and 10% low endotoxin calf serum (HyClone Laboratories, Logan, UT). The HC were incubated either in this standard medium or with the addition of recombinant murine tumor necrosis factor alpha (rmTNFα, Genzyme Corporation, Cambridge, MA), lipopolysaccharide (LPS from *Escherichia coli* 0111:B_4; Difco Laboratories, Detroit, MI), glucagon (Calbiochem Corp., San Diego, CA), or other chemicals (all from Sigma) as indicated in the Results section. The rmTNFα preparation had a specific activity of 40 U/ng. It was added at concentrations of 500 U/mL and 2000 U/mL, which equals 12.5 ng/mL and 50 ng/mL, respectively. For measurement of mitochondrial respiration, HC were placed in 75-cm^2 flasks at 12 mL/flask at a concentration of 1×10^6 cells/mL. Except for time course experiments, all incubations were carried out over a period of 18 hours. The culture supernatants were then harvested for determination of HC enzyme and nitrite/nitrate release, and the cells were collected for mitochondrial respiration measurements. To detach the HC, the cultures were incubated for 5 minutes with ethylenediaminetetra-acetic acid (EDTA)-trypsin (Gibco), followed by the addition of 10% calf serum to inactivate the trypsin. For determination of radiolabeled leucine incorporation and superoxide production, HC were plated at 2×10^5 cells/mL on gelatin (Sigma)-coated 96-well tissue culture trays at 0.1 mL/well.

Respiration Measurements

Respiration medium (pH 7.2) contained 250 mmol/L sucrose, 2.0 mmol/L H_2KPO_4, 10 mmol/l MgCl, 20 mmol/L HEPES, 0.5 mmol/L EDTA, 1 mmol/L adenosine diphosphate, and 0.7% bovine serum albumin. All these and the following reagents used for respiration measurements were purchased from Sigma. The plasma membrane of the HC was made permeable to the substrates by exposure to 0.0075% digitonin for 1 minute followed by three washes at 400*g* and 4°C.[17] Oxygen consumption was measured using a Clark-type electrode (Yellow Springs Instruments Co., Yellow Springs, OH). As shown in Figure 1, state 3 respiration[18] was initiated by the addition of glutamate and malate (final concentration, 5 mmol/L) resulting in the production of nicotinamide-adenine dinucleotide phosphate (NADH), which serves as a substrate for nicotinamide-adenine dinucleotide phosphate–ubiquinone oxidoreductase (complex I) of the mitochondrial electron transport chain. To measure state 3 respiration mediated by succinate-ubiquinone oxidoreductase (complex II), electron flow from complex I was inhibited by rotenone (100 nmol/L), and succinate (5 mmol/L) was added. Electron flow from complex III to cytochrome C was blocked by the addition of antimycin A (20 nmol/L) to determine state 3 respiration rate of cytochrome oxidase (complex IV). N,N,N′, N′-tetramethyl-p-phenylenediamine (TMPD 0.2 mmol/L) in combination with ascorbate (1 mmol/L) served as an artificial electron donor system for complex IV. Oxygen consumption was calculated from an initial air-saturated $[0_2]$ = 195 nmol/mL.[19] State 4 respiration was initiated

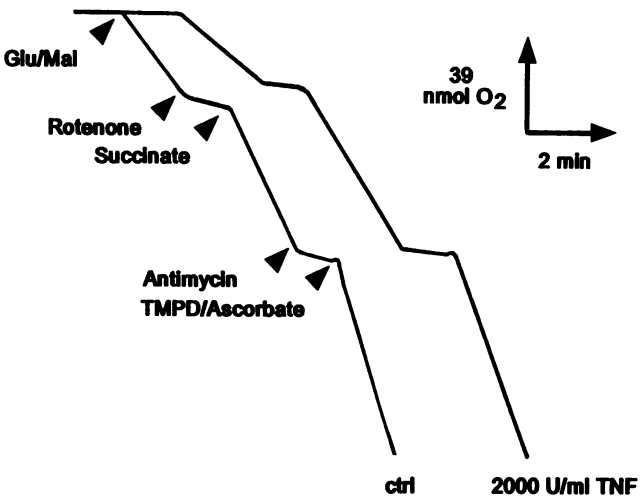

FIG. 1. Schematic presentation of an oxygen-electrode recording showing substrate-specific oxygen consumption by permeabilized rat HC. Respiration rates mediated by complex I of the enzyme complexes of the mitochondrial electron transport chain were determined by addition of glutamate/malate (5 mmol/L). After inhibition of complex I activity with rotenone (100 mmol/L), complex II activity was assayed by the addition of succinate (5 mmol/L). Antimycin A (20 nmol/L) was used to block electron transport from complex III to complex IV, allowing selective measurement of complex IV activity with TMPD/ascorbate (0.2 mmol/L/1 mmol/L) as an artificial electron donor.

by blocking adenosine triphosphatases of the mitochondrial inner membrane with the addition of oligomycin (2 μg/mL). The acceptor control index (ACI) was calculated as the ratio of state 3/state 4 respiration.[18] The treatment with digitonin resulted in an ACI for complex I—mediated respiration of 9.6 ± 0.4 of fresh HC, indicating that mitochondrial function was adequate.[17]

Determination of Protein Synthesis

Total protein synthesis was measured using a 4-hour labeling interval with [^{3}H] leucine (5.0 Ci/mmol/L, 1.0 μ Ci/well; New England Nuclear, Boston, MA) added in Minimal Essential Medium without L-leucine (Gibco). [^{3}H] leucine incorporation was determined by liquid scintillation counting.

Determination of Superoxide Production

Superoxide production was measured by the superoxide-dismutase-sensitive rate of reduction of cytochrome c.[20] Cytochrome c (Sigma) was added at 100 μmol/L to the incubation medium. The rate of cytochrome c reduction was determined as the change of absorbance at 550 nm in the absence or presence of 360 U/mL superoxide-dismutase. Positive controls were Kupffer cells plated at 1×10^6 cells/mL and stimulated with 1 μg/ml phorbol myristate acetate for 2 hours.

Determination of Nitrite/Nitrate and Enzyme Release

As stable end products of N = 0 biosynthesis, nitrite (NO_2^-) and nitrate (NO_3^-) were measured in culture supernatants using an automated procedure based on the Griess reaction.[21] Positive controls were HC stimulated with a specific combination of cytokines and LPS.[11] Glutamate-oxaloacetate-transaminase (GOT) and glutamate-pyruvate-transaminase (GPT) levels were determined using a Technitron RA-500 analyzer. Cell lysis was performed with 1×10^6 cells/mL by three cycles of rapid freeze–thawing.

Statistical Analysis

Results represent means ± standard error of the mean of three experiments unless otherwise stated. Significance between groups was determined using the Student's unpaired t test.

Results

To determine whether rat HC mitochondrial respiratory activity is altered by LPS or TNFα, HC were placed in culture under standard conditions or stimulated with 10 μg/mL LPS, 500 U/mL rmTNFα, or 2000 U/mL rmTNFα. Respiratory activity mediated by complex I, complex II, or complex IV of the mitochondrial electron transport chain was measured after an 18-hour incubation period. Exposure to TNFα, but not to LPS, inhibited the activity of all three enzyme complexes in a concentration-dependent fashion (Table 1). After exposure to 2000 U/mL TNFα complex I, complex II and complex IV mediated oxygen consumption was reduced to 67.0 ± 3.5%, 69.8 ± 6.0% and 83.8 ± 4.9% that of untreated HC. For subsequent experiments only complex I activity was determined.

Previous studies have shown that TNFα-induced mitochondrial swelling develops within several hours.[22] Time course experiments in the current study showed a constant decrease of HC complex I activity starting as early as 3 hours after exposure to TNFα (Fig. 2). A difference between 500 and 2000 U/mL rmTNFα became manifest after 18 hours. The inhibition of mitochondrial respiration induced by 500 U/mL rmTNFα plateaued between 12 and 18 hours, whereas 2000 U/mL induced a continuous decline in HC oxygen consumption, even beyond 12 hours. The ACI was determined as a parameter of intactness of the mitochondrial membrane.[17] In parallel with the inhibition of state 3 respiration, TNFα induced a 33.7% reduction of the ACI. Because the degree of ACI reduction corresponds in these experiments to the inhibition of state 3 respiration, it can be concluded that the inhibitory effect of TNFα on mitochondrial respiration is not due to electron leakage at the mitochondrial inner membrane.

As parameters of general metabolic function and morphologic integrity, total protein synthesis and the release of intracellular enzymes were determined after 18 hours of incubation. In contrast to mitochondrial respiration, TNFα significantly increased HC total protein synthesis as measured by leucine incorporation (Table 2). Tumor necrosis factor alpha also increased the release of intracellular enzymes, specifically glutamic-oxaloacetic trans-

TABLE 1. *Inhibtion of Substrate-specific Hepatocyte Mitochondrial Respiration by TNFα**

Additions to the Culture Medium	Oxygen Consumption		
	Complex I	Complex II	Complex IV
	nmol/min/10^6 HC		
None	26.5 ± 1.3	45.8 ± 0.8	65.9 ± 2.7
10 μg/mL LPS	27.9 ± 2.2	44.5 ± 1.7	66.9 ± 2.0
500 U/mL TNFα	20.7 ± 1.7†	40.5 ± 1.3	58.9 ± 1.1
2000 U/mL TNFα	17.3 ± 1.3†	31.8 ± 2.4†	43.9 ± 6.5

* Freshly harvested HC were incubated under the conditions listed above. After 18 hours, the activities of three enzyme complexes of the mitochondrial respiratory chain were measured as oxygen consumption after the addition of specific substrates as described in the Materials and Methods section.

† $p < 0.01$ *vs.* untreated HC.

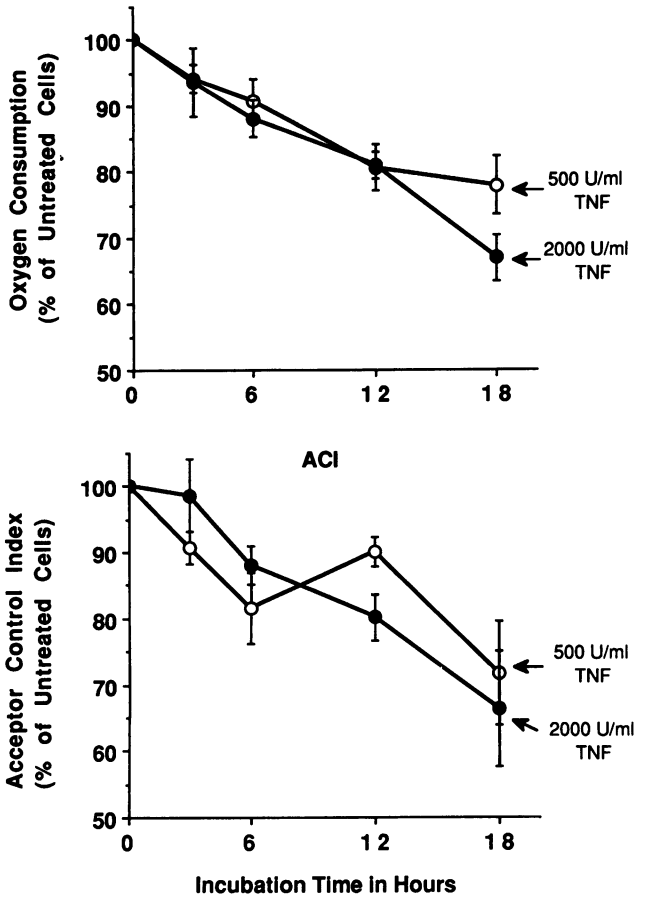

FIG. 2. Time courses of inhibition of complex I-mediated respiration parameters induced by exposure to TNFα. Freshly harvested HC were placed in culture, and malate/glutamate-dependent oxygen consumption was measured after the incubation periods indicated above. The acceptor control index was calculated as the ratio of state 3 to state 4 respiration. State 4 respiration was initiated by the addition of oligomycin (2 μg/mL) to the respiration medium. The results represent four experiments.

aminase and glutamic pyruvate transaminase. However, the amount of enzymes found in the supernatants represents only a very small portion of the total enzyme content of lysed hepatocytes, indicating that cell death did not occur to a major extent under these conditions.

Tumor necrosis factor alpha–stimulated production of oxygen radicals has been implicated as a possible mechanism of TNFα-induced tumor cell cytotoxicity.[23,24] Another proposed mechanism of tumor cytotoxicity is the generation of nitric oxide. This short-lived radical was shown to specifically inhibit mitochondrial respiration in various cell types.[11,19,25] To determine whether either one of these mechanisms is involved in TNFα-induced alteration of HC metabolism, we measured the release of superoxide and the stable endproducts of nitric oxide biosynthesis, nitrites and nitrates, from TNFα-treated HC. Exposure to TNFα, however, did not result in detectable HC superoxide release nor the generation of NO_2^- and NO_3^- (Table 3).

TABLE 2. *TNFα Enhances Protein Synthesis and the Release of Intracellular Enzymes by Hepatocytes**

Culture Conditions	Protein Synthesis		
TNFα Concentrations (U/mL)	[^{3}H]-Leucine Incorporation (cpm)	Enzyme Release (U/L)	
		GPT	GOT
—	42,607 ± 1876	61 ± 10	369 ± 23
500	49,274 ± 1954†	57 ± 10	510 ± 65†
2000	49,340 ± 4587	87 ± 14	539 ± 52†
Cell lysis	ND	398 ± 65	5993 ± 296

* After 18 hours of incubation with the various concentrations of TNFα HC, total protein synthesis was determined as incorporation of radiolabeled leucine, and the release of intracellular enzymes from 10^6 HC/mL into the culture supernatants was measured. The same number of HC/mL was lysed by three cycles of rapid freeze thawing to determine the total content of these enzymes in the HC. The results represent five experiments.

† $p < 0.05$ *vs.* incubations without TNFα.

To evaluate the role of oxygen radicals in the inhibition of mitochondrial respiration by TNFα more thoroughly, oxygen radical scavengers were added to the incubation media. The addition of ascorbate as a superoxide scavenger or mannitol as a hydroxyl radical scavenger almost completely prevented the inhibitory effect of TNFα on mitochondrial respiration (Fig. 3). Ascorbate also significantly reduced the release of intracellular enzymes, while the effect of mannitol on enzyme release was not significant (not shown). 1,3-bis (choroethyl)-1-nitrosourea, an inhibitor of glutathione reductase, was used to investigate whether the availability of reduced glutathione is important for hepatocellular defense against the effect of TNFα. This seems not to be the case, because addition of 200 μmol/L 1,3-bis (choroethyl)-1-nitrosourea did not change respiration rates in the presence or absence of TNFα. The amount of 1,3-bis (choroethyl)-1-nitrosourea used in this

TABLE 3. *TNFα Does not Induce Release of Superoxide Radicals or Nitric Oxide Radicals by Hepatocytes*

Culture Conditions	Radical Release	
TNFα Concentration (U/mL)	O_2^- (nmol/10^6 HC)	$\cdot N{=}O$ (nmol NO_2^- + NO_3^-/10^6 HC)
—	0.69 ± 0.43	8.5 ± 1.6
500	0.43 ± 0.43	8.5 ± 1.7
2000	0.46 ± 0.43	7.4 ± 2.6
Positive controls	4.46 ± 1.28	228.8 ± 15.4

For determination of superoxide as well as nitric oxide release, HC were incubated for 18 hours. Kupffer cells that were stimulated with phorbol myristate acetate for 2 hours served as positive controls for superoxide production. Positive controls for nitric oxide production were HC that were exposed to a specific combination of cytokines and LPS as described in the Materials and Methods section.

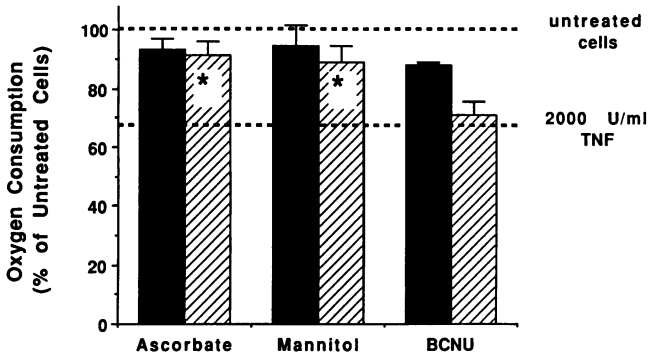

FIG. 3. Oxygen radicals prevent TNFα-induced suppression of complex I-mediated respiration rates. HC were incubated for 18 hours either in regular medium (solid columns) or in medium supplemented with 2000 U/mL TNFα (hatched columns). Ascorbate (1 mmol/L) and mannitol (10 mmol/L) were added as exogenous oxygen radical scavengers. The role of endogenous defense mechanisms against oxygen radicals was tested by addition of 1,3-bis(chlorethyl)-1-nitros-urea (BCNU; 200 μmol/L). Complex I activity was measured as malate/glutamate-dependent oxygen consumption. *$p < 0.01$ *vs.* 2000 U/mL TNFα.

experiment was shown to effectively block glutathione reduction (unpublished observations).

In tumor cells, the effect of TNFα is dramatically increased by inhibition of protein synthesis.[10,26] Cycloheximide, which inhibits protein synthesis by blocking translation, had no significant effect when administered alone for 18 hours, but almost completely suppressed complex I activity in combination with TNFα (Table 4). Enzyme release was increased, but again not to a level that could explain the suppression of mitochondrial enzyme activity by cell death. Neither a combination of superoxide dismutase (300 U/mL) and catalase (1000 U/mL) nor oxygen radical scavengers were able to reverse the inhibition of complex I activity by TNFα plus cycloheximide. The added enzymes, however, can only act extracellularly, because they cannot penetrate the plasma membrane.

For all kinds of chemical signals, receptor expression is important to regulate the efficiency of signal transduction. In various tumor cell lines, interferon-g (IFNg) and cyclic adenosine monophosphate (cAMP) have been shown to up-regulate TNFα receptors on the cell surface.[27,28] Therefore, experiments were undertaken to investigate whether IFNg or cAMP were able to enhance inhibition of HC mitochondrial respiration by TNFα. Although IFNg had no influence on inhibition of complex I activity by TNFα, dibutryl-cyclic AMP (DBcAMP), a membrane permeable version of cAMP, synergistically increased the effect of TNFα (Fig. 4). The combination of TNFα with glucagon, which acts through intracellular cAMP elevation,[29] also resulted in a relatively decreased HC complex I activity of 57.6 ± 6.5% compared with incubations with glucagon alone. The rise in respiratory activity induced by glucagon is a well-established effect of this hormone.[30] The addition of oxygen radical scavengers partially prevented the inhibition of mitochondrial respiration by TNFα in combination with DBcAMP or glucagon.

Discussion

Tumor necrosis factor alpha is a very well-studied molecule, which has proven to be responsible for a variety of pathophysiologic events. Once thought to preferentially kill tumor cells, TNFα is now known to also mediate many of the deleterious consequences of major trauma and sepsis.[4,31] To this list we can now add TNFα-induced inhibition of energy metabolism of HC *in vitro.*

A variety of different effects of TNFα on cells *in vitro* have been described, including DNA fragmentation,[32] adenosine diphosphate ribosylation,[33] increased permeability of plasma membranes,[34] and inhibition of mitochondrial respiration.[10] All these alterations might be responsible for or at least contribute to TNFα-induced cytotoxicity. We have focused on the effect of TNFα on

TABLE 4. *Inhibition of Protein Synthesis by Cycloheximide Potentiates Suppression of Energy Metabolism and Enzyme Release by Hepatocytes**

Culture Conditions			Oxygen Consumption (Complex I Mediated) (% of Untreated HC)	Enzyme Release (GOT) (% of Lysed HC)
TNFα (2000 U/mL)	Cycloheximide (10 μg/mL)	Other Additions		
		–		
–	–	–	—	6.3 ± 0.4
–	+	–	94.7 ± 5.4	7.4 ± 1.1
+	–	–	67.0 ± 3.5†	8.2 ± 0.7†
+	+	Superoxide dismutase + catalase	6.9 ± 1.6‡	12.7 ± 1.8‡
+	+	(300 U/mL + 1000 U/mL)	5.3 ± 1.9	ND
+	+	Ascorbate + mannitol (10 mmol/L + 10 mmol/L)	5.7 ± 3.2	ND

* Mitochondrial respiration rates and enzyme release into the culture supernatants were determined after 18-hour incubations under the indicated conditions. The amount of enzymes in the supernatants is calculated as the percentage of enzyme activity that was determined in cell lysates of equal numbers of HC.

† $p < 0.01$ *vs.* untreated HC.

‡ $p < 0.001$ *vs.* untreated HC.

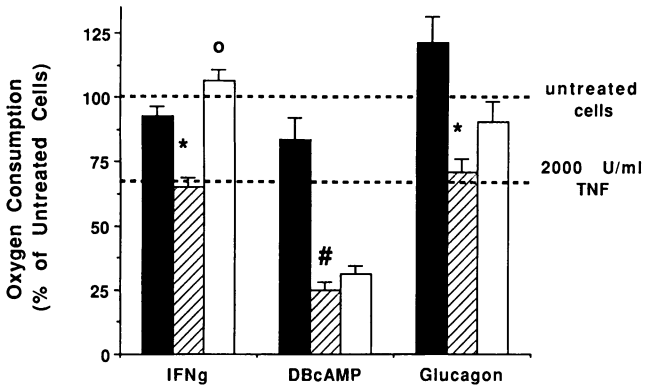

FIG. 4. Cyclic AMP synergistically enhances TNFα-induced inhibition of complex I activity. HC were incubated for 18 hours in regular medium (solid columns), in medium containing 2000 U/mL TNFα (hatched columns), or in medium supplemented with 2000 U/mL TNFα, 10 mmol/L ascorbate, and 10 mmol/L mannitol (open columns). In an attempt to up-regulate TNF receptors, IFNg (100 U/mL), dibutryl-cyclic AMP (1 mmol/L), or glucagon (10 μmol/L) was added. *$p < 0.05$ *vs.* IFNg or glucagon alone. °$p < 0.005$ *vs.* IFNg + TNFα. #$p < 0.001$ *vs.* 2000 U/mL TNFα.

mitochondrial function of normal cultured HC, because several previous studies had shown deteriorated hepatic mitochondrial function and morphology in endotoxin-treated animals.[8,9,35] It is well accepted that many of the effects of endotoxin are mediated by TNFα.[1,3,4] Furthermore, studying the effects of endotoxin and cytokines on hepatocellular metabolism is of clinical relevance because liver dysfunction often—but not always—accompanies the multiple organ failure syndrome seen in surgical and traumatized patients.[36] In our experiments, exposure of HC to a relatively high concentration of LPS (10 μg/mL) had no effect on mitochondrial respiration. Tumor necrosis factor alpha significantly inhibited the activity of specific enzyme complexes of the mitochondrial respiratory chain in a concentration-dependent manner. These *in vitro* findings suggest that mitochondrial dysfunction after *in vivo* administration of LPS may at least in part be due to the release of TNFα.

A general problem of *in vitro* experiments using cell culture techniques is the determination of concentrations of mediators that reflect physiologic conditions. Because TNFα acts primarily in a paracrine and autocrine fashion, TNFα receptors in the local environment of the TNFα-producing cell will be fully saturated before the cytokine spills over into the systemic circulation. This may be specifically true for the hepatic sinusoids, although to our knowledge TNFα concentrations in the sinusoids have never been determined. Therefore, serum levels may not provide optimal guidelines for choosing *in vitro* concentrations of cytokines. Nevertheless, ng/mL concentrations of TNFα in serum have been described in humans.[37,38] In previous experiments, we have found TNFα levels of up to 500 U/L × 10^6 cells (12.5 ng/mL) in the supernatants of cultured Kupffer cells.[39] In the current study, mitochondrial inhibition was found to be enhanced when the concentration of TNFα was increased from 500 to 2000 U/mL. This finding suggests that a concentration of 500 U/mL does not fully saturate the HC TNFα receptors.

For the evaluation of mitochondrial damage, the ACI is an important parameter. The ACI is calculated as the ratio of state 3 to state 4 respiration[17,18] and reflects enzyme activity (state 3) as well as intactness of the mitochondrial inner membrane in maintaining the proton gradient (state 4). Time course experiments demonstrated that the decrease of state 3 respiration parallels that of the ACI. This observation indicates that there was no major damage to the mitochondrial membranes rather than a specific inhibition of the enzyme complexes of the electron transport chain. Other defects, such as the inhibition of mitochondrial ATPase, also may contribute to mitochondrial dysfunction in sepsis.[40]

The release of intracellular enzymes was increased in the presence of TNFα, but not to an extent that would correspond to the inhibition of mitochondrial function. It is not clear at this point whether the elevation of enzyme release reflects cell death, increased permeability of the cell membranes, or both. In any case, cell damage does not appear to be a major factor in our experiments because the exposure to TNFα led to a rise in HC total protein synthesis, too. This finding corresponds to other reports that propose a role for TNFα in the induction of the hepatic acute phase response.[41,42]

The mechanisms by which TNFα kills cells are essentially not known. In terms of the inhibitory effect on mitochondrial respiration, two categories of agents are likely to be involved: (1) reactive oxygen intermediates, mainly superoxide and hydroxyl radicals, and (2) intermediates of oxidative nitrogen metabolism, specifically the nitric oxide radical. Both types of radical species have been shown to inhibit mitochondrial respiration in a similar fashion as that observed in our experiments.[11,25,43] Furthermore, in other cell types TNFα has been implicated in both oxygen radical production and induction of nitric oxide synthesis.[44] By measuring the stable end products of nitric oxide synthesis, we could not detect any difference between TNFα-treated and untreated HC. Therefore, nitric oxide does not seem to play a role in TNFα-induced inhibition of mitochondrial function. These results support the previous finding that only a specific combination of several cytokines will induce nitric oxide production in cultured HC.[39]

In terms of the involvement of reactive oxygen intermediates the results appeared to be contradictory. Superoxide release by HC was not detected in response to TNFα; however, oxygen radical scavengers prevented most of the effects of TNFα on HC mitochondrial respiration. There are several explanations for this obvious disparity. One is that TNFα induces HC synthesis of other oxygen radical

species than superoxide, which are able to inhibit the enzyme complexes of the mitochondrial electron transport chain[43] without being detected by our assay. This explanation is unlikely, however, because in biologic systems most reactive oxygen intermediates are derived from superoxide.[45] A better explanation takes into consideration that our assay for superoxide production only detects superoxide that is released from the cells.[20] Because HC have a great capacity to scavenge oxygen radicals,[45] the superoxide may have been absorbed within the cells before detectable amounts reach the extracellular medium. In any case, our results are supported by the finding that in tumor cells scavenging of oxygen radicals also protects from TNFα-mediated cytotoxicity.[23,46]

A large variety of hepatotoxic substances, including ethanol, are known to induce the production of reactive oxygen intermediates.[47] Similar to TNFα, these compounds lead to increased HC release of intracellular enzymes, which is prevented by ascorbate or other oxygen radical scavengers. Other drugs or manipulations, such as fasting, have been shown to suppress hepatocellular defense mechanisms against oxygen toxicity.[45] In light of these observations, we have focused on the interactions of TNFα with other hepatotoxic substances in ongoing studies. It is tempting to speculate that the effects of TNFα may be enhanced by a suppression of cellular defense mechanisms. In the presented experiments inhibition of glutathione reductase, however, had no influence on TNFα-induced mitochondrial dysfunction. Therefore, other pathways of oxygen radical detoxification may be affected by the action of TNFα.

Another important consideration in terms of protection against TNFα is that the sensitivity toward TNFα-induced cytotoxicity is increased by inhibitors of protein synthesis in a variety of cell types.[10,26] The current study confirms this mechanism for the inhibition of HC respiration by TNFα, too. The preferred explanation for this finding is that TNFα itself induces the synthesis of one or several proteins that protect the cell against its adverse effects.[6] Up-regulation of specific gene expression has been demonstrated after TNFα binding, including those for manganese superoxide dismutase,[48] the ferritin heavy chain,[49] heat shock proteins,[50] and others that may have protective effects. In contrast, the hepatocellular catalase activity is decreased by TNFα treatment.[51] Addition of superoxide dismutase alone or in combination with catalase did not overcome the profound mitochondrial dysfunction induced by TNFα and cycloheximide in the current study. Because these two enzymes cannot cross the plasma membrane, this finding demonstrates again that only the generation of intracellular reactive oxygen intermediates seems to be of relevance. Using other methods, such as anti-sense techniques, we will try to elucidate the role of induction of manganese superoxide dismutase by TNFα in further studies.

The effectiveness of cytokines and other chemical signals on target cells is regulated by various factors, including receptor expression. Binding studies have shown that HC express relatively few receptors for TNFα and that receptor expression correlates with sensitivity to TNFα-induced cytotoxicity.[52] Interferon-g and cAMP are known to increase TNF receptor expression in various tumor cell lines.[27,28] Both substances were therefore used in an attempt to increase the effects of TNFα on HC. However, combination of TNFα with IFNg did not enhance the inhibitory effect of TNFα, whereas the combination with cAMP synergistically increased TNFα-induced HC mitochondrial dysfunction. The latter finding appears to be of physiologic significance, because many hormones, such as glucagon[29] and catecholamines,[53] increase intracellular cAMP levels. Most of these hormones are elevated in trauma and sepsis, and synergism with TNFα could result in major damage. The current study demonstrates that the inhibitory effect of TNFα in combination with glucagon is indeed increased compared with TNFα alone. Experiments are underway to determine whether cAMP and cAMP-inducing agents act through HC TNF receptor up-regulation or whether they enhance the effect of TNFα through other mechanisms.

In conclusion, the presented experiments demonstrate that TNFα inhibits mitochondrial respiration of normal rat HC by a mechanism that involves the generation of reactive oxygen intermediates. This observation may have immediate clinical implications in patients suffering from major trauma or sepsis, because a variety of oxygen radical scavengers can be safely administered. Other factors that increase TNFα-induced mitochondrial dysfunction have been identified, including inhibition of protein synthesis and possibly up-regulation of TNFα receptors. Both conditions also may be influenced by therapeutic strategies available in the near future.

References

1. Carswell EA, Old LJ, Casse RL, et al. An endotoxin-induced serum factor that causes necrosis of tumors. Proc Natl Acad Sci USA 1975; 72:3666–3670.
2. Old LJ. Tumor necrosis factor. *In* Bonavida B, Granger G, eds. Tumor Necrosis Factor: Structure, Mechanism of Action, Role in Disease and Therapy. Basel: Karger, 1990, pp 1–30.
3. Beutler B, Cerami A. Cachectin (tumor necrosis factor): an endogenous mediator of shock and inflammatory response. *In* Oppenheim JJ, Shevach EM, eds. Immunology: The Role of Cells and Cytokines in Immunity and Inflammation. New York: Oxford University Press, 1990, pp 226–237.
4. Beutler B. Orchestration of septic shock by cytokines: the role of cachectin (tumor necrosis factor). *In* Roth BL, Nielsen TB, McKee AE, eds. Molecular and Cellular Mechanisms of Septic Shock. New York: Liss, 1989, pp 219–235.
5. Loetscher H, Pan YE, Lahm HW, et al. Molecular cloning and expression of the human 55 kd tumor necrosis factor receptor. Cell 1990; 61:351–359.
6. Larrick JW, Wright SC. Cytotoxic mechanisms of tumor necrosis factor-α. FASEB J 1990; 4:3215–3223.
7. Schumer W, Das Gupta TK, Moss GS, Nyhus LM. Effect of en-

dotoxemia on liver cell mitochondria in man. Ann Surg 1970; 171:875–882.
8. Mela L, Bacalzo LV Jr, Miller LD. Defective oxidative metabolism of rat liver mitochondria in hemorrhagic and endotoxin shock. Am J Physiol 1971; 220:571–577.
9. Mela L, Miller LD, Diaco JF, Sugerman HJ. Effect of *E. coli* endotoxin on mitochondrial energy-linked functions. Surgery 1970; 68:541–549.
10. Lancaster JR Jr, Laster SM, Gooding LR. Inhibition of target cell mitochondrial electron transfer by tumor necrosis factor. FEBS Lett 1989; 248:169–174.
11. Stadler J, Billiar TR, Curran RD, et al. Effect of exogenous and endogenous nitric oxide on mitochondrial respiration of rat hepatocytes. Am J Physiol 1991; 260:C910–C916.
12. Keller GA, West MA, Cerra FB, Simmons RL. Multiple systems organ failure: modulation of hepatocyte protein synthesis by endotoxin activated Kupffer cells. Ann Surg 1985; 201:87–95.
13. Keller GA, West MA, Wilkes LA, et al. Modulation of protein synthesis by endotoxin-activated Kupffer cells: II. Mediation by soluble transferrable factors. Ann Surg 1985; 201:429–435.
14. Keller GA, West MA, Harty JT, et al. Modulation of hepatocyte protein synthesis by endotoxin-activated Kupffer cells: III. Evidence for the role of a monokine similar to but not identical with interleukin-1. Ann Surg 1985; 201:436–443.
15. Bankey P, Carlson A, Ortiz M, et al. Tumor necrosis factor production by Kupffer cells requires protein kinase C activation. J Surg Res 1990; 49:256–261.
16. Ertel W, Morrison MH, Ayala A, et al. Passive immunization against cachectin (TNFα) prevents hemorrhage induced suppression of Kupffer cell function. Surg Forum 1990; 41:91.
17. Moreadith RW, Fiskum G. Isolation of mitochondria from ascites tumor cells permeabilized with digitonin. Anal Biochem 1984; 137:360–367.
18. Chance B, Williams GR. Respiratory enzymes in oxidative phosphorylation: III. The steady state. J Biol Chem 1955; 217:409–427.
19. Granger DL, Lehninger AL. Sites of inhibition of mitochondrial electron transport in macrophages-injured neoplastic cells. J Cell Biol 1982; 95:527–535.
20. Pick E, Mizel D. Rapid microassays for the measurement of superoxide and hydrogen peroxide production by macrophages in culture using an automatic enzyme immunoassay reader. J Immunol Methods 1981; 46:211–226.
21. Green LC, Wagner DA, Glogowski J, et al. Analysis of nitrate, nitrite, and [15N] nitrate in biological fluids. Anal Biochem 1982; 126: 131–138.
22. Matthews N. Anti-tumor cytotoxin producted by human monocytes: studies on its mode of action. Br J Cancer 1983; 48:405–410.
23. Matthews N, Neale ML, Jackson SK, Stark JM. Tumor cell killing by tumour necrosis factor: inhibition by anaerobic conditions, free-radical scavengers and inhibitors of arachidonate metabolism. Immunology 1987; 62:153–155.
24. Zimmermann RJ, Marafino BJ Jr, Chan A, Landre P, Winkelhake JL. The role of oxidant injury in tumor cell sensitivity to recombinant human tumor necrosis factor *in vivo*. J Immunol 1989; 142:1405–1409.
25. Stuehr DJ, Nathan CF. Nitric oxide: a macrophage product responsible for cytostasis and respiratory inhibition in tumor target cells. J Exp Med 1989; 169:1543–1555.
26. Kull FC Jr, Cuatrecasas P. Possible requirement of internalization in the mechanism of in vitro cytotoxicity in tumor necrosis serum. Cancer Res 1981; 41:4885–4890.
27. Aggarwal BB, Eessalu TE, Hass PE. Characterization of receptors for human tumour necrosis factor and their regulation by g-interferon. Nature 1985; 318:665–667.
28. Scheurich P, Köbrich G, Pfizenmaier K. Antagonist control of tumor necrosis factor receptors by protein kinases A and C. J Exp Med 1989; 170:947–958.
29. Wakelam MJO, Murphy GJ, Hruby VJ, Hanslay MD. Activation of two signal-transducing systems in hepatocytes by glucagon. Nature 1986; 323:68–71.
30. Yamazaki RK. Glucagon stimulation of mitochondrial respiration. J Biol Chem 1975; 250:7924–7930.
31. Tracey KJ, Beutler B, Lowry SF, et al. Shock and tissue injury induced by recombinant human cachectin. Science 1986; 234:470–474.
32. Rubin BY, Smith LJ, Hellerman GR, et al. A correlation between the anticellular and DNA fragmenting activities of tumor necrosis factor. Cancer Res 1988; 48:6006–6010.
33. Agarwal S, Drysdale B-E, Shin HS. Tumor necrosis factor-mediated cytotoxicity involves ADP-ribosylation. J Immunol 1988; 140: 4187–4192.
34. Brett J, Gerlach H, Nawroth P, et al. Tumor necrosis factor/cachectin increases permeability of endothelial cell monolayers by a mechanism involving regulatory G proteins. J Exp Med 1989; 169: 1977–1991.
35. Levy E, Path FC, Slusser RJ, Ruebner BH. Hepatic changes produced by a single dose of endotoxin in the mouse. Am J Pathol 1968; 52:477–490.
36. Cerra FB. Hypermetabolism, organ failure, and metabolic support. Surgery 1987; 101:1–14.
37. Damas P, Reuter A, Gysen P, et al. Tumor necrosis factor and interleukin-1 serum levels during severe sepsis in humans. Crit Care Med 1989; 17:975–978.
38. Girardin E, Grau GF, Dayer J-M, et al. Tumor necrosis factor and interleukin-1 in the serum of children with severe infectious purpura. N Engl J Med 1988; 319:397–400.
39. Curran RD, Billiar TR, Stuehr DJ, et al. Multiple cytokines are required to induce hepatocyte nitric oxide production and inhibit total protein synthesis. Ann Surg 1990; 212:462–471.
40. Mela L, Miller LD, Bacalzo LV, et al. Role of intracellular variations of lysosomal enzyme activity and oxygen tension in mitochondrial impairment in endotoxemia and hemorrhage in the rat. Ann Surg 1973; 178:727–735.
41. Perlmutter DH, Dinarello CA, Punsal PI, Colten HR. Cachectin/tumor necrosis factor regulates hepatic acute-phase gene expression. J Clin Invest 1986; 78:1349–1354.
42. Bankey PE, Mazuski JE, Ortiz M, et al. Hepatic acute phase response is indirectly regulated by tumor necrosis factor. J Trauma 1990; 30:1181–1188
43. Zhang Y, Marcillat O, Giulivi C, et al. The oxidative inactivation of mitochondrial electron transport chain components and ATPase. J Biol Chem 1990; 265:16330–16336.
44. Ding AH, Nathan CF, Stuehr DJ. Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. J Immunol 1988; 141:2407–2412.
45. Weiss SJ. Oxygen, ischemia and inflammation. Acta Physiol Scand 1986; 548(Suppl):9–37.
46. Zimmerman RJ, Chan A, Leadon SA. Oxidative damage in murine tumor cells treated in vitro by recombinant human necrosis factor. Cancer Res 1989; 49:1644–1648.
47. Videla L. Hepatic antioxidant-sensitive respiration. Effect of ethanol, iron and mitochondrial uncoupling. Biochem J 1984; 223:885–891.
48. Wong GHW, Goeddel DV. Induction of manganous superoxide dismutase by tumor necrosis factor: possible protective mechanism. Science 1988; 242:941–944.
49. Torti SV, Kwak El, Miller SC, et al. The molecular cloning and characterization of murine ferritin heavy chain, a tumor necrosis factor-inducible gene. J Biol Chem 1988; 263:12638–12644.
50. Kaufmann SHE. Heat shock proteins and the immune response. Immunol Today 1990; 11:129–132.
51. Yasmineh WG, Parkin JL, Caspers JI, Theologides A. Tumor necrosis factor/cachectin decreases catalase activity of rat liver. Cancer Res 1991; 51:3990–3995.
52. Kull FC Jr, Jacobs S, Cuatrecasas P. Cellular receptor for ^{125}I-labeled tumor necrosis factor: specific binding, affinity labelling, and relationship to sensitivity. Proc Natl Acad Sci USA 1985; 82:5756–5760.
53. Lefkowitz RJ, Stadel JM, Caron MG. Adenylate cyclase-coupled beta-adrenergic receptors: structure and mechanisms of activation and desensitization. Annu Rev Biochem 1983; 52:159–186.