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Tumor Necrosis Factor Alpha Alters the Cytotoxic Effect of Hydrogen Peroxide in Cultured Hepatocytes

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We examined whether tumor necrosis factor-α (TNF) affects the cytotoxic capacity of reactive oxygen species on rat hepatocytes in culture. Both TNF and reactive oxygen species are involved in many inflammatory events including hepatic ischemia/reperfusion injury and endotoxic shock. Synchronous treatment of hepatocytes with both TNF and H_2O_2 demonstrated that TNF (2000 ng/ml) enhanced the cytotoxic effect of H_2O_2 (500 μM). By contrast, pretreatment with TNF (2000 ng/ml) for 24 h followed by exposure to H_2O_2 (1000 μM) reduced the reactive oxygen-induced cytotoxicity. We conclude that TNF increases the effects of reactive oxygen-induced cytotoxicity when exposed synchronously, whereas TNF pretreatment induces a cytoprotective effect to reactive oxygen species, presumably by up-regulation of the reduced form of glutathione levels in hepatocytes.

Tumor necrosis factor-α (TNF) is now recognized as an important mediator of hepatic ischemia/reperfusion injury (1–3), and endotoxic shock (4,5). We have previously shown that TNF is released during ischemia/reperfusion injury and is involved in the associated pathophysiologic alterations of the liver: characterized by neutrophilic infiltration, leakage of hepatic enzymes, and hepatocyte necrosis (1). TNF generation is associated with reactive oxygen species, and several studies have implicated reactive oxygen species in hepatic ischemia/reperfusion injury (6–10). Recent studies revealed that TNF produces oxidative damage in several cell lines (11, 12) and also in hepatocytes (13). Furthermore, TNF enhances the deleterious effects of H_2O_2 on pulmonary vascular endothelial cells (14). We have recently shown that TNF and reactive oxygen species are both involved in, and interact in, hepatic ischemia/reperfusion (15). To date, there are no studies examining the combined cytotoxicity of TNF and reactive oxygen species in cultured hepatocytes *in vitro*.

We investigated whether TNF has a direct cytotoxic effect on hepatocytes, a synergistic effect with reactive oxygen species, or both. Experiments were designed to determine the cytotoxic effect on isolated hepatocytes of recombinant human TNF alone, synchronous TNF and H_2O_2 exposure, and TNF pretreatment followed by H_2O_2 exposure. TNF enhanced the reactive oxygen-induced cytotoxicity when the cells were exposed to TNF and H_2O_2 synchronously. Interestingly, we found that TNF pretreatment had a protective effect on hepatocytes that were subsequently exposed to H_2O_2. In addition, we assessed the effects of TNF on hepatocyte glutathione (GSH) and catalase activity, which are major intracellular oxygen radical buffering agents, in order to examine how TNF induces a cytoprotective effect to H_2O_2.

MATERIALS AND METHODS

Hepatocyte isolation. Hepatocytes were isolated using a modification of the method previously described by Seglen (16). Briefly, anesthetized male Sprague-Dawley rats (200–250 g, Charles River Breeding Laboratories, Portage, MI) were subjected to laparotomy and the liver was perfused via the portal vein with calcium- and magnesium-free HBSS containing 10 mM Hepes (Gibco Laboratories, Grand Island, NY) at 37°C. The perfusate was then changed to DMEM/F12 (Gibco) containing 0.05% collagenase type IV (Sigma Chemical Co., St. Louis, MO) for 10 min. The liver was excised and the cells were dispersed by shaking. The resulting crude preparation was filtered through sterile gauze and the collagenase was inactivated with 5% fetal calf serum. Repeated low-speed centrifugation was performed to minimize contamination by non-parenchymal cells. Hepatocyte viability was consistently more than 85%, which was confirmed by trypan blue exclusion. The cells were plated at a density of 1.25×10^5 cells per well in 24-well tissue culture plates (Primaria, Becton Dickinson & Co., Lincoln Park, NJ), which was equivalent to 1.59×10^5 cells/cm^2, in 1 ml of Media 199 (Gibco) containing 10 mM Hepes, 10^5 U/l penicillin G, 10^5 μg/l streptomycin, 1.6 U/l insulin, 400 nM dexamethasone, 10% calf serum. The cultures were incubated in a humidified chamber at 37°C in an atmosphere of 5% CO_2 for 24 h and the following studies were performed.

Hepatocyte treatment with H_2O_2, TNF, or TNF + H_2O_2. Cells were incubated with H_2O_2 (Mallinckrodt Specialty Chemicals Co., Paris, KY) at a dose range of 10–1000 μM to examine the kinetics

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of cell cytotoxicity, for periods of 0.25, 0.5, 1, 3, 6, or 24 h. Hepatocytes were exposed to recombinant human TNF (specific bioactivity of 2×10^7 U/mg, R&D Systems Inc., Minneapolis, MN) at a dose range of 0.2–2000 ng/ml for a period of 24 h. We also assessed the cytotoxicity of synchronous treatment with both TNF and H_2O_2 (500 μM) for 24 h, and of TNF pretreatment for 24 h followed by H_2O_2 (1000 μM) treatment for 5 h. In some studies, we used heat-inactivated TNF (90°C, 30 min), polyclonal goat anti-human TNF antibody (R&D Systems Inc.), and a nonrelevant control goat IgG (R&D Systems Inc.) to confirm that the observed effects were due to TNF itself. In some experiments, the γ-glutamyl cysteine synthetase inhibitor, DL-buthionine-[S, R]-sulfoximine (BSO) was used to deplete GSH in hepatocytes.

Cell cytotoxicity. Hepatocyte cytotoxicity was determined using an MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazoliumbromide, Sigma) method (17). Following experimental treatments, the cell supernatants were replaced by 1.0 ml of fresh media. Then 50 μl of 5 mg/ml MTT was added to each well and the cells were incubated at 37°C for 1 h. Viable cells convert the soluble yellow MTT to insoluble purple formazan by the action of mitochondrial dehydrogenases. A 750 μl aliquot of the supernatant was replaced with 0.04 N HCl/ isopropanol. After the MTT crystals had solubilized (3 h), 200 μl triplicate samples were pipetted into a 96-well plate. The optical density was measured at 550 nm (OD_{550}) with a microplate reader (EL311; Bio Tek Instruments Inc., Winooski, VT). The percentage cytotoxicity was calculated as follows; % specific cytotoxicity $= [1-(A-B)/(C-B)] \times 100$ where $A = OD_{550}$ of the treatment sample (e.g., rTNF, H_2O_2, etc.), $B = OD_{550}$ of a completely lysed sample, and $C = OD_{550}$ of the control sample.

Glutathione and catalase assays. Intracellular GSH, oxidized glutathione (GSSG) and catalase were measured after treatment with TNF (2000 ng/ml). After hepatocytes were treated with or without TNF, the cells were washed with HBSS and immediately detached with trypsin/EDTA (Gibco), then lysed with 100 μl of 1% Triton X-100 (Sigma). GSH and GSSG were measured by the enzymatic recycling assay described by Anderson (18). After 50 μl of 10% sulfosalicylic acid (Sigma) was added to each 100 μl aliquot of cell lysate, the samples were centrifuged (10,000 g) for 5 min at 4°C to precipitate the protein. To assay total glutathione, a 25 μl aliquot of supernatant was added to a mixture of 700 μl of 0.3 mM NADPH (Sigma), 100 μl of 6 mM DTNB (5,5′-dithiobis(2-nitrobenzoic acid), Aldrich Chemical Co., Milwaukee, WI), and 175 μl of H_2O. After adding 10 μl of 50 U/ml glutathione reductase (Sigma), the rate of change in optical density at 405 nm was assessed. To assay GSSG, the GSH present in the sample was derivatized by adding 2 μl of 2-vinylpiridine (Aldrich) and 6 μl of triethanolamine (Sigma) to a 100 μl aliquot of supernatant. After 60 min incubation at 25°C, GSSG was measured as the same manner as GSH. Both GSH and GSSG values are expressed as a percentage of the control value. To determine the extent to which BSO treatment depletes GSH in hepatocytes, GSH and GSSG were assayed after cells were exposed to BSO for periods of 0, 3, 8, and 24 h. Cellular catalase values were determined using the method described by Góth (19). Briefly, after hepatocytes were washed with HBSS, they were treated with trypsin/EDTA and then lysed with 200 μl of 1% Triton X-100. In an incubator at 37°C, 1.0 ml of H_2O_2 substrate (65 μmol/ml H_2O_2 in 60 mmol/l sodium-potassium phosphate buffer, pH = 7.4) was catabolized with 200 μl of sample for 60 s. The enzymatic degradation of H_2O_2 was stopped by addition of 1.0 ml of 32.4 mmol/l ammonium molybdate (Sigma). The yellow complex of ammonium molybdate and H_2O_2 was measured spectrophotometrically at 405 nm.

Statistics. All data are expressed as the mean ± SEM. The statistical significance between groups was assessed by Student's *t* test for single comparisons and by one-way analysis of variance (ANOVA) for multiple comparisons.

RESULTS

H_2O_2 cytotoxicity. The kinetic study of H_2O_2 cytotoxicity showed that 15 to 30 min incubation with H_2O_2 is not cytotoxic to hepatocytes at doses of up to 1000 μM, whereas a 1 to 24 h incubation with H_2O_2 is cytotoxic in a dose-dependent manner at doses from 10 to 1000 μM (Fig. 1).

Cytotoxic effects on hepatocytes of TNF and H_2O_2, separately or in combination. Treatment with TNF alone was not cytotoxic for hepatocytes at doses up to and including 2000 ng/ml for a period of 24 h. However, when hepatocytes were treated with TNF and H_2O_2 (500 μM) synchronously for 24 h, TNF potentiated the cytotoxic effect of H_2O_2 in a dose-dependent manner (Fig. 2). Incubation with H_2O_2 alone caused only 26.7 ± 7.3% cytotoxicity, whereas synchronous incubation with 2000 ng/ml TNF and H_2O_2 significantly increased the specific cytotoxicity to 73.3 ± 7.4% ($p = 0.01$). Heat-inactivated TNF did not enhance the cytotoxic effect of H_2O_2 (27.0 ± 5.5% cytotoxicity; no significant difference compared to H_2O_2 alone). Pretreatment of TNF with anti-TNF antibody abolished the cytotoxicity-enhancing effect (32.4 ± 4.6% cytotoxicity; no significant difference compared to H_2O_2 alone), but pretreatment of TNF with a control IgG did not reduce the cytotoxicity-enhancing effect (69.3 ± 3.0% cytotoxicity; $p<0.01$ compared to H_2O_2 alone). These findings confirmed that TNF itself enhanced the deleterious effect of H_2O_2.

In contrast, when cells were pretreated with TNF for 24 h and subsequently incubated with H_2O_2 (1000 μM) for 5 h, TNF provided a cytoprotective effect for the cells in a dose-dependent manner (Fig. 3). Preincubation without TNF followed by H_2O_2 treatment caused 59.1 ± 3.0% cytotoxicity, whereas preincubation with TNF (2000 ng/ml) and subsequent H_2O_2 treatment significantly decreased the cytotoxicity to 31.3 ± 2.3% ($n=5$, $p < 0.01$). Heat-inactivated TNF did not render a cytoprotective effect against H_2O_2 (62.2 ± 5.5% cytotoxicity; no significant difference compared to H_2O_2 alone). Pretreatment of TNF with anti-TNF antibody abolished the protective effect against H_2O_2 (60.7 ± 5.3% cytotoxicity; no significant difference compared to H_2O_2 alone), whereas pretreatment of TNF with a control IgG did not abolish the cytoprotective effect (33.1 ± 3.7% cytotoxicity; $p < 0.01$ compared to H_2O_2 alone). These results confirmed that TNF itself provided a cytoprotective effect against H_2O_2 if used as a pretreatment. Pretreatment with both TNF (2000 ng/ml) and BSO (0.1 mM) simultaneously decreased the cytoprotective effect against subsequent H_2O_2 (1000 μM) treatment (53.0 ± 4.7% cytotoxicity; $p < 0.01$ compared to 2000 ng/ml TNF pretreatment). This result suggests that intracellular GSH might be related to the cytoprotective effect provided by TNF pretreatment.

Effects of TNF or BSO on hepatocyte GSH and GSSG. After incubation with TNF (2000 ng/ml), intracellular

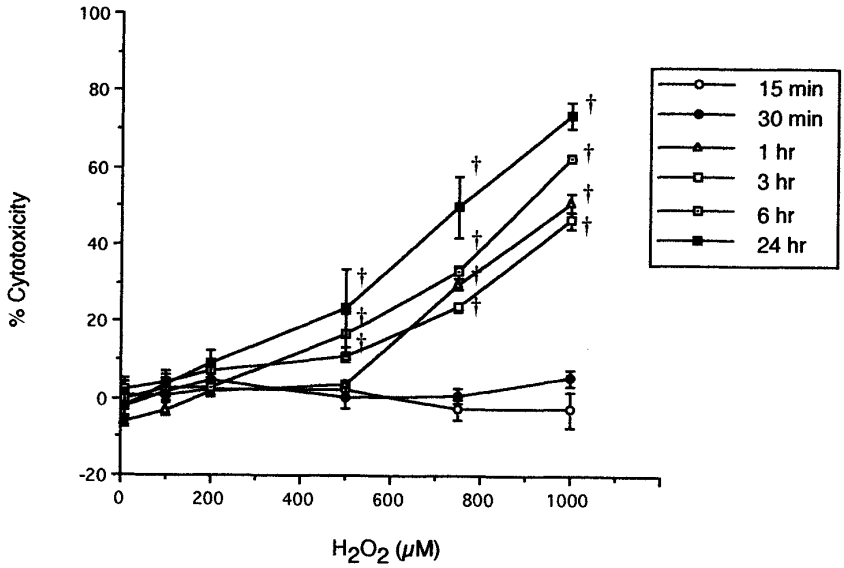

FIG. 1. Cytotoxic effect of H_2O_2 on hepatocytes at the indicated incubation times. Hepatocytes were incubated with H_2O_2 at concentrations of 10–1000 μM for 15 min–24 h. Cell viability was assessed by MTT assay. Specific cytotoxicity was calculated as follows; % cytotoxicity = $[1-(A-B)/(C-B)] \times 100$ where A = OD_{550} of the treatment sample, B = OD_{550} of a completely lysed sample, and C = OD_{550} of the control sample. Values are the mean ± SEM of more than five separate experiments. The differences between incubation times were compared by ANOVA and Student's *t* test. † indicates $p < 0.05$ compared to the control (without H_2O_2).

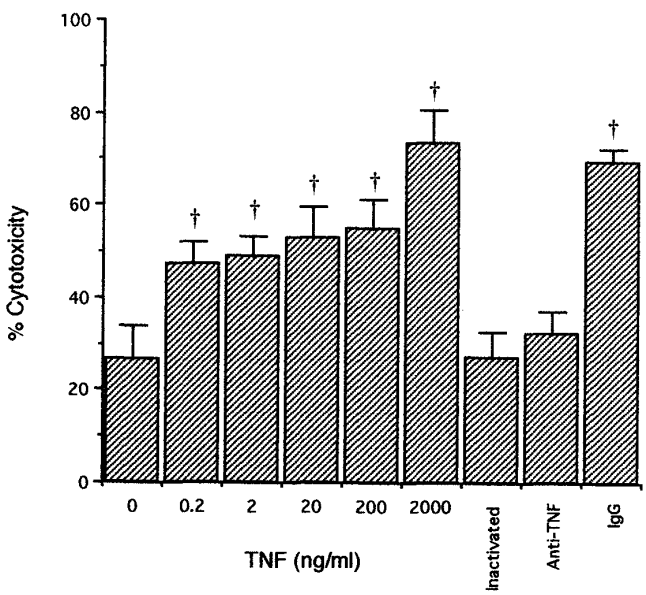

FIG. 2. Cytotoxic effect on hepatocytes of simultaneous TNF and H_2O_2 treatment for 24 h. Hepatocytes were incubated with various concentrations of TNF (0–2000 ng/ml) and H_2O_2 (500 μM) simultaneously for 24 h. Three additional, treatments were also used: Inactivated, heat-inactivated TNF (90°C, 30 min) together with H_2O_2 (500 μM); Anti-TNF, 30 min pretreatment of TNF with polyclonal goat anti-human TNF antibody then combined with H_2O_2 (500 μM) for 24 h hepatocyte incubation; IgG, 30 min pretreatment of TNF with nonrelevant control goat IgG then combined with H_2O_2 (500 μM) for 24 h hepatocyte incubation. Values are the mean ± SEM of five separate experiments. Results were compared with samples without TNF by Student's *t* test. † indicates $p < 0.05$ compared to controls without TNF.

GSH increased in a time-dependent manner (Fig. 4). After exposure to TNF for 24 h GSH had increased about two fold (222 ± 41%) compared with untreated cells. On the other hand, GSSG, which indicates the extent of oxidant stress, increased about two fold (193 ± 33%) after 3 h of TNF incubation and then gradually decreased.

Effect of TNF on hepatocyte catalase activity. We next examined hepatocyte catalase activity following TNF exposure to determine if this played a role in the protective mechanism. The catalase activity did not change after TNF exposure compared to controls.

DISCUSSION

It has been demonstrated previously that the cytotoxic mechanism of TNF involves oxidant damage in several cell lines (12) and hepatocytes (13). TNF and reactive oxygen species have been shown to be intimately related and to act synergistically in the course of inflammation, such as hepatic ischemia/reperfusion injury (15) and septic shock (20). Therefore, we investigated whether or not TNF has a deleterious effect on hepatocytes synergistically with reactive oxygen species *in vitro*.

In this study of TNF cytotoxicity, the maximal dose used was 2000 ng/ml (4×10^4 U/ml) of rh-TNF. The results showed that exposure to such a high concentration of TNF did not damage cultured hepatocytes, as determined by MTT assay. We could not determine the relevance to the physiological state in the liver of such

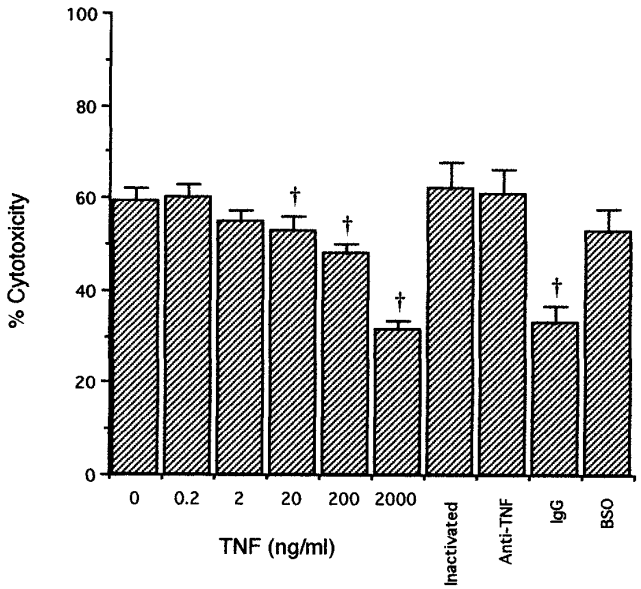

FIG. 3. Effect on hepatocytes of TNF for 24 h pretreatment followed by H_2O_2 for 5 h. Hepatocytes were preincubated with various concentrations of TNF (0–2000 ng/ml). Inactivated, heat-inactivated TNF (2000 ng/ml); Anti-TNF, TNF + neutralizing antibody; IgG, TNF + control IgG; BSO, TNF (2000 ng/ml) + BSO (0.1 mM). After these 24 h pretreatments, cells were incubated with H_2O_2 (1000 μM) for 5 h. Values are the mean ± SEM of five separate experiments. Results were compared to samples without TNF by Student's *t* test. † indicates $p < 0.05$ compared to controls without TNF.

a high concentration of TNF. We assume that a high concentration of TNF might exist at the sinusoidal level, since Kupffer cells release TNF when they are stimulated in the liver. Stadler et al. (21) reported that incubating hepatocytes with 2000 U/ml TNF (corresponding to 100 ng/ml TNF used in this study) causes a minor release of intracellular enzymes, with a rise in protein synthesis. Furthermore, Adamson and Billings (13) showed that 10 μg/ml TNF (corresponding to 31 μg/ml TNF used in this study: the difference is derived from various specific activity per amount) is not directly cytotoxic to hepatocytes over a 24 h exposure period, as determined by lactate dehydrogenase leakage. Our data are consistent with the suggestion that hepatocytes are very resistant to TNF, since the cytotoxicity of TNF seems to be mediated by oxidant radicals, and hepatocytes have a strong capacity for scavenging by antioxidants.

While TNF seems to have deleterious effects on tumor cell lines, resistance to TNF is also known to be induced by exposure to TNF. The mechanisms inducing TNF resistance have not been thoroughly elucidated. The TNF-resistant tumor cell line L929 was produced by up-regulating GSH with *in vitro* passaging of parental TNF-sensitive cells in the presence of TNF (12). In the same report, TNF incubation was shown to increase intracellular GSH in several other tumor cell lines, suggesting that TNF exposure increases the cells' protection against TNF itself. Additionally, several reports have suggested that TNF resistance is conferred by up-regulation of the expression of specific genes, including manganese superoxide dismutase (22), and others that may have protective effects, such as ferritin heavy chain (23), heat shock proteins (24), and plasminogen activator inhibitor type-2 (25).

We demonstrated here that protection against H_2O_2 is induced in hepatocytes by TNF pre-exposure. We

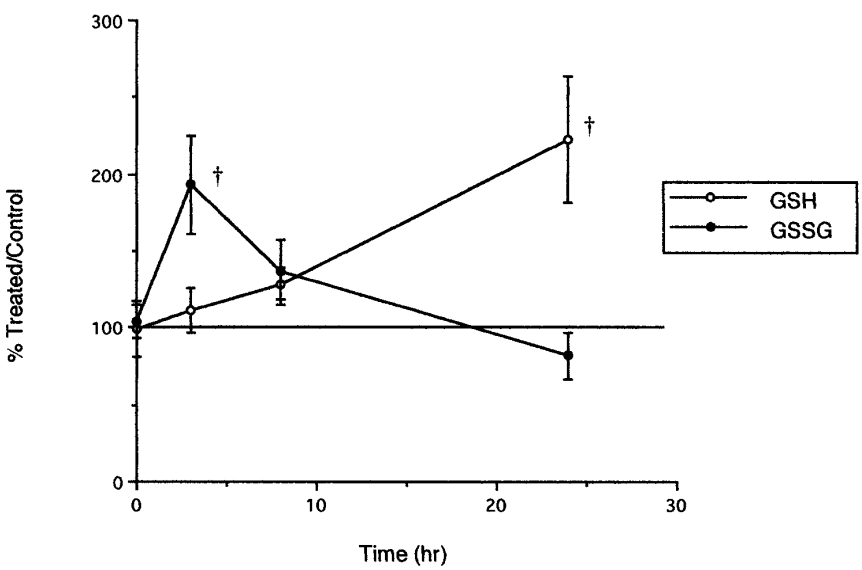

FIG. 4. Effect of TNF on the time course of hepatocyte GSH and GSSG. Cells were incubated with or without TNF (2000 ng/ml) for the indicated time periods. GSH and GSSG were determined using an enzymatic recycling assay. Values are the mean of % treated/control ± SEM of four separate experiments. Open circles represent GSH and closed circles represent GSSG. The levels of GSH and GSSG after 3, 8, and 24 h of TNF incubation were compared with the 0 h level by Student's *t* test. † indicates $p < 0.05$ compared to time 0 h.

also investigated whether TNF-mediated cytoprotection against H_2O_2 is due to the glutathione system and catalase, which have been reported to be major protective factors against H_2O_2 (26). Although the antioxidant system of GSH consists of several factors including GSH, GSSG, GSH peroxidase, GSSG reductase, and others, it has been reported that TNF susceptibility depends on the GSH content of tumor cells (12,27). We attempted to determine whether BSO, which is a GSH inhibitor, would suppress the protective effect of TNF toward H_2O_2 (Fig. 4). We demonstrated that GSH suppression by BSO during simultaneous TNF incubation did suppress the cytoprotective effect against H_2O_2 compared to TNF alone. We also confirmed that 24 h incubation with BSO (0.1 mM) does not have a toxic effect on hepatocytes. Furthermore, the extent of GSH suppression by BSO (0.1 mM) was determined to be 46.0%, compared to the control, after 24 h exposure.

We studied the effect of TNF exposure on hepatocyte GSH and catalase activity directly. The results demonstrated that GSH, but not catalase, is up-regulated, to 222%, in hepatocytes during exposure to TNF. A rise in intracellular GSSG after a 3 h incubation with TNF indicates that TNF produces oxidant stress in hepatocytes. A likely mechanism of GSH up-regulation is that TNF causes intracellular oxidant formation which increases conversion of GSH to GSSG resulting in rebound formation of GSH by an increase in the activity of the transport system for the amino acids cystine and glutamate (28). In contrast to the TNF-mediated GSH increase in hepatocytes, TNF incubation did not alter intracellular catalase activity. This result was consistent with other reports that TNF does not modify catalase activity in endothelial cells (29,30).

In summary, we have shown here that 24 h hepatocyte treatment with TNF alone is not cytotoxic at doses up to and including 2000 ng/ml. Synchronous treatment of hepatocytes with both TNF and H_2O_2 appeared to have a deleterious effect but, paradoxically, TNF pretreatment had a cytoprotective effect against subsequent H_2O_2 exposure. Synchronous treatment with TNF and H_2O_2, caused sudden damage to hepatocytes by oxygen free radicals without activation of protective mechanisms. The cytoprotective effect of TNF seems likely to be induced by GSH up-regulation in response to TNF-mediated oxidant stress, since GSH depletion by BSO reduced the cytoprotective effect induced by TNF pretreatment. We conclude that TNF has divergent effects in hepatocytes, producing a deleterious increase in susceptibility to H_2O_2 during simultaneous exposure, but inducing a protective effect against H_2O_2, presumably by up-regulating GSH levels, during sequential exposure.

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