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Identification of Tumor Necrosis Factor as a Transcriptional Regulator of the Phosphoenolpyruvate Carboxykinase Gene following Endotoxin Treatment of Mice

MOLLY R. HILL AND RODERICK E. McCALLUM†*

Department of Microbiology and Immunology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73190

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The decreased synthesis of hepatic phosphoenolpyruvate carboxykinase (PEPCK), the rate-limiting enzyme of gluconeogenesis, that occurs during endotoxemia was shown previously in rats to occur at the transcriptional level. In the current study, the exogenous administration of human recombinant tumor necrosis factor (TNF), a proximal mediator of endotoxic shock, reduced the PEPCK transcription rate, $mRNA^{PEPCK}$ levels, and PEPCK enzyme activity in a time- and dose-dependent manner in CD-1 mice. Comparable amounts of circulating TNF were measured in mice 2 h after injection of human recombinant TNF (10^5 U) or a 50% lethal dose of *Escherichia coli* endotoxin (20 mg/kg). Direct action of TNF to decrease the PEPCK transcription rate was confirmed in vitro with H-4-II-E Reuber hepatoma cells, in which a dose-dependent inhibition of PEPCK transcription was observed with 1 to 100 U of TNF per ml. A role for TNF-elicited changes in PEPCK gene expression during endotoxemia was confirmed by the protective effect of rabbit polyclonal antibodies to recombinant murine TNF. C57BL/6 mice passively immunized with anti-TNF 4 h prior to endotoxin challenge exhibited normal PEPCK enzyme activity. Neutralization of circulating TNF with anti-TNF failed, however, to prevent the hypoglycemia commonly observed during endotoxemia, suggesting the participation of other mediators. Anti-TNF treatment reduced circulating interleukins 1 and 6 at 3 and 6 h after endotoxin treatment, respectively. These results suggest that during endotoxemia, the development of hypoglycemia is multifaceted and that several cytokines are most likely involved. The findings from the Reuber hepatoma cell model afford an opportunity in future work to map putative cytokine response elements in the PEPCK promoter responsible for perturbed hormonal regulation of the gene during endotoxemia.

Disrupted carbohydrate homeostasis, evidenced by hypoglycemia, is a common feature of infectious disease and intoxication. Although several mechanisms have been proposed to account for endotoxic hypoglycemia (36, 58), we have focused our efforts on the molecular mechanisms underlying the diminished gluconeogenesis that occurs (37). Berry et al. (5) first observed in mice an inhibitory effect of endotoxin on the glucocorticoid induction of the rate-limiting enzyme of gluconeogenesis, phosphoenolpyruvate carboxykinase (PEPCK). We observed subsequently that mice given a 50% (LD_{50}) lethal dose of *Salmonella typhimurium* endotoxin were hyperglycemic at 1 h after treatment; however, the animals became hypoglycemic at 3 and 6 h (39). Coincident with this pattern, hepatic PEPCK activity decreased as early as 3 h after endotoxin. Furthermore, dexamethasone induction of PEPCK enzyme synthesis was substantially reduced in endotoxic animals. Subsequent studies revealed that decreased PEPCK activity during endotoxemia was mediated by soluble factors produced by macrophages. We observed an inhibitor of hepatocyte PEPCK induction produced by Kupffer cells stimulated with endotoxin (36), and Moore et al. (41) performed numerous studies characterizing a macrophage product termed glucocorticoid-antagonizing factor that is responsible for reduced hormonal induction of PEPCK both in vivo and in vitro. A role for monokines in the hypoglycemia resulting from endotoxic shock was suggested by passive transfer experiments by Hill et al. (28), who observed that diminished hepatic glucocorticoid receptor function during endotoxemia was plasma mediated. Results from additional studies revealed that the cytokines interleukin 1 (IL-1; 27, 38), tumor necrosis factor (TNF; 26, 38), and IL-6 (29) mediated impaired hormonal regulation of PEPCK synthesis both in vivo and in vitro.

Substantial interest has recently focused on TNF as the proximal mediator of septic and endotoxic shock (reviewed in reference 8). A central role for TNF in the pathogenesis of septic shock was first suggested by results of protection experiments by Beutler et al. (7) and Tracey et al. (55). In these experiments, anti-TNF antibodies were administered prior to endotoxic challenge. Furthermore, the injection of recombinant TNF into rats or mice results in hypoglycemia (2, 54) as well as a variety of metabolic changes similar to those observed in sepsis (reviewed in reference 23). Chajek-Shaul et al. (13) reported that adrenalectomy significantly sensitized rats to lethal hypoglycemia and hypothermia. Treatment with human recombinant TNF (hurTNF) caused a significant reduction in liver PEPCK and glucose-6-phosphatase activities, although no changes in the activities of these enzymes were observed in sham-operated controls given the same dose of TNF.

Vogel and Havell (57) reported that antibody prepared against murine TNF (murTNF) abolished endotoxin-induced TNF activity yet failed to abrogate endotoxin-induced hypoglycemia. Silverstein et al. (48) observed that hydrazine sulfate (a compound that stimulates pituitary-adrenal hormone release [49]) protected mice from endotoxin lethality

* Corresponding author.
† Present address: Department of Medical Microbiology and Immunology, Texas A&M Health Science Center, College Station, TX 77843.

and prevented a decrease in hepatic PEPCK activity, although there was no corresponding improvement in levels of glucose in plasma. The participation of multiple cytokines in the hypoglycemia characteristic of sepsis and endotoxic shock is suggested by the recent work of Vogel et al. (58) and Mengozzi et al. (40), in which hypoglycemia resulting from endotoxin, IL-1, or TNF was prevented in animals given recombinant IL-1 receptor antagonist.

We recently examined the effect of a nonlethal dose of *Escherichia coli* endotoxin on PEPCK gene expression in fasted rats (26). Five hours after endotoxin treatment, the PEPCK transcription rate and the amount of $mRNA^{PEPCK}$ were significantly decreased at a time when the insulin/glucagon molar ratio and the levels of corticosterone in plasma were elevated. Factors in addition to increased insulin (a deinducer of the PEPCK gene [45]) were suggested to underlie the observed changes in PEPCK transcription, since similar findings were obtained in rats with streptozoticin-induced diabetes. The administration of recombinant IL-6, a potent inflammatory mediator in the liver, was shown to decrease cyclic AMP (cAMP) induction of PEPCK gene expression. These results indicated that during endotoxemia, PEPCK gene expression is regulated at the transcriptional level by inflammatory mediators.

This study was undertaken to examine the role of TNF as a transcriptional inhibitor of the PEPCK gene. Mice treated with hurTNF displayed reduced PEPCK transcription rates and $mRNA^{PEPCK}$ amounts similar to those resulting from endotoxin injection. The in vitro addition of TNF to hepatoma cell cultures reduced PEPCK transcription rates. Administration of polyclonal antibody to murTNF ablated the plasma TNF response after endotoxin injection and the subsequent reduction in PEPCK gene expression. A role for additional cytokines (IL-1 and IL-6) in disrupted carbohydrate metabolism is suggested, however, by the failure of anti-TNF to protect endotoxic mice from hypoglycemia.

MATERIALS AND METHODS

Animals. Male CD-1 random outbred mice (18 to 20 g) were purchased from Charles Rivers Laboratories (Wilmington, Mass.), and male C57BL/6 inbred mice (20 to 25 g) were purchased from Sasco (Omaha, Nebr.). Animals were given food and water ad libitum and maintained under National Institutes of Health guidelines for the care and use of laboratory animals (31). All experimental protocols were approved by the Institutional Animal Care and Utilization Committee at the University of Oklahoma Health Sciences Center.

Cell culture. H-4-II-E Reuber rat hepatoma cells (RHC) and L929 mouse fibroblasts were obtained from the American Type Culture Collection (Rockville, Md.). RHC and L929 cells were maintained as monolayers in 75-cm^2 flasks containing Iscove's modified Dulbecco medium (GIBCO, Grand Island, Nebr.) with 10% fetal calf serum and gentamicin at 37°C in a humidified 5% CO_2 incubator. B9 hybridoma cells were obtained from Lucien Aarden (Central Laboratory of The Netherlands Red Cross, Amsterdam) and were maintained in suspension culture in Iscove's modified Dulbecco medium containing 5% heat-inactivated fetal calf serum, glutamine, mercaptoethanol, penicillin, and streptomycin and supplemented with 10 to 100 U of human recombinant IL-6 (R&D Systems, Minneapolis, Minn.) per ml. For in vitro cytokine experiments, RHC were cultured in 150-cm^2 plastic dishes for 5 to 7 days. Eighteen hours before use, the medium was removed and replaced with serum-free medium. For cytokine assays, L929 cells and B9 cells were subcultured to 96-well plates.

Endotoxin, recombinant cytokines, and anti-TNF antibody preparation. Endotoxin was prepared from *E. coli* serotype O111:B4 by hot aqueous-phenol extraction, ultracentrifugation, and alcohol precipitation (61). The preparation contained <3% RNA and <5% protein, and the LD_{50}s determined by the method of Reed and Muench (44) were approximately 20 and 10 mg/kg for CD-1 and C57BL/6 strain mice, respectively. hurTNF (lot R9071AX; specific activity, 4.75×10^7 U/mg) and murTNF (lot 4296-17; specific activity, 1.2×10^7 U/mg were kindly provided by Genentech, Inc. (South San Francisco, Calif.). Rabbit polyclonal antiserum to murTNF was elicited by using the protocol recommended for the Ribi adjuvant system. Rabbits were immunized with 50 μg of murTNF in adjuvant (0.5 mg each of monophosphoryl lipid A, trehalose dimycolate, and cell wall skeleton in 0.2% Tween 80) (Ribi Immunochem Research, Inc., Hamilton, Mont.). Antigen was injected subcutaneously and intramuscularly at several sites. Animals were boosted 3 weeks following primary immunization and bled 10 days later, and subsequent booster injections were administered at 3-week intervals. Following immunization with a total of 200 μg of murTNF, the serum immunoglobulin G (IgG) fraction was isolated, and the neutralizing activity was determined with the L929 cell cytotoxicity assay. One neutralizing unit (NU) of anti-TNF antibody is defined as the reciprocal of the dilution that resulted in 50% inhibition of cell cytotoxicity resulting from treatment with 500 U of murTNF per ml. The immune rabbit IgG fraction was found to contain 1.35×10^5 NU/ml, or 4.25×10^3 NU per mg of protein. Normal rabbit IgG (Sigma Chemical Co., St. Louis, Mo.) was used for a control and demonstrated no TNF-neutralizing activity by the L929 assay.

Time course of endotoxin and hurTNF effects in CD-1 mice. In order to compare the effects of endotoxin injection (20 mg/kg of body weight, given intraperitoneally [i.p.]) with hurTNF administration (10^4 or 10^5 U per mouse, given intravenously [i.v.]) on hepatic PEPCK gene expression, CD-1 random outbred mice were treated and then monitored at 0, 2, 4, 6, and 8 h after injection. Food was withdrawn the evening before experimentation, and fasting was continued throughout each experiment to induce PEPCK. At intervals after treatment, animals were anesthetized with methoxyflurane (Metafane; Pitman-Moore, Inc., Washington Crossing, N.J.) and decapitated to obtain trunk blood for TNF, glucose, and insulin assays. Liver samples were obtained for (i) nucleus isolation for PEPCK transcription run-on assay, (ii) $mRNA^{PEPCK}$ isolation and measurement, and (iii) cytosol preparation for PEPCK enzyme assay. Samples from three mice per treatment were pooled at each time interval, and the experiment was repeated three times.

Effect of anti-TNF antibody treatment of C57BL/6 mice on lipopolysaccharide-induced responses. In a preliminary series of experiments, the effect of rabbit polyclonal anti-murTNF on endotoxin lethality was determined in C57BL/6 mice. Five or six mice per group were passively immunized 4 h before endotoxin challenge (25, 10, 5, or 1 mg/kg). A total of 6.75×10^4 NU per mouse was injected i.p. (0.25 ml) and i.v. (0.25 ml). Control animals were administered 31.7 mg of normal rabbit IgG in a similar fashion. The animals were observed for 48 h, and the mean time to death was calculated for each treatment group.

To evaluate the role of TNF in endotoxin-induced changes in PEPCK transcription, anti-murTNF was administered to C57BL/6 inbred mice 4 h before endotoxin challenge (10

mg/kg, i.p.). A total of 6.75×10^4 NU per mouse was injected i.p. (0.25 ml) and i.v. (0.25 ml). Control animals were either untreated or administered 31.7 mg of normal rabbit IgG. Plasma was obtained for TNF assay at 90 min and for IL-1 assay at 3 h after treatment by the insertion of a heparinized microhematocrit tube into the ophthalmic venous plexus while mice were under methoxyflurane anesthesia. At 6 h after treatment, all animals were anesthetized and decapitated to obtain trunk blood for IL-6 and glucose assays. Liver samples were obtained for cytosol preparation for PEPCK enzyme assay. Samples obtained from five or six mice per treatment were used.

Effect of hurTNF on PEPCK transcription in RHC in vitro. Following overnight incubation of cells in serum-free medium, PEPCK was induced in RHC by the addition of 125 nM dexamethasone, 125 μM 8-(4-chlorophenylthio)-adenosine 3′:5′-cyclic monophosphate (CPT-cAMP), and 50 μM 3-isobutyl-1-methylxanthine (IBMX; a phosphodiesterase inhibitor). hurTNF (1, 10, or 100 U/ml) was added at the same time as inducers, and the cultures were incubated at 37°C for 1 h. Cells were scraped from the plates and homogenized in Tris-sucrose buffer, nuclei were isolated and purified, and PEPCK transcription run-on assays were performed as described below. Insulin (1 nM), a deinducer of the PEPCK gene (45), was added as a positive control.

PEPCK nuclear run-on assay. The PEPCK transcription elongation assay was carried out according to the procedure of Sasaki et al. (45). Following homogenization of liver tissue, nuclei were isolated through a series of centrifugations in sucrose buffers. Isolated nuclei (200 μg) were incubated at 26°C for 10 min in 75 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) at pH 8.0; 100 mM KCl; 2.5 mM $MgCl_2$; 25% glycerol; 0.1 mM phenylmethylsulfonyl fluoride; 0.05 mM EDTA; 4 mM dithiothreitol; 25 U of RNasin; 0.5 mM each ATP, CTP, and GTP; and 200 μCi of [α-^{32}P]UTP (400 to 600 Ci/mmol). The reaction was terminated by digestion with DNase I and proteinase K, and after phenol extraction and alcohol precipitation, the RNA samples were hybridized with nitrocellulose-bound pPC112.R3 (provided by Daryl Granner, Vanderbilt University) or pBR322 as a control. pPC112.R3 is a plasmid containing a 5.8-kb *Eco*RI site of pPR322 (3). All of the insert represents PEPCK DNA that is transcribed into precursor mRNA. The results are expressed as parts of PEPCK mRNA transcribed per million parts of total RNA transcribed. Modifications for nuclei isolated from RHC were made as described in reference 45.

Quantitative measurement of mRNA^{PEPCK}. Following experimental treatment, total liver RNA was isolated by the Chirgwin et al. method (14). RNA samples were denatured in 20× standard saline citrate plus 37% formaldehyde. After being heated at 60°C for 15 min, 2.5 μl of each sample was applied under vacuum to a nylon membrane with an ABN slot blot apparatus (American Bionetics, Hayward, Calif.). The blots were air dried, heated at 80°C for 1 h, and prehybridized for 6 to 18 h. The blots were hybridized overnight with a cDNA probe for PEPCK (pPC116 [3], also provided by D. Granner) or a cDNA probe for actin (*Drosophila melanogaster*; obtained from Sarah Tobin, University of Oklahoma Health Sciences Center) labeled with [^{32}P]dCTP by random priming with a Prime-A-Gene kit (Amersham Corp., Arlington Heights, Ill.). Following hybridization, the blots were washed under stringent conditions, and autoradiographs were prepared. The intensities of hybridized bands were determined by laser scan densitometry (Molecular Dynamics, Sunnyvale, Calif.), and the results are expressed as relative integrator units adjusted to a constant amount (optical density at 260 nm) of total RNA applied to the blot.

TNF, IL-1, and IL-6 assays. For TNF assay, L929 cells were plated at 2×10^4 cells per well in 96-well microtiter plates and incubated overnight at 37°C in 5% humidified CO_2. Plasma was serially diluted in medium containing actinomycin D (1 μg/ml) and added to the cells. Negative controls consisted of cells incubated with medium plus actinomycin D alone, and positive controls consisted of cells incubated with medium containing serial dilutions of murTNF. Following incubation for 18 h, 3-(4,5dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 5 mg/ml in phosphate-buffered saline) was added (25 μl/well), and incubation was continued for an additional 2 h. The microtiter plates were centrifuged at $600 \times g$ for 2 min at 4°C and the medium was removed. Lysing buffer (50% dimethyl formamide and 20% sodium dodecyl sulfate; 100 μl per well) was added, and the plates were incubated 4 h at 37°C. The optical density at 570 nm was read with a Dynatech MR-700 microtiter plate reader (Dynatech Laboratories, Inc., Alexandria, Va.), and the survival data were linearized by logit transformation and plotted against the log of the plasma dilution. TNF content is expressed as units per milliliter relative to results with the murTNF standard included on each plate.

Levels of IL-1 in the plasma of endotoxic mice pretreated with anti-TNF antibody were determined at 3 h after endotoxin administration by enzyme-linked immunosorbent assay (ELISA) with the InterTest-1αX kit (Genzyme Corp., Cambridge, Mass.). This assay is highly specific for mouse IL-1α, and the results are expressed in picograms per milliliter.

The level of IL-6 in plasma was measured with the B9 proliferation assay as described by Helle et al. (25). Briefly, B9 cells were plated in 96-well microtiter plates at 5,000 cells per well in Iscove's modified Dulbecco medium containing 15% fetal calf serum. Plasma was serially diluted, and 1, 0.1, 0.01, and 0.001% dilutions were added to the cells, which were incubated at 37°C for 3 days in a 5% humidified CO_2 incubator. The indicator, MTT, was added as described above for the TNF assay, and the optical density at 570 nm was determined. The picograms per milliliter were interpolated from a standard curve prepared with human recombinant IL-6.

Enzyme, hormone, and metabolite assays. Liver cytosol preparations were obtained by homogenizing livers in 0.25 mM Tris-sucrose (pH 7.4) containing 5 mM dithiothreitol and centrifuging them at $468{,}000 \times g$ for 10 min in a TL-100 tabletop ultracentrifuge (Beckman Instruments, Inc., Palo Alto, Calif.). PEPCK enzyme activity was measured by a ^{14}C radiometric method as described previously (39). Units of enzyme activity correspond to nanomoles of oxaloacetate formed from phosphoenolpyruvate per 15 min per milligram of cytosol protein. Protein was determined by using the bicinchoninic acid protein assay kit (50) (Pierce Biochemicals, Rockford, Ill.). Levels of insulin in plasma were measured by using a radioimmunoassay kit from Ventrex Laboratories (Cambridge, Mass.), and the results are expressed in microunits per milliliter. Levels of glucose in plasma were determined by a colorimetric glucose oxidase method (Sigma), and the results are expressed as milligrams per deciliter.

Statistical analysis. Statistical analysis of the data was performed by using Student's *t* test for significant differences

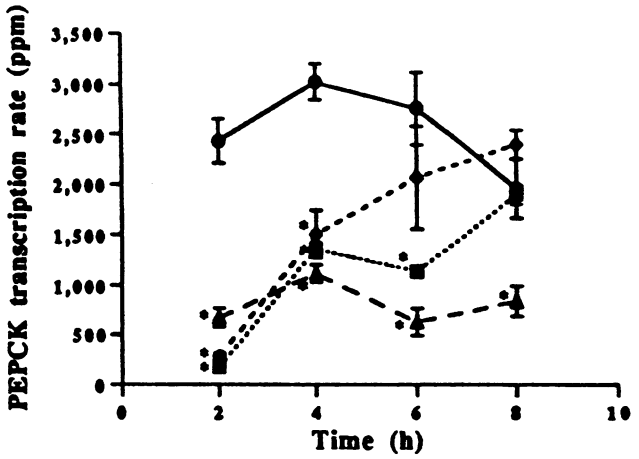

FIG. 1. Hepatic PEPCK transcription rate in CD-1 mice treated with endotoxin (20 mg/kg) or hurTNF (100,000 or 10,000 U). At 2, 4, 6, and 8 h after treatment, hepatic nuclei were prepared as described in Materials and Methods, and nuclear run-on assays were performed. Results represent means ± standard errors of the means for nuclei pooled from three mice at each time interval in four separate experiments and are expressed as parts of PEPCK mRNA transcribed per million parts of total RNA transcribed. Symbols: ●, control; ▲, endotoxin; ◆, 100,000 U of TNF; ■, 10,000 U of TNF; *, significantly different ($P \leq 0.05$) from fasted control value.

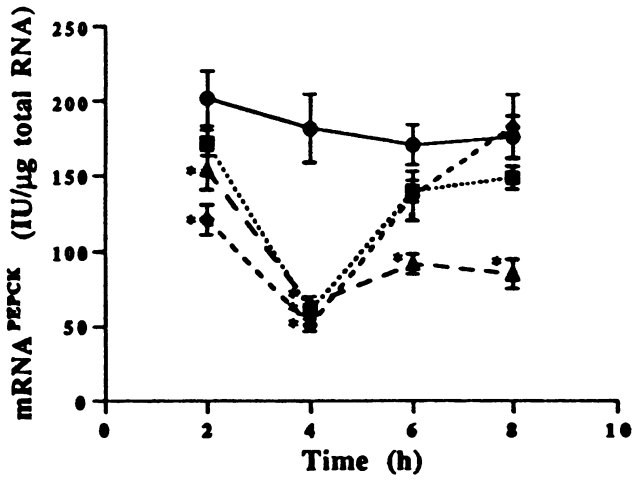

FIG. 2. Hepatic mRNA^{PEPCK} levels in CD-1 mice treated with endotoxin (20 mg/kg) or hurTNF (100,000 or 10,000 U). At 2, 4, 6, and 8 h after treatment, total RNA was prepared from liver homogenates as described in Materials and Methods, and slot blot hybridization with a cDNA probe for PEPCK was performed. Results represent means ± standard errors of the means for livers pooled from three mice at each time interval in four separate experiments and are expressed as relative integrator units (IU) per microgram of total RNA as determined by laser densitometry of autoradiographs. Symbols: ●, control; ▲, endotoxin; ◆, 100,000 U of TNF; ■, 10,000 U of TNF; *, significantly different ($P \leq 0.05$) from fasted control value.

($P \leq 0.05$) with StatWorks on a Macintosh SE30 microcomputer (Apple Computer, Inc., Cupertino, Calif.).

RESULTS

Exogenous administration of hurTNF to CD-1 mice reduces hepatic PEPCK gene expression in a manner similar to endotoxin treatment. We showed previously that endotoxin treatment of rats decreased gene expression of PEPCK (26). In addition, IL-1, TNF, and IL-6 treatment of RHC in vitro has been demonstrated to down regulate hormonal induction of PEPCK (29, 38). Since circumstantial and direct evidence implicates TNF as the proximal mediator in endotoxic shock (6, 7, 55), we carried out the following experiments to determine if TNF administration mimicked the effects of bacterial endotoxin on PEPCK gene expression. Following fasting overnight to induce PEPCK, CD-1 mice were injected with an LD_{50} of endotoxin (20 mg/kg) or hurTNF (10^4 or 10^5 U). Groups of animals were killed at 2-h intervals after treatment, and blood and liver tissue were obtained.

Endotoxin treatment of fasted mice significantly decreased the PEPCK transcription rate at 2 h, an effect that continued through the 8-h observation period (Fig. 1). The PEPCK transcription rate in endotoxin-treated mice at 2 h was similar to that seen in fed control mice (641 ± 13 ppm), indicating that endotoxin treatment completely blocked the enhanced PEPCK transcription rate due to fasting. Following treatment with 10^5 U of hurTNF, the PEPCK transcription rate at 2 h was also decreased to nonfasting rates; however, a gradual recovery to normal transcription rate was observed by 8 h after treatment. Interestingly, a smaller dose of hurTNF (10^4 U) had effects similar to those of 10^5 U, yet animals demonstrated a slower return to normal.

Reduced transcription of the PEPCK gene in endotoxin- and hurTNF-treated mice was reflected by reduced amounts of hepatic mRNA^{PEPCK} (Fig. 2). Compared with endotoxin- and TNF-induced changes in the PEPCK transcription rate noted at 2 h (Fig. 1), substantial reduction of mRNA^{PEPCK} levels was not observed until 4 h after treatment. A slight but significant reduction in mRNA^{PEPCK} (control = 202 ± 19 integrator units per μg) was seen at 2 h in mice treated with endotoxin (154 ± 13 integrator units per μg) or with 10^5 U of TNF (121 ± 10 integrator units per μg). However, by 4 h after treatment, mice given endotoxin, 10^5 U of TNF, or 10^4 U of TNF had mRNA^{PEPCK} levels only 36, 28, and 34% of the control values, respectively. Thus, an approximately 2-h delay between maximal reductions in PEPCK transcription rate and substantial decreases in mRNA^{PEPCK} was seen in endotoxin- and TNF-treated mice. TNF-treated animals demonstrated a return of mRNA^{PEPCK} levels to control values at 6 and 8 h, whereas mRNA^{PEPCK} levels in endotoxic mice remained at approximately 50% of that in controls throughout the 8-h course of treatment. Endotoxin or TNF treatment did not have an effect on total RNA metabolism, as indicated by no significant differences between treatment groups in hepatic levels of mRNA of the housekeeping gene, actin (data not shown).

The biological result of reduced PEPCK transcription rate and mRNA^{PEPCK} was observed 8 h following treatment as a substantial reduction in hepatic PEPCK enzyme activity (Table 1). Although slight decreases in activity were seen at 4 and 6 h after endotoxin or hurTNF treatment (data not shown), significantly less PEPCK activity ($P \leq 0.05$) was not seen until 8 h. Regardless of dose, endotoxin and 10^4 or 10^5 U of hurTNF decreased enzyme activity to less than 50% of that of the fasted control. The actual number of PEPCK enzyme units induced due to fasting in endotoxin- or TNF-treated mice was less than 25% of the control value.

The exogenous administration of 10^5 U of hurTNF mimicked the circulating-TNF response seen in mice 2 h after endotoxin treatment (Table 2). TNF levels in plasma were

TABLE 1. Hepatic PEPCK activity in CD-1 mice 8 h after treatment with endotoxin or hurTNF

Treatment	PEPCK	
	Activity (U/mg)	U induced
Fed	466 ± 37[a]	
Fasted[b]	1,204 ± 36	738
Fasted + endotoxin (20 mg/kg)[c]	600 ± 61[d]	134
Fasted + hurTNF (10^5 U/mouse)[e]	549 ± 66[d]	83
Fasted + hurTNF (10^4 U/mouse)[e]	643 ± 42[d]	177

[a] Mean ± standard error of the mean for 9 to 12 animals per group.
[b] Fasted overnight plus an additional 8 h after the start of the experiment at 8:00 a.m.
[c] RNA-free lipopolysaccharide extracted (Westphal) from *E. coli* O111:B4 and administered i.p.
[d] Significantly different from fasted control value ($P \leq 0.05$).
[e] hurTNF administered i.v.

assayed by the L929 cytotoxicity assay, and comparable amounts of the cytokine were seen in animals treated with endotoxin or 10^5 U of hurTNF (482 ± 76 and 604 ± 32 U/ml, respectively). A 10-fold-lower dose of hurTNF (10^4 U) resulted in a 10-fold-lower amount of circulating TNF at 90 min after injection.

Both endotoxin and hurTNF elicit an early elevation of plasma insulin. Since we previously observed increased insulin levels in plasma of rats following endotoxin treatment (26), similar measurements were performed in endotoxin- and hurTNF-injected mice. As can be seen in Fig. 3, increases in levels of insulin in plasma occurred as early as 2 h after treatment with either endotoxin or hurTNF. The mild elevation (only double the control values) in levels of insulin in plasma of endotoxic mice returned to normal by 6 h, yet mice treated with 10^5 U of hurTNF manifested slightly increased levels of insulin until 8 h following treatment.

In vitro addition of hurTNF to RHC down regulates PEPCK transcription. In order to determine if TNF acted directly to down regulate PEPCK transcription in the liver, RHC were treated with the cytokine in vitro at the same time that inducers (dexamethasone and cAMP) of PEPCK gene expression were added. As can be seen in Fig. 4, a dose-dependent inhibition of PEPCK induction was seen in RHC treated with 1 to 100 U of hurTNF per ml for 1 h. A statistically significant effect of TNF on PEPCK transcription rate was noted with 100 U/ml, an amount that produced

TABLE 2. TNF activity in plasma of CD-1 mice 2 h after treatment with endotoxin or hurTNF

Treatment	Amt	TNF activity in plasma (U/ml)
Fasted[a]	NA[b]	—[c]
Fasted + endotoxin	20 mg/kg[d]	482 ± 76[e]
Fasted + hurTNF	10^5 U/mouse[f]	604 ± 32
Fasted + hurTNF	10^4 U/mouse[f]	65 ± 9[g]

[a] Fasted overnight plus an additional 8 h after the start of the experiment at 8:00 a.m.
[b] NA, not applicable.
[c] —, not detected.
[d] RNA-free lipopolysaccharide extracted (Westphal) from *E. coli* O111:B4 and administered i.p.
[e] Mean ± standard error of the mean for 9 to 12 animals per group.
[f] hurTNF administered i.v.
[g] Significantly less than the value for the 10^5 U per mouse group ($P \leq 0.05$).

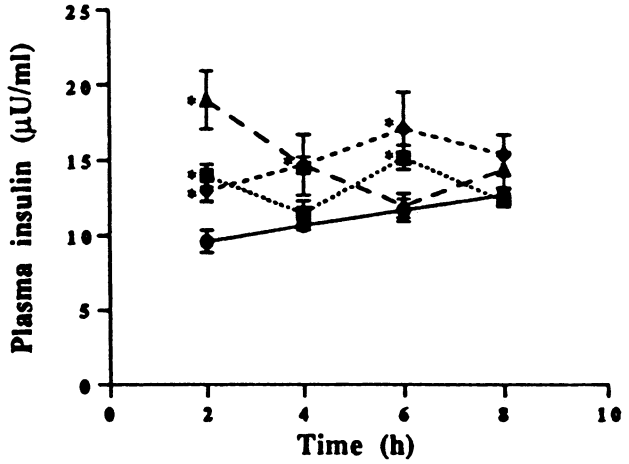

FIG. 3. Levels of insulin in plasma of CD-1 mice treated with endotoxin (20 mg/kg) or hurTNF (100,000 or 10,000 U). At 2, 4, 6, and 8 h after treatment, blood was obtained and insulin levels in plasma were determined by commercial radioimmunoassay as described in Materials and Methods. Results represent means ± standard errors of the means for plasma pooled from three mice at each time interval in four separate experiments. Symbols: ●, control; ▲, endotoxin; ◆, 100,000 U of TNF; ■, 10,000 U of TNF; *, significantly different ($P \leq 0.05$) from fasted control value.

the same degree of inhibition as that seen after insulin addition. The decreased PEPCK transcription rates in RHC treated with hurTNF and insulin were reflected further in decreases in PEPCK enzyme activity at 4 h after treatment. TNF (10 U/ml) caused a decrease in enzyme activity to 49%

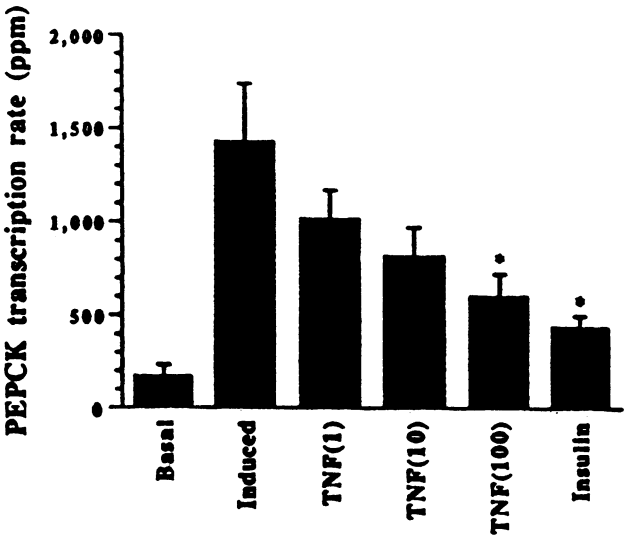

FIG. 4. PEPCK transcription rate in H-4-II-E RHC cultured for 1 h in the presence of hurTNF (1, 10, or 100 U/ml). PEPCK was induced by the simultaneous addition of CPT-cAMP, IBMX, and TNF. Nuclei were prepared as described in Materials and Methods, and nuclear run-on assays were performed. Results represent means ± standard errors of the means for triplicate determinations on cells from four separate experiments and are expressed as parts of PEPCK mRNA transcribed per million parts of total RNA transcribed. *, significantly different ($P \leq 0.05$) from fasted control value.

of that of the induced control, and insulin decreased activity to 24% of that of the induced control.

In other experiments, hurTNF treatment of RHC caused a decrease in the basal transcription rate of the PEPCK gene (not treated with hormonal inducers). Control RHC demonstrated a PEPCK transcription rate of 300 ± 42 ppm, whereas RHC treated with hurTNF (1 U/ml) or insulin (1 nM) had transcription rates of 139 ± 3 and 117 ± 33 ppm, respectively.

Reduction of endotoxin lethality in C57BL/6 mice pretreated with rabbit polyclonal anti-TNF. Since antibody to TNF can diminish endotoxin lethality by neutralizing TNF and its actions (7, 55), we tested the effect of passive immunization of endotoxic mice with anti-TNF on PEPCK induction. Initially, the effect of anti-TNF on endotoxin lethality was assessed. Passive immunization of C57BL/6 mice with 6.75 × 10^4 NU of anti-TNF 4 h before challenge with 1 LD_{50} (10 mg/kg) of endotoxin reduced mortality from 67% to zero (data not shown). Survival was not improved, however, in anti-TNF-treated mice challenged with a lethal dose (25 mg/kg) of endotoxin. All immunized mice died after lethal endotoxin challenge, exhibiting a mean time to death of 23.4 h. The dose-response curve for endotoxin lethality in C57BL/6 mice was very narrow, since no mortality was noted in animals given either 1 or 5 mg of endotoxin per kg without antibody pretreatment. In additional experiments (data not shown), mice receiving 6.75 × 10^4 NU of anti-TNF demonstrated >1:640 dilution of TNF-neutralizing capacity in their plasma as late as 6 h after endotoxin treatment (10 h after being passively immunized with anti-TNF). The amount of anti-TNF administered was about fivefold higher than the minimal amount of antibody required to neutralize circulating-TNF levels in mice given 10 mg of endotoxin per kg.

Effect of anti-TNF pretreatment on PEPCK transcription and developing hypoglycemia in endotoxic C57BL/6 mice. As can be seen in Fig. 5A, anti-TNF immunization prevented the change in hepatic PEPCK transcription elicited by endotoxin. Mice were administered normal rabbit IgG or anti-TNF (6.75 × 10^4 NU) 4 h before challenge with endotoxin, and PEPCK activity was measured 6 h after endotoxin treatment. With 1 LD_{50} (10 mg/kg) or 0.1 LD_{50} (1 mg/kg) of endotoxin, PEPCK activity in mice given normal rabbit IgG was reduced from approximately 3,200 to 2,200 U. PEPCK activity in endotoxic mice that did not receive normal rabbit IgG was no different from that in IgG-treated animals (data not shown). Anti-TNF blocked the decrease in PEPCK activity in mice given 1 LD_{50} of endotoxin and caused a significant inductive effect (30% increase to 4,200 U) in animals given 0.1 LD_{50} of endotoxin.

Anti-TNF pretreatment of C57BL/6 mice failed to prevent the characteristic hypoglycemia observed 6 h following endotoxin (either 10 or 1 mg/kg) (Fig. 5B). All mice challenged with endotoxin (regardless of whether they received normal rabbit IgG or anti-TNF) exhibited levels of glucose in plasma at 6 h ranging between 55 and 63% of that of the untreated control. The levels of glucose in plasma of endotoxic mice that did not receive normal rabbit IgG were no different from those in IgG-treated animals (data not shown). Therefore, anti-TNF treatment was effective in preventing decreases in PEPCK activity due to endotoxin, yet the event was dissociated from hypoglycemia, since levels of glucose in plasma were not improved in endotoxic mice pretreated with anti-TNF.

Effect of anti-TNF pretreatment on levels of circulating cytokine in endotoxic mice. Although TNF is regarded as a primary mediator in endotoxic shock, multiple cytokines are most likely involved in many of the reactions that occur. Since anti-TNF pretreatment was effective in preventing PEPCK decreases following endotoxin yet failed to prevent hypoglycemia, we measured levels of TNF, IL-1, and IL-6 in plasma of mice receiving normal rabbit IgG or anti-TNF (6.75 × 10^4 NU). It can be seen in Fig. 6A that anti-TNF pretreatment of C57BL/6 mice completely prevented the appearance of TNF at 90 min after endotoxin. Mice were bled at 90 min because TNF levels in plasma are increased only transiently, and peak production is usually seen between 1.5 and 2 h after endotoxin (6, 7). A dose-dependent increase in the level of TNF in plasma was observed in

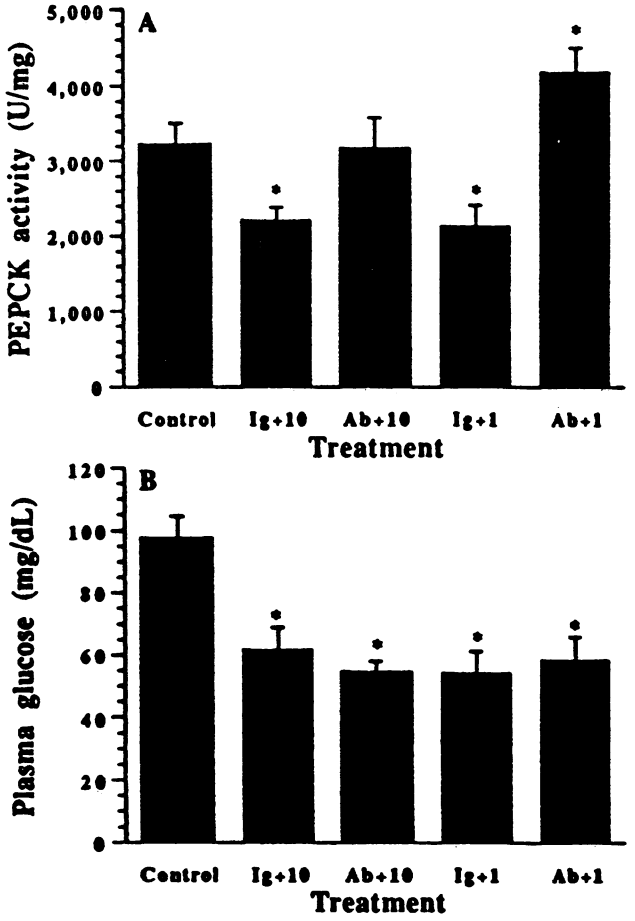

FIG. 5. Hepatic PEPCK activity (A) and levels of glucose in plasma (B) in C57BL/6 mice passively immunized with rabbit polyclonal anti-TNF (IgG fraction) and challenged 4 h later with endotoxin. PEPCK activity was measured by a ^{14}C radiometric assay, and units are expressed as nanomoles of oxaloacetate formed from phosphoenolpyruvate per 15 min per milligram of cytosol protein. Levels of glucose in plasma were measured by the glucose oxidase method. In order to induce PEPCK, all mice were fasted overnight before the start of the experiment at 8:00 a.m. Treatment groups: Ig+10, normal rabbit IgG followed by challenge with endotoxin (10 mg/kg; 1 LD_{50}); Ab+10, anti-TNF followed by challenge with endotoxin (10 mg/kg; 1 LD_{50}); Ig+1, normal rabbit IgG followed by challenge with endotoxin (1 mg/kg; nonlethal); Ab+1, anti-TNF followed by challenge with endotoxin (1 mg/kg; nonlethal. Results represent means ± standard errors of the means for five or six mice per group. *, significantly different ($P \leq 0.05$) from fasted control group value.

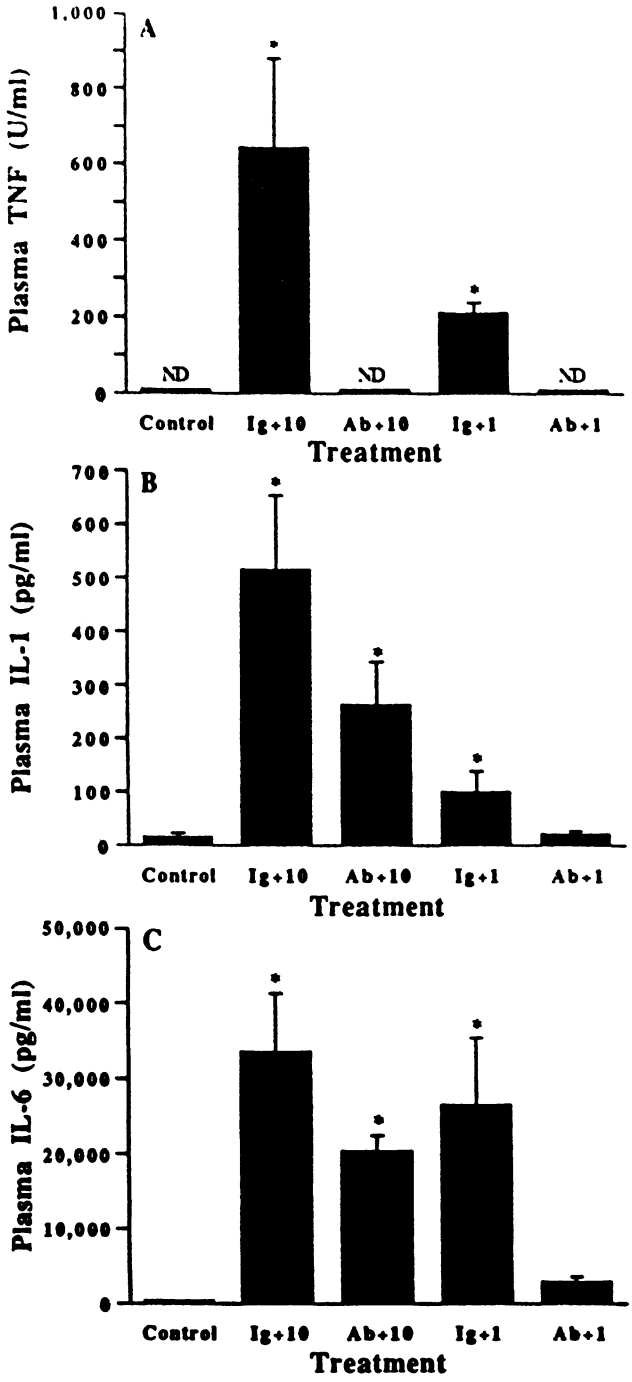

FIG. 6. Levels of TNF (A), IL-1 (B), and IL-6 (C) in plasma of C57BL/6 mice passively immunized with rabbit polyclonal anti-TNF (IgG fraction) and challenged 4 h later with endotoxin. TNF activity was measured by the L929 cell cytotoxicity assay, IL-1 was determined with a commercial ELISA kit, and IL-6 was measured by the B9 hybridoma cell proliferation assay. In order to induce PEPCK, all mice were fasted overnight before the start of the experiment at 8:00 a.m. Treatment groups: Ig+10, normal rabbit IgG followed by challenge with endotoxin (10 mg/kg; 1 LD_{50}); Ab+10, anti-TNF followed by challenge with endotoxin (10 mg/kg; 1 LD_{50}); Ig+1, normal rabbit IgG followed by challenge with endotoxin (1 mg/kg; nonlethal); Ab+1, anti-TNF followed by challenge with endotoxin (1 mg/kg; nonlethal). Results represent means ± standard errors of the means for five or six mice per group. *, significantly different ($P \leq 0.05$) from fasted control group value; ND, not detected.

control mice pretreated with normal rabbit IgG. The mean level measured in mice given 1 LD_{50} of endotoxin was approximately three times the amount assayed in mice that received 0.1 LD_{50}.

Anti-TNF pretreatment significantly decreased the amount of IL-1 produced at 3 h in response to endotoxin (Fig. 6B). The 3-h sampling interval was chosen because levels of IL-1 in plasma frequently peak after TNF release peaks (17). The IL-1 response was reduced by 50%, from 515 ± 138 to 263 ± 80 pg/ml, in passively immunized mice given 1 LD_{50} of endotoxin. IL-1 levels in mice pretreated with anti-TNF and challenged subsequently with 0.1 LD_{50} were neutralized completely.

Similar results were seen with levels of IL-6 in plasma of mice pretreated with anti-TNF (Fig. 6C). When 1 LD_{50} of endotoxin was used, IL-6 levels at 6 h were reduced from 33,638 ± 7,793 pg/ml in IgG-treated mice to 20,422 ± 2,095 pg/ml in animals pretreated with anti-TNF. The 6-h sampling interval was chosen for IL-6 measurement because the cytokine was previously observed to peak after TNF and IL-1 peak (26). IL-6 production was blocked in mice immunized with anti-TNF and challenged with the lower endotoxin dose (0.1 LD_{50}) (26,605 ± 8,910 pg/ml for IgG control versus 2,976 ± 587 pg/ml for anti-TNF). Levels of circulating cytokines (TNF, IL-1, and IL-6) in endotoxic mice pretreated with normal rabbit IgG were no different from those in mice given endotoxin alone (data not shown).

DISCUSSION

The results of this study offer clear evidence that TNF affects PEPCK gene expression at the transcriptional level. PEPCK plays a pivotal role in intermediary carbohydrate metabolism, and regulation of PEPCK gene expression is complex. Glucagon, acting through cAMP, and glucocorticoids regulate PEPCK by increasing the transcription rate of the gene (24) and by stabilizing $mRNA^{PEPCK}$ (30, 43). Insulin negatively regulates PEPCK by decreasing the transcription rate (22), an effect that is dominant over the inductive effect of cAMP and glucocorticoids in cell culture when all three hormones are present (45). The rapid response of the PEPCK gene to hormones is due to a relatively short-lived mRNA (half-life of ~40 min for rat liver $mRNA^{PEPCK}$ [4]). The chronology of PEPCK gene down regulation following endotoxin or TNF treatment of mice can be seen in the data presented in Fig. 1 and 2 and Table 1. A significant depression in the transcription rate of the hepatic PEPCK gene seen at 2 h and a substantial decrease in the amount of $mRNA^{PEPCK}$ produced at 4 h were reflected subsequently in decreased enzyme activity by 8 h. The injection of recombinant TNF mimicked endotoxin treatment in down regulating PEPCK gene expression. Circulating-TNF levels in mice given endotoxin or 10^5 U of TNF were approximately equal at 2 h after treatment (Table 2). Mice given 10^4 U of TNF demonstrated 10-fold-lower levels of circulating TNF. Interestingly, mice given 1 LD_{50} of endotoxin showed a sustained depression of PEPCK transcription through 8 h, whereas animals given a bolus injection of recombinant TNF demonstrated a transient depression of the PEPCK transcription rate but full recovery by 8 h. This suggests that endotoxin-treated animals continued to produce either TNF throughout the 8-h observation period or additional mediators that sustained reduced PEPCK transcription. The mild elevation of levels of circulating insulin seen at 2 h in both endotoxin- and TNF-treated mice (Fig. 3) suggests the possibility that insulin was responsible for deinduction of the PEPCK gene.

It is possible that TNF induced elevated levels of insulin either directly by affecting pancreatic β cells or indirectly by stimulation of production of IL-1, which is highly active in the pancreas (17). Our previous results suggested a possible role for elevated insulin levels in the endotoxin inhibition of PEPCK transcription; however, rats with streptozoticin-induced diabetes that were challenged with endotoxin yielded results similar to those with nondiabetic controls (26). An interesting feature of the hormonal changes that occur in endotoxic mice is that elevated levels of circulating glucagon and corticosterone (hormones that normally induce PEPCK synthesis) are unable to reverse the block in PEPCK induction (26).

Definitive proof of a direct effect of TNF at the PEPCK transcriptional level was obtained from experiments in which we used RHC cultures (Fig. 4). A dose-dependent inhibition of the hormonal induction (cAMP plus dexamethasone) of PEPCK was seen in hepatoma cells treated with recombinant TNF. TNF is known to affect the transcription of a wide variety of genes in different cell types. The majority of genes studied are up regulated by TNF, and common transcription factors, e.g., $NF_{\kappa}B$, AP-1, IRF-1, NF-IL-6, and NF-GMa, are involved (reviewed in reference 56). Of interest is the effect of down regulatory activity of TNF on several important genes. Thrombomodulin production by endothelial cells (35), collagen synthesis in fibroblasts (32), and albumin production by hepatocytes (10) have been shown to be inhibited by TNF at the transcriptional level. Considerable evidence exists for transcriptional inhibition by TNF of a number of adipocyte enzymes, including acyl-coenzyme A synthetase (60), stearoyl-coenzyme A synthetase (60), hormone-sensitive lipase (53), and lipoprotein lipase (21). A role for TNF in the down regulation of the lipoprotein lipase gene during cachexia is well documented (6). Stephens and Pekala (52) reported recently that the insulin resistance noted in 3T3-L1 adipocytes chronically treated with TNF is due to decreased transcription of the glucose transporter protein GLUT4, which in turn is due to a significant decrease in the accumulation of mRNA for the transcription factor C/EBP. The mechanism(s) by which TNF modulates gene expression is incompletely understood, but the involvement of multiple second-messenger pathways is suspected (11). Our demonstration of the down regulation of hepatic PEPCK gene transcription by TNF is consistent with those of other investigators. A full understanding of the molecular mechanisms of TNF action will provide a basis for a pharmacological approach intended to inhibit or potentiate selected biological actions of this cytokine. Our results obtained from the RHC culture model afford us the opportunity in future studies to map putative cytokine response elements in the PEPCK promoter region responsible for perturbed regulation during endotoxemia.

A role for TNF-mediated transcriptional inhibition of PEPCK gene expression in endotoxic shock is confirmed by the results of protection studies in which mice were treated with rabbit polyclonal anti-TNF prior to endotoxin challenge. Antibody treatment completely prevented the decrease in PEPCK activity that normally follows endotoxin challenge (Fig. 5A). Five times the amount of anti-TNF calculated to prevent the appearance of detectable levels of TNF in plasma 2 h after endotoxin injection (6.75×10^4 NU per mouse) was used to prevent changes in PEPCK activity. It is interesting that anti-TNF did not protect C57BL/6 mice from a lethal endotoxin challenge (25 mg/kg). However, animals were protected completely from challenge with 1 LD_{50} dose (10 mg/kg). The endotoxin dose-response curve for the strain of mice used was very narrow, with no mortality seen at doses below 5 mg/kg. These results are similar to those in the original study by Beutler et al. (7), in which anti-TNF treatment did not protect mice given endotoxin doses of >500 μg per mouse (25 mg/kg). In other words, excessive amounts of endotoxin continue to be lethal in passively immunized animals. Nevertheless, the results of our protection experiments indicate that the changes in PEPCK gene expression that usually occur in animals administered 1 LD_{50} of endotoxin are prevented with antibodies against TNF.

The failure of anti-TNF antibodies to reverse endotoxic hypoglycemia (Fig. 5B) is interesting and is consonant with the results of others (48, 49, 57). The dynamic nature of developing hypoglycemia during sepsis is underscored by the results of Schumer and Srivenugopal (46), who used rats subjected to cecal ligation and puncture-induced sepsis. At 2 h after surgery, a direct correlation among increases in PEPCK gene expression, PEPCK activity, and level of glucose was observed. At 4 h, decreased PEPCK gene expression, PEPCK activity, and level of glucose in serum were directly correlated. By 6 h after surgery, however, a negative correlation between PEPCK gene expression and continuing hypoglycemia was observed.

Causes other than diminished gluconeogenesis may account for hypoglycemia during sepsis and shock. Endotoxin-tolerant rats were infused with endotoxin-induced monokines or TNF by Bagby et al. (1), and an increase in glucose rate of appearance was observed at 3 h, paralleling changes in glucoregulatory hormones. Lee et al. (34) demonstrated that TNF-cachectin increased glycogenolysis, glucose uptake, and lactate production in myotubes of the L-6 muscle cell line. Other reports indicate that TNF causes increased glucose uptake (recycling) by peripheral tissues, particularly those rich in macrophages and endothelial cells (19, 51). We consider the changes in PEPCK transcription due to TNF to play a key role in diminished gluconeogenic potential at a time in the course of sepsis and shock when increased energy reserves are required to carry out the metabolism necessary for an effective inflammatory response. The fact that the PEPCK gene is down regulated in vivo at a time after endotoxin treatment when counterregulatory hormones that normally up regulate PEPCK gene expression are elevated suggests dysregulation of PEPCK at the molecular level. Further studies are required to elucidate the underlying mechanism(s).

Although TNF seems to play an important role in the pathogenesis of sepsis and shock, there is evidence for the involvement of multiple cytokines and mediators in the responses noted. For this reason, we measured levels of two other important cytokines, IL-1 and IL-6, in plasma obtained from mice given anti-TNF before endotoxin challenge. Although anti-TNF treatment ablated the TNF response after endotoxin treatment (Fig. 6A), reduced levels of IL-1 (Fig. 6B) and IL-6 (Fig. 6C) persisted. The response was dependent on the challenge dose of endotoxin. In mice challenged with a nonlethal dose of endotoxin (1 mg/kg), anti-TNF pretreatment completely prevented an elevation of levels of circulating IL-1 and IL-6. However, anti-TNF pretreatment resulted in only a 50% reduction in levels of IL-1 and IL-6 in mice given a 10-fold-greater endotoxin dose (10 mg/kg). Fong et al. (20) noted a similar attenuation of IL-1 and IL-6 levels in bacteremic baboons pretreated with anti-TNF antibodies.

Because of the cell-associated nature of IL-1α, its detection during sepsis or endotoxemia is more variable than the

elevation of IL-1β (the secreted form of the cytokine) that is consistently seen. Cannon et al. (12) did not detect circulating IL-1α in 15 septic patients, and <40 pg/ml was found in four of six human volunteers given bacterial endotoxin. In baboons made bacteremic by injection of *E. coli*, serum IL-1α was undetected, although dramatic increases in serum IL-1β were seen (20). On the other hand, Wakabayshi et al. (59) noted both IL-1α and IL-1β production in rabbits given heat-killed *Staphylococcus epidermidis* or *E. coli* or endotoxin. Clark et al. (15) detected IL-1α in a wide variety of tissues obtained from endotoxic rabbits by using an improved radioimmunoassay. The level of IL-1α increased within 1 to 2 h of stimulation, reaching a peak at 2 h and decreasing to undetectable levels by 24 h. In their study, IL-1α levels were consistently higher (two to three times) than those of circulating IL-1β. The exogenous administration of IL-1α to primates was shown to duplicate many of the hemodynamic, metabolic, and hormonal changes produced by endotoxin injection (18).

The dynamics of the appearance of circulating cytokine following endotoxin treatment have been well characterized (16, 47, 62), and the usual order of appearance is TNF > IL-1 > IL-6; however, multiple interactions can occur. Furthermore, it is important to note that cytokines detectable in serum represent the excess of mediators produced which have not been trapped by target cells (42). In this sense, locally produced cytokines may be more important in the tissue-specific responses that occur. IL-1 is well known for its effects on pancreatic β cells, and its activity as a hypoglycemic agent during endotoxemia is established (17, 27). Vogel et al. (58) found that pretreatment of mice with IL-1 receptor antagonist afforded complete protection from hypoglycemia following endotoxin challenge. Similar findings were reported by Mengozzi et al. (40). IL-1-induced increases in glucose utilization by peripheral tissues have been shown to be insulin dependent (33), and IL-1 treatment specifically increases the gene expression of glucose transporters in fibroblasts (9). The exact role of IL-6 in endotoxin-induced hypoglycemia is less clear, although it is likely that multiple cytokine interactions underlie endotoxic hypoglycemia.

In summary, we determined that during experimental endotoxic shock in mice, TNF was a potent transcriptional inhibitor of PEPCK gene expression. This was reflected by a reduced transcription rate of the PEPCK gene, decreased amounts of $mRNA^{PEPCK}$, and significantly diminished enzyme activity. These findings were confirmed in vitro in RHC. Anti-TNF antibodies given to mice before endotoxin challenge blocked the appearance of circulating TNF and prevented the transcriptional inhibition of PEPCK synthesis. The failure of anti-TNF to prevent hypoglycemia in endotoxic mice suggested the possible involvement of other mediators, which was evidenced by incomplete neutralization of IL-1 and IL-6 in plasma by anti-TNF.

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