Interleukin-1 and Nitric Oxide Protect Against Tumor Necrosis Factor α -Induced Liver Injury Through Distinct Pathways

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Mice sensitized with D-galactosamine (GalN) and challenged with recombinant murine tumor necrosis factor α (TNF α) developed severe apoptotic and secondary necrotic liver injury as assessed by histology, measurement of cytosolic DNA fragments, and determination of liver specific enzymes in plasma. Pretreatment with recombinant human interleukin-1 β (IL-1) rendered mice insensitive to this TNF α toxicity. Coadministration of the liver-specific transcriptional inhibitor GalN with IL-1 prevented the development of tolerance, implicating *de novo* synthesis of liver specific proteins in the induction of tolerance. Pretreatment of mice with IL-1 resulted in elevated levels of nitrite/nitrate in serum and in enhanced nitric oxide synthase (NOS) activity in liver cells isolated from these animals. In addition, pharmacological doses of the nitric oxide (NO) donor sodium nitroprusside conferred complete protection against TNF α -induced liver injury in galactosamine-sensitized mice, suggesting a possible link between IL-1- and NO-induced protection. However, prevention of NO-synthesis by N^G-monomethyl-L-arginine (NMMA) did not abolish IL-1-induced tolerance to TNF α *in vivo*. Cytotoxicity of TNF α to isolated hepatocytes sensitized with actinomycin D (ActD) was not significantly altered by inhibition of endogenous nitrite release. Also, enhanced NO production elicited *in vitro* by glycerol trinitrate or *ex vivo* by pretreatment with IL-1 had no significant effect in this system. We conclude that IL-1- and NO-induced protection of mice against TNF α -mediated liver damage follow distinct pathways.

Bacterial endotoxins (lipopolysaccharides [LPS]), as well as constituents from gram-positive bacteria, fungi, protozoa, or viruses, exert their proinflammatory effects by inducing the production of a variety of endogenous mediators in the host. Tumor necrosis factor α (TNF α) has been recognized as one of the key inflammatory mediators in various experimental settings and has been shown to mediate lethal shock and multiorgan failure (for review see reference 1).

Various animal models have been developed to study organ injury induced by TNF α . We and others have used mice sensitized to LPS or TNF α by transcriptional inhibitors such as D-galactosamine (GalN)^{2,3} or actinomycin D (ActD).⁴ In analogy to data from experiments carried out *in vivo*, ActD has been shown to sensitize cell lines up to 10,000-fold towards TNF α . An essential feature of the *in vivo* models is that during transcriptional inhibition otherwise subtoxic doses of TNF α result in a relatively selective liver damage which allows quantitative assessment of TNF α -toxicity by measurement of plasma enzymes.³ In the GalN model, animals can be completely desensitized to an otherwise lethal challenge of either LPS or TNF α by pretreatment with LPS, TNF α , or interleukin-1 β (IL-1).⁵ Of these agents, IL-1 was shown to be the most potent tolerance-inducing agent to TNF α challenge in GalN-sensitized animals.⁵

Although the mediators and mechanisms of tolerance may have important clinical implications, the phenomenon is not well understood. Tolerance is best characterized at the level of macrophages, which release a reduced spectrum of mediators upon LPS stimulation after desensitization with LPS.⁶ However, this mechanism alone is unlikely to account for tolerance to direct administration of TNF α *in vivo*.⁵

Circumstantial evidence suggests the involvement of nitric oxide (NO) (for review see reference 7), a pleiotropic endogenous mediator, in the development of tolerance to LPS- or cytokine-induced toxicity. Stimulation with cytokines was shown to induce NO production by endothelial cells,⁸ macrophages,⁹ hepatocytes,¹⁰⁻¹² and Ito cells.¹³ Moreover, it was shown that endogenously produced NO reduced organ injury or mortality in various models of septic organ damage and shock, such as endotoxin-induced liver damage, glomerular thrombosis, or endotoxin shock.¹⁴⁻¹⁷

Abbreviations: LPS, lipopolysaccharides; TNF α , tumor necrosis factor α ; GalN, D-galactosamine; ActD, actinomycin D; IL-1, interleukin-1 β ; NO, nitric oxide; NOS, NO synthase; SNP, sodium nitroprusside; ELISA, enzyme-linked immunosorbent assay; LDH, lactic dehydrogenase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; NMMA, N^G-monomethyl-L-arginine.

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Because IL-1 is both a strong inducer of NO synthases (NOS)^{18,19} and a potent agent for induction of tolerance,^{5,20,21} in this study we investigated whether there is a causal relation between IL-1-mediated tolerance to TNF α -induced liver damage and NO formation. The first aim of this study was to check whether tolerance induction results in an increase in NOS-activity. Secondly, we examined whether or not NOS induction was responsible for IL-1-mediated tolerance to TNF α in GalN-sensitized mice, i.e., in a model in which toxicity is unlikely to be primarily initiated by LPS-induced endogenous cytokine production.

MATERIALS AND METHODS

Animal Experiments. Male Bagg Albino/c mice were supplied by the animal house of the University of Konstanz, Germany. Animals were kept at 22°C and 55% relative humidity in a 12-hour day-night rhythm with free access to food (Altromin 1313; Altromin Co., Lage, Germany) and water. 16 hours before *in vivo* experiments food was withdrawn. For hepatocyte isolation, fed Bagg Albino/c mice were used. All animals received humane care in compliance with the guidelines of the local authorities.

Treatment Schedules. All substances were administered in pyrogen-free saline. LPS (Roth, Karlsruhe, Germany), sodium nitroprusside (SNP) (Fluka, Buchs, Switzerland), and GalN (Roth) were injected intraperitoneally in a volume of 200 μ L, recombinant human IL-1 β (a gift from Dr K. Vosbeck, Ciba-Geigy, Basel, Switzerland), recombinant murine TNF α (a gift from Dr Adolf, Bender & Co., Vienna, Austria) and NMMA (Sigma Chemical Co., St. Louis, MO) were injected intravenously in a volume of 300 μ L. Animals were killed by cervical dislocation 8 hours after challenge, and blood was withdrawn by cardiac puncture into heparinized syringes.

DNA-Fragmentation. Livers were perfused for 10 seconds with cold perfusion buffer (50 mmol/L phosphate, 120 mmol/L NaCl, 10 mmol/L ethylenediaminetetraacetic acid, pH 7.4), before they were excised. One slice of the large anterior lobe was immediately fixed in 4% formalin for histological studies. The remaining parts of the liver were disintegrated with three strokes of an Elvehjem type homogenizer. The 20% homogenate (in perfusion buffer) was centrifuged at 13,000g for 20 minutes. The supernatant was further diluted 250-fold. DNA fragmentation was quantified by measuring cytosolic oligonucleosome-bound DNA using an enzyme-linked immunosorbent assay (ELISA) kit (Boehringer, Mannheim, Germany).²² Briefly, the cytosolic fraction (13,000g supernatant) from about 75 μ g of liver was used as antigen source in a sandwich ELISA with a primary antihistone antibody coated to the microtiter plate and a secondary anti-DNA antibody coupled to peroxidase.

Histology. Formalin-fixed liver slices were imbedded in paraplast. 2- to 3- μ m sections were stained with hematoxylin/eosin.

Cell Culture and Incubation Conditions. Hepatocytes were isolated from 12-week-old male Bagg Albino/c mice by the two-step collagenase perfusion method according to Seglen,²³ modified by Klaunig et al.²⁴ Nonparenchymal liver cells, containing about 50% macrophages, were prepared by differential centrifugation according to Doolittle and Richter.²⁵ Viability of cells exceeded 80% as determined by Trypan blue exclusion. Hepatocytes were plated in 200 μ L of RPMI 1640 medium supplemented with 10% newborn calf serum (Bio-

chrom, Berlin, Germany) in 24-well plates at a density of 8×10^4 hepatocytes per well. Nonparenchymal cells were plated in the same medium as hepatocytes at a density of 2×10^5 cells per well in 24-well plates. Cells were allowed to adhere to culture plates for 5 hours before the medium was exchanged for RPMI 1640 medium without serum. Substances were added as stock solutions in a volume of 50 μ L of RPMI 1640 per well. Incubations were performed for the periods indicated at 37°C in an atmosphere consisting of 5% CO₂, 40% O₂, and 55% N₂.

Enzyme Assays. Activities of alanine aminotransferase, aspartate aminotransferase, and sorbitol dehydrogenase in plasma and lactate dehydrogenase (LDH) in cell supernatants and lysates were determined according to Bergmeyer,²⁶ using an Eppendorf (Hamburg, Germany) ACP 5040 enzyme analyzer.

Cell Viability Assays. Cytotoxicity was expressed as the proportion of LDH released into medium compared with the total amount of LDH present in the cells as determined by lysis of cells with 0.1% Triton X-100.²⁷

The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay was performed essentially according to Mosmann.²⁸ Briefly, hepatocytes cultured in 24-well plates were incubated for 30 minutes with MTT (0.4 mg/mL final concentration, Sigma Chemical Co), medium was discarded and the cells were lysed in 200 μ L 95/5: i-propanol/formic acid. An aliquot of 100 μ L was transferred to 96-well microtiter plates and measured at 560/690 nm on an ELISA-reader. MTT-toxicity was defined as:

$$\text{toxicity}[\%] = (100\%) - (100\%) \times \frac{\text{Absorbance}_{\text{tox}}}{\text{Absorbance}_{\text{control}}}$$

TNF α Assay. TNF α in liver cell supernatants was determined essentially as described in Espevik and Nissen-Meyer,²⁹ using recombinant murine TNF α as a standard.

Nitrite Determination in Cell Culture Supernatants. For determination of nitrite release cells were incubated for 20 hours in RPMI 1640 medium without phenol red. Culture supernatants were assayed for nitrite by the Griess reaction: briefly, 200 μ L culture supernatant were transferred to 96-well microtiter plates, 20 μ L sulfanilamide (1% in 1.2 mol/L HCl, Sigma Chemical Co.), and 20 μ L N-(1-naphthyl) ethylenediamine (0.1% in H₂O, Sigma Chemical Co.) were added and absorbance was read after 3 minutes' incubation time at 560/690 nm on an ELISA-reader. The nitrite content in the culture supernatant increased linearly within the time span of 20 hours investigated.

Nitrite Determination in Serum. Nitrite in serum was measured essentially according to the method of Misko et al,³⁰ using the Griess assay instead of the fluorimetric assay described. Briefly, plasma obtained by cardiac puncture into heparinized syringes was filtered through an Ultrafree-MC microcentrifuge filter unit (Millipore, Bedford, MA) for 1 hour at 14,000 rpm to remove hemoglobin released by cell lysis, which might interfere with the colorimetric assay. Because nitrite in serum is mostly oxidized to nitrate by reaction with the iron-heme center of hemoglobin, the resulting nitrate was first reduced to nitrite by incubation with nitrate reductase from aspergillus species (Sigma Chemical Co). Fourteen million units of nitrate reductase (in 20 mmol/L Tris/HCl, pH 7.6) were added to 10 μ L of filtrate, and the reaction was started by addition of nicotinamide adenine dinucleotide phosphate in Tris/HCl-buffer to a final concentration of 40 μ mol/L and a final volume of 50 μ L. After 5 minutes incuba-

FIG. 1. IL-1-induced tolerance against TNF α -mediated liver injury in galactosamine-sensitized mice. Animals were challenged with rmuTNF α (10 μ g/kg, intravenous) and GalN (700 mg/kg, intraperitoneal) 4 h after pretreatment (Pret.). IL-1 was administered intravenously in a dose of 10 μ g/kg either alone or together with GalN (700 mg/kg, intraperitoneal). ALT, aspartate-aminotransferase (AST) and sorbitol dehydrogenase (SDH) were measured 8 hours after GalN/TNF α challenge. In control experiments, in which mice were treated with GalN/IL-1 and a second dose of GalN without TNF α , no liver damage was observed. Data are expressed as mean values $\pm$ SEM; $n = 6$. * $P < .05$ vs. pretreatment with saline.

tion at room temperature, the reaction was terminated by dilution with 50 μ L of distilled water. The whole reaction volume was transferred to 96-well microtiter plates, 10 μ L sulfanilamide (1% in 1.2 mol/L HCl), and 10 μ L *N*-(1-naphthyl) ethylenediamine (0.1% in H₂O) were added and absorbance was read after 3 minutes' incubation time at 560/690 nm on an ELISA reader.

Statistics. Data from *in vivo* experiments are given as mean $\pm$ SEM, all other data as mean $\pm$ SD. Data for transaminases and DNA-fragmentation were analyzed by nonparametric analysis of variance (Kruskal-Wallis). Where there were differences among the groups ($P < .05$), these were subjected to one-sided nonparametric multiple comparisons of the control against all other groups.³¹ Statistical analysis of nitrite/nitrate concentrations (Tables 2 and 3) was done using the parametric Waller-Duncan test. $P < .05$ was considered to be significant.

RESULTS

IL-1-Induced Tolerance to GalN/TNF-Mediated Liver Damage. Intravenous administration of recombinant murine TNF α (10 μ g/kg) to GalN-sensitized male BALB/c mice induced severe necrotic liver damage as determined by elevated plasma levels of liver enzymes 8 hours after challenge (Fig. 1). Apoptotic changes preceded liver necrosis by several hours. After 5 hours, oligonucleosomal cytosolic DNA-fragments in livers of GalN/TNF challenged mice were increased 2.9 ± 0.7 -fold compared with livers treated with GalN only. Eight hours after challenge, i.e., at the time when blood was withdrawn for assessment of liver injury by clinical chemistry, DNA-fragmentation was increased 3.6 ± 0.4 -fold compared with controls (data $\pm$ SEM, n

= 3, $P < .05$ vs. GalN-treated control). Histological examination confirmed these findings and their interpretation; 3.5 hours after injection of TNF α to GalN-sensitized mice, hyperchromatic nuclear membranes and formation of apoptotic bodies were observed. Eight hours after TNF, numerous apoptotic bodies were identified between necrotic hepatocytes (Fig. 2). In contrast, pretreatment of the animals with 10 μ g/kg recombinant human IL-1 β intravenously 4 hours before challenge led to a completely refractory state with respect to TNF α -induced cytotoxicity in GalN-sensitized mice; i.e., no significant transaminase release into plasma occurred at 5 hours or at 8 hours after TNF α administration (Fig. 1) and DNA-fragmentation was not significantly increased at 5 hours ($130 \pm 10\%$ of GalN control) and 8 hours ($110 \pm 10\%$). Nevertheless, numerous infiltrating neutrophils were present in the liver of IL-1-pretreated mice 8 hours after challenge with GalN and TNF (Fig. 2). IL-1-pretreated mice went through a period of illness characterized by reduced motility and piloerection. However, the animals recovered from these symptoms within a few hours. When we varied the interval between IL-1 pretreatment and challenge, we found that tolerance started as early as 1 hour after IL-1 pretreatment and lasted for at least 48 hours (data not shown).

Prevention of IL-1 Induced Tolerance by Inhibition of Hepatic Transcription. Cytokine producing macrophages were not rendered refractory by IL-1 pretreatment. In fact, spontaneous TNF α release from liver macrophages prepared from tolerant mice was significantly enhanced (1.2 ± 0.8 ng/ 10^6 cells) compared with cells from untreated controls (0.7 ± 0.2 ng/ 10^6 cells). LPS-induced TNF α -release from IL-1-pretreated mice was not significantly different from that released from Kupffer cells of control mice (9.0 ± 1.8 vs. 9.9 ± 2.8 ng/ 10^6 cells). These data suggest that tolerance induced by IL-1 may be confined to the target of TNF α -mediated cytotoxicity, i.e., liver parenchymal cells. To study the role of hepatic transcription in the development of tolerance, the liver-specific transcriptional inhibitor GalN was applied together with IL-1. This treatment protocol prevented IL-1-induced tolerance to a subsequent challenge with GalN and TNF α (Fig. 1), i.e., we observed fulminant hepatic failure caused by GalN/TNF α in spite of a preceding treatment with a dose of IL-1 that otherwise would have provided complete protection against TNF α challenge.

Protection Against GalN/TNF α -Induced Liver Damage by Administration of SNP. Liver NOS is one of the putatively protective proteins¹⁴⁻¹⁷ known to be induced by IL-1.^{10,12,19} To study possible participation of NO in the development of tolerance, we first tested the efficacy of the pharmacological NO donor SNP against liver damage induced by TNF α in GalN-sensitized mice. GalN-sensitized mice treated with SNP (1.7 mg/kg, 15 minutes before challenge) were protected against liver damage induced by recombinant murine TNF α (10 μ g/kg) (Table 1). Thus, endogenous NO pro-

FIG. 2. Histopathology of $\text{TNF}\alpha$ -induced liver damage in D-galactosamine-sensitized mice. Influence of IL-1 pretreatment. Liver sections were prepared after treatment of mice for (A) 8 hours with GalN or (B) 8 hours with GalN/ $\text{TNF}\alpha$ (hyperchromatic nuclear membranes [h] and numerous apoptotic bodies [a] as signs of hepatocellular apoptosis are shown between necrotic hepatocytes) or (C) for 8 hours with GalN/ $\text{TNF}\alpha$ after IL-1 pretreatment. Numerous infiltrating neutrophils (n) but neither apoptotic nor necrotic liver damage are visible.

duction from exogenous precursors has the potential to protect against $\text{TNF}\alpha$ -mediated toxicity. Although the animals showed very pronounced hypotension and vasodilatation over a period of 20 minutes after SNP administration, no elevated nitrate/nitrite levels were detectable in plasma 15 minutes or 2 hours after injection of SNP. This is explained by the fact that the maximal attainable NO concentration ($9 \mu\text{mol/L}$) after this dose of SNP (170 nmol/mouse) is below the detection limit of the Griess assay.

TABLE 1. Protection by SNP-Pretreatment Against $\text{TNF}\alpha$ -Induced Liver Failure in Galactosamine-Sensitized Mice

Pretreatment*	ALT† (U/L)	AST (U/L)	SDH (U/L)	n‡
Saline	8470 ± 4120	2920 ± 1100	3650 ± 1580	4
SNP	$111 \pm 25§$	$446 \pm 423§$	$123 \pm 28§$	5

* SNP was given intraperitoneally in a dose of 1.7 mg/kg 15 minutes before challenge (700 mg/kg GalN, intraperitoneal, plus $10 \mu\text{g/kg}$ $\text{TNF}\alpha$, intravenous).

† ALT, aspartate aminotransferase (AST), and sorbitol dehydrogenase (SDH) were measured 8 hours after challenge, data are expressed as means $\pm$ SEM.

‡ n = number of animals per group.

§ $P < .05$.

IL-1-Induced Endogenous NO Production. Because pharmacologically delivered NO provided protection against $\text{TNF}\alpha$ toxicity *in vivo*, we checked whether NO was produced endogenously upon treatment of mice with IL-1. Male BALB/c mice were injected with either saline or IL-1 ($10 \mu\text{g/kg}$). Serum nitrite/nitrate levels were elevated 6 hours after administration of IL-1 as compared with saline-treated animals (Table 2). To identify the source of IL-1-induced endogenous NO production, hepatocyte and nonparenchymal liver cell cultures from IL-1- or saline-treated animals were prepared 4 hours after pretreatment. Endogenous NO production was determined by measuring the amount of nitrite released into the culture supernatant. Hepatocytes were left untreated or were further stimulated by incubation with LPS ($10 \mu\text{g/mL}$) or IL-1 (50 ng/mL). Hepatocytes isolated from saline-treated animals released less than $1 \text{ nmol nitrite}/10^6 \text{ cells}$ within 20 hours whereas *in vivo* pretreatment with IL-1 led to an increased spontaneous *ex vivo* nitrite production in liver cell cultures by a factor of at least five (Table 3). This augmented basal release was further enhanced by more than fourfold when these cells were incubated with IL-1 *in vitro* (Table 3). To characterize the requirements for induction of NOS activity by IL-1, animals were simul-

TABLE 2. Effect of the NOS Inhibitor NMMA on IL-1-Induced Tolerance Against Liver Injury Caused by TNF in Galactosamine-Sensitized Mice

Pretreatment*	Treatment†	ALT (U/L)	Nitrite/nitrate ($\mu\text{mol/L}$)	n
saline	GalN/TNF α	10130 $\pm$ 2400	35 $\pm$ 11	5
saline	GalN	90 $\pm$ 9‡	36 $\pm$ 9	3
IL-1	GalN/TNF α	22 $\pm$ 24‡	32 $\pm$ 6	5
IL-1	saline	18 $\pm$ 15‡	68 $\pm$ 9§	9
IL-1	GalN	29 $\pm$ 16‡	23 $\pm$ 4	5
IL-1 + NMMA	GalN/TNF α + NMMA	35 $\pm$ 23‡	34 $\pm$ 10	5
IL-1 + NMMA	NMMA	68 $\pm$ 78‡	27 $\pm$ 12	5
NMMA	GalN/TNF α + NMMA	10380 $\pm$ 3370	27 $\pm$ 8	5

NOTE. n = number of animals per group. ALT was measured 8 hours after challenge, levels of nitrite/nitrate in plasma were determined 6 hours after pretreatment. Nitrite levels in untreated animals were 30 $\mu\text{mol/L}$. Data are means $\pm$ SEM.

* Animals were pretreated 4 hours before challenge by an intravenous dose of 10 $\mu\text{g/kg}$ IL-1 $\pm$ NMMA (175 mg/kg) or by injection of saline.

† GalN (700 mg/kg) was administered intraperitoneally simultaneously with TNF α (10 $\mu\text{g/kg}$, intravenous) and NMMA (175 mg/kg, intravenous).

‡ $P < .05$ vs. GalN/TNF α control.

§ $P < .05$ vs. all other groups.

taneously treated with the transcriptional inhibitor GalN or with the translational inhibitor cycloheximide, 4 hours before the preparation of hepatocytes. Coadministration of GalN and IL-1 did not abrogate the subsequent nitrite production from hepatocytes. Cells from these animals showed an even greater spontaneous release of NO that was further increased more than fourfold by *in vitro* stimulation with IL-1 (Table 3). In contrast, cells from cycloheximide/IL-1-treated animals showed no increased basal or IL-1 inducible release of nitrite compared with controls (Table 3). When cycloheximide (50 $\mu\text{mol/L}$) was present *in vitro* together with the stimulus, the further induction of NOS-activity by IL-1 was completely prevented in all experiments (data not shown). Additional experiments showed that nonparenchymal liver cell cultures prepared from the same mice by differential centrifugation and plastic adhesion produced less than 5% per cell of the nitrite found in hepatocyte cultures, suggesting that the nitrite determined in the above experiments was predominantly derived from hepatocytes.

No Impairment of Tolerance by Inhibition of NOS *In Vivo*. To study the relevance of endogenously formed NO to the development of tolerance *in vivo*, we inhibited endogenous NO production by administration of *N*^Gmonomethyl-L-arginine (NMMA) (2×175 mg/kg), a competitive inhibitor of NOS. NMMA reduced the elevated nitrate/nitrite levels caused by IL-1 pretreatment to values observed in control animals. However, administration of NMMA caused neither an altered toxicity of TNF α *in vivo* nor any modification of the IL-1-induced tolerance in GalN-sensitized mice (Table 2). Interestingly, injection of GalN with or without TNF α 4 hours after administration of IL-1 already prevented the increase of nitrite/nitrate concentrations in serum. Because GalN is a liver specific transcriptional inhibitor, this suggests that the liver is the major source of IL-1-induced nitrite/nitrate release into plasma.

Absence of Ex Vivo Tolerance. To further explore the mechanisms of IL-1-induced target cell tolerance, we studied whether the state of tolerance induced by IL-

TABLE 3. *Ex Vivo* Nitrite Production by Hepatocytes of IL-1 Pretreated and Control Mice

Pretreatment* (<i>in vivo</i>)	Control	Stimulus (<i>in vitro</i>)		ActD (333 nM) + TNF (100 ng/mL)‡
		LPS (10 $\mu\text{g/mL}$)	IL-1 (50 ng/mL)	
Saline	<1†	1.2 $\pm$ 0.2	2.8 $\pm$ 0.6	<1
IL-1	4.8 $\pm$ 1.2§	6.7 $\pm$ 0.6§	19.8 $\pm$ 2.9§	7.9 $\pm$ 0.7§
GalN/IL-1	7.5 $\pm$ 0.9§#	10.7 $\pm$ 0.5§#	33.7 $\pm$ 2.1§#	2.3 $\pm$ 0.3§#
CHX/IL-1	<1	<1	3.1 $\pm$ 0.8	<1

* Animals were pretreated 4 hours before preparation of hepatocytes. IL-1 was administered intravenously in a dose of 10 $\mu\text{g/kg}$, GalN, and CHX were given intraperitoneally in a dose of 700 mg/kg or 40 mg/kg respectively, simultaneously with IL-1.

† Hepatocytes were incubated for 20 hours before the amount of nitrite was assayed in the supernatant. Data are means (nmol nitrite/ 10^6 cells) $\pm$ SD of triplicate incubations.

‡ ActD was added 30 minutes before TNF α .

§ $P < .05$ vs. saline pretreatment.

$P < .05$ vs. IL-1 pretreatment.

1 *in vivo* was still present *ex vivo* in hepatocytes. Liver cells prepared from tolerant and nontolerant mice were compared with respect to their susceptibility to TNF α cytotoxicity after sensitization with the transcriptional inhibitor ActD. In this *ex vivo* experiment—in contrast to the protection observed that was caused by IL-1 *in vivo*—hepatocyte death induced by ActD/TNF α occurred to a similar extent regardless of the pretreatment (Fig. 3). Thus, the difference in spontaneous NO production between hepatocytes from IL-1-pretreated mice and cells from control animals (Table 3) had no significant influence on the sensitivity of liver cells to TNF α *in vitro*.

Lack of Correlation Between Nitrite Release and Cytotoxicity of ActD/TNF α In Vitro. The finding that TNF-induced hepatocytotoxicity in ActD pretreated cells occurred independently of hepatocyte NO production was further corroborated by experiments using the NOS inhibitors NMMA or *N* ω -nitro-L-arginine (0.1 mmol/L). In control experiments, these inhibitors were shown to completely block nitrite release from hepatocytes prepared from tolerant mice. Nevertheless, these NOS inhibitors did not significantly affect the cytotoxicity of TNF α in hepatocytes in the presence of ActD (Table 4). Also, the NO donor glycerol trinitrate neither protected nor sensitized hepatocytes against ActD/TNF α -induced hepatocytotoxicity (Table 4).

DISCUSSION

A large variety of experimental models have been used to study possible mechanisms of septic shock and ensuing organ failure. LPS, as the primary initiator of

FIG. 3. TNF cytotoxicity on ActD-sensitized hepatocytes from IL-1 pretreated or control mice. Hepatocytes were prepared 4 hours after pretreatment (10 μ g/kg IL-1, or saline). Cells were incubated with 333 nmol/L of ActD for 30 minutes before addition of TNF (1.5 to 100 ng/mL). LDH release was determined after 20 hours of incubation as an indicator of cell death. Treatment with ActD alone did not induce LDH release significantly higher than that of untreated hepatocytes. Data are expressed as mean $\pm$ SD of triplicate incubations.

TABLE 4. Nitrite Release and Cytotoxicity Induced by TNF in Hepatocytes Sensitized With ActD

<i>In Vivo</i> Pretreatment*	<i>In Vitro</i> Effector†	Percentage of Toxicity‡ After Incubation With TNF/ActD	NO ₂ -Release§ by Hepatocytes
IL-1	Control	35 $\pm$ 2	4.8 $\pm$ 1.2
IL-1	NMMA	36 $\pm$ 0	<1
IL-1	<i>N</i> ω NA	29 $\pm$ 3	<1
Saline	Solvent control	38 $\pm$ 2	<1
Saline	Glycerol trinitrate	35 $\pm$ 3	31 $\pm$ 4.3

NOTE. Data are means $\pm$ SD of triplicate incubations. There is no correlation between NO₂-release by hepatocytes and toxicity of TNF α /ActD (Pearson correlation coefficient: 0.08, *P* = .9).

* Animals were pretreated 4 hours before preparation of hepatocytes. IL-1 was administered intravenously in a dose of 10 μ g/kg.

† Incubation of hepatocytes with drugs (NMMA, 0.1 mmol/L; *N* ω NA, 0.1 mmol/L; nitroglycerin 55 μ mol/L in 0.1% EtOH) and stimuli was started 4 hours after hepatocyte preparation.

‡ Determined by MTT viability assay after 20 hours' incubation. ActD (333 nmol/L) was added 30 minutes before TNF α (15 ng/mL). Toxicity was compared with hepatocytes only treated with ActD.

§ Amount of nitrite released into the medium in nmol/10⁶ cells 20 h after start of incubation.

a cytokine response, or TNF α , as a distal mediator of LPS toxicity, also in GalN-sensitized mice,³ are commonly used to elicit the systemic inflammatory response. However, pretreatment with minute amounts of either one of these agents protects mice from a second, otherwise lethal, challenge. Besides TNF α , a variety of other cytokines, such as IL-10, transforming growth factor- β , or IL-1, have been implicated in tolerance induction.^{4,5,20,32} In the present study, we used IL-1 to induce tolerance to the TNF α challenge in the low-dose model of the GalN-sensitized mouse. This study design allowed us to investigate mechanisms of target cell tolerance independent of the suppression of TNF α release from macrophages. The dose of TNF α used in our experiments with GalN-sensitized mice was chosen to cause severe necrotic liver injury at about 8 hours after challenge. This protocol allows us to study acute inflammatory injury of the liver.³³ In this model, we found that IL-1 caused tolerance against GalN/TNF α when given at any time between 1 hour and 48 hours before challenge. We chose 4 hours as the pretreatment interval to leave sufficient time between the two doses of GalN that were administered in some of the experiments (prevention of IL-1-induced tolerance by inhibition of hepatic transcription).

As with the liver failure observed in the ActD/TNF α model,²² a salient feature of the GalN/TNF α model is apoptotic and secondary necrotic liver cell death.³⁴ Therefore, we measured both cytosolic DNA fragments and hepatocyte specific enzymes to quantitate organ injury. According to these parameters, IL-1 completely protected mice against both forms of hepatic injury induced by TNF α . Because the development of tolerance

could be completely abolished by coadministration of GalN, which selectively impairs the hepatic RNA synthesis³⁴ via trapping of uridine nucleotides and subsequent depletion of the uridine triphosphate pool,³⁵ we conclude that IL-1-induced tolerance is an active process requiring hepatic transcription. This view is further corroborated by our finding that cytokine release of Kupffer cells prepared from IL-1-treated, tolerant animals was not impaired compared with controls. Several IL-1-inducible intrahepatic or secretory liver proteins have been described as being associated with the development of tolerance, e.g., manganese superoxide dismutase,^{36,37,38} or acute phase proteins like ferritin³⁹ or α_1 -acid glycoprotein.⁴⁰ These observations imply an active biosynthetic intrahepatic process as a cause of tolerance development.

Another putatively protective protein known to be induced by IL-1 is NOS, an enzyme producing the potent vasodilator NO.¹⁴⁻¹⁷ Our finding that NOS activity is increased by incubation of murine liver cell cultures with IL-1 *in vitro* is in agreement with previous findings showing an induction of NOS messenger RNA in rat and human hepatocytes under similar conditions.^{12,19} In our experiments, enhancement of NOS activity in murine hepatocytes was suppressed by incubation with cycloheximide, suggesting induction via *de novo* protein synthesis. In analogy to the *in vitro* experiment, treatment of mice with IL-1 *in vivo* led to elevated levels of nitrite/nitrate in serum and to an increased NOS activity in liver cells prepared from these animals. However, the induction of NOS under the latter conditions did not render these cells tolerant to the cytotoxic action of TNF α in the presence of ActD. Furthermore, NOS activity *ex vivo* was still enhanced in cells from IL-1-treated animals in which tolerance induction was blocked by coadministration of GalN. In contrast to these *ex vivo* observations, *in vivo* the administration of GalN 4 hours after treatment of mice with IL-1 prevented the IL-1-induced increase in plasma nitrate/nitrite. Two explanations seem feasible for this obvious difference between our *in vivo* and *ex vivo* findings. In contrast to the accumulation of nitrite *in vitro*, nitrate and nitrite may be eliminated via urinary excretion *in vivo*, necessitating continuous RNA and protein synthesis to maintain elevated nitrate/nitrite plasma levels. Alternatively, the nitrite release *ex vivo* might be explained by a rapid repletion of cellular uridine pools during the *in vitro* incubation, i.e., an abolition of a persistent GalN effect on transcription. In any case, our *in vivo* and *ex vivo* experiments provide additional evidence that the phenomenon of tolerance and NOS induction are separate events. Our data confirm that IL-1 induces NOS both *in vivo* and *in vitro*. However, the induction of NOS and development of tolerance seem to be simultaneous events occurring independently from each other.

The cytotoxicity of TNF α in hepatocyte cultures containing ActD was neither affected by inhibition of nitrite release nor by enhancement of NO-formation by

glycerol trinitrate. These findings suggest that, on the one hand NO is not required for the toxicity of TNF in murine hepatocytes. On the other hand, NO did not prevent cytotoxicity in this *in vitro* model when present in excess. The lack of protection by exogenously applied NO in this *in vitro* model compared with the protection seen *in vivo* may be explained by physiological reactions that cannot take place *in vitro* such as prevention of ischemia/reperfusion by SNP-induced vasodilatation.

The reason why hepatocytes prepared from IL-1-tolerant mice are sensitive to ActD/TNF α induced cytotoxicity is not known. It is conceivable that the protection conferred by proteins synthesized in the liver is indirectly mediated through the interaction with extrahepatic cells, e.g., liver-derived proteins such as α_1 acid glycoprotein, may cause the release of anti-inflammatory glucocorticoids from the adrenals. Alternatively, protection might be mediated directly by plasma proteins synthesized by the liver.⁴⁰ Such proteins may not be present in sufficient amounts in cell cultures.

The role of endogenously produced NO in septic shock and other inflammatory models is not yet clarified. To study the significance of NO in TNF α -induced liver failure in GalN-sensitized mice and to further investigate the role of endogenously formed NO for the development of tolerance, we examined whether injection of an NO-releasing agent (SNP) *in vivo* could protect against TNF α toxicity and what effects inhibition of NOS might have on IL-1-induced tolerance. The finding that SNP protected against TNF α -induced hepatotoxicity is consistent with findings in the GalN/LPS model where various vasodilators were protective.⁴¹ An increased production of NO is probably responsible for the detrimental decrease in blood pressure seen in septic shock,^{42,43} and knock-out mice lacking the inducible NOS show decreased sensitivity towards LPS-induced lethality.^{43,44} Nevertheless, beneficial effects of this mediator in sepsis have also been described. For instance, an aggravation of LPS-induced hepatic damage in *Corynebacterium parvum*-sensitized mice by administration of NMMA, a competitive inhibitor of NOS, was reported^{14,15} and inhibition of endogenous NO production increased lethality in staphylococcal enterotoxin-B-induced shock in mice.⁴⁵ Furthermore, whereas NMMA administered to a patient in septic shock increased blood pressure as expected, it finally led to intravascular coagulation and death.⁴⁶ The aggravation of septic complications by inhibition of NO formation on the one hand and the protection against TNF α conferred by SNP on the other hand suggest beneficial effects of endogenously produced NO. However, we observed that NMMA administration did not abolish IL-1-induced tolerance although the increase in serum nitrite/nitrate levels induced by IL-1 pretreatment was shown to be completely prevented. This argues against a predominant role of endogenously produced NO in this inflammatory

model and against a role of NO in the development of tolerance.

In summary, IL-1 pretreatment protected GalN-sensitized mice against TNF α induced hepatotoxicity. Although IL-1 increased NO release of liver cells *in vivo* and *ex vivo* and although treatment with the NO donor SNP was protective against TNF α toxicity, endogenously produced NO had no effect on IL-1 induced tolerance *in vivo* nor on TNF α mediated cytotoxicity *in vitro* in the presence of ActD. We conclude that IL-1 induces expression and/or release of tolerance-inducing proteins different from NOS and that increased production of endogenous NO is not the mechanism responsible for tolerance development.

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REFERENCES

1. Waage A. Presence and involvement of TNF in septic shock. In: Beutler B, ed. Tumor necrosis factors. The molecules and their emerging role in medicine. New York: Raven, 1992:275-283.
2. Lehmann V, Freudenberg MA, Galanos C. Lethal toxicity of lipopolysaccharide and tumor necrosis factor in normal and D-galactosamine-treated mice. *J Exp Med* 1987;165:657-663.
3. Tiegs G, Wolter M, Wendel A. Tumor necrosis factor is a terminal mediator in galactosamine/endotoxin-induced hepatitis in mice. *Biochem Pharmacol* 1989;38:627-631.
4. Wallach D, Holtmann H, Engelmann H, Nophar Y. Sensitization and desensitization to lethal effects of tumor necrosis factor and IL-1. *J Immunol* 1988;140:2994-2999.
5. Libert C, van Bladel S, Brouckaert P, Shaw A, Fiers W. Involvement of the liver, but not of IL-6, in IL-1-induced desensitization to the lethal effects of tumor necrosis factor. *J Immunol* 1991;146:2625-2632.
6. Freudenberg MA, Galanos C. Induction of tolerance to lipopolysaccharide (LPS)-D-galactosamine lethality by pretreatment with LPS is mediated by macrophages. *Infect Immunol* 1988;56:1352-1357.
7. Moncada S, Palmer RMJ, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol Rev* 1991;43:109-142.
8. Ignarro LJ, Bugy GM, Wood KS, Byrns RE, Chaudhuri G. Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc Natl Acad Sci U S A* 1987;84:9265-9269.
9. Stuehr DJ, Marletta MA. Induction of nitrite/nitrate synthesis in murine macrophages by BCG infection, lymphokines or interferon- γ . *J Immunol* 1987;139:518-525.
10. Stadler J, Bergonia HA, Di Silvio M, Sweetland MA, Billiar TR, Simmons RL, Lancaster JR Jr. Nonheme iron-nitrosyl complex formation in rat hepatocytes: detection by electron paramagnetic resonance spectroscopy. *Arch Biochem Biophys* 1993;302:4-11.
11. Adamson GM, Billings RE. Cytokine toxicity and induction of NO synthase activity in cultured mouse hepatocytes. *Toxicol Appl Pharmacol* 1993;119:100-107.
12. Geller DA, Lowenstein CJ, Shapiro RA, Nussler AK, Di Silvio M, Wang SC, Nakayama DK, et al. Molecular cloning and expression of inducible nitric oxide synthase from human hepatocytes. *Proc Natl Acad Sci U S A* 1993;90:3491-3495.
13. Helyar L, Bundschuh DS, Laskin JD, Laskin DL. Induction of hepatic Ito cell nitric oxide production after acute endotoxemia. *HEPATOLOGY* 1994;20:1509-1515.
14. Harbrecht BG, Billiar TR, Stadler J, Demetris AJ, Ochoa JB, Curran RD, Simmons RL. Nitric oxide synthesis serves to reduce hepatic damage during acute murine endotoxemia. *Crit Care Med* 1992;20:1568-1574.
15. Harbrecht BG, Billiar TR, Stadler J, Demetris AJ, Ochoa J, Curran RD, Simmons RL. Inhibition of nitric oxide synthesis during endotoxemia promotes intrahepatic thrombosis and an oxygen radical-mediated hepatic injury. *J Leukoc Biol* 1992;52:390-394.
16. Cobb JP, Natanson C, Hoffman WD, Lodato RF, Banks S, Koev CA, Solomon MA, et al. *N*^w-amino-L-arginine, an inhibitor of nitric oxide synthase, raises vascular resistance but increases mortality rates in awake canines challenged with endotoxin. *J Exp Med* 1992;176:1175-1182.
17. Shultz PJ, Raji L. Endogenously synthesized nitric oxide prevents endotoxin-induced glomerular thrombosis. *J Clin Invest* 1992;90:1718-1725.
18. Eizirik DL, Björklund A, Welsh N. Interleukin-1-induced expression of nitric oxide synthase in insulin-producing cells is preceded by *c-fos* induction and depends on gene transcription and protein synthesis. *FEBS Lett* 1993;317:62-66.
19. Geller DA, Nussler AK, Di Silvio M, Lowenstein CJ, Shapiro RA, Wang SC, Simmons RL, et al. Cytokines, endotoxin, and glucocorticoids regulate the expression of inducible nitric oxide synthase in hepatocytes. *Proc Natl Acad Sci U S A* 1993;90:522-526.
20. Sheppard BC, Norton JA. Tumor necrosis factor and interleukin-1 protection against the lethal effects of tumor necrosis factor. *Surgery* 1991;109:698-705.
21. Leon P, Redmond HP, Shou J, Daly JM. Interleukin-1 and its relationship to endotoxin tolerance. *Arch Surg* 1992;127:146-151.
22. Leist M, Gantner F, Böhlinger I, Germann PG, Tiegs G, Wendel A. Murine hepatocyte apoptosis induced in vitro and in vivo by TNF- α requires transcriptional arrest. *J Immunol* 1994;153:1778-1788.
23. Seglen PO. Preparation of isolated rat liver cells. *Methods Cell Biol* 1976;13:29-83.
24. Klaunig JE, Goldblatt PJ, Hinton DE, Lipsky MM, Chacko J, Trump BF. Mouse liver cell culture I. Hepatocyte isolation. *In Vitro* 1981;17:913-925.
25. Doolittle RL, Richter GW. Isolation and culture of Kupffer cells and hepatocytes from single rat livers. With observations on iron-loaded Kupffer cells. *Lab Invest* 1981;45:558-566.
26. Bergmeyer HU. Methods of enzymatic analysis. Weinheim: Verlag Chemie, 1984.
27. Kingsley ED, Gray P, Tolman KG, Tweedale R. The toxicity of metabolites of sodium valproate in cultured hepatocytes. *J Clin Pharmacol* 1983;23:178-185.
28. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983;65:55-63.
29. Espevik T, Nissen-Meyer J. A highly sensitive cell line WEHI 164 clone 13, for measuring cytotoxic factor/tumor necrosis factor from human monocytes. *J Immunol Methods* 1986;95:99-105.
30. Misko TP, Schilling RJ, Salvemini D, Moore WM, Currie MG. A fluorimetric assay for the measurement of nitrite in biological samples. *Anal Biochem* 1993;214:11-16.
31. Zar JH. Biostatistical analysis. New Jersey: Prentice-Hall, 1984.
32. Cavaillon JM, Pitton C, Fitting C. Endotoxin tolerance is not a LPS-specific phenomenon: partial mimicry with IL-1, IL-10 and TGF β . *J Endotox Res* 1994;1:21-29.
33. Wendel A. Biochemical pharmacology of inflammatory liver injury in mice. *Methods Enzymol* 1990;186:675-680.
34. Leist M, Gantner F, Böhlinger I, Tiegs G, Germann P-G, Wendel A. Tumor necrosis factor-induced hepatocyte apoptosis precedes liver failure in experimental murine shock models. *Am J Pathol* 1995;146:1220-1234.
35. Decker K, Keppler D. Galactosamine hepatitis: key role of the nucleotide deficiency period in the pathogenesis of cell injury and cell death. *Rev Physiol Biochem Pharmacol* 1974;71:77-100.

36. Wong GHW, Elwell JH, Oberley LW, Goeddel DV. Manganous superoxide dismutase is essential for cellular resistance to cytotoxicity of tumor necrosis factor. *Cell* 1989;58:923-931.
37. Hirose K, Longo DL, Oppenheim JJ, Matsushima K. Overexpression of mitochondrial manganese superoxide dismutase promotes the survival of tumor cells exposed to interleukin-1, tumor necrosis factor, selected anticancer drugs, and ionizing radiation. *FASEB J* 1993;7:361-368.
38. Masuda A, Longo DL, Kobayashi Y, Appella E, Oppenheim JJ, Matsushima K. Induction of mitochondrial manganese superoxide dismutase by interleukin 1. *FASEB J* 1988;2:3087-3091.
39. Rogers JT, Bridges KR, Durmowicz GP. Translational control during the acute phase response. *J Biol Chem* 1990;65:14572-14578.
40. Libert C, Brouckaert P, Fiers W. Protection by α_1 -acid glycoprotein against tumor necrosis factor-induced lethality. *J Exp Med* 1994;180:1571-1575.
41. Wendel A, Tiegs G, Werner C. Evidence for the involvement of a reperfusion injury in galactosamine/endotoxin-induced hepatitis in mice. *Biochem Pharmacol* 1987;36:2637-2639.
42. Petros A, Lamb G, Leone A, Moncada S, Bennett D, Vallance P. Effects of a nitric oxide synthase inhibitor in humans with septic shock. *Cardiovasc Res* 1994;28:34-39.
43. MacMicking JD, Nathan C, Hom G, Chartrain N, Fletcher DS, Trumbauer M, Stevens K, et al. Altered responses to bacterial infection and endotoxic shock in mice lacking inducible nitric oxide synthase. *Cell* 1995;81:641-650.
44. Wel X-Q, Charles IG, Smith A, Ure J, Feng G-J, Huang F-P, Xu D, et al. Altered immune responses in mice lacking inducible nitric oxide synthase. *Nature* 1995;375:408-411.
45. Florquin S, Amraoui Z, Dubois C, Decuyper J, Goldman M. The protective role of endogenously synthesized nitric oxide in staphylococcal enterotoxin B-induced shock in mice. *J Exp Med* 1994;180:1153-1158.
46. Petros A, Bennett D, Vallance P. Effect of nitric oxide synthase inhibitors on hypotension in patients with septic shock. *Lancet* 1991;338:1557-1558.