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Tolerance against tumor necrosis factor α (TNF)-induced hepatotoxicity in mice: the role of nitric oxide

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Abstract

D-Galactosamine-sensitized mice challenged with tumor necrosis factor α (TNF) 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 of mice with interleukin-1β (IL-1) resulted in elevated levels of nitrite/nitrate in serum and rendered mice insensitive towards TNF toxicity. Pharmacological doses of the nitric oxide (NO) donor sodium nitroprusside (SNP) also conferred complete protection against TNF toxicity, suggesting a possible link between IL-1- and NO-induced protection. However, NO-synthesis inhibition by N^G-monomethyl-L-arginine failed to abrogate IL-1-induced tolerance against TNF toxicity. We conclude that IL-1 and NO protect against TNF-induced liver injury through distinct pathways.

Keywords: Interleukin-1; Sodium nitroprusside; Liver injury; Necrosis; Apoptosis; Galactosamine

1. Introduction

Tumor necrosis factor α (TNF) has been recognized as one of the key inflammatory mediators of bacterial lipopolysaccharides (LPS) and has been shown to mediate lethal shock and multi-organ failure. Transcriptional inhibitors such as the liver-specific agent D-galactosamine (GalN) or actinomycin D (ActD) sensitize mice several thousand-fold towards LPS or TNF [1]. In these models animals develop a relatively selective liver damage which allows quantitative assessment of TNF-toxicity by measurement of liver-specific plasma enzymes [1]. Pretreatment of mice by either LPS, or TNF, or interleukin-1β (IL-1) confers complete tolerance to an otherwise lethal second challenge with LPS or TNF. Tolerance was suggested to be due to one or several protective proteins synthesized within the liver [2].

Circumstantial evidence suggested the involvement of nitric oxide synthase (NOS) in the development of tolerance towards LPS- or cytokine-induced toxicity. NO was shown to be produced in considerable amounts by murine hepatocytes stimulated with cytokines [3]. Moreover, it was shown that endogenously produced NO reduced endotoxin-induced lethality as well as hepatic or renal organ damage [4–6]. Since

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IL-1 is both a strong inducer of NOS [7] as well as a potent agent for induction of tolerance [2], we investigated whether there might exist a causal relation between IL-1-mediated NO formation and tolerance to TNF-induced liver damage in GalN-sensitized mice.

2. Materials and methods

2.1. Animal experiments

Male BALB/c mice from the animal house of the University of Konstanz, Germany, received humane care in compliance with the legal requirements in Germany. All substances were administered in pyrogen-free saline. Sodium nitroprusside (SNP, 1.7 mg/kg, Fluka, Buchs, Switzerland) and GalN (700 mg/kg, Roth, Karlsruhe, Germany) were injected i.p. in a volume of 200 μl, rhuIL-1β (10 μg/kg, a gift from Dr. K. Vosbeck, Ciba Geigy, Basel, Switzerland), rmuTNFα (10 μg/kg, a gift from Dr. Adolf, Bender & Co, Vienna, Austria) and N^Gmonomethyl-L-arginine (NMMA, 175 mg/kg, Sigma Chemical Co., St. Louis, MO) were injected i.v. in a volume of 300 μl. Animals were sacrificed by cervical dislocation 5 or 8 h after challenge and blood was withdrawn by cardiac puncture into heparinized syringes.

2.2. Histology and DNA fragmentation

Livers were perfused for 10 s as described [8] before they were excised. One slice of the large anterior lobe was immediately immersed in 4% formalin and imbedded in paraplast. Sections (2–3 μm) were stained with hematoxylin/eosin. The remaining parts of the liver were homogenized. The homogenate was centrifuged at $13\,000 \times g$ for 20 min. The supernatant (final dilution 1250-fold) was used to measure DNA fragmentation with an ELISA kit (Boehringer, Mannheim, Germany) as described [8].

2.3. Cell culture and incubation conditions

Hepatocytes and non-parenchymal liver cells were isolated from 12-week-old male BALB/c mice by a two-step collagenase perfusion method and cultured as described [8].

2.4. Enzyme assays

Activities of alanine-aminotransferase (ALT) in plasma were determined according to [9].

2.5. Nitrite determination in cell culture supernatants

For determination of nitrite release, cells were incubated for 20 h in RPMI 1640 medium without phenol red. Culture supernatants were assayed for nitrite according to the Griess reaction.

2.6. Nitrite determination in serum

Nitrite in serum was measured essentially according to the method of Misko et al. [10], using the Griess assay.

2.7. Statistics

Data from in vivo experiments are given as means $\pm$ S.E.M., all other data as means $\pm$ S.D. Data for transaminases and DNA fragmentation were analysed by non-parametric analysis of variance (Kruskal-Wallis). Statistical analysis of serum nitrite/nitrate concentrations (Table 1) was done using the parametric Waller-Duncan-Test. $P < 0.05$ was considered to be significant.

3. Results

3.1. IL-1-induced tolerance against GalN/TNF-mediated liver damage

Intravenous administration of 10 μg/kg rmuTNFα to GalN-sensitized mice induced severe necrotic liver damage as determined by elevated plasma levels of ALT 8 h after challenge (6260 ± 1010 U/l vs. untreated control: 40 ± 20 U/l, $n = 6$, $P < 0.05$). Liver necrosis was preceded by apoptotic changes. DNA fragmentation was increased 2.9 ± 0.7-fold 5 h after GalN/TNF administration compared to GalN controls. Eight h after challenge DNA fragmentation was increased 3.6 ± 0.4*-fold compared to controls (data $\pm$S.E.M., $n = 3$, $^*P < 0.05$). Histological examination confirmed these findings: 3.5 or 5 h after injection of TNF to GalN-sensitized mice, hyperchromatic nuclear membranes and formation of apoptotic bodies were seen. Eight h after GalN/TNF, numerous apoptotic bodies were identified between necrotic hepatocytes [11].

Table 1
Effect of the NOS inhibitor NMMA on IL-1-induced tolerance against liver injury caused by TNFα in galactosamine-sensitized mice

Pretreatment[a]	Treatment[b]	ALT[c] (U/l)	Nitrite/nitrate[c] (μM)	n
Saline	GalN/TNF	10 130 ± 2400	35 ± 11	5
IL-1	GalN/TNF	22 ± 24*	32 ± 6	5
IL-1 + NMMA	GalN/TNF + NMMA	35 ± 23*	34 ± 10	5
NMMA	GalN/TNF + NMMA	10 380 ± 3370	27 ± 8	5
IL-1	Saline	17 ± 11*	77 ± 14**	5
Saline	GalN	90 ± 9*	36 ± 9	3

[a] Animals were pretreated 4 h before challenge by an i.v. dose of 10 μg/kg IL-1 ± NMMA (175 mg/kg) or by injection of saline.
[b] GalN (700 mg/kg) was administered i.p. simultaneously with TNF (10 μg/kg, i.v.) and NMMA (175 mg/kg, i.v.).
[c] Plasma ALT was measured in U/l 8 h after challenge, levels of nitrite/nitrate in plasma were determined 6 h after pretreatment. Data are means ± S.E.M.
* $P < 0.05$ vs. GalN/TNF control.
** $P < 0.05$ vs. all other groups.
n, number of animals per group.

Pretreatment of the animals with 10 μg/kg rhuIL-1β i.v. 4 h before challenge led to a completely refractory state against TNF-induced cytotoxicity in GalN-sensitized mice, i.e. neither ALT release into plasma nor DNA fragmentation were significantly increased.

3.2. Prevention of IL-1-induced tolerance by inhibition of hepatic transcription

In order to study the role of hepatic biosynthesis in the development of tolerance, the liver-specific transcriptional inhibitor GalN was given together with IL-1 4 h prior to GalN/TNF-challenge. Under this experimental condition we observed severe liver failure 8 h after challenge (ALT: 8440 ± 1940 U/l). Mice treated with IL-1/GalN and challenged 4 h later with GalN alone developed no liver injury. These observations imply active biosynthetic hepatic processes as a requirement for IL-1-induced tolerance.

3.3. Protection against GalN/TNF-induced liver damage by administration of SNP

Liver NOS is one of the putatively protective proteins [4–6] known to be induced by IL-1 [7]. In order to study a 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 in GalN-sensitized mice. Pretreatment with SNP (1.7 mg/kg i.p., 15 min before challenge) protected against liver damage induced by GalN/TNF as shown by significantly reduced levels of plasma ALT compared to GalN/TNF controls (110 ± 25 U/l vs. 8470 ± 4120 U/l, $n = 5$, $P < 0.05$). These results indicate that endogenous NO production from exogenous precursors has the potential to protect against TNF-mediated toxicity.

3.4. IL-1-induced endogenous NO production

Since pharmacologically delivered NO provided protection against TNF toxicity in vivo, we checked whether NO was produced endogenously upon treatment of mice with IL-1. Serum nitrite/nitrate levels were elevated 6 h after administration of IL-1 as compared to saline-treated animals (Table 1). In order to identify the source of IL-1-induced endogenous NO production, hepatocyte and non-parenchymal liver cell cultures from IL-1 or saline-treated animals were prepared 4 h 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 IL-1 (50 ng/ml). In vivo pretreatment with IL-1 lead to an increased ex vivo nitrite production in liver

Table 2
Ex vivo nitrite production by hepatocytes of IL-1-pretreated and control mice

Pretreatment[a] (in vivo)	Stimulus (in vitro)	
	None	IL-1 (50 ng/ml)
Saline	<1[b]	2.8 ± 0.6
IL-1	4.8 ± 1.2	19.8 ± 2.9

[a] Animals were pretreated 4 h before preparation of hepatocytes. IL-1 was given i.v. in a dose of 10 μg/kg.
[b] Hepatocytes (8×10^4 per well) were incubated in RPMI 1640 for 20 h before the amount of nitrite was assayed in the supernatant. Data are means (nmol nitrite/10^6 cells) ± S.D. of triplicate incubations.

cell cultures as compared to hepatocytes from saline-treated control animals (Table 2). This augmented basal release was further enhanced more than 4-fold when these already activated cells were incubated with IL-1 in vitro (Table 2). Additional experiments with the same mice showed that non-parenchymal liver cell cultures prepared from cells 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 (data not shown).

3.5. No impairment of tolerance by inhibition of NOS in vivo

In order to study the relevance of endogenously formed NO for development of tolerance in vivo, endogenous NO production was inhibited by administration of NMMA (2×175 mg/kg), a competitive inhibitor of NOS, together with IL-1 pretreatment and together with a subsequent TNF challenge. Administration of NMMA caused neither an altered toxicity of TNF in vivo nor any modification of the IL-1-induced tolerance in GalN-sensitized mice (Table 1).

4. Discussion

A large variety of experimental models have been used in order to study possible mechanisms of septic shock and ensuing organ failure. LPS as the primary initiator of a cytokine response or TNF as a distal mediator of LPS toxicity are commonly used to elicit the systemic inflammatory response. However, pretreatment of mice with minute amounts of either one of these agents protects them from a second, otherwise lethal challenge. In the present study we used IL-1 to induce tolerance against TNF challenge in the low-dose model of the GalN-sensitized mouse.

A salient feature of the GalN/TNF model is apoptotic and secondary necrotic liver cell death [11]. We therefore measured cytosolic DNA fragments and hepatocyte-specific enzymes to quantitate organ injury. According to these parameters, IL-1 completely protected mice against hepatic injury induced by TNF. Since the development of tolerance could be completely abolished by co-administration of GalN, which selectively impairs the hepatic RNA synthesis [11] we conclude that IL-1-induced tolerance is an active process requiring hepatic transcription.

One putatively protective protein known to be induced by IL-1 is NOS, an enzyme producing the potent vasodilatator NO. 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 mRNA in human hepatocytes under similar conditions [7]. In analogy to the in vitro experiments, 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 ex vivo.

The role of endogenously produced NO in septic shock and other inflammatory models is not yet clarified. In order to study the significance of NO in TNF-induced liver failure in GalN-sensitized mice and to further investigate the role of endogenously formed NO for the development of tolerance, we examined in vivo whether injection of an NO-releasing agent (SNP) could substitute for IL-1 in the protection against TNF toxicity and which effects inhibition of NOS might have on IL-1-induced tolerance. The protection by SNP against TNF-induced hepatotoxicity is consistent with findings in the GalN/LPS model where various vasodilatators prevented liver injury [1]. Though an increased production of NO is probably responsible for the

detrimental fall in blood pressure seen in septic shock [12], beneficial effects of this mediator in septic complications have also been described. For instance, an aggravation of LPS-induced hepatic damage in *C. parvum*-sensitized mice by administration of NMMA, a competitive inhibitor of NOS, was reported [5]. However, we found that NMMA administration at a dose described to block the endogenous NO formation [5] did not abolish IL-1-induced tolerance. 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.

We conclude that IL-1 induces expression and/or release of tolerogenic proteins different from NOS and that increased production of endogenous NO is not the mechanism responsible for tolerance development.

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