Biochemical Pharmacology, Vol. 38, No. 4, pp. 627–631, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

TUMOR NECROSIS FACTOR IS A TERMINAL MEDIATOR IN GALACTOSAMINE/ENDOTOXIN-INDUCED HEPATITIS IN MICE

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(*Received* 29 *June* 1988; *accepted* 16 *September* 1988)

Abstract—Intravenous injection of murine recombinant tumor necrosis factor α (TNF-α) to male NMRI albino mice in doses greater than 4 μg/kg (specific activity 4×10^7 U/mg) resulted in a fulminant hepatitis when animals had been sensitized 1 hr before by intraperitoneal administration of 700 mg/kg galactosamine. Liver injury was assessed by measurement of serum transaminases as well as sorbitol dehydrogenase activity 8 hr after administration of TNF-α. Pretreatment with either galactosamine or 40 μg/kg TNF-α alone did not cause hepatitis. Pretreatment of galactosamine/TNF-α-injured mice with 800 mg/kg uridine or with 6 mg/kg calmidazolium fully protected the animals, while administration of either verapamil or nifedipine (100 mg/kg, respectively) had no significant effect. The following inhibitors of generation or action of leukotriene D_4, which were previously shown to block galactosamine/endotoxin-induced hepatitis in mice, failed to protect against galactosamine/TNF-α-induced intoxication: 200 μg/kg dexamethasone, 174 mg/kg BW 755 C or 13×10 mg/kg FPL 55712. In addition, unlike in the galactosamine/endotoxin model no prevention was achieved by pretreatment of galactosamine/TNF-α-injured animals with the following substances blocking the development of an ischemia/reperfusion syndrome: 2×100 mg/kg allopurinol, 3.3×10^4 U/kg superoxide dismutase, 10^6 U/kg catalase or 10 μg/kg iloprost. We conclude from our results that tumor necrosis factor α is likely to act as a final mediator of endotoxin action in a sequence of events which includes formation of leukotriene D_4 and reactive oxygen species.

A large variety of autacoids seems to be involved in the pathogenesis of endotoxemia. Among them, peptido-leukotrienes have been suggested to mediate endotoxin-induced hepatic injury [1]. After administration of endotoxin to rodents or monkeys, peptido-leukotrienes were found in the bile [2, 3]. Moreover, endotoxin-induced liver injury in galactosamine sensitized mice was shown to be mediated by leukotriene D_4 [4]. We also provided evidence that the pathogenic mechanism of galactosamine/endotoxin-induced hepatitis includes a transient ischemia induced by the vasoconstrictor LTD_4,§ followed by reflow and reoxygenation as soon as LTD_4 has been degraded [5]. Recent interest in the mechanism of endotoxin action focused on cytokines such as tumor necrosis factor α (TNF-α). This cytokine is released from macrophages upon an endotoxin-stimulus [6]. In addition, TNF-α was found in sera of endotoxin-treated rabbits [7]. Moreover, it has been shown that passive immunisation against TNF-α partially protected mice from lethal effect of endotoxin [8]. Submicrogram amounts of human recombinant TNF-α could substitute for endotoxin in inducing lethality in GalN-sensitized mice [9]. The aim of this study was to demonstrate the causal involvement of TNF-α in GalN/E-induced hepatitis and to deduce the sequence of the hitherto recognized pathogenic mediators.

MATERIALS AND METHODS

Male NMRI albino mice were purchased from the Lippische Versuchtierzucht, Extertal, F.R.G. They were kept at least one week on the standard diet C 1000 (Altromin, Lage, F.R.G.) under environmentally controlled conditions with free access to food and water. The animals received intraperitoneally a dose of 700 mg/kg D-galactosamine HCl (Serva, Heidelberg) at 8 a.m. together with 1 μg/kg *Salmonella abortus* equi endotoxin (Sigma, St. Louis, MO), or 1 hr after GalN-administration murine recombinant tumor necrosis factor α (specific activity 4×10^7 U/mg; Genzyme, Boston) intravenously instead of endotoxin. TNF-α was a generous gift by Dr. Martin Schönharting, Hoechst AG, Werk Albert, Wiesbaden. Nine hours after intoxication the animals were killed by cervical dislocation. Blood was withdrawn by heart puncture into 2.5% heparin. Liver injury was assessed by measurement of serum alanin aminotransferase (SGPT), serum aspartate aminotransferase (SGOT) and sorbitol dehydrogenase (SDH) activities. Every GalN/TNF-α-experiment was accompanied by a GalN/E-control (with and without inhibitor). Uridine (Sigma, St. Louis, MO) was injected intraperitoneally in phosphate buffered saline (PBS)

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§ Abbreviations used: LTD_4, leukotriene D_4; GalN, D-galactosamine; E, endotoxin; TNF-α, tumor necrosis factor α; SGPT, serum alanine amino transferase (EC 2.6.1.1.); SGOT, serum aspartate amino transferase (EC 2.6.1.2.); SDH, sorbitol dehydrogenase (EC 1.1.1.14.); PBS, phosphate-buffered saline.

Table 1. Dose-dependence of murine recombinant (mr) TNF-α-induced hepatitis in male, galactosamine-sensitized mice and control experiments

Pretreatment	SDH (U/l)	SGOT (U/l)	SGPT (U/l)	N	m
None	40 ± 4	75 ± 16	40 ± 4	6	0
700 mg/kg GalN	70 ± 9	110 ± 15	50 ± 6	11	0
40 μg/kg TNF-α	20 ± 14	50 ± 6	10 ± 6	3	0
700 mg/kg GalN + 0.4 μg/kg TNF-α	100 ± 57	180 ± 53	90 ± 42	4	0
700 mg/kg GalN + 4 μg/kg TNF-α	400 ± 174*	410 ± 137*	370 ± 148*	4	0
700 mg/kg GalN + 10 μg/kg TNF-α	1210 ± 731*	750 ± 387*	1770 ± 1098*	3	0
700 mg/kg GalN + 15 μg/kg TNF-α	2310 ± 545*	1170 ± 271*	3510 ± 901*	4	2
700 mg/kg GalN + 20 μg/kg TNF-α	2320 ± 92*	1140 ± 243*	3180 ± 534*	3	2
700 mg/kg GalN + 40 μg/kg TNF-α	2830 ± 557*	2830 ± 596*	4240 ± 965*	11	9
700 mg/kg GalN + 15 μg/kg TNF-α + 800 mg/kg uridine	50 ± 17†	255 ± 44†	75 ± 21†	8	0

mr TNF-α (spec. act. 4×10^7 units/mg) was injected i.v. 1 hr after i.p.-administration of GalN in pyrogenfree solution. Uridine was intraperitoneally injected together with GalN. Serum enzyme activities, SDH, SGOT and SGPT, were determined 9 hr after GalN-administration.

* $P \leq 0.05$ vs untreated group or GalN-control group.

† $P \leq 0.05$ vs control group.

N = number of animals per group, m = number of animals which died within 9 hr. Data are expressed as mean values ± SEM.

together with GalN. Dexamethasone (Sigma, St. Louis, MO) was administered i.p. 1 hr before endotoxin or 90 min before TNF-α. BW 755 C (3-amino-1(3(trifluoromethyl) - phenyl) - 2 - pyrazoline, Wellcome Laboratories, Beckenham, U.K.) was orally given 2 hr before, or either indomethacin or verapamil or nifedipine (all from Sigma, St Louis, MO), 1 hr before endotoxin or TNF-α, respectively, in 1% tylose.

Administration of FPL 55712 (7-(3-(4-acetyl-3-hydroxy-2-propylptienoxyl)-2-hydroxypropoxy)-4-oxo-8-propyl-4H-1-benzopyran-2-carboxylic acid, Fisons, Loughborough, U.K.) was carried out by i.p. injection every 30 min for 6 hr starting simultaneously with the first injection together with GalN. Iloprost was a gift by Schering A.G., Berlin, and was given i.p. in PBS as a bolus injection 10 min before endotoxin or TNF-α. Allopurinol (Sigma, St Louis, MO) was dissolved by titrating an aqueous suspension to pH 11.5 by addition of 2 N NaOH. This solution was injected i.p. in a volume of 200 μl at a concentration of 15 mg/ml 24 hr and 1 hr before endotoxin or TNF-α to yield a final dose of 2×100 mg/kg. Control animals received PBS made up to pH 11.5. Bovine superoxide dismutase (Grünenthal GmbH, Aachen) or catalase (Boehringer Mannheim) were administered intravenously 1 hr before endotoxin or TNF-α in PBS-solution. Bovine serum albumin of the highest purity grade (Sigma, St Louis, MO) was used for control experiments in equivalent protein concentration compared to SOD or catalase. The calcium antagonist calmidazolium (Compound R 24571; Sigma, St Louis, MO) was given intraperitoneally 2 hr before endotoxin or TNF-α. Galactosamine and TNF-α as well as all inhibitors or antagonists were administered in pyrogen-free, aqueous solutions (Ampuwa, Fresenius, Bad Homburg).

Statistics. The results were analyzed according to Student's *t*-test. Data are expressed as mean values ± SEM; $P \leq 0.05$ was considered to be significant.

RESULTS

The administration of TNF-α in doses of 4 μg/kg or higher to GalN-sensitized mice led to a fulminant hepatitis. In higher doses, TNF-α caused lethality within 9 hr. Pretreatment with either GalN or 40 μg/kg TNF-α alone did not induce hepatitis detectable by enzyme release into the circulation (Table 1). GalN was previously shown to deplete hepatic uridine nucleotides and thus led to an inhibition of RNA- and consequently protein biosynthesis [10]. In order to check whether TNF-α induced liver injury requires UTP depletion, we treated the animals additionally with an equivalent dose of uridine which restores the uridine nucleotide pool [10]. When TNF-α was administered to mice pretreated with uridine, they were fully protected against liver injury (Table 1). In our previous studies on GalN/E-hepatitis in mice, a variety of anti-inflammatory compounds had been investigated concerning their protective potency [4, 11]. Among them, substances which inhibit the synthesis of leukotrienes or antagonize the biological activity of LTD_4 were highly effective. Table 2 shows a comparison between GalN/TNF-α- and GalN/E-induced liver injury. In contrast to the situation in the GalN/E model (Table 2, right-hand panel), neither the 5-lipoxygenase inhibitor BW 755 C, nor FPL 55712, a LTD_4 receptor antagonist, nor the cyclooxygenase- and phospholipase A_2-inhibitor [12] indomethacin, showed any significant protection against GalN/TNF-α (Table 2, left hand side panel). The anti-phospholipase-inducing steroid dexamethasone significantly reduced the enzyme release; however, it did not fully prevent GalN/TNF-α-induced hepatic damage.

In a previous paper [5], we suggested a transient hepatic ischemia/reperfusion episode as the consequence of the endotoxin-induced leukotriene D_4 production and/or release in our animals. The evidence was based on the inhibitory effectiveness of allopurinol or intravenously administered catalase or

Table 2. Influence of compounds interfering with the eicosanoid metabolism on mr TNF-α-or endotoxin-induced hepatitis in male GalN-sensitized mice

Pretreatment	GalN/TNF-α SGPT (U/l	N	m	GalN/E SGPT (U/l)	N	m
None	40 ± 4	6	0	40 ± 4	6	0
Disease control	4080 ± 455	24	8	3870 ± 455	35	11
200 μg/kg dexamethasone	1580 ± 540*	14	0	30 ± 7*	4	0
174 mg/kg BW 755 C	2700 ± 830	15	2	250 ± 61*	3	0
13 × 10 mg/kg FPL 55712	2830 ± 997	7	0	215 ± 116*	4	0
9 mg/kg indomethacin	3540 ± 973	8	3	248 ± 100*	3	0

700 mg/kg GalN were injected intraperitoneally together with 1 μg/kg salmonella abortus equi endotoxin (E), 15 μg/kg mr TNF-α were given by i.v.-administration 1 hr after 700 mg/kg GalN. Dexamethasone was administered i.p. 1 hr before E or 90 min before mr TNF-α, BW 755 C was given p.o. 2 hr before E or mr TNF-α and FPL 55712 was i.p. injected between 0 and 6 hr, every 30 min, beginning with the first injection together with GalN. Indomethacin was p.o. administered 1 hr before E or mr TNF-α.

* $P \leq 0.05$; SGPT was determined 9 hr after GalN, analogous data were obtained by determination of SGOT and SDH.

Data are expressed as mean values ± SEM.

superoxide dismutase, as well as on the antagonizing capability of the vasodilator iloprost. Consequently, we studied now the effect of this pharmacological intervention comparatively in the GalN/TNF-α and in the GalN/E model.

Data in the right panel of Table 3 show in agreement with our previous results [5] that these interventions fully protected the animals against GalN/E-induced liver injury. In contrast, they failed to protect against TNF-α induced hepatitis in GalN-sensitized mice (Table 3, left panel). Appropriate control experiments as to the effect of solvents or inert protein are included into Table 3.

Since Ca^{2+} has been implicated into the activation of phospholipase A_2 as well as the 5-lipoxygenase pathway, smooth muscle contraction, final cell death and many more functions, we finally studied the influence of the Ca^{2+}/calmodulin antagonist calmidazolium as well as of the Ca^{2+}-channel blocker verapamil or nifedipine on GalN/TNF-α induced liver injury. The data in Table 4 demonstrate that verapamil or nifedipine, which are known to block Ca^{2+}-channels in smooth muscle plasma membranes [12], were only effective within the GalN/E-model, where LTD_4-induced vasoconstriction is likely to be involved. On the other hand, we observed a powerful antagonistic effect of calmidazolium on GalN/E- as well as on GalN/TNF-α-induced hepatitis. This means that intracellular Ca^{2+}-increase plays a pivotal role in TNF-α-induced liver cell death. We cannot decide, however, from these whole animal experiments, whether this Ca^{2+}-dependent process takes place within the liver or affects primarily leukocytes.

DISCUSSION

The ability of intravenous TNF-α to induce fulminant hepatitis in GalN-sensitized mice instead of endotoxin showed the conventional dose-dependence needed to conclude that this lymphokine is

Table 3. Effect of the prostacyclin analogue iloprost, the xanthin oxidase inhibitor allopurinol or reactive oxygen scavenging enzymes on mr TNF-α- or endotoxin-induced hepatitis in male GalN-sensitized mice

Pretreatment	GalN/TNF-α SGPT (U/l)	N	m	GalN/E SGPT (U/l)	N	m
None	40 ± 4	6	0	40 ± 4	6	0
Disease control	4080 ± 455	24	8	3870 ± 455	35	11
10 μg/kg iloprost	2203 ± 965	8	0	230 ± 51*	10	0
Disease control + PBS pH 11.5	3090 ± 1140	5	2	8230 ± 1854	4	0
2 × 100 mg/kg allopurinol	2230 ± 360	8	2	60 ± 8*	5	0
Disease control + BSA	3580 ± 970	5	2	6550 ± 4220	4	1
3.3×10^4 U/kg SOD	3560 ± 717	6	2	160 ± 109*	4	0
10^6 U/kg catalase	5960 ± 1411	8	6	30 ± 8*	4	0

Iloprost was administered i.p. as a bolus injection 10 min before endotoxin (E) or mr TNF-α, 100 mg/kg allopurinol were intraperitoneally injected 24 hr and 1 hr before E or mr TNF-α, BSA, SOD or catalase were administered i.v. 1 hr before E or mr TNF-α. Pretreatment with GalN, E or mr TNF-α c.f. Table 2.

* $P \leq 0.05$, SGPT was determined 9 hr after GalN, analogous data were obtained by determination of SGOT and SDH. Data are expressed as mean values ± SEM.

Table 4. Influence of the calcium-antagonist calmidazolium or the calcium channel blockers verapamil and nifedipine on mr TNF-α or endotoxin-induced hepatitis in GalN-sensitized mice

Pretreatment	GalN/TNF-α SGPT (U/l)	N	m	GalN/E SGPT (U/l)	N	m
None	40 ± 4	6	0	40 ± 4	6	0
Disease control	4270 ± 1846	6	1	3560 ± 1514	4	2
Verapamil	2370 ± 533	16	7	80 ± 43*	8	0
Nifedipine	3130 ± 1521	8	4	150 ± 45*	8	0
Calmidazolium	280 ± 135*	8	0	220 ± 117*	5	0

6 mg/kg calmidazolium were administered i.p. 2 hr before endotoxin (E) or mr TNF-α; 100 mg/kg verapamil or 100 mg/kg nifedipine were given orally 1 hr before endotoxin (E) or mr TNF-α. Pretreatment with GalN, E or mr TNF-α, cf. Table 2.

* $P < 0.05$; SGPT was determined 9 hr after GalN, SGOT and SDH showed analogous values.

Data are expressed as mean values ± SEM.

involved in the pathogenesis of this kind of liver injury. Actually, we found a peak serum TNF-α-level of 5–6000 units per animal 2 hr after endotoxin administration. (U. Schade, G. Tiegs and A. Wendel, unpublished). Our minimal effective dose of 4 μg/kg given as a bolus injection corresponds to a total of 4800 units of TNF-α per animal. This means that our experiments operate in a pathophysiologically relevant concentration. For the further studies we chose a dose of 15 μg/kg in order to obtain reproducibility on the one hand and to work within a range allowing inhibitor studies on the other hand.

The comparison of various inhibitors in the GalN/E and the GalN/TNF-α model leads to the compulsory conclusion that events associated with eicosanoid metabolism or reactive oxygen species are localized upstream to the action of TNF-α. Pretreatment of the animals by dexamethasone showed a significant reduction in the severity of liver injury, even though no complete protection was achieved. This finding could be due to the capability of dexamethasone to decrease TNF-receptor affinity [14] by a yet unknown mechanism.

Although our results identify TNF-α as the preliminary terminal mediator in the GalN/E-induced hepatitis model, we cannot exclude multiple interactions with other lymphokines such as interleukines or mutual stimulation between eicosanoid lipid mediators and leukotactic oxygen species such as O_2^-.

Until now, any mechanistic information on the interaction of TNF-α and liver cells is lacking. Some clues, however, may be provided by the studies in Table 4 dealing with effectors of Ca^{2+} homeiostasis. The protective effectiveness of verapamil and nifedipine in the GalN/E model and their ineffectiveness

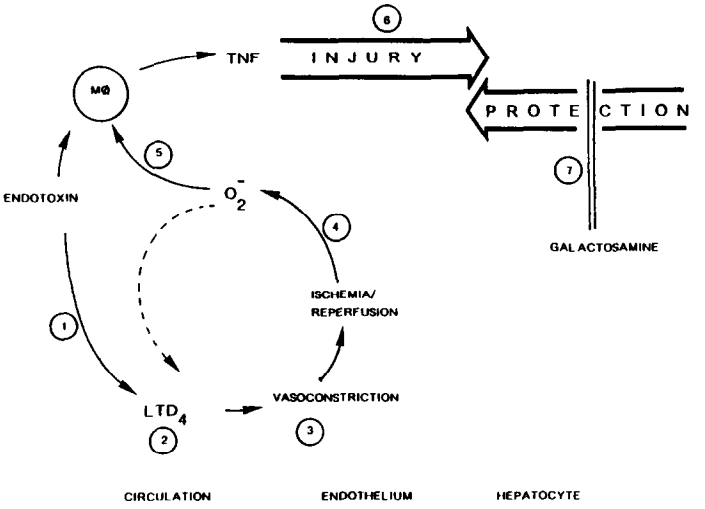

Fig. 1. Possible interactions between endotoxin-induced pathogenic mediators in galactosamine-sensitized mice. MQ: macrophage/monocyte. Inhibitors used in this study: (1) Dexamethasone, BW 755 C, indomethacine; (2) FPL 55712; (3) iloprost, verapamil, nifedipine; (4) allopurinol; (5) superoxide dismutase; (6) calmidazolium; (7) uridine.

in the GalN/TNF-α model are in agreement with the view that a process acting upon specific Ca^{2+} channels mediates endotoxin-induced lesions upstream from the site of action of TNF-α. In contrast, the general blockade of the Ca^{2+}/calmodulin system resulted in an apparent protection of the animals also against TNF-α. From these findings we conclude that an intracellular, Ca^{2+}-sensitive processes is involved in the terminal action of TNF-α. Before an attempt is made to explain these findings, it should be recalled that the hemodynamics of the hepatic microcirculation are known to depend on the activity of smooth muscle cells around the arterioles and precapillary sphincters [15]. It seems therefore feasible that contraction of these structures by LTD_4 in the GalN/E-model was prevented by the Ca^{2+} channel blockers verapamil and nifedipine. As a consequence, the previously postulated [5] transient ischemia following LTD_4-induced vasoconstriction did not occur, including the allopurinol- or superoxide-dismutase-sensitive reperfusion phase. This in turn leads to assume that the deleterious pathogenic cascade does not propagate unless superoxide is produced and/or released from yet unidentified sites or cells. A conjective diagrammatic summary of our present interpretation of these and the previous findings is given in Fig. 1 which includes also the sites of action of the inhibitory agents used. We emphasize our awareness of deriving a mechanistic proposal of indirect pharmacological evidence obtained by *in vivo* experiments.

A further consideration included in Fig. 1 is required as to the role of galactosamine in affecting a process which normally protects against endotoxin-induced as well as TNF-α-induced liver injury. The known UTP depletion and hence inhibition of hepatic protein synthesis by GalN and its reversal by uridine administration suggests that an intact protein synthesis of the liver still counteracts the deleterious effects of the terminal mediator TNF-α (cf. data in Table 1). *In-vitro* evidence on the cytotoxicity of TNF-α on SV 80 cells only in the presence of cycloheximide has been recently published [16].

The results of this study may also bear some practical pharmacological consequences, as they combine the relevance of the *in-vivo* design with the possibility to differentiate the biochemical action mechanism of drugs in three acutely inflammatory liver injury models, i.e. GalN/endotoxin-, GalN/LTD_4-, and GalN/TNF-α-induced hepatitis in the mouse.

Acknowledgements—Supported by the Deutsche Forschungsgemeinschaft Schwerpunkt-Programm "Eicosanoide", grant We 686/11-1. Stimulating suggestions came from Dr. H. Kolb, Düsseldorf, Dr. M. Schönharting, Wiesbaden, and Dr. U. Schade, Borstel. Special thanks go to Dr. Schönharting for providing TNF-α.

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