Biochemical Pharmacology, Vol. 37, No. 13, pp. 2569–2573, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00
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LEUKOTRIENE-MEDIATED LIVER INJURY*

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(*Received* 3 *November* 1987; *accepted* 28 *December* 1987)

Abstract—The pathogenic mechanism of fulminant hepatitis induced by 700 mg/kg D-galactosamine *plus* 33 μg/kg endotoxin was investigated in male NMRI mice. The extent of liver injury was assessed by measurement of serum transaminases and sorbitol dehydrogenase activities 9 hr after intoxication, as well as by histopathological evaluation. When the hepatic glutathione content of galactosamine endotoxin-treated animals had been decreased by more than 90% following administration of 250 mg/kg phorone or 400 mg/kg diethyl maleate given three times, no signs of liver injury were observed. Since different agents interfering with the leukotriene synthesis pathway also prevented galactosamine/endotoxin-induced hepatitis, we suspected that a glutathione-derived peptidoleukotriene may be the pathogenic metabolite. *In vivo* inhibition of the catabolism of leukotriene C_4 by administration of 50 mg/kg of the glutamyl transpeptidase inhibitor AT 125 (Acivicin) also protected the animals against liver injury. In order to elucidate which metabolite of leukotriene C_4 was responsible for the observed hepatotoxicity we intravenously injected leukotrienes into animals that had received only galactosamine. Injection of 50 μg/kg leukotriene E_4 1 hr after galactosamine had no effect. The same dose of leukotriene D_4 led to a fulminant hepatitis which was prevented when the leukotriene D_4 antagonist FPL 55 712 had been given before. In contrast, lipoxygenase inhibitors or AT 125 did not protect against galactosamine + LTD_4. Galactosamine/endotoxin-induced and galactosamine/leukotriene D_4-induced hepatitis resulted in similarly localized histopathological changes, i.e. diffuse necrosis in the organ.

We conclude from our results that galactosamine/endotoxin-induced hepatitis is mediated by a leukotriene D_4-dependent mechanism.

Among the eicosanoids, leukotrienes represent extremely potent mediators of inflammatory or anaphylactic reactions. In general, chemotactic potency is ascribed to mono- and dihydroxylated leukotriene species such as the HETEs and LTB_4, while mainly vasoconstrictive properties are attributed to the peptido-leukotrienes such as LTC_4 and LTD_4 [1]. Despite increasing knowledge on the systemic action profile of leukotrienes only little is known on their organotropy. On the basis of the detection of cysteinyl leukotrienes in rodent and monkey bile [2, 3] after endotoxin administration, it has been suggested that leukotrienes are involved in the pathogenesis of hepatic injury. This proposal was indirectly supported by the observation that compounds interfering with leukotriene synthesis or action prevented endotoxin-induced lethality [4] or GalN/endotoxin-induced† hepatitis in mice in a dose-dependent manner [5]. However, direct experimental proof as well as identification of the primary pathogenic species is lacking. The aim of this study was to verify the hypothesis of LT-mediated liver injury by extending our previous studies on the mechanism of GalN/E-induced hepatitis in mice [5] and the role of intravenously administered leukotrienes [6] in this model.

MATERIALS AND METHODS

Male albino mice (strain NMRI, Han) were purchased from the Zentral-Institut für Versuchstiere, Hannover, F.R.G. They were kept at least one week on the standard diet C 1000 (Altromin, Lage, F.R.G.) in macrolone cages under environmentally controlled conditions with free access to food and water. Experiments were started at 7 a.m. with animals that had an average weight of 35 g. After 9 hr the animals were sacrificed by cervical dislocation. Blood was withdrawn by heart puncture into 2.5% heparin. Livers were perfused for 1 min with ice-cold 0.9% NaCl via the portal vein. For biochemical assays, livers were immediately frozen in liquid nitrogen after removal, and stored for no longer than two days. For histopathological evaluation, livers were fixed after the experiment in 10% phosphate-buffered formalin. They were transferred into 4% formalin after one day. Sections were stained with hematoxilin/eosin and graded by Dr. D. Mayer, Deutsches Krebsforschungs-Zentrum, Heidelberg. Serum enzyme activities were determined according to Bergmeyer [7]. Total soluble glutathione was measured according to a modified procedure of the GSSG-reductase assay [8].

Chemicals. Leukotrienes were purchased from ICN Biomedicals (Eschwege, F.R.G.) and purified on a Spectra-Physics SP 2000 high performance liquid

* Dedicated to Prof. Leopold Flohé on the occasion of his 50th birthday.

† Abbreviations used: GalN, D-galactosamine; E, endotoxin; GSH, glutathione; SGPT, serum alanine amino transferase, EC 2.6.1.1; SGOT, serum aspartate amino transferase, E.C. 2.6.1.2; SDH, sorbitol dehydrogenase EC 1.1.1.14; PBS, phosphate buffered saline.

chromatograph A Novapak C_{18} reversed phase column (Waters) was run isocratically with acetonitrile/methanol/water/acetic acid (33.6/5.4/61/1) at a flow of 1 ml/min. Leukotrienes were detected at 280 nm. The leukotrienes were intravenously injected via the tail vein within 1 min in a total volume of 400 μl per animal at a concentration of 3.7 μg/ml in phosphate-buffered saline (PBS) containing 0.07% ethanol. 700 mg/kg D-galactosamine (Serva, Heidelberg) was injected i.p. 1 hr prior to the leukotrienes or was given together with 50 μg/kg *Salmonella abortus* equi endotoxin (Sigma Chemicals, St Louis, MO). Phorone (di-isopropylidene acetone, Fluka, Buchs, Switzerland) was given i.p. in vegetable oil. Diethyl maleate (Fluka) was i.p. injected three times 20 min apart in oil as a 20% solution. Thirty minutes after the last injection the animals were treated with GalN/E. Buthionine sulfoximine, given i.p. 8 hr prior to GalN/E, was synthesized according to Ref. 11. For the phorone and diethyl maleate experiments, the animals had been starved overnight, for the buthionine sulfoximine experiment for 21 hr. AT 125 (= Acivicin, Upjohn, Kalamazoo) was injected i.v. 1 hr before GalN/E was given. Ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one, Nattermann & Cie, GmbH, Köln) was given p.o. in 1% tylose 1 hr prior to GalN/E or simultaneously with GalN 1 hr before LTD_4 was injected. Nafazatrom (3-methyl-1[2-(2-naphthyloxy) ethyl]-2-pyrazoline-5-one, Bayer A. G., Leverkusen), REV 5901 (Revlon, Tucahoe, NY), aspirin (Bayer), ibuprofen (Klinge, München), and WEB 2086, 3-{4(2-chlorophenyl)9-methyl-6H-thieno-[3,2f]- [1,2,4-triazolo]- [4,3-a]- [1,4]-diacetine-2-yl}-1-(4-morpholinyl)-1-propanone (Boehringer, Ingelheim) were given p.o. in 1% tylose 1 hr prior to GalN/E. BW 755 C (3-amino-1-[3(trifluoromethyl)-phenyl]-2-pyrazoline. Wellcome Laboratories, Beckenham, U.K.) was given p.o. 2 hr prior to GalN/E. FPL 55 712 (Fisons, Loughborough, U.K.) was i.p. injected every 30 min for 6 hr starting simultaneously with the first injection together with GalN. Diethylcarbamazine (Sigma Chemicals) was analogously administered every 45 min. Dexamethasone (Merck, Darmstadt, F.R.G.) was given i.p. 1 hr before GalN/E in PBS. The results were statistically analyzed according to Student's *t*-test. Data are given in mean values ± SEM; $P < 0.05$ was considered to be significant.

RESULTS

Mice treated with GalN/E developed a fulminant hepatitis which led to serum transaminases or sorbitol dehydrogenase activities of several thousand units per liter after 9 hr. Administration of either agent alone did not result in such symptoms of liver damage. The results in Table 1 demonstrate that pharmacological intervention with different agents leading to a block in the leukotriene pathway resulted in an apparent protection against GalN/E hepatitis. These protective agents included the anti-phospholipase-active steroid dexamethasone, the lipoxygenase inhibiting compounds BW 755 C, nafazatrom, RV 5901 and ebselen, the LTA_4 synthesis blocker diethyl carbamazine, and the leukotriene receptor antagonist FPL 55 712 [9]. On the other hand, the prostanoid synthesis inhibitors ibuprofen or aspirin had no significant effect on GalN/E hepatitis. Also, pretreatment of the animals with the platelet-activating factor antagonist WEB-2086 failed to exert a significant influence on GalN/E hepatitis.

Since these results indicated that leukotrienes might be causally involved in the pathogenesis of GalN/E-hepatitis, we were interested to identify the type(s) of mediators which lead to organ injury. The following experiment was intended to discriminate between mono- or polyhydroxylated leukotrienes, e.g . HETEs or LTB_4, and peptidoleukotrienes as potential hepatotoxic mediators. When mice were treated with the established GSH depletors phorone or diethyl maleate [10] their hepatic glutathione content dropped within the first 30 min after injection from about 20 nmoles/mg to around 1 nmol/mg. This GSH-depleted state was maintained until 150 min after injection of the depletors, and then gradually started to recover up to 50% after 8 hr (Fig. 1). When the animals received GalN/E at the nadir of the GSH depletion, i.e. 90 min after GalN/E they

Table 1. Influence of various agents interfering with eicosanoid synthesis or action on galactosamine/endotoxin-induced hepatitis in mice

Treatments	SDH	SGOT	SGPT	N	M
None	40 ± 4	75 ± 16	40 ± 4	6	0
Disease control	3960 ± 940	2430 ± 390	4950 ± 1090	15	6
200 μg/kg Dexamethasone	110 ± 21**	140 ± 35**	100 ± 21**	8	0
220 mg/kg Aspirin	3060 ± 920	2180 ± 660	3730 ± 1070	8	4
45 mg/kg Ibuprofen	5460 ± 2090	2830 ± 890	7160 ± 2480	10	0
78 mg/kg Diethylcarbamazine	190 ± 36**	270 ± 28**	290 ± 57**	10	0
174 mg/kg BW 755C	120 ± 65**	170 ± 60**	140 ± 78**	8	0
100 mg/kg REV 5901	160 ± 46**	200 ± 21**	220 ± 42**	8	0
60 mg/kg Ebselen	350 ± 85*	180 ± 37**	510 ± 130**	8	0
75 mg/kg Nafazatrom	160 ± 30**	160 ± 19**	180 ± 47**	7	0
13 × 10 mg/kg FPL 55712	160 ± 65*	140 ± 29**	150 ± 86*	6	0
20 mg/kg WEB-2086	9340 ± 3530	4570 ± 1950	12130 ± 3730	8	3

* $P \leq 0.05$.
** $P \leq 0.01$.
SDH, SGOT and SGPT in U/liter; data means ± SEM.
N = number of animals; M = number of animals who died within 9 hr after intoxication.

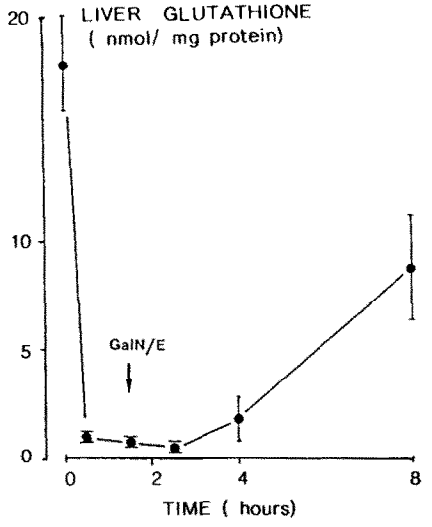

Fig. 1. Time course of hepatic glutathione depletion after administration of 250 mg/kg phorone to male mice. Data are means ± SD (N = 6). The arrow indicates the time, when galactosamine/endotoxin was given in the experiments shown in Table 2.

were fully protected against hepatotoxicity in this model (Table 2). In contrast, inhibition of glutathione synthesis by buthionine sulfoximine pretreatment [11] which also led to a moderate decrease of the hepatic glutathione content, did not prevent GalN/E-hepatitis (Table 2). These findings allow the conclusion that a leukotriene species which needs the availability of GSH has to be formed before pathogenic processes take place.

The next step in our study was to discriminate between the individual cysteinyl-leukotrienes C_4, D_4 and E_4 as potential mediators of GalN/E-hepatitis. When the conversion of LTC_4 to LTD_4 was blocked *in vivo* by pretreatment of the animals with the γ-glutamyl transpeptidase inhibitor AT 125 [12], the mice were also protected (Table 3). Therefore it seems likely that a metabolite of LTC_4 represented the ultimately hepatotoxic agent. The results of the experiments shown in Table 4 clearly identify LTD_4 as this species, in an experimental variation where direct injections of either LTD_4 or its metabolite

Table 3. Influence of the inhibition of LTC_4 catabolism on GalN/E-induced hepatitis in mice

Vehicle control	40 ± 4	6	0
GalN/E	3860 ± 1440	9	1
GalN/E + AT 125[a]	175 ± 40**	16	0

** $P \leqslant 0.01$; data means ± SEM.

[a] Given intravenously 1 hr prior to GalN/E; 50 mg/kg i.v. Analogous data were obtained for SGOT and SDH activities.

Table 4. Effect of i.v. injected leukotrienes on the development of hepatitis in *starved* mice

Treatment	SGPT	N	M
PBS	40 ± 4	6	0
GalN/PBS	50 ± 6*	11	0
GalN/LTD_4	1950 ± 490	13	2
LTD_4	60 ± 4*	5	0
GalN/LTD_4 + FPL 55712	80 ± 10*	5	0
GalN/LTD_4 + Ebselen	1950 ± 1000	5	1
GalN/LTD_4 + AT 125	1770 ± 1310	5	1
GalN/LTE_4	150 ± 40*	5	0
LTE_4	120 ± 10*	5	0

Data (mean ± SEM) were assessed 9 hr after administration of the compounds.

* $P \leqslant 0.05$ compared to disease controls.

Leukotriene dose: 50 μg/kg 1 hr after GalN, other compounds as in Table 1.

Analogous data were obtained for SGOT and SDH activities.

LTE_4 replaced endotoxin treatment in our model. The experiment shows furthermore, that unlike in the GalN/E experiments, neither of the leukotriene metabolism inhibitors studied (i.e. ebselen or AT 125) protected against the GalN/LTD_4 combination. On the other hand, GalN/LTD_4-induced hepatitis in mice was antagonized by FPL 55 712, suggesting the required specificity for the interpretation given above.

Although these latter findings would argue against it, it might have been possible that minute and virtually undetectable amounts of endotoxin in our solvents may have caused liver symptoms when administered intravenously. Therefore we

Table 2. Influence of liver glutathione status of galactosamine-sensitized starved mice on endotoxin-induced hepatitis

Treatment	SGPT	Liver glutathione[a]	N	M
Vehicle control	40 ± 4	18 ± 1	6	0
Disease control	2210 ± 460	18 ± 1	16	15
Phorone + GalN/E	50 ± 10**	1 ± 0.04	8	0
DEM + GalN/E	90 ± 10**	1.5 ± 0.1	8	0
BSO + GalN/E	3170 ± 960	11.4 ± 1.5	8	6

Depletion: 250 mg/kg phorone i.p. 90 min prior to GalN/E. 400 mg/kg diethyl maleate (DEM) in oil i.p. three times every 20 min within the first hour prior to GalN/E.

Synthesis block: 900 mg/kg buthione sulfoximine (BSO) i.p. 8 hr prior to GalN/E.

** $P \leqslant 0.01$, data mean ± SEM.

[a] At the time GalN/E was given; analogous data were obtained for SGOT and SDH activities; glutathione in nmol per mg protein.

Table 5. Sensitivity of mice to galactosamine and endotoxin after intraperitoneal and intravenous administration and potentiation of a subtoxic endotoxin dose by LTD_4

Treatment	Mode	SGPT	N	M
GalN + 0.05 μg/kg E	i.v.	150 ± 33[a]	8	0
GalN + 0.05 μg/kg E + LTD_4	i.v.	1110 ± 270*[a]	8	0
GalN	i.p.	50 ± 6[b]	11	0
50 μg/kg E	i.p.	60 ± 17[b]	9	0

Data: means ± SEM.
* $P \leq 0.05$.
[a] Determined after 30 hr.
[b] Determined after 9 hr

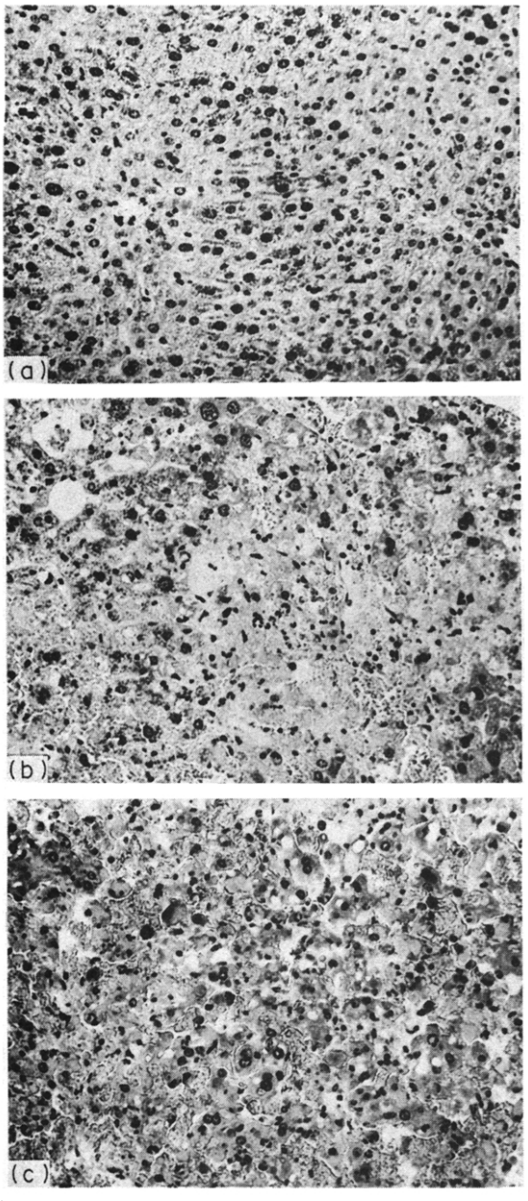

Fig. 2. Livers of mice treated 9 hr before excision with 700 mg/kg galactosamine (a), or 700 mg/kg galactosamine + 50 μg/kg leukotriene D_4 (b), or 700 mg/kg galactosamine + 50 μg/kg endotoxin (c). Fixation: 4% phosphate-buffered saline; staining: hematoxylin/eosine; magnification: 148-fold.

performed some control experiments where a dose of endotoxin was given i.v. which was one thousand times lower than the administered i.p. dose. The results in Table 5 demonstrate that, if endotoxin in trace amounts had been present, its deleterious effects would have been greatly potentiated by LTD_4.

Some livers of animals with moderate transaminase levels, i.e. several hundred U/l, were taken from the GalN/E and the GalN/LTD_4-treated groups in order to compare the histopathologically visible lesions. In Fig. 2, three out of nine livers examined are shown. Both groups of injured animals showed diffuse large necrotic areas with inflammatory infiltrations and nuclear atypia (kariorexis). In both groups, the immediate periportal zone showed a normal pattern whereas advanced necrosis originated centrally. A morphological discrimination between GalN/LTD_4 (Fig. 2b) or GalN/E (Fig. 2c)-induced necrosis was not possible.

DISCUSSION

The inhibitory pattern of compounds interfering with eicosanoid metabolism (Fig. 3) in this and earlier studies [4, 5] on GalN/E-induced hepatitis was indicative for a specifically leukotriene-mediated

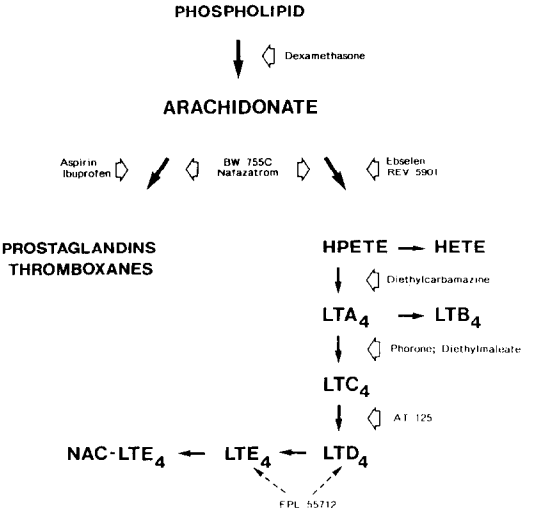

Fig. 3. Schematic diagram of the site of interaction of agents used in this study to modulate eicosanoid metabolism.

organ injury. This experimental model differs considerably from other chemically or drug-induced hepatopathies in which secondary reactions of a reactive intermediate are thought to represent the primary injurious mechanism. In these models which include paracetamol-, halogenated hydrocarbon-, or allyl alcohol-induced liver damage, depletion of hepatic GSH potentiated the hepatotoxicity of the xenobiotics [13–15]. The findings of this study provide a novel example of an opposite effect of GSH depletion, i.e. a protection against GalN/E hepatitis. We interpret this in terms of the inability of the GSH-depleted cells to form peptidoleukotrienes, either due to lack of GSH, or due to inhibition of GSH transferase by phorone or diethyl maleate. Several *in vitro* studies seem to support this interpretation: (a) a reduced biosynthesis of slow reacting substance (= LTC_4/D_4) at low GSH levels in leukemia cells was reported [16]; (b) in macrophages, depletion of GSH selectively inhibited LTC_4 synthesis [17]; (c) for isolated LTC_4 synthetase for leukemia cells, K_m values of the enzyme for GSH were determined to range around intracellular GSH concentrations, i.e. from 3 to 6 mmol/l at a cosubstrate concentration of 20 μmol/l LTA_4 [18]. These kinetic conditions could also underly our finding that lowering the intrahepatic GSH levels by synthesis inhibition did not block GalN/E hepatitis (Table 2). In addition to these theoretical considerations, preferential effects of GSH depletors on individual liver cell types may be equally important.

Since AT 125 was reported to inhibit the metabolism of LTC_4 *in vitro* [19], we chose a dose sufficient to inhibit γ-glutamyl transpeptidase *in vivo* [12] in order to prevent endotoxin-induced LTD_4 synthesis in the liver. Therefore the conclusion that under these conditions the lack of LTD_4 formation accounted for the observed protection seems justified. In fact, the direct leukotriene injection experiments corroborated the set of assumptions derived from the previous experiments. The lack of protection by AT 125 against GalN/LTD_4 (Table 4 vs Table 3) represent a control which suggests that side effects of AT 125 other than inhibiting the formation of LTD_4 seem unlikely in this model.

Our experimental protocol does not enable us to decide whether LTD_4 alone or the combination of minute amounts of endotoxins plus leukotriene trigger a sequence of events which finally lead to hepatitis. It is highly likely that some more unknown components are involved in metabolic processes associated with specialized white blood cells rather than with those in the liver. In any case, a potentiation of a subtoxic endotoxin stimulus may well reflect a pathophysiological condition and would lead to the concordant conclusion, i.e. that LTD_4 mediates GalN/E-induced hepatitis. As to the final pathogenic mechanism of LTD_4 injury, we have evidence that at least in the GalN/E model, an ischemia/reperfusion syndrome is involved [20]. Since LTD_4 has vasoconstrictive properties in other organs, it is likely to induce a local spasm in hepatic vessels and thus leads to a transient hypoxia followed by a deleterious reoxygenation phase [21].

The role of galactosamine in sensitizing the liver towards LTD_4 or endotoxin requires further experiments. It is known for a long time that GalN-induced UTP depletion leads to an instantaneous block of hepatic protein synthesis [22]. Therefore, hepatic proteins with a high turnover are likely to be primarily affected by GalN pretreatment, and excellent candidates of this class are acute-phase proteins.

Acknowledgements—This work was financially supported by the Deutsche Forschungesgemeinschaft, Grant We 686/10.

Helpful suggestions and intensive discussions contributed by Dr D. Keppler, Heidelberg, and Dr L. Flohé, Aachen, are gratefully acknowledged. Thanks go to Dr V. Ullrich, Konstanz and Dr E Graf, Köln, for providing leukotrienes. Indispensable support was received from Dr P. Bannasch and Dr D. Mayer, Heidelberg, with the histopathological evaluation of the livers.

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