TNF- α dependent production of inducible nitric oxide is involved in PGE₁ protection against acute liver injury

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Abstract

Background—Tumour necrosis factor α (TNF- α) and nitric oxide modulate damage in several experimental models of liver injury. We have previously shown that protection against D-galactosamine (D-GalN) induced liver injury by prostaglandin E₁ (PGE₁) was accompanied by an increase in TNF- α and nitrite/nitrate in serum.

Aims—The aim of the present study was to evaluate the role of TNF- α and nitric oxide during protection by PGE₁ of liver damage induced by D-GalN.

Methods—Liver injury was induced in male Wistar rats by intraperitoneal injection of 1 g/kg of D-GalN. PGE₁ was administered 30 minutes before D-GalN. Inducible nitric oxide synthase (iNOS) was inhibited by methylisothiourea (MT), and TNF- α concentration in serum was lowered by administration of anti-TNF- α antibodies. Liver injury was evaluated by alanine aminotransferase activity in serum, and histological examination and DNA fragmentation in liver. TNF- α and nitrite/nitrate concentrations were determined in serum. Expression of TNF- α and iNOS was also assessed in liver sections.

Results—PGE₁ decreased liver injury and increased TNF- α and nitrite/nitrate concentrations in serum of rats treated with D-GalN. PGE₁ protection was related to enhanced expression of TNF- α and iNOS in hepatocytes. Administration of anti-TNF- α antibodies or MT blocked the protection by PGE₁ of liver injury induced by D-GalN.

Conclusions—This study suggests that prior administration of PGE₁ to D-GalN treated animals enhanced expression of TNF- α and iNOS in hepatocytes, and that this was causally related to protection by PGE₁ against D-GalN induced liver injury.

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Keywords: tumour necrosis factor α ; nitric oxide; prostaglandin E₁; methylisothiourea; D-galactosamine; liver injury

Hepatic injury induced by D-galactosamine (D-GalN) is a suitable experimental model of liver failure.¹ D-GalN induces inhibition of RNA and protein synthesis thus altering hepatocellular function.² Both effects may be a consequence of a depleted intracellular pool of uracil nucleotides.² Several studies have re-

ported that prostaglandin E reduces hypertransaminasaemia observed during in vivo hepatotoxicity induced by D-GalN,³ thioacetamide,⁴ aflatoxin B₁,⁵ carbon tetrachloride,⁶ bile duct ligation,⁷ fat enriched and choline deficient diets,⁸ viral hepatitis,⁹ and complement mediated hepatic necrosis.¹⁰ Furthermore, prostaglandin E₁ (PGE₁) has a beneficial effect on fulminant viral hepatitis in humans with a decrease in levels of transaminases and improvement in encephalopathy and coagulation factors.^{11,12} Prostacyclin also reduces serum transaminases in hepatic necrosis induced by D-GalN in rats.¹³ Several hypotheses have been presented to explain this protective effect of prostaglandins. It has been suggested that protection by PGE₁ against liver injury induced by ischaemia-reperfusion¹⁴ or bile duct ligation⁷ may occur at the vascular level. PGE₁ reduces plasma membrane microviscosity induced by lipid peroxidation in rat hepatocytes, suggesting that it has a stabilising effect.¹⁵ Marinovich and colleagues¹⁶ have shown that prostaglandin E₂ modifies the arachidonic acid cascade profile after activation of phospholipase A₂ by carbon tetrachloride in rat hepatocyte cultures. The antifibrotic activity of prostaglandin E₂ has also been proved.⁸

Tumour necrosis factor α (TNF- α) is a multifunctional cytokine mostly secreted by inflammatory cells and is involved in numerous pathological states.¹⁷ In particular, it has been shown that TNF- α enhances liver injury when D-GalN toxicity is already established.¹⁸ Nevertheless, D-GalN treated animals can be protected from an otherwise lethal challenge of either lipopolysaccharide or TNF- α toxicity by pretreatment with a sublethal dose of TNF- α .¹⁹

Nitric oxide is a labile and highly reactive compound that plays a physiological role in blood pressure regulation, neurotransmission, tumour cell killing, immunity, and inflammatory processes.²⁰ Two constitutive forms of nitric oxide synthase (cNOS) (eNOS in endothelial cells and nNOS in neurones) and one inducible form (iNOS) (in hepatocytes, Kupffer cells, macrophages, fibroblasts, chondrocytes, and endothelial cells) have been described.²¹ Expression of iNOS is modulated by cytokines secreted during inflammation.²² The cytoprotection or cytotoxicity of nitric

Abbreviations used in this paper: TNF- α , tumour necrosis factor α ; D-GalN, D-galactosamine; PGE₁, prostaglandin E₁; iNOS, inducible nitric oxide synthase; cNOS, constitutive nitric oxide synthase; MT, methylisothiourea; ALT, alanine aminotransferase; PBS, phosphate buffered solution.

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oxide has been reported by several authors.^{23–24} Administration of a nitric oxide donor induces tolerance against a lethal combination of TNF- α and D-GalN treatment.²⁵

In a previous study²⁶ we observed that protection by PGE₁ against D-GalN induced liver injury was associated with an increase in TNF- α and nitrite/nitrate in serum. Prior administration of both inflammatory mediators has been shown to induce tolerance against lipopolysaccharide or cytokine induced liver toxicity.^{19–25} The aim of the present study was to evaluate if TNF- α and/or nitric oxide participate in the protective effect of PGE₁ on D-GalN induced liver injury in rats.

Materials and methods

EXPERIMENTAL DESIGN

Male Wistar rats (175–225 g) were kept under standard conditions and received a regular diet (Piensos equilibrados, CIA, Ebro Agrícolas, Utrera, Spain) and water ad libitum. Animal care and experimental protocols were in accordance with the *Guide for the Care and Use of Laboratory Animals* prepared by the National Academy of Sciences and published by the National Institute of Health (NIH publication No 86–23, revised 1985).

To evaluate the involvement of iNOS on D-GalN injury and its protection by PGE₁, animals (n=80) were divided into eight groups: control; PGE₁; D-GalN; PGE₁+D-GalN; methylisothiourea (MT); MT+PGE₁; MT+D-GalN; and MT+PGE₁+D-GalN. D-GalN (Sigma Chemical Corporation, St Louis, Missouri, USA) was dissolved in 0.9% NaCl just before intraperitoneal administration (1 g/kg). MT (Sigma Chemical Corporation) was used as a highly specific inhibitor of iNOS.²⁷ The lowest dose and pattern of administration of MT that effectively blocked the production of nitric oxide induced by lipopolysaccharide (10 mg/kg) were determined in a preliminary study (unpublished data). MT was dissolved in 0.9% NaCl and administered (25 mg/kg) intraperitoneally 30 minutes and one hour before D-GalN, and four and eight hours after D-GalN administration. PGE₁ was dissolved in 10% ethanol and administered (250 μ g/kg) intraperitoneally 30 minutes before D-GalN. The amount (100 μ l) of 10% ethanol used had no effect on the variables of the study in the presence or absence of hepatotoxin. Accordingly, the control group received the corresponding amount of 0.9% NaCl intraperitoneally. The dose of PGE₁ administered in the present study was within the range used in several experimental studies^{4–5,8} and during clinical trials.¹¹ After administration of the treatments, rats were maintained in a fasted state with access to water ad libitum. The animals were killed 12 hours after D-GalN administration. Those animals who did not receive D-GalN were killed 12 hours after PGE₁ or the second administration of MT. The experimental design scheme is shown diagrammatically in fig 1A. Blood samples were obtained by cardiac puncture and serum was frozen for measurement of alanine aminotransferase (ALT),

Figure 1 Experimental design scheme. (A) Pattern of administration of methylisothiourea (MT), prostaglandin E₁ (PGE₁), and D-galactosamine (D-GalN). (B) Pattern of administration of anti-tumour necrosis factor α (anti-TNF- α) antibodies, PGE₁, and D-GalN.

TNF- α , and nitrite/nitrate levels. Endotoxin content was also measured in serum from control and D-GalN treated animals.

To evaluate the involvement of TNF- α in D-GalN induced liver injury and its protection by PGE₁, animals (n=40) were divided into eight groups: control; PGE₁; D-GalN; PGE₁+D-GalN; anti-TNF- α +control; anti-TNF- α +PGE₁; anti-TNF- α +D-GalN; and anti-TNF- α +PGE₁+D-GalN. Polyclonal rabbit antihuman TNF- α antibodies (Genzyme Diagnostics, Cambridge, Massachusetts, USA) administered to animals can neutralise rat and human TNF- α forms, as indicated by the manufacturer. Antibodies were dissolved in phosphate buffered solution (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄) at pH 7.4 and administered (5 mg/kg) intraperitoneally one hour before D-GalN. D-GalN and PGE₁ were administered under the conditions previously described. The amount of ethanol used, as solvent for PGE₁, had no effect on the variables of the study in the presence or absence of the hepatotoxin. Accordingly, the control group received the corresponding amount of 0.9% NaCl intraperitoneally. Non-immunised rabbit IgG (Sigma Chemical Corporation) as a negative control of anti-TNF- α antibodies had no effect on the variables of the study in the presence or absence of hepatotoxin. After administration of the treatments, rats were maintained in a fasted state with access to water ad libitum. The animals were killed 12 hours after D-GalN administration. Those animals who did not receive D-GalN were killed 12 hours after PGE₁ or anti-TNF- α treatment. The experimental design scheme is shown diagrammatically in fig 1B. Blood samples were obtained by cardiac puncture and serum was frozen for ALT and nitrite/nitrate measurements. Serum samples for evaluation of the efficacy of anti-TNF- α antibodies in reducing TNF- α concentrations were also obtained. For this purpose, serum was filtered through HiTrap Protein A cartridges (Pharmacia Biotech AB, Uppsala, Sweden) to remove antibodies, thus avoiding interference with ELISA. It was observed that TNF- α concentrations in serum were undetectable by ELISA using the dose and pattern of administration of anti-TNF- α antibodies described above (data not shown). Several liver specimens were also removed and fixed in buffered 4% formalin in PBS (Panreac, Barcelona, Spain) and embedded in paraffin. Thin sections (3 μ m) were obtained for evaluation of histological

damage and expression of TNF- α and iNOS. Other liver specimens were immediately frozen to evaluate DNA fragmentation and TNF- α content.

HISTOLOGICAL EXAMINATION

Liver injury was evaluated in liver fixed sections embedded in paraffin and stained with haematoxylin and eosin.

DNA FRAGMENTATION IN THE LIVER

Detection of the classical laddering of DNA fragmentation in liver extracts is related to apoptosis. Frozen livers (1 g) were homogenised (Ultra-turrax T25; Janke and Kunkel IKA-Laboratory) in lysis solution (1% SDS, 10 mM Tris HCl, 50 mM EDTA), pH 7.4. Samples were incubated with RNaseA (1 mg/ml) (Sigma Chemical Corporation) for two hours at 37°C and proteinase K (1 mg/ml) (Sigma Chemical Corporation) for 45 minutes at 48°C. DNA was obtained by phenol:chloroform: isoamyl alcohol 25:24:1 (Sigma Chemical Corporation) extraction and precipitated with isopropanol (v/v) and 0.5 M NaCl for 12 hours at -20°C. DNA was recovered by centrifugation at 15 000 *g* for 20 minutes, and the pellet was washed with 70% ethanol, dried, and resuspended in Tris-EDTA buffer (10 mM Tris, 50 mM EDTA), pH 7.4. Samples (250 μ g DNA) were applied and analysed on 2% agarose gel with ethidium bromide (0.5 μ g/ml).

BIOCHEMICAL PARAMETERS

Activity of serum ALT was measured using routine laboratory methods²⁸ in samples obtained from experimental animals.

EXPRESSION OF TNF- α IN LIVER

TNF- α content in liver evaluated by western blot

Frozen livers (1 g) were homogenised (Ultra-turrax T25; Janke and Kunkel IKA-Laboratory) in lysis solution (25 mM HEPES, 10 mM MgCl₂, 2 mM EGTA, 1 mM PMSF, 1 μ g/ml aprotinin, and 1 μ g/ml leupeptin), pH 7.5. Polyclonal goat antihuman TNF- α antibodies (R&D Systems, Minneapolis, USA) used in the assay also detected rat TNF- α forms, as indicated by the manufacturer. Recombinant human TNF- α (25 pg) (Genzyme, Diagnostics) was used as standard. Samples (50 μ g) were applied to 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Membranes were treated with blocking solution (20 mM Tris HCl, 150 mM NaCl, 0.1% Tween 20, and 5% milk powder), pH 7.6, for one hour and further incubated with primary antibodies for one hour and with alkaline phosphatase linked secondary antibodies (Sigma Chemical Corporation) for one hour. TNF- α was visualised incubating the membranes with the alkaline phosphatase substrate (BCIP-NBT) for 20 minutes. TNF- α content of each sample was determined by quantitative densitometry using the TDI's 1D Manager version 2.0 software included in a Gel Printer Plus System (Tecnología para Diagnóstico e Investigación, Madrid, Spain).

TNF- α immunolocalisation in liver sections

Cell expression of TNF- α was also evaluated by immunohistochemistry in rat liver fixed sections (3 μ m) embedded in paraffin, as described above. Deparaffinised sections were treated with 3% H₂O₂ for 30 minutes at room temperature to eliminate the interference of endogenous peroxidases. Non-specific immunostaining was blocked by treatment with 1% fetal bovine serum and 10% rat serum for one hour at room temperature. TNF- α expression was assessed using polyclonal goat antihuman TNF- α antibodies (R&D Systems) which cross react with rat TNF- α forms, as described by the manufacturer. Liver sections were incubated with primary antibodies (5 μ g/ml) for 24 hours at 4°C and with biotinated polyclonal antibody against goat IgG (Zymed, San Francisco, USA) for one hour at 37°C. Samples were incubated with streptavidin-biotin-peroxidase complex (Dako A/S, Copenhagen, Denmark) and visualised with 50 mM diaminobenzidine solution, pH 6.0, and 0.003% H₂O₂ for eight minutes at room temperature co-stained with haematoxylin.

The degree of immunostaining of TNF- α was evaluated semiquantitatively using four categories: (1) no expression; (2) mild overexpression or less than 25% of hepatocytes stained; (3) moderate overexpression or 25–50% of hepatocytes stained; and (4) intense overexpression or more than 50% of hepatocytes stained.

ENDOTOXIN MEASUREMENT

Endotoxin content was measured in serum obtained from control and D-GalN treated rats using a commercial kit (E-toxate, Sigma Chemical Corporation).

TNF- α MEASUREMENT IN SERUM

TNF- α was quantified in serum by competitive ELISA.²⁶ The following parameters were determined: for reproducibility, interassay precision profile (1.2 $\pm$ 0.03%), intra-assay precision profile (2.9 $\pm$ 0.90%), and intra-assay coefficient of variation (1.9%); for parallelism, slope ratio (0.94 $\pm$ 0.030%); and for sensitivity, minimum detectable dose (0.08 pg/ml). A representative

Figure 2 Representative standard curve for the tumour necrosis factor α (TNF- α) ELISA. B/Bo represents the ratio between the absorbance obtained from each TNF- α concentration of the standard curve (B) and the absorbance obtained in the absence of TNF- α antigen (Bo).

standard curve for the TNF- α ELISA is shown in fig 2. Polyclonal rabbit antihuman TNF- α antibodies (Genzyme Diagnostics) used in the assay were also useful in detecting rat TNF- α forms, as indicated by the manufacturer. Recombinant human TNF- α (Genzyme Diagnostics) used as a standard in the assay was calibrated against the related NIBSC reference standard (87/650). Briefly, wells of ELISA plates were coated with 0.5 ng of TNF- α for one hour at 37°C. The wells were blocked with 2% bovine serum albumin in 10 mM PBS-0.05% Tween (PBS-Tween) at pH 7.4. The samples and anti-TNF- α antibodies (32.5 ng/ml) were incubated for two hours at 37°C, transferred to antigen coated wells, and incubated for two hours at 37°C. The wells were washed with PBS-Tween and incubated with the biotinated secondary antibodies (125 pg/ml) (Zymed) for one hour at 37°C. The wells were then washed with Tris buffer (100 mM) at pH 7.6 and incubated with streptavidin-alkaline phosphatase solution (Master Diagnostica, Granada, Spain) for 30 minutes at 37°C. Finally, the wells were washed and incubated with *p*-nitrophenyl phosphate (Sigma Chemical Corporation) as alkaline phosphatase substrate for one hour at 37°C. Later, the wells were read at 405 nm in a titrated Organon Teknika 510 ELISA reader.

MEASUREMENT OF NITRIC OXIDE METABOLITES

Production of nitric oxide was measured by quantification of its related end products, nitrite/nitrate. In the assay, nitrate was converted to nitrite by nitrate reductase (EC 1.6.6.2) and total nitrite was measured using the Griess reaction.²⁹ Briefly, samples were incubated with nitrate reductase (0.2 U/ml), FAD (5 mM), and NADPH (50 mM) for 20 minutes at 37°C. The reaction was stopped by addition of sodium pyruvate (10 mM) and lactate dehydrogenase (24 mg/ml) for five minutes at 37°C, and precipitated with 1.4% ZnSO₄. Total nitrite reacted with Griess reagent (1% sulphanilamide, 2.5% PO₄H₃, 0.1% *n*-naphthylethylene-diamine) for 10 minutes at 37°C, and was read using the 540 nm filter in a titrated Organon Teknika 510 ELISA reader.

EXPRESSION OF INDUCIBLE NITRIC OXIDE SYNTHASE IN LIVER

Cell expression of iNOS was evaluated by immunohistochemistry in rat liver fixed sections (3 μ m) embedded in paraffin, as described above. Deparaffinised sections were treated with microwaves at 700 W for 10 minutes in 0.01 M citrate buffer at pH 6.0 for antigen retrieval before immunostaining. Non-specific immunostaining was blocked by treatment with 0.3% casein and 0.5% Tween 20 for 30 minutes before incubation with normal goat serum. iNOS expression was assessed with a specific monoclonal antibody against rat iNOS (Transduction Laboratories, Lexington, Kentucky, USA) at a 1:20 dilution for 16 hours at 4°C. iNOS was visualised using a standard multilink streptavidin-biotin-alkaline phosphatase kit (Master Diagnostica).

The degree of immunostaining of iNOS was evaluated semiquantitatively using four categories:

(1) no expression; (2) mild overexpression or less than 25% of hepatocytes stained; (3) moderate overexpression or 25–50% of hepatocytes stained; (4) intense overexpression or more than 50% of hepatocytes stained.

STATISTICAL ANALYSIS

Results are expressed as mean (SEM). Comparisons were made using ANOVA with the least significant difference test (LSD). Statistical significance was set at $p \leq 0.05$.

Results

LIVER INJURY INDUCED BY D-GALACTOSAMINE AND ITS PROTECTION BY PGE₁

Administration of D-GalN induced serious liver injury with numerous apoptotic bodies in hepatic sections (fig 3C) and an increase in DNA fragmentation in liver extract (fig 4, lane 3) compared with control animals (fig 3A; fig 4, lane 2, respectively). Biochemical parameters of liver injury, such as ALT activity in serum, were also significantly enhanced by D-GalN (1109 (172.0) U/l) compared with the control group (19 (0.9) U/l) (fig 5) ($p \leq 0.05$). Prior administration of PGE₁ reduced all histological markers of necrosis and apoptosis induced by D-GalN (fig 3D; fig 4, lane 5; fig 5).

TNF- α EXPRESSION AND SYNTHESIS FOLLOWING D-GALACTOSAMINE AND/OR PGE₁

D-GalN significantly enhanced TNF- α content in liver extract (0.133 (0.0070) pg/ μ g protein) (fig 6A, lane 3) that was correlated with higher expression of TNF- α in the cytoplasm of hepatocytes (degree 3) (fig 6D) than TNF- α content (0.116 (0.0005) pg/ μ g protein) (fig 6A, lane 2) and TNF- α staining (degree 2) (fig 6B) in control livers ($p \leq 0.05$). Particularly interesting was the strong staining of TNF- α in apoptotic bodies in liver sections from D-GalN treated rats (fig 6D). PGE₁ alone enhanced TNF- α expression in the liver (fig 6A, lane 4; fig 6C). Prior administration of PGE₁ to D-GalN treated animals gave rise to a significant increase in hepatic TNF- α content (0.230 (0.0033) pg/ μ g protein) (fig 6A, lane 5) and an overall higher TNF- α diffused expression in hepatocytes (degree 4) (fig 6E) ($p \leq 0.05$).

It was confirmed that the presence of endotoxin, as a potential inducer of TNF- α release, in serum from control and D-GalN treated animals was undetectable (data not shown). TNF- α serum concentrations are shown in fig 7. TNF- α concentrations in serum were significantly higher in D-GalN (21 (1.9) pg/ml) and PGE₁ (20 (2.9) pg/ml) groups than in the control group (11 (1.3) pg/ml) ($p \leq 0.05$). An additional significant effect on TNF- α levels in serum was observed when PGE₁ and D-GalN were administered together ($p \leq 0.05$).

NITRIC OXIDE MODULATES THE EFFECTS OF D-GALACTOSAMINE AND PGE₁ ON LIVER INJURY AND TNF- α SYNTHESIS

PGE₁ administration significantly increased nitrite/nitrate concentrations in PGE₁ (29 (2.9) μ M) and PGE₁+D-GalN (22 (1.5) μ M) groups compared with control (17 (0.9) μ M) and D-GalN (18 (1.0) μ M) groups, respectively

Figure 3 Effects of prior administration of prostaglandin E₁ (PGE₁) on histological liver injury induced by D-galactosamine (D-GalN) observed by light microscopy. (A) Control, (B) PGE₁, (C) D-GalN, and (D) PGE₁+D-GalN treated rats. PGE₁ reduced the numerous apoptotic bodies exhibiting compacted chromatin (a, arrow) observed with D-GalN. (Original magnification $\times 100$.)

($p \leq 0.05$) (fig 8). To study the role of nitric oxide on D-GalN liver injury and its protection by PGE₁, MT was administered to inhibit iNOS activity. Nitric oxide concentrations in serum were reduced significantly in all MT treated groups ($p \leq 0.05$) (fig 8). The increase in ALT activity in serum by D-GalN was not affected by MT treatment (fig 5). However, protection by PGE₁ on ALT release induced by D-GalN in the PGE₁+D-GalN (1609 (290.9) U/l) group with MT treatment was significantly abolished compared with values obtained with PGE₁+D-GalN (547 (104.7) U/l) without iNOS inhibition (fig 5) ($p \leq 0.05$).

It was also observed that inhibition of iNOS activity by MT modified TNF- α concentrations in serum obtained from experimental animals (fig 7). In this sense, MT significantly enhanced TNF- α levels in serum from all groups compared with their respective controls ($p \leq 0.05$).

Figure 4 Effects of prior administration of prostaglandin E₁ (PGE₁) on DNA fragmentation in liver extract induced by D-galactosamine (D-GalN) with or without prior administration of anti-tumour necrosis factor α (TNF- α) antibodies. Lane 1: DNA molecular weight marker (base pairs); lane 2: control; lane 3: D-GalN; lane 4: PGE₁; lane 5: PGE₁+D-GalN; lane 6: anti-TNF- α +control; lane 7: anti-TNF- α +D-GalN; lane 8: anti-TNF- α +PGE₁; and lane 9: anti-TNF- α +PGE₁+D-GalN. Administration of PGE₁ or anti-TNF- α antibodies reduced DNA fragmentation induced by D-GalN.

Figure 5 Effects of prior administration of prostaglandin E₁ (PGE₁) on alanine aminotransferase (ALT) activity in serum induced by D-galactosamine (D-GalN) with (+) or without (-) inhibition of iNOS activity by methylisothiourea (MT). PGE₁ only reduced ALT in serum induced by D-GalN in the absence of MT. Values are mean (SEM) ($n=10$). Groups with different superscript letters are significantly different ($p \leq 0.05$).

ANTI-TNF- α ANTIBODIES MODULATE THE EFFECTS OF D-GALACTOSAMINE AND PGE₁ ON LIVER INJURY

Administration of anti-TNF- α antibodies to D-GalN treated animals sharply reduced DNA fragmentation in liver extract (fig 4, lane 7) compared with that found in D-GalN treated rats (fig 4, lane 3) without additional treatment. ALT activity in serum was also significantly lower in D-GalN treated rats when anti-TNF- α antibodies were administered (397 (68.2) U/l) compared with values in the D-GalN group without antibodies (1109 (172.0) U/l) ($p \leq 0.05$) (table 1). Interestingly, no protection by PGE₁ on DNA fragmentation

Figure 6 Effects of prior administration of prostaglandin E_1 (PGE_1) on expression of tumour necrosis factor α ($TNF-\alpha$) in the liver of animals treated with D-galactosamine (D-GalN). (A) $TNF-\alpha$ in liver extract measured by western blot. Lane 1: standard (25 pg); lane 2: control; lane 3: D-GalN; lane 4: PGE_1 ; and lane 5: PGE_1 +D-GalN. Western blot is representative of five different experiments. Prior administration of PGE_1 strongly enhanced $TNF-\alpha$ expression in the liver extract induced by D-GalN. $TNF-\alpha$ expression in liver sections evaluated by immunohistochemistry: (B) control; (C) PGE_1 ; (D) D-GalN; and (E) PGE_1 +D-GalN treated rats. Images are representative of five different experiments. PGE_1 enhanced $TNF-\alpha$ staining in the nucleus of control hepatocytes. Numerous apoptotic bodies (a, arrow) induced by D-GalN showed strong $TNF-\alpha$ staining. Prior administration of PGE_1 to D-GalN treated rats induced enhancement of diffused $TNF-\alpha$ staining. (Original magnification $\times 100$.)

Figure 7 Effects of prior administration of prostaglandin E_1 (PGE_1) on tumour necrosis factor α ($TNF-\alpha$) in serum of rats treated with D-galactosamine (D-GalN) with (+) or without (–) inhibition of iNOS activity by methylisothiourea (MT). Prior administration of PGE_1 strongly enhanced $TNF-\alpha$ concentration in serum induced by D-GalN. MT administration raised $TNF-\alpha$ in all groups. Values are mean (SEM) ($n=10$). Groups with different superscript letters are significantly different ($p \leq 0.05$).

(fig 4, lane 9) or ALT release (1093 (130.1) U/l) (table 1) induced by D-GalN was observed when anti- $TNF-\alpha$ antibodies were administered compared with DNA fragmentation (fig 4, lane 5) and ALT (547 (104.7) U/l) in the PGE_1 +D-GalN group without antibodies ($p \leq 0.05$) (table 1).

We also evaluated if anti- $TNF-\alpha$ antibody treatment had an effect on hepatic iNOS (fig 9) expression and nitrite/nitrate (table 2) concentrations in serum. The liver from control rats showed no expression of iNOS (degree 1) (fig 9A). Administration of anti- $TNF-\alpha$ antibodies to PGE_1 +D-GalN rats clearly reduced expression of iNOS in hepatocytes (degree 2) (fig 9C) compared with that observed in the PGE_1 +D-GalN (degree 3) group (fig 9B). In contrast, administration of $TNF-\alpha$ antibodies significantly enhanced nitrite/nitrate concentrations in serum in control animals but did not change their levels in the other groups compared with their respective control groups ($p \leq 0.05$) (table 2).

Figure 8 Effects of prior administration of prostaglandin E₁ (PGE₁) on nitrite/nitrate concentration in serum of rats treated with D-galactosamine (D-GalN) with (+) or without (-) inhibition of iNOS activity by methylisothiourea (MT). PGE₁ enhanced nitrite/nitrate concentrations in serum in the absence of MT. MT administration reduced nitrite/nitrate values in all groups. Values are mean (SEM) (n=10). Groups with different superscript letters are significantly different ($p \leq 0.05$).

Table 1 Effects of administration of anti-tumour necrosis factor α (TNF- α) antibodies on alanine aminotransferase (ALT) activity in serum induced by D-galactosamine (D-GalN) treated rats and its reduction by prostaglandin E₁ (PGE₁)

Treatment	ALT (U/I)
Control	19 (0.9) ^c
PGE ₁	16 (1.5) ^c
D-GalN	1109 (172.0) ^a
PGE ₁ +D-GalN	547 (104.7) ^b
Anti-TNF- α +control	29 (1.4) ^c
Anti-TNF- α +PGE ₁	24 (2.2) ^c
Anti-TNF- α +D-GalN	397 (68.2) ^b
Anti-TNF- α +PGE ₁ +D-GalN	1093 (130.1) ^a

Administration of anti-TNF- α antibodies reduced ALT activity in serum induced by D-GalN and abolished the protection by PGE₁ of D-GalN dependent liver injury.

Values are mean (SEM) (n=5).

Groups with different superscript letters are significantly different ($p \leq 0.05$).

Discussion

Using different experimental interventions we have shown that TNF- α participates in D-GalN induced liver injury and PGE₁ dependent protection. Our data suggest that this protective effect of PGE₁ is related to enhanced expression of TNF- α and iNOS in hepatocytes.

D-GalN has been shown to be a suitable experimental model of liver injury.¹ D-GalN reduces the intracellular pool of uracil nucleotides in hepatocytes thus inhibiting the synthesis of RNA and proteins.^{1,2} Intraperitoneal administration of D-GalN causes an increase in the level of transaminases in serum, hepatic necrosis, and coma in rats.^{3,26,30-32} As previously described,^{26,33} liver injury (figs 3-5) induced by D-GalN correlated with an increase in TNF- α in serum (fig 7). In addition, our study showed that this increase in TNF- α concentration in serum was associated with enhanced TNF- α expression in hepatocytes with strong staining in apoptotic bodies (fig 6). The direct involvement of TNF- α in D-GalN liver injury was demonstrated when prior administration of anti-TNF- α antibodies reduced apoptosis (fig 4) and necrosis (table 1).

Figure 9 Effects of administration of anti-tumour necrosis factor α (TNF- α) antibodies on expression of inducible nitric oxide synthase (iNOS) in liver sections induced by prior administration of prostaglandin E₁ (PGE₁) to D-galactosamine (D-GalN) treated rats. (A) Control, (B) PGE₁+D-GalN, and (C) anti-TNF- α +PGE₁+D-GalN treated rats. Images are representative of five different experiments. Anti-TNF- α antibodies reduced enhancement of iNOS expression induced by PGE₁ to D-GalN treated animals. (Original magnification $\times 200$.)

Nevertheless, treatment with anti-TNF- α antibodies diminished D-GalN induced ALT release by only 65%. This result may indicate that factors other than TNF- α are also mediating liver injury by D-GalN. Under our conditions we can assume that D-GalN was directly responsible for TNF- α production as no endotoxin was detected in samples from control and D-GalN treated rats and endotoxaemia appears 36 hours after D-GalN administration.³⁴ Nevertheless, stimulation of inflammatory cells by D-GalN cannot be excluded as hepatotoxin stimulated TNF- α release one minute after portal infusion.³³

The relationship between D-GalN liver damage and TNF- α has been reported previously in a murine model of septic liver

Table 2 Effects of administration of anti-tumour necrosis factor α (TNF- α) antibodies on nitrite/nitrate concentrations in serum with D-galactosamine (D-GalN) and/or prostaglandin E₁ (PGE₁) treatments

Treatment	Nitrite/nitrate (μ M)
Control	17 (0.9) ^d
PGE ₁	29 (2.9) ^b
D-GalN	18 (1.0) ^d
PGE ₁ +D-GalN	22 (1.5) ^c
Anti-TNF- α +control	45 (8.5) ^a
Anti-TNF- α +PGE ₁	31 (7.3) ^{ab}
Anti-TNF- α +D-GalN	17 (5.3) ^{cd}
Anti-TNF- α +PGE ₁ +D-GalN	30 (7.3) ^{abc}

Administration of anti-TNF- α antibodies enhanced nitrite/nitrate concentrations in the control group.

Values are mean (SEM) (n=5).

Groups with different superscript letters are significantly different ($p \leq 0.05$).

failure.³⁵⁻³⁷ TNF- α participates as a primary mediator in the pathogenesis of infection, injury, and inflammation, and in the beneficial processes of host defence and tissue homeostasis. In this sense, acute systemic release of TNF- α during endotoxaemia plays a central role in liver injury and lethality.³⁸⁻⁴⁰ In contrast, when low amounts of TNF- α are released in tissues, the beneficial effects may predominate.⁴¹ Under our conditions, although D-GalN induced a moderate increase in TNF- α (fig 7) concentration in serum compared with that found during endotoxaemia,³⁸ expression of low amounts of TNF- α in hepatocytes may enhance cell death during D-GalN induced transcriptional arrest. An increase in TNF- α in serum has also been associated with enhancement of liver damage in other diseases, such as alcoholic hepatitis,⁴² and ischaemia-reperfusion and allograft rejection after liver transplantation.⁴³⁻⁴⁴ In contrast, enhanced expression of TNF- α during viral hepatitis⁴⁵⁻⁴⁷ may be beneficial due to its antiviral associated activity.⁴⁸

D-GalN administration alone did not modify nitrite/nitrate (fig 8) concentrations in serum and inhibition of iNOS activity by MT did not enhance ALT release induced by D-GalN (fig 5). As a consequence, our studies showed that nitric oxide was not involved in D-GalN induced liver injury. Nevertheless, a clear tendency to reduce nitrite/nitrate production by D-GalN was observed when PGE₁ was also administered (fig 8). Such an effect may be due to haemodynamic disturbances⁴⁹ and/or reduction in nitric oxide production from hepatocytes by D-GalN.⁵⁰ It has been observed that expression of iNOS in hepatocytes is associated with liver injury induced by an oxidative stress related model such as acetaminophen administration,⁵¹ chronic viral hepatitis,⁵² and allograft rejection in liver transplantation.⁵³ Liver damage induced by endotoxaemia was exacerbated by non-selective inhibition of nitric oxide synthase (cNOS and iNOS)⁵⁴ but reduced by selective iNOS inhibition.⁵⁵ The authors postulated that although low production of nitric oxide by cNOS was cytoprotective and maintained microvascular patency, excess nitric oxide production by iNOS was cytotoxic. In this sense, vasoactive properties of nitric

oxide are also associated with liver protection during experimental alcoholic disease⁵⁶ and ischaemia-reperfusion injury.⁵⁷ Under our conditions, inhibition of iNOS in D-GalN treated rats enhanced liver injury (fig 5) when PGE₁ was also administered (MT+PGE₁+D-GalN group) and TNF- α (fig 7) reached the highest concentration in serum among all groups. Florquin and colleagues⁵⁸ have indicated that several cytokines (for example, TNF- α , interleukin 1 α , or interferon γ) could be involved in the increase in liver injury induced by iNOS inhibition during experimental septic liver failure.

Several reports have shown that prostaglandin E decreases liver damage induced by D-GalN.¹⁵⁻²⁶ As previously described,²⁶ administration of PGE₁ 30 minutes before D-GalN reduced necrosis and apoptosis (figs 3-5) and enhanced TNF- α (fig 7) concentrations in serum. We showed that PGE₁ alone and with D-GalN increased TNF- α expression in hepatocytes (fig 6). Pretreatment with sublethal doses of TNF- α protected D-GalN treated mice from an otherwise lethal challenge of lipopolysaccharide or TNF- α .¹⁹ In the present study, administration of anti-TNF- α antibodies to PGE₁+D-GalN treated animals showed that TNF- α is essential for PGE₁ protection against D-GalN induced liver injury (table 1, fig 4). As described above, TNF- α was also important during D-GalN induced liver injury. Accordingly, recovery of liver damage (table 1, fig 4) by D-GalN in anti-TNF- α -PGE₁+D-GalN treated rats also indicated that factors other than TNF- α were mediating liver injury in this experimental group. Modulation of TNF- α expression by PGE₁ in other cell types, such as inflammatory cells, has been studied extensively. In this sense, PGE₁ may reduce⁵⁹⁻⁶⁰ or enhance⁶¹⁻⁶² in vitro TNF- α release from inflammatory cells in relation to the concentration range of the prostanoid used. Administration of an inhibitor of prostaglandin synthesis, such as indomethacin, increased TNF- α levels in serum and enhanced gastric mucosal damage in rats.⁶³ Also, other authors have shown that indomethacin does not modify lipopolysaccharide induced TNF- α release in mice.⁶⁴ Under our experimental conditions, the effect of PGE₁ administered 30 minutes before D-GalN may have been different from that found in other studies in which it was administered after induction of liver injury. Controversial results on the in vivo effect of PGE₁ on TNF- α release could also be due to the concentration range of the prostanoid, animal model (toxic or endotoxaemia), type of cells studied, animal species, and pattern of administration.

In addition to TNF- α ,¹⁹ prior administration of nitric oxide donors²⁵ has also been shown to protect against liver injury. In fact, enhanced expression of iNOS or nitric oxide donor inhibits apoptosis in cultured hepatocytes.⁶⁵⁻⁶⁶ Under our conditions, PGE₁ enhanced expression of iNOS (fig 9) in hepatocytes and nitrite/nitrate (fig 8) concentrations in serum from D-GalN treated rats. The important role of iNOS during PGE₁ protection against D-GalN

liver injury was also demonstrated when inhibition of iNOS by MT abolished ALT (fig 5) reduction in these animals. iNOS expression in hepatocytes during lipopolysaccharide treatment *in vivo*^{67,68} and *in vitro*⁶⁹ is regulated by TNF- α . In agreement with this, anti-TNF- α antibody administration showed that TNF- α was responsible for induction of iNOS (fig 9) expression in hepatocytes by PGE₁ in D-GalN treated rats.

In conclusion, we have shown that protection of D-GalN induced liver injury by PGE₁ is related to its capacity to increase TNF- α and iNOS expression in hepatocytes. Our data strongly support the beneficial effect of potential therapeutic mediators that could enhance expression of iNOS in hepatocytes during liver failure.

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