Tumour necrosis factor-alpha and nitric oxide mediate apoptosis by D-galactosamine in a primary culture of rat hepatocytes: Exacerbation of cell death by cocultured Kupffer cells

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BACKGROUND: Prostaglandin E_1 (PGE_1) reduces cell death in experimental and clinical liver dysfunction.

OBJECTIVES: Whether PGE_1 protects against D-galactosamine (D-GalN)-associated hepatocyte cell death by the regulation of tumour necrosis factor-alpha (TNF-alpha) and/or nitric oxide (NO) in hepatocytes or cocultured Kupffer cells was examined.

METHODS: Anti-TNF-alpha antibodies were used to evaluate the role of TNF-alpha during D-GalN cytotoxicity and its protection by PGE_1 in cocultured hepatocytes and Kupffer cells. Cell apoptosis and necrosis were assessed by DNA fragmentation and lactate dehydrogenase release, respectively. Nitrite+nitrate (NOx), as NO end products, and TNF-alpha concentrations were measured in the culture medium. The role of NO was determined by measuring inducible NO synthase (iNOS) expression and the effect of its inhibition during D-GalN cytotoxicity and its protection by PGE_1.

RESULTS: D-GalN enhanced hepatocyte cell death associated with high TNF-alpha and NOx levels in a culture medium. Anti-TNF-alpha and iNOS inhibition suggested that TNF-alpha was mediating apoptosis, but not necrosis, through the stimulation of NO production. The antiapoptotic activity of PGE_1 was associated with a reduction of NO production, but was blocked by iNOS inhibition. This apparent contradiction was explained by the ability of PGE_1 to enhance iNOS expression shortly after its administration and inhibit it later during D-GalN treatment. Anti-TNF-alpha antibodies did not reduce the exacerbation of D-GalN-associated cell death in hepatocytes by cocultured Kupffer cells.

CONCLUSION: TNF-alpha mediates D-GalN-induced apoptosis via NO production in cultured hepatocytes. The protective effect of PGE_1 against D-GalN-induced apoptosis is probably through the induction of low iNOS expression that was followed by a reduction of iNOS expression and NO production induced by the hepatotoxin. The exacerbation of hepatocyte cell death by Kupffer cells was not related to TNF-alpha and NO.

Key Words: *Cell death; Hepatocytes; Kupffer cells; Nitric oxide; Prostaglandin E_1; Tumour necrosis factor-alpha*

Résumé à la page suivante

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Le facteur de nécrose tumorale alpha et l'oxyde nitrique médient l'apoptose par la D-galactosamine dans une culture primaire d'hépatocytes de rat : accélération de la mort des cellules par les cellules de Kupffer en coculture

CONTEXTE : La prostaglandine E1 (PE1) diminue la mort des cellules dans le dysfonctionnement clinique et expérimental du foie.
OBJECTIF : Vérifier si la PE1 protège contre la mort des hépatocytes associée à la D-galactosamine (D-Gal) par la régulation du facteur de nécrose tumorale alpha (TNF alpha) et/ou de l'oxyde nitrique (NO) dans les hépatocytes ou les cellules de Kupffer en coculture.
MÉTHODE : Nous nous sommes basés sur les anticorps anti-TNF alpha pour évaluer le rôle du TNF alpha durant la cytotoxicité de la D-Gal et sa protection par la PE1 dans les hépatocytes et les cellules de Kupffer en coculture. L'évaluation de l'apoptose et de la nécrose cellulaires s'est faite à l'aide de la fragmentation de l'ADN et de la libération de lactate-déshydrogénase respectivement. Les concentrations de nitrite et nitrate (NOx), comme produits terminaux du NO, et du TNF alpha ont également été mesurées dans le milieu de culture. Nous avons déterminé le rôle du NO en mesurant l'expression de la NO-synthase inductible (NOSi) et l'effet de son inhibition durant la cytotoxicité de la D-Gal et sa protection par la PE1.
RÉSULTATS : La D-Gal a accéléré la mort des hépatocytes associée à des concentrations élevées de TNF alpha et de NOx dans le milieu de culture. L'inhibition des anti-TNF alpha et de la NOSi donne à penser que le TNF alpha médie l'apoptose, mais non la nécrose, des cellules en stimulant la production de NO. L'activité antiapoptotique de la PE1 a été associée à une diminution de la production de NO, mais elle a été bloquée par l'inhibition de la NOSi. Cette apparente contradiction s'explique par la capacité de la PE1 d'accroître l'expression de la NOSi peu de temps après son administration et de l'inhiber par la suite durant le traitement à la D-Gal. Les anticorps anti-TNF alpha n'ont pas ralenti l'accélération de la mort des cellules associée à la D-Gal dans les hépatocytes par les cellules de Kupffer en coculture
CONCLUSION : Le TNF alpha médie l'apoptose des cellules provoquée par la D-Gal en stimulant la production de NO dans les hépatocytes en culture. L'effet protecteur de la PE1 contre l'apoptose induite par la D-Gal est probablement lié à l'apparition d'une expression de faible intensité de la NOSi, suivie de la diminution de son expression et de la production de NO causées par l'hépatotoxine. L'accélération de la mort des hépatocytes par les cellules de Kupffer n'était pas liée au TNF alpha et au NO.

Tumour necrosis factor-alpha (TNF-alpha) is mostly synthesized and released by stimulated phagocytes (1). It is involved in the pathogenesis of shock, control of tumour-cell growth, inflammation, acute-phase gene expression and normal cell proliferation (2). In addition, acute systemic release of TNF-alpha during septic liver failure causes liver injury and death (3,4).

Kupffer cells are the largest population of sessile tissue macrophages, uniquely positioned in the liver sinusoids. They are the first immunological cell type to come into contact with gut-derived endotoxin, releasing potent inflammatory mediators such as cytokines and prostaglandins. Prostaglandin E_2 (PGE_2) exerts an autocrine regulatory feedback with suppression of endotoxin-induced TNF-alpha synthesis in rat Kupffer cells (5). PGE has cytoprotective properties in different experimental models of liver dysfunction (6). PGE reduces the hypertransaminasemia induced in vivo by D-galactosamine (D-GalN) (7), thioacetamide (8), aflatoxin B_1 (9), carbon tetrachloride (10), bile duct ligation (11), fat-enriched and choline-deficient diet (12), viral hepatitis (13) and complement-mediated hepatic necrosis (14). Furthermore, PGE_1 has a beneficial effect on fulminant viral hepatitis in humans, with a decrease in the levels of transaminases and improvement of encephalopathy and coagulation factors (15,16).

Nitric oxide (NO) is a labile, highly reactive compound involved in blood pressure regulation, neurotransmission, tumour-cell killing, immunity and inflammation (17). The inducible form of NO synthase (iNOS) is expressed in hepatocytes, Kupffer cells, macrophages, fibroblasts, chondrocytes and endothelial cells (17). The iNOS produces large amounts of NO in response to cellular stimulation by cytokines (18). The protective or cytotoxic properties of NO have been observed in various experimental models of liver injury (19). The inhibition of NO synthase reduces (20-23) or exacerbates (23-27) liver damage in vivo.

The present article focuses on the role of TNF-alpha derived from Kupffer cells in the induction of cell death by D-GalN in primary cultures of rat hepatocytes. We also assessed whether the protective effect of exogenous PGE_1 on D-GalN-associated cell death was linked to regulation of TNF-alpha and NO production.

MATERIALS AND METHODS

Materials

All reagents were from Sigma Chemical Co (USA) unless otherwise stated. William's medium E was from AppliChem (Germany). Antibiotics-antimycotic solution and fetal bovine serum were from Life Technologies Inc (United Kingdom).

Kupffer cell isolation

The yield and purity of the Kupffer cell population obtained by either pronase or collagenase digestion of the liver followed by purification through counterflow elutriation were compared. Kupffer cells were first isolated by the classical method based on the pronase digestion of the liver (28). Kupffer cell content of the cellular suspension was evaluated using fluorescein isothiocyanate-labelled ED-1 antibodies (Serotec Ltd, England) detected by flow cytometry (Immunocytometry system, Becton Dickinson, USA) analysis. Following this method, the highest purity and yield of the Kupffer cell population were obtained at a flow rate of 100 mL/min (60% and 3.7×10^6 cells, respectively) and the total number of Kupffer cells isolated was 7×10^6. To improve the purity of the Kupffer cell suspension, the classical method for hepatocyte isolation based on the collage-

nase perfusion of livers described by Seglen (29) was attempted. This procedure led to obtaining hepatocytes and Kupffer cells from a single experimental animal. Briefly, livers were perfused in situ through the portal vein, first with oxygenated solution I (10 mM HEPES, 6.7 mM potassium chloride, 145 mM sodium chloride and 2.4 mM EGTA), pH 7.4, at 37°C at a flow of 40 mL/min for 10 min, and then with solution II (100 mM HEPES, 6.7 mM potassium chloride, 67 mM sodium chloride, 10 g/L albumin, 4.8 mM calcium chloride and 0.05% collagenase A), pH 7.4, at 37°C at a flow of 20 mL/min for 10 min. Thereafter, the liver was gently minced in a Petri dish and filtered through nylon mesh (60 µm). The cell suspension was centrifuged at 50 × *g* for 5 min. The supernatant was mostly composed of nonparenchymal cells. The procedure for the purification of hepatocytes contained in the pellet is described below. The supernatant containing Kupffer cells was centrifuged at 300 × *g* for 5 min at 4°C, and the pellet was resuspended in 5 mL of Gey's balanced salt solution (GBSS) (1.5 mM calcium chloride dihydrate, 4.96 mM potassium chloride, 0.22 mM potassium dihydride phosphate, 0.28 mM magnesium sulphate heptahydrate, 0.120 mM sodium chloride, 0.027 M sodium bicarbonate, 1.06 mM sodium dihydrogen phosphate and 5.551 mM glucose), pH 7.4. This volume of cell suspension was carefully layered on 7 mL of 30% metrizamide (2-[3-acetamido-5-N-methylacetamido-2,4,6-triiodobenzamido]-2-deoxy-D-glucose) (Sigma) prepared in GBSS without sodium chloride and centrifuged at 1400 × *g* for 10 min at 4°C. The pellet (cell debris and erythrocytes) was discarded and the supernatant (mostly nonparenchymal cells) was washed twice with GBSS at 300 × *g* for 5 min at 4°C and 5 mL of cell suspension was immediately injected to the three-way teflon valve of the elutriation centrifuge (JE-5, Beckman Coulter, Inc, USA) previously stabilized at 600 × *g* at 20°C and 0.9 mL/min flow rate of the pump using GBSS as eluent. A range of volumes was recovered by increasing the flow rate of the pump from 20 to 250 mL/min. Each recovered cell suspension was centrifuged at 600 × *g* for 5 min at 4°C and the pellet was resuspended in 100 mM phosphate buffer solution (PBS) (137 mM sodium chloride, 2.7 mM potassium chloride and 4.3 mM sodium phosphate dibasic), pH 7.4. The viability, measured by trypan blue exclusion, exceeded 85% in all cases. Kupffer cell content in each solution was evaluated by fluorescein isothiocyanate-labelled ED-1 antibodies and detected by flow cytometry analysis. The highest purity and yield of the Kupffer cell population were obtained in the suspensions ranging from 150 to 200 mL/min flow rate (90% to 95% and 3.5×10^6 cells, respectively). The total amount of Kupffer cells isolated was 6.5×10^6. The high purity and yield of Kupffer cells obtained were sufficient to validate this method. The selected purified Kupffer cell-containing solutions were pooled and washed twice with William's medium E, pH 7.4, supplemented with 1 µM insulin, 0.6 µM hydrocortisone, 15 mM HEPES, 100 U/mL penicillin, 100 mg/mL streptomycin, 0.25 µg/mL amphotericin, 2 mM glutamine and 26 mM sodium bicarbonate. Kupffer cells (4×10^5, 85,000 cells/cm^2) were plated in transwells with a 0.4 µm pore size collagen type I-coated polytetrafluoroethylene membrane (Corning Costar Corporation, USA) and cultured in supplemented William's medium E, pH 7.4, containing 5% fetal bovine serum. After 2 h, the medium was removed and replaced by fresh supplemented medium without fetal bovine serum and the culture was maintained for 24 h without treatment.

Preparation of primary hepatocytes and cell culture

The hepatocyte population obtained above was washed twice with William's medium E, pH 7.4, supplemented with 1 µM insulin, 0.6 µM hydrocortisone, 15 mM HEPES, 100 U/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL amphotericin, 2 mM glutamine and 26 mM sodium bicarbonate. Cell viability was consistently greater than 85%, as determined by trypan blue exclusion. Contamination of hepatocyte cultures with Kupffer cells was not detected morphologically, through latex bead ingestion (3 µm) or by fluorescein isothiocyanate-labelled ED-1 antibodies. Hepatocytes (1.4×10^6, 150,000 cells/cm^2) were plated in a collagen type I cellware 6-well plate (Biocoat Cell Environments, Becton Dickinson Labware, England) and cultured in supplemented William's medium E, pH 7.4, containing 5% fetal bovine serum. After 2 h, the medium was removed and replaced by fresh supplemented medium without fetal bovine serum and the culture was maintained for 24 h without treatment. The hepatocyte and Kupffer coculture was performed at the ratio 1.4×10^6 hepatocytes to 4×10^5 Kupffer cells (3.5:1), as found in the normal liver (30).

Experimental design

The present study was designed to evaluate if TNF-alpha and NO participate in the induction of cell death in hepatocytes by D-GalN and the protective effect of PGE_1 in the presence or absence of cocultured Kupffer cells. PGE_1 (1 µM) (Alprostadil, Pharmacia & Upjohn, Belgium) was administered 2 h before D-GalN (5 mM). Polyclonal goat antirat TNF-alpha antibodies (0.3 µg/mL) (R&D System, USA) were administered 2 h before the prostanoid or 4 h before the hepatotoxin. Nonimmunized goat immunoglobulin G (R&D System, USA) as a negative control of anti-TNF-alpha antibodies had no effect on the variables of the study in the presence or absence of the hepatotoxin or Kupffer cells. All the parameters were evaluated 24 h after the administration of D-GalN.

The expression of iNOS and the effect of its inhibition by N-omega-nitro-L-arginine methyl ester (L-NAME) (0.5 mM) were evaluated in the experimental conditions in which NO was shown to play a role.

Measurement of lactate dehydrogenase release

Lactate dehydrogenase (LDH) activity in the culture medium was measured by modification of a colorimetric routine laboratory method (31). Briefly, a volume (50 to 200 µL) of culture medium was incubated with 0.2 mM beta-NADH

and 0.4 mM pyruvic acid diluted in PBS, pH 7.4. LDH activity in the sample was proportional to the linear decrease in the absorbance at 334 nm. LDH was calculated using a commercial standard.

DNA fragmentation

The whole hepatocyte population, including the floating cells obtained from collected culture medium, was treated with 1 mL of lysis buffer (100 mM trishydroxymethylaminomethane buffer containing hydrochloric acid, 5 mM EDTA, 150 mM sodium chloride and 0.5% sarkosyl), pH 8.0, at 4°C for 10 min. Samples were incubated with ribonuclease (50 µg/mL) at 37°C for 2 h and proteinase K (100 µg/mL) at 48°C for 45 min. DNA was obtained by phenol:chloroform:isoamyl alcohol (25:24:1) (Sigma) extraction and precipitated with 0.3 M sodium chloride and cold isopropanol (1:1) at –20°C for 12 h. DNA was recovered by centrifugation of the sample at 20,800 × *g* at 4°C for 10 min. Thereafter, the precipitate was washed with 70% ethanol, dried and resuspended in trishydroxymethylaminomethane buffer containing EDTA (10 mM trishydroxymethylaminomethane, 1 mM EDTA) at pH 8.0. Samples (100 µg DNA) were analyzed on a 1.5% agarose gel with ethidium bromide (0.5 µg/mL).

Measurement of NO production

The release of NO was assessed by the quantification of its related end products, nitrite+nitrate (NOx). 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 (32). Briefly, the samples were incubated with 0.2 U/mL nitrate reductase, 5 mM flavin adenine dinucleotide and 50 mM NADH phosphate at 37°C for 20 min. The reaction was stopped by the addition of 10 mM sodium pyruvate and 24 mg/mL LDH at 37°C for 5 min, and precipitated with 1.4% zinc sulphate. Total nitrite reacted with Griess reagent (1% sulphanilamide, 2.5% phosphoric acid and 0.1% n-naphthyl-ethylene-diamine) at 37°C for 10 min and it was read using the 540-nm filter in a titrated Organon Teknika 510 ELISA reader (Organon, Belgium).

TNF-alpha measurement in culture medium

A volume of culture medium (800 µL) was treated with 50 µL of protein A Sepharose 4 fast flow (Amersham Pharmacia Biotech AB, Sweden) for 1 h at 4°C to remove antirat TNF-alpha antibodies. Thereafter, the samples were centrifuged at 12,000 × *g* for 20 s and the supernatants were used for the measurement of TNF-alpha. TNF-alpha was quantified by ELISA following a previously described method (33). Briefly, the wells of ELISA plates were coated with 0.5 ng of rat TNF-alpha (R&D Systems, USA) for 1 h at 37°C. They were then blocked with 2% bovine serum albumin in 10 mM PBS-0.05% Tween (PBS-Tween) at pH 7.4. The samples and antirat TNF-alpha antibodies (R&D Systems, USA) (32.5 ng/mL) were incubated for 2 h at 37°C, transferred to the antigen-coated wells and incubated for 2 h at 37°C. The wells were washed with PBS-Tween and incubated with the biotinated secondary antibodies (125 pg/mL) (Zymed, USA) for 1 h at 37°C. They were then washed with trishydroxymethylaminomethane buffer (100 mM) at pH 7.6 and incubated with streptavidin-alkaline phosphatase solution (Master Diagnóstica, Spain) for 30 min at 37°C. Finally, they were washed and incubated with *p*-nitrophenyl phosphate (Sigma) as alkaline phosphatase substrate for 1 h at 37°C. The wells were read at 405 nm in a titrated Organon Teknika 510 ELISA reader.

Assay for caspase-3-like activity

The whole hepatocyte population, including the floating cells obtained from collected culture medium, was treated with 1 mL of lysis solution (50 mM trishydroxymethylaminomethane buffer containing hydrochloric acid pH 7.5, 2 mM EDTA, 100 mM sodium chloride, 1% nonidet NP-40, 1 mM phenylmethylsulfonyl fluoride, 20 µg/mL aprotinin, 20 µg/mL leupeptin and 20 µg/mL pepstatin A) at 4°C for 10 min, transferred to microfuge tubes and centrifuged at 20,800 × *g* at 4°C for 5 min. The caspase-3-like activity in the cell extract (25 µg) was measured by colorimetric assay using the peptide-based substrate ac-N-acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide (Bachem AG, Switzerland). The increase in absorbance of enzymatically released *p*-nitroanilide was measured at 405 nm for 10 min in a DU 640 Spectrophotometer (Beckman Coulter, Inc, USA).

Evaluation of iNOS expression

The whole hepatocyte population, including the floating cells obtained from collected culture medium, was treated with 1 mL of lysis solution (50 mM trishydroxymethylaminomethane buffer containing hydrochloric acid pH 7.5, 2 mM EDTA, 100 mM sodium chloride, 1% nonidet NP-40, 1 mM phenylmethylsulfonyl fluoride, 20 µg/mL aprotinin, 20 µg/mL leupeptin and 20 µg/mL pepstatin A) at 4°C for 10 min, transferred to microfuge tubes and centrifuged at 20,800 × *g* at 4°C for 5 min. Proteins (100 µg) were separated by 12% sodium dodecyl sulphate-polyacrylamind gel electrophoresis and transferred to nitrocellulose. The membranes for measuring iNOS expression were incubated with anti-iNOS antibodies (BD Transduction Laboratories, Belgium) as primary antibodies and anti-mouse-immunoglobin G-horseradish peroxidase (Santa Cruz Biotechnology, Inc, USA) as secondary antibody revealing protein content by enhanced chemiluminiscence.

Statistical analysis

Results are expressed as the mean ±SEM of eight independent cell culture experiments. Data were evaluated by one-way ANOVA. Because the homogeneity of variances assessed by the Bartlett test was sufficient, groups were subjected to the multiple comparison least significant differences test. Statistical significance was set at P=0.05 or less.

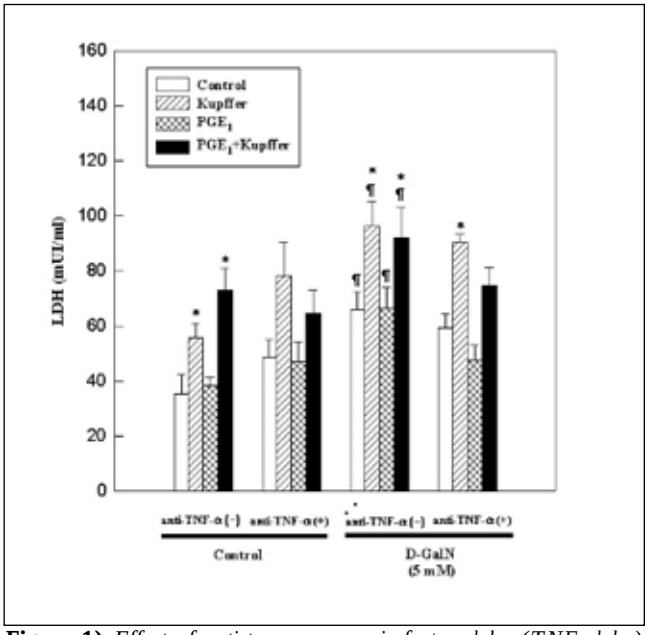

Figure 1) *Effect of anti-tumour necrosis factor-alpha (TNF-alpha) antibodies, prostaglandin E_1 (PGE_1) and Kupffer cells in the release of lactate dehydrogenase (LDH) induced by D-galactosamine (D-GalN) in a primary culture of rat hepatocytes. Kupffer cells enhanced necrosis in hepatocytes in both the presence and abscence of D-GalN. PGE_1 and anti-TNF-alpha antibodies treatment did not affect cell necrosis. Data are the mean ±SEM of eight independent cell culture experiments. *P≤0.05 compared with the corresponding value of the group without Kupffer cells. ¶P≤0.05 compared with the corresponding value of the group without D-GalN treatment*

RESULTS

Effect of PGE_1 and anti-TNF-alpha antibodies on D-GalN-induced necrosis in hepatocyes cocultured with Kupffer cells

D-GalN induces necrosis measured by LDH release and trypan blue exclusion in a primary culture of rat hepatocytes (34,35). In the present study, D-GalN also increased LDH release (66±6.5 mIU/mL versus 35±6.9 mIU/mL in controls) (P≤0.0001) (Figure 1). Kupffer cells exacerbated hepatocyte necrosis in nearly all groups, although this was not significant in the groups in which PGE_1 and anti-TNF-alpha antibodies were coadministered. PGE_1 or anti-TNF-alpha antibody treatment did not affect the LDH release induced by D-GalN or Kupffer cells.

Effect of PGE1 and anti-TNF-alpha antibodies on d-GalN-induced apoptosis in hepatocytes cocultured with Kupffer cells.

The administration of PGE_1 reduces D-GalN induced apoptosis measured by DNA fragmentation and caspase-3 activity (34). In our conditions, PGE_1 was also able to reduce DNA fragmentation induced by D-GalN in hepatocytes (Figure 2). The coculture of hepatocytes with Kupffer cells increased DNA fragmentation, especially in control hepatocytes. Anti-TNF-alpha antibodies reduced DNA fragmentation in all experimental conditions. PGE_1 and anti-TNF-alpha did not abolish the raise induced by Kupffer cells.

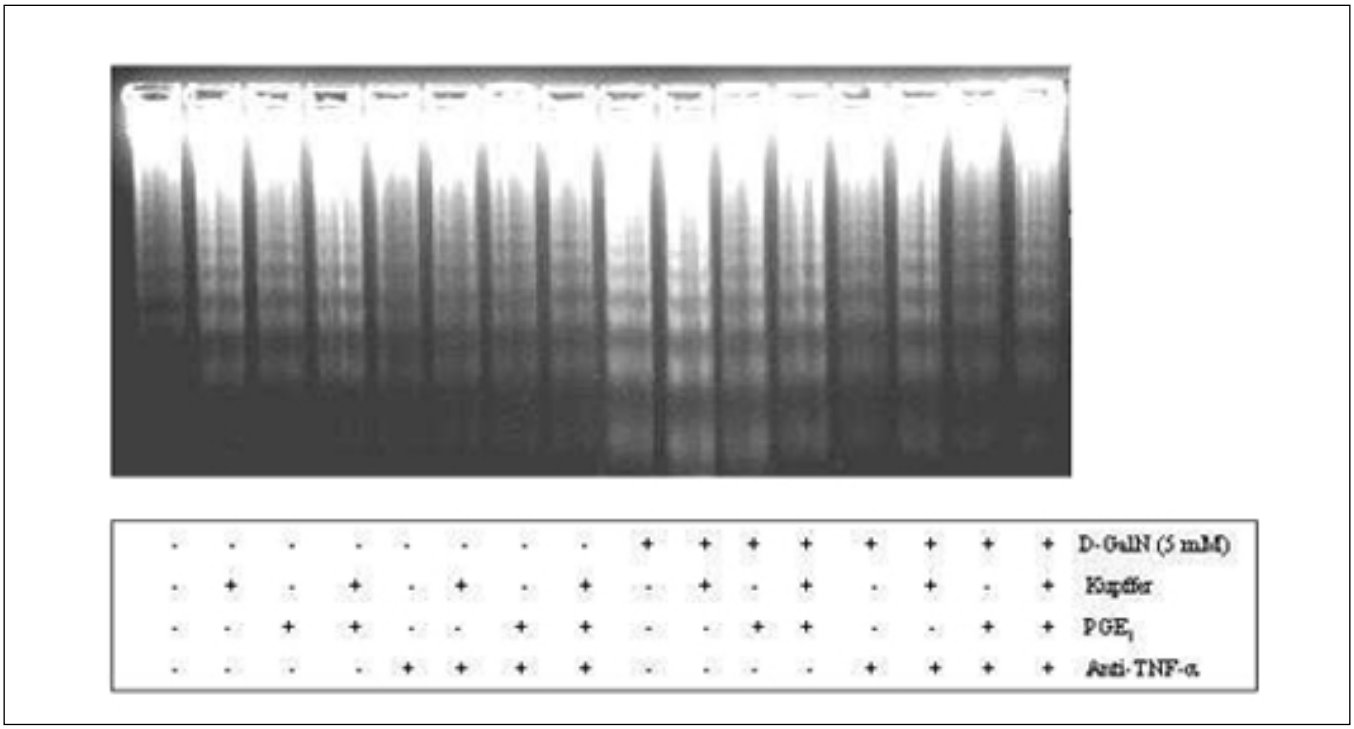

Figure 2) *Effect of anti-tumour necrosis factor-alpha (TNF-alpha) antibodies, prostaglandin E_1 (PGE_1) and Kupffer cells in DNA fragmentation induced by D-galactosamine (D-GalN) in a primary culture of rat hepatocytes. The absorbance of a representative band of DNA fragmentation has been measured in each sample. PGE_1 was also able to reduce DNA fragmentation induced by D-GalN in hepatocytes. The coculture of hepatocytes with Kupffer cells increased DNA fragmentation, especially in control hepatocytes. Anti-TNF-alpha antibodies reduced DNA fragmentation in all experimental conditions. PGE_1 and anti-TNF-alpha did not abolish the raise induced by Kupffer cells. The image is representative of eight independent cell culture experiments*

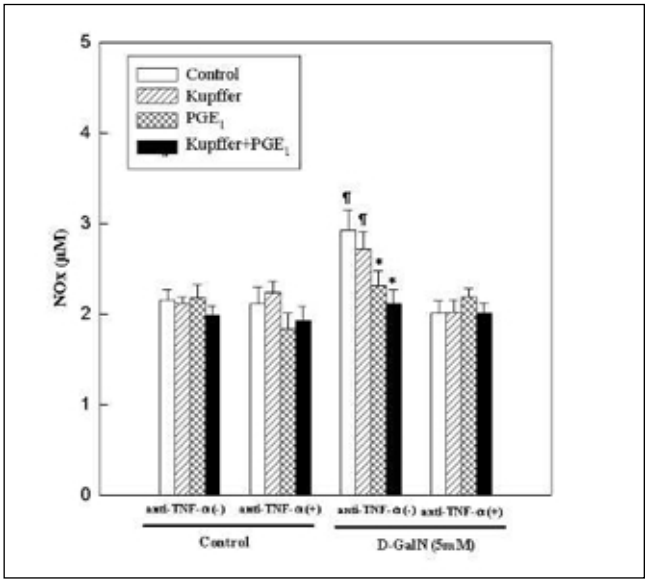

Figure 3) *Effect of anti-tumour necrosis factor-alpha (TNF-alpha) antibodies, prostaglandin E_1 (PGE_1) and Kupffer cells in the concentration of nitrite+nitrate (NOx) induced by* D*-galactosamine (*D*-GalN) in a primary culture of rat hepatocytes.* D*-GalN enhanced the concentration of NOx in culture medium. Kupffer cells did not modify the concentration of NOx in culture medium. PGE_1 and anti-TNF-alpha antibodies abolished the rise in NOx induced by* D*-GalN. Data are the mean ±SEM of eight independent cell culture experiments. *P≤0.05 compared with the corresponding value of the group without PGE_1 treatment. ¶P≤0.05 compared with the corresponding value of the group without* D*-GalN treatment*

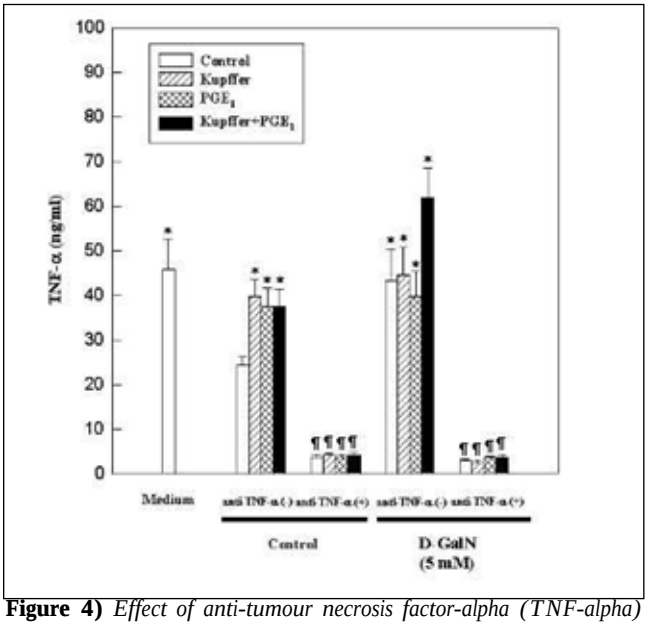

Figure 4) *Effect of anti-tumour necrosis factor-alpha (TNF-alpha) antibodies, prostaglandin E_1 (PGE_1) and Kupffer cells on the concentration of TNF-alpha in medium from cultured hepatocytes treated with* D*-galactosamine (*D*-GalN). Control hepatocytes diminished the concentration of TNF-alpha obtained in the culture medium treated with fetal bovine serum in absence of cultured hepatocytes and Kupffer cells.* D*-GalN, PGE_1 and Kupffer cells enhanced the concentration of TNF-alpha in culture medium from control hepatocytes. Anti-TNF-alpha antibodies reduced TNF-alpha concentration in all experimental groups studied. Data are the mean ±SEM of eight independent cell culture experiments. *P≤0.05 compared with the corresponding value of the control group without* D*-GalN treatment. ¶P≤0.05 compared with the corresponding value of the group without anti-TNF-alpha treatment.*

Effect of PGE1 and anti-TNF-alpha antibodies on d-GalN-induced rise in the NOx concentration in culture medium from hepatocytes cocultured with Kupffer cells

NO production was evaluated by measuring the concentration of NOx in culture medium (Figure 3). D-GalN significantly increased NOx content (2.92±0.221µM versus 2.15±0.114 µM in controls) (P≤0.002). Kupffer cells did not modify the concentration of NOx in culture medium. PGE_1 and anti-TNF-alpha antibodies abolished the rise in NOx induced by D-GalN.

Effect of PGE1and anti-TNF-alpha antibodies on the concentration of TNF-alpha in culture medium from d-GalN-treated hepatocytes cocultured with Kupffer cells

TNF-alpha concentration in culture medium treated with fetal bovine serum in the absence of cultured hepatocytes or Kupffer cells was 46±6.7 ng/mL (Figure 4). Control hepatocytes reduced this concentration to 24±1.8 ng/mL (P≤0.007). The addition of Kupffer cells, PGE_1 and D-GalN abolished the capacity of the control hepatocytes to reduce the extracellular concentration of TNF-alpha (Figure 4). Anti-TNF-alpha antibodies reduced TNF-alpha content in all groups studied (Figure 4).

Role of iNOS inhibition in d-GalN-induced cell death and its protection by PGE1

PGE_1 reduced the rise in the concentration of NOx in culture medium and caspase-3-like activity in hepatocytes induced by D-GalN (Table 1). An inhibitor of iNOS (L-NAME) was used to study the link between NO and D-GalN-induced cell death and its protection by PGE_1. L-NAME significantly reduced the NOx content in culture medium in all conditions (Table 1) (P≤0.001). L-NAME did not change cell necrosis (data not shown). L-NAME reduced caspase-3-like activity induced by D-GalN (Table 1) (P≤0.001). L-NAME also blocked PGE_1 protection against D-GalN-induced caspase-3-like activity (Table 1) (P≤0.05).

Regulation of iNOS expression by d-GalN and/or PGE1 treatment

The expression of iNOS is shown in Figure 5. It was observed as an important enhancement of iNOS expression 12 h after D-GalN administration (Figure 5B) in comparison with that observed at 3 h (Figure 5A). In contrast, PGE_1 already raised the expression of iNOS in D-GalN-treated hepatocytes 3 h after the administration of the hepatotoxin (Figure 5A). Nevertheless, at longer hepato-

TABLE 1
Nitric oxide-derived end product, nitrite+nitrate, concentration in culture medium and caspase-3-like activity in hepatocytes after D-galactosamine (D-GalN) and/or prostaglandin E_1 (PGE_1) treatments in presence or absence of an iNOS inhibitor such as N-omega-nitro-L-arginine methyl ester (L-NAME) in primary culture of rat hepatocytes

Groups	Nitric oxide (μM)	Caspase-3-like activity (Abs/h/mg protein)
Control	12.47±0.659	0.571±0.0493
PGE_1	12.61±0.855	0.806±0.0513
L-NAME	4.38±0.232*	0.769±0.0688
L-NAME+PGE_1	4.15±0.286*	0.809±0.2680
D-GalN	16.76±1.338†	2.177±0.1083†
PGE_1+D-GalN	13.38±0.961‡	1.393±0.1295‡
L-NAME+D-GalN	5.04±0.265*	1.135±0.2069*
L-NAME+PGE_1+D-GalN	3.49±0.185*	1.797±0.0556*

*Values represent means ±SE of eight independent cell culture experiments. Data were compared using ANOVA with the least significant difference test. *P≤0.05 compared with the corresponding value of the group without L-NAME treatment. †P≤0.05 compared with the corresponding value of the group without D-GalN treatment. ‡P≤0.05 compared with the corresponding value of the group without PGE_1 treatment. Abs Absorbance*

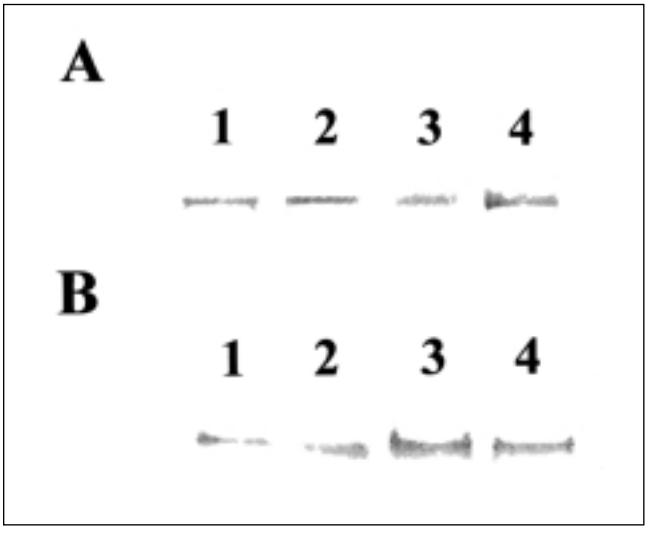

Figure 5) *Modulation of inducible nitric oxide synthase (iNOS) expression induced by D-galactosamine (D-GalN) and prostaglandin E_1 (PGE_1) at 3 h* **(A)** *and 12 h* **(B)** *after D-GalN treatment.* **1)** *Control,* **2)** *PGE_1,* **3)** *D-GalN and* **4)** *PGE_1+D-GalN. iNOS expression was evaluated in hepatocytes as described in the Materials and Methods section. PGE_1 enhanced iNOS expression at 3 h after D-GalN* **(A)** *but reduced the rise on iNOS expression at 12 h after D-GalN* **(B)**. *The images are representative of five independent cell culture experiments*

toxin exposure (12 h), PGE_1 clearly reduced iNOS expression induced by D-GalN (Figure 5B).

DISCUSSION

Our study evaluated the role of TNF-alpha and NO during PGE_1 protection against D-GalN-induced cell death in a primary culture of rat hepatocytes and Kupffer cells. The treatment with anti-TNF-alpha antibodies showed that TNF-alpha mediates D-GalN apoptosis, but not necrosis, in hepatocytes, probably through the enhancement of NO production. The involvement of NO during D-GalN-induced apoptosis in cultured hepatocytes was supported by the cytoprotective effect of iNOS inhibition. PGE_1 protection against D-GalN apoptosis was associated with a reduction of NO production but not with an alteration of the TNF-alpha concentration. Surprisingly, PGE_1 protection was also abolished by iNOS inhibition. This apparent contradiction was explained by the ability of PGE_1 to enhance iNOS expression shortly after its administration and to inhibit it later during D-GalN treatment. The exacerbation of D-GalN-associated apoptosis and necrosis in hepatocytes by Kupffer cells was unrelated to TNF-alpha and NO.

D-GalN is a suitable experimental model of liver injury (36). D-GalN reduces the intracellular pool of uracil nucleotides in hepatocytes, thus inhibiting the synthesis of RNA and proteins (36). The administration of D-GalN induces cell death in vivo (33,37-41) and in vitro (34,35,42) in rat hepatocytes. NO is a key bioregulator of cell death (43), promoting (44) or reducing (44-46) cell death induced by various agents in cultured hepatocytes. It has been shown that TNF-alpha induces NO release (47) and cell death (48) in cultured hepatocytes. In our conditions, anti-TNF-alpha antibodies reduced apoptosis in hepatocytes (Figure 2) and NOx in culture medium induced by D-GalN (Figure 3). The studies on iNOS expression in hepatocytes (Figure 5), and the effect of iNOS inhibition on D-GalN apoptosis (Table 1), suggested that NO was mediating apoptosis induced by the hepatotoxin in cultured hepatocytes. McMillan (49,50) has found that D-GalN is able to reduce or enhance NO production, whether the hepatotoxin is administered at either 2 h or 10 h after cell isolation, respectively. In concordance with data not included in this study, McMillan (49) has not found any protective effect of iNOS inhibitor on hepatocyte necrosis induced by D-GalN . The differences observed in the effect of D-GalN in NO production may be the consequences of the underlying important role of NO during hepatocyte isolation (51,52). In our conditions, all treatments were added to the culture 12 h after cell isolation when hepatocytes showed the optimal phenotype. It is observed that high NO production enhances mitochondrial dysfunction and apoptosis (53,54). Nevertheless, the low extracellular NOx concentration in the culture medium (Figure 3), the absence of mitochondrial membrane potential disturbances (34), and caspase-9 activation (data not shown) induced by D-GalN (5 mM) suggest that NO is mediating apoptosis through a pathway unrelated to mitochondrial disturbances.

Kupffer cells enhanced apoptosis (Figure 2) and necrosis (Figure 1) induced by D-GalN in hepatocytes. This noxious effect of Kupffer cells was associated with a rise in the TNF-alpha concentration (Figure 4), but not the NOx

(Figure 3) concentration, in the culture medium. Nevertheless, the failure of anti-TNF-alpha antibodies to abolish apoptosis and necrosis induced by Kupffer cells suggested that TNF-alpha is not involved in D-GalN hepatocyte cell death by Kupffer cells. In other experimental conditions using a low hepatocyte to Kupffer cell ratio (1:5), the stimulation of Kupffer cells with endotoxins enhances TNF-alpha and NO production and causes mild damage and protein synthesis inhibition in hepatocytes (55,56). Kurose et al (57) also showed that Kupffer cell-derived NO mediates the suppression of cell proliferation and induces apoptosis in cocultured hepatoma cells (ratio 1:1). In contrast, other authors have found a lack of effect of iNOS inhibition in the exacerbation of hepatocyte damage in macrophage and hepatocytes cocultures (58). In our experimental conditions using a high hepatocyte to Kupffer ratio (3.5:1) observed in control rats (30), we did not find a role for TNF-alpha and NO during the exacerbation of D-GalN-associated hepatocyte damage by Kupffer cells.

PGE reduces liver damage in fulminant viral hepatitis in humans (15,16) and in different experimental in vivo models (7-14,33,38,40) and in vitro (10,34) models of liver injury. We have previously shown that the protective effect of PGE_1 against in vivo liver injury induced by D-GalN is related to its capacity to stimulate the expression of iNOS in hepatocytes (33). In the present study, although NO promoted apoptosis by D-GalN, and PGE_1 protection was associated with a reduction of NO production, the inhibition of iNOS also blocked PGE_1 cytoprotection against D-GalN-induced apoptosis (Table 1). This apparent contradiction was explained by the ability of PGE_1 to enhance iNOS expression shortly after its administration and to inhibit it later during D-GalN treatment (Figure 5). It is interesting to note that iNOS inhibition during PGE_1 and D-GalN treatments was associated with high hepatocyte apoptosis. PGE_1 may also have a proapoptotic effect in the absence of NO. In fact, PGE_1 was able to slightly enhance DNA fragmentation (Figure 2) and caspase-3-like activity (Table 1) in control cells. Nevertheless, more studies are necessary to confirm this issue.

CONCLUSIONS

TNF-alpha mediates D-GalN-induced apoptosis in hepatocytes, probably through the stimulation of NO production. The protection provided by PGE_1 against D-GalN apoptosis is associated with its capacity to block the induction of iNOS expression and NO production induced by the hepatotoxin. Nevertheless, this protective effect of PGE_1 is also blocked by iNOS inhibition, suggesting that the enhancement of iNOS expression shortly after prostanoid administration is essential for its protective effect. The exacerbation of hepatocyte cell death by Kupffer cells was unrelated to TNF-alpha and NO.

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