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HEPATOLOGY

PGE_1 abolishes the mitochondrial-independent cell death pathway induced by D-galactosamine in primary culture of rat hepatocytes

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Abstract

Background and Aim: PGE_1 reduces *in vivo* and *in vitro* D-galactosamine (D-GalN)-induced cell death in hepatocytes. The present study was undertaken to elucidate the intracellular pathway by which D-GalN induces cell death in cultured hepatocytes. In addition, we evaluated if PGE_1 was able to modulate different parameters related to D-GalN-induced apoptosis in cultured rat hepatocytes.

Methods: Hepatocytes were isolated from male Wistar rats (225–275 g) by the classical collagenase procedure. PGE_1 (1 μM) was administered 2 h before D-GalN (5 mM) in primary culture of rat hepatocytes. Apoptosis was determined by DNA fragmentation and caspase-3, -6, -8 and -9 activation in hepatocytes. Caspase activation was evaluated by the detection of the related cleaved product and its associated activity. Cell necrosis was determined by the measurement of lactate dehydrogenase (LDH) activity in culture medium. To elucidate the role of mitochondria, we measured neutral (nSMase) and acid (aSMase) sphingomyelinase, as well as the expression of cytochrome c in mitochondria and cytoplasm fractions from D-GalN treated hepatocytes.

Results: D-GalN induced caspase-3 activation and DNA fragmentation in hepatocytes. This apoptotic response was not associated with the activation of caspase-6, -8 or -9. The use of specific inhibitors confirmed that only caspase-3 was involved in D-GalN-induced apoptosis. D-GalN did not modify nSMase and aSMase activities, nor mitochondrial cytochrome c release in hepatocytes.

Conclusions: D-GalN induced apoptosis through caspase-3 activation but without modification of the activity of caspase-6, -8, -9, SMases or cytochrome c release. PGE_1 appears to prevent D-GalN-induced apoptosis by a mitochondria-independent mechanism.

Key words: apoptosis, D-galactosamine, hepatocytes, mitochondria, PGE_1, sphingomyelinase.

INTRODUCTION

Apoptosis and necrosis are two different types of cell death. Necrosis is a non-controlled cellular disruption as a consequence of extreme noxious conditions. In contrast, apoptosis is a genetically controlled cell death pathway that can occur under physiological or pathological conditions. Apoptosis plays an important role in the maintenance of liver tissue homeostasis as well as during the pathogenesis of liver diseases.[1,2] Apoptosis can occur through different intracellular pathways: the death receptor-mediated pathway (extrinsic pathway, type I cells) and the mitochondria-mediated pathway (intrinsic pathway, type II cells).[3] Both pathways converge to the activation of proteases or caspases that mediate the morphological and biochemical features of apoptosis. In type I cells, the stimulation of Fas or tumor necrosis factor (TNF)-α receptors triggers autoproteolytic activation of procaspase-8[4–6] that leads to the further processing of downstream executioner caspases, such as caspase-3 and caspase-6.[7] In type II cells, different extracellular and/or intracellular signals stimulate the release of mitochondrial cytochrome c to the cytosol that, when in the presence of apoptotic protease-activating factor (APAF-1) and adenosine triphosphate (ATP), stimulates the autoproteolysis of pro-

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caspase-9.[8–10] Activated caspase-9 is also able to induce the processing of procaspase-3.[11] Whether the death program takes place through the extrinsic or intrinsic pathway is determined by the death signal and cell type. Different intracellular key mediators, such as Bid[12] or ceramide,[13] have been recently related to the activation of the intrinsic pathway. Ceramide is generated from sphingomyelin hydrolysis by neutral (nSMase) or acidic (aSMase) sphingomyelinases that act as regulators of intracellular ceramide levels and consequently ceramide-mediated functions. Ceramide has been shown to induce hepatocyte cell death through disruption of mitochondrial function in rat hepatocytes.[14]

Hepatic injury induced by D-galactosamine (D-GalN) is a suitable experimental model of human liver failure.[15] D-GalN induces inhibition of RNA and protein synthesis by reduction of the intracellular pool of uracil nucleotides.[15] D-GalN enhances different parameters of liver injury in experimental animals.[16–18] Different isoforms of prostaglandins (PG), such as PGE_1, PGE_2 or PGI_2, exert cytoprotection in numerous experimental models of liver injury.[19] In particular, PGE_1 reduces apoptosis and necrosis induced by D-GalN in rat liver[20–22] and cultured hepatocytes.[23,24]

The aim of the present study was to investigate different parameters related to the intrinsic or extrinsic apoptotic pathways induced by D-GalN, to clarify the intracellular mechanism by which PGE_1 reduces D-GalN-induced cell death in primary culture of rat hepatocytes.

METHODS

Materials

All chemical reagents were obtained from Sigma Chemical (St Louis, MO, USA) unless otherwise indicated. William's medium E was obtained from AppliChem (Darmstadt, Germany). Antibiotics-antimycotic solution and fetal bovine serum were purchased from Life Technologies (Paisley, UK). Ac-DEVD-pNA, Ac-VEID-pNA, Ac-IEPD-pNA and Ac-LEHD-AFC, and substrates of caspases-3, -6, -8 and -9 were purchased from Bachem AG (Bubendorf, Switzerland). Ac-DMQD-CHO, Ac-VEID-CHO, Ac-IEPD-CHO and Ac-LEHD-CHO, inhibitors of caspases-3, -6, -8 and -9 came from Bachem AG (Bubendorf, Switzerland). Antibodies against caspase-3 (H-277), caspase-6 (K-20), caspase-8 (H-134), caspase-9 (H-170), and cytochrome c (H-104) were from Santa Cruz Biotechnology (CA, USA). Experimental protocols were approved by the corresponding Ethics Committee of the Institution and conformed to the provisions of the Declaration of Helsinki in 1995 (as revised in Edinburgh 2000). Animal care followed the Institutional Guidelines for animal usage in research.

Isolation and culture of rat hepatocytes

Male Wistar rats (200–250 g) were anesthetized with sodium thiopental by intraperitoneal administration. Hepatocytes were isolated by the non-recirculating collagenase perfusion of livers cannulated through the portal vein, in accordance with Seglen *et al*.[25] Livers were perfused *in situ* first with an oxygenated solution I (10 mM HEPES, 6.7 mM KCl, 145 mM NaCl and 2.4 mM EGTA), pH 7.4 at 37°C at a flow rate of 40 mL/min for 10 min, and then with solution II (100 mM HEPES, 6.7 mM KCl, 67 mM NaCl, 10 g/L albumin, 4.8 mM $CaCl_2$ and 0.05% collagenase A), pH 7.4 at 37°C at a flow rate of 20 mL/min for 10 min. Thereafter, they were gently minced in a Petri dish and filtered through a nylon mesh. Hepatocytes were centrifuged and washed three times at 50 *g* for 5 min in 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 $NaHCO_3$. Cell viability was consistently >85%, as determined by trypan blue exclusion. Contamination of hepatocyte cultures with Kupffer cells was not detected morphologically, through latex bead ingestion (3 mm) or by fluorescein isothiocyanate-labeled ED-1 (Serotec, Oxford, UK). Hepatocytes (150.000 cells/cm^2) were plated in a Petri dish coated with collagen type I (Iwaki, Gyouda, Japan) 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 a fresh supplemented medium without fetal bovine serum and the culture was maintained for 24 h without treatment. PGE_1 (1 μM) was added 2 h before D-GalN (5 mM). The caspase inhibitors (200 μM) were coadministered together with D-GalN. The hepatocytes were harvested at several times.

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 Tris-HCl, 5 mM EDTA, 150 mM NaCl, 400 μg/mL proteinase K and 0.5% sarkosyl), pH 8.0 at 4°C for 10 min and after were incubated at 48°C for 45 min. DNA was obtained by phenol : chloroform : isoamyl alcohol (25:24:1) extraction and precipitated with 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 Tris-EDTA buffer (10 mM Tris, 1 mM EDTA) at pH 8.0. After RNAse (50 μg/mL) incubation at 37°C for 2 h, samples (100 μg DNA) were analyzed on 1.5% agarose gel with ethidium bromide (0.5 μg/mL).

Analysis of caspase-3, -6, -8 and -9 processing

The whole hepatocyte population, including the floating cells obtained from collected culture medium, was treated with 1 mL of lysis solution (50 mM Tris-HCl pH 7.5, 2 mM EDTA, 100 mM NaCl, 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 Eppendorf tubes and centrifuged at 20 800 *g* at 4°C for 5 min. Samples (100 μg) were separated by 12% SDS-PAGE and transferred to nitrocellulose. The membranes were incubated with anticaspase-3, -6, -8 or -9 antibodies as primary antibodies and antirabbit-IgG-horseradish peroxidase (Santa Cruz Biotechnology) as secondary antibody, revealing protein content by enhanced chemiluminescence (ECL).

Analysis of caspase-3, -6, -8 and -9-like activities

The whole hepatocyte population, including the floating cells obtained from collected culture medium, was treated with 1 mL of lysis solution (50 mM Tris-HCl pH 7.5, 2 mM EDTA, 100 mM NaCl, 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 Eppendorf tubes and centrifuged at 20 800 *g* at 4°C for 5 min. Caspase-like activities in cell extract (100 μg protein) diluted in incubating buffer (50 mM HEPES pH 7.4, 1 mM EDTA, 100 mM NaCl, 10% sucrose, 0.1% CHAPS, 5 mM dithiothreitol) were measured using the corresponding peptide-based substrates (100 μM). The enzymatically released *p*NA (caspase-3, -6 and -8 substrates) or AFC (caspase-9 substrate) were recorded during 1 h at 37°C by the linear increase in absorbance (405 nm) or fluorescence (Ex 400 nm, Em 505 nm) using a GENios Reader (TECAN, Salzburg, Austria).

Measurement of lactate dehydrogenase release

Lactate dehydrogenase (LDH) activity in the culture medium was measured by modification of a colorimetric routine laboratory method.[26] Briefly, a sample (0–400 μL) was incubated with 0.2 mM β-NADH and 0.4 mM pyruvic acid diluted in PBS pH 7.4. LDH concentration in culture medium was proportional to β-NADH consumption measured by the linear decrease in absorbance (334 nm). LDH concentration was calculated using a commercial standard.

Analysis of mitochondrial cytochrome c release

The whole hepatocyte population, including the floating cells obtained from collected culture medium, was washed with ice-cold phosphate-buffered saline. Subcellular fractionation was conducted according to the method of Gross *et al.*[12] Cells were kept in ice-cold water for 1 min, and homogenized in an equal volume of 500 mM sucrose solution using a micropestle (Eppendorf AG, Hamburg, Germany) for 30 s. Cytosolic fraction was obtained by centrifugation at 100 000 *g* for 1 h at 4°C. The precipitate was resuspended in 250 mM sucrose solution and centrifuged at 1000 *g* for 10 min at 4°C. The supernatant was used as a mitochondria-enriched heavy-membrane fraction. Samples (50 μg protein) from cytosolic and mitochondria-enriched fraction were separated by 14% SDS-PAGE and transferred to nitrocellulose. The membranes were incubated with anti-cytochrome c antibodies as primary antibodies and antirabbit-IgG-horseradish peroxidase (Santa Cruz Biotechnology) as secondary antibody revealing protein content by ECL.

Assays for sphingomyelinase activities

The measurement of aSMase and nSMase activities were carried out following the procedure described by Martin *et al.*[27] The whole hepatocyte population, including the floating cells obtained from collected culture medium, was treated with lysis solution (25 mM Tris-HCl, pH 7.4, 0.5% Triton X-100, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 2 μg/mL aprotinin and 2 μg/mL leupeptin) for 10 min on ice. After incubation, the cell lysates were centrifuged at 2000 *g* for 5 min at 4°C. The supernatant (15 μL, 30 μg of protein) was mixed with either nSMase buffer (100 μL) (50 mM Tris-HCl, pH 7.4, 0.05% Triton X-100, 10 mM $MgCl_2$, and 5 mM dithiothreitol) or aSMase buffer (100 μL) (50 mM sodium acetate, pH 5.0, 0.2% Triton X-100), respectively, and incubated with cold sphingomyielin (1 μL of 10 nmol solution) and 0.5 μL [methyl-^{14}C]sphingomyelin (specific activity 10 000 cpm/nmol) (Amersham, Spain) for 30 min at 37°C. After incubation, the reaction was stopped by adding 900 μL chloroform : methanol (2:1) and 200 μL distilled water. Tubes were vortexed and centrifuged at 1500 *g* for 5 min to achieve the separation of both phases. [^{14}C]Phosphorylcholine present in the aqueous phase was quantified using a liquid scintillation counter (Beckman, Fullerton, CA, USA). SMase activity was expressed as cpm/μg protein/min.

Statistical analysis

Results are expressed as mean ± SE of 3–8 different experiments. Data were compared using ANOVA with the least significant difference (LSD) test as posthoc multiple comparison analysis. A P-value ≤0.05 was considered significantly different.

RESULTS

D-galactosamine induces cell death in cultured rat hepatocytes

In concordance with previous studies,[23,24] D-GalN induced apoptosis and necrosis in primary culture of rat hepatocytes (Fig. 1a,b). In the present study, apoptosis by D-GalN was already evident 2 h after hepatotoxin administration (Fig. 1a). In contrast, the increase in cell necrosis by D-GalN appeared to be significant at the latest time-point of the study (24 h) (Fig. 1b). The rise

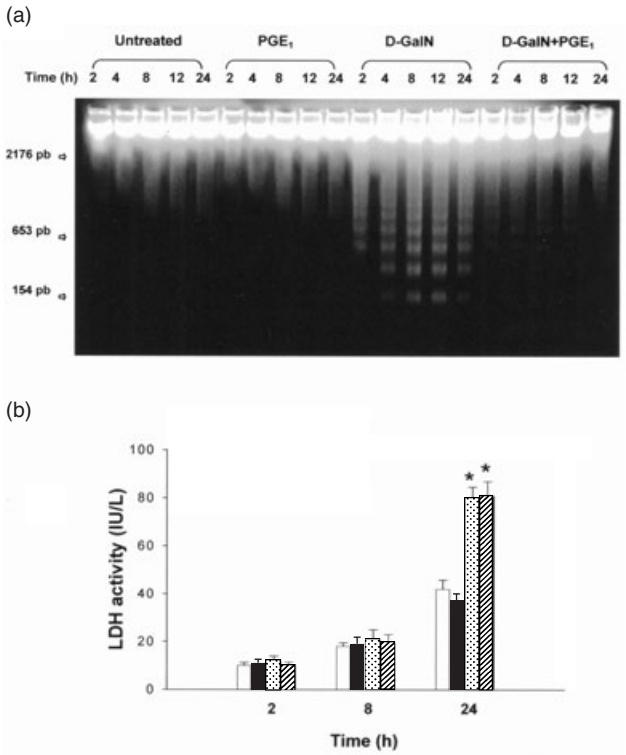

Figure 1 DNA fragmentation in (a) hepatocytes and (b) lactate dehydrogenase (LDH) activity in culture medium induced by (▨) D-galactosamine (D-GalN) or (■) PGE_1 in primary culture of rat hepatocytes. DNA fragmentation and LDH activity were measured, as described in the Methods section. D-GalN induced apoptosis and necrosis in cultured hepatocytes. PGE_1 abolished apoptosis, but not necrosis, in D-GalN-treated hepatocytes. The image of DNA fragmentation is representative of five independent experiments. LDH activity is presented as mean ± SE of eight independent experiments. $*P < 0.001$ (D-GalN-treated groups *vs* their related control groups). (□) Untreated; (▨) D-GalN + PGE_1.

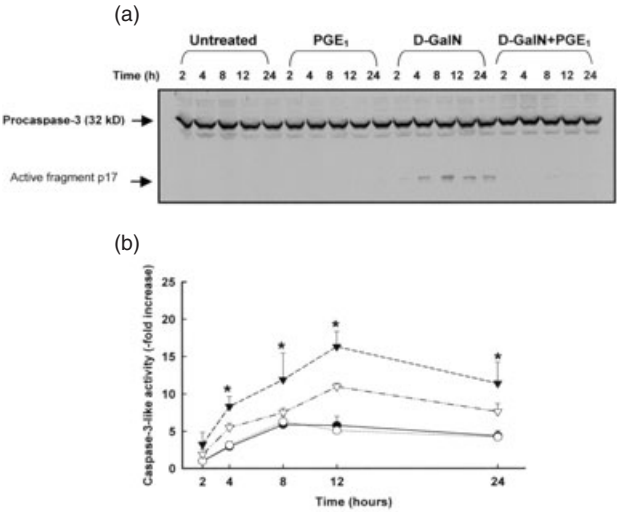

Figure 2 Kinetic study of caspase-3 (a) processing and (b) activity induced by (▼) D-galactosamine (D-GalN) or (○) PGE_1 in primary culture of rat hepatocytes. Caspase-3 activation was measured, as described in the Methods section. PGE_1 inhibited caspase-3 activation induced by D-GalN. The image of caspase-3 processing is representative of five independent experiments. Caspase-3 activity is presented as mean ± SE of eight independent experiments. $*P < 0.05$ (D-GalN *vs* (▽) D-GalN + PGE_1 group). (●) Untreated.

in necrosis was related to a reduction in apoptosis by D-GalN in cultured hepatocytes.

Examination of caspase-3, -6, -8 and -9 activation in D-galactosamine-induced apoptosis

The activation of caspase processing has been demonstrated to be involved during *in vivo and in vitro* cell death in hepatocytes.[11,28] The induction of apoptosis by D-GalN was related to a significant increase in caspase-3 processing (Fig. 2a) and activity (Fig. 2b) ($P \leq 0.05$). In our experimental conditions, we have not observed the activation of caspase-6 (Fig. 3a), caspase-8 (Fig. 3b) or caspase-9 (Fig. 3a). The participation of caspase-3 in D-GalN-induced apoptosis was confirmed using Ac-DMQD-CHO (Figs 4,5). The caspase-3 inhibitor abolished DNA fragmentation (Fig. 4), caspase-3 processing (Fig. 5a) and activity (Fig. 5b) observed in D-GalN-treated hepatocytes. The addition of caspase-6, -8 and -9 inhibitors did not reduce DNA fragmentation and caspase-3 activation induced by the hepatotoxin (Figs 4,5).

D-galactosamine did not induce mitochondrial cytochrome c release in cultured hepatocytes

Mitochondrial dysfunction plays an important role in different experimental models of cell death.[11,14] In this process, cytochrome c release from the mitochondria to the cytosol is essential for the amplification of the apoptotic response. We examined whether D-GalN (5 mM) induces the release of cytochrome c in cultured hepatocytes. Western blot analysis of mitochondrial and cytosolic fractions showed that cytochrome c was not released from mitochondria at any time-points studied (Fig. 6). Cytochrome c was only located in the mitochondrial fraction (Fig. 6).

Activity of neutral and acid sphingomyelinases in cultured hepatocytes

Ceramide has been implicated in the intracellular signaling pathway, leading to apoptosis in cultured rat hepatocytes.[14] We evaluated the activity of nSMase and aSMase that have been involved in ceramide generation. Detectable nSMase (Fig. 7a) and aSMase (Fig. 7b) was

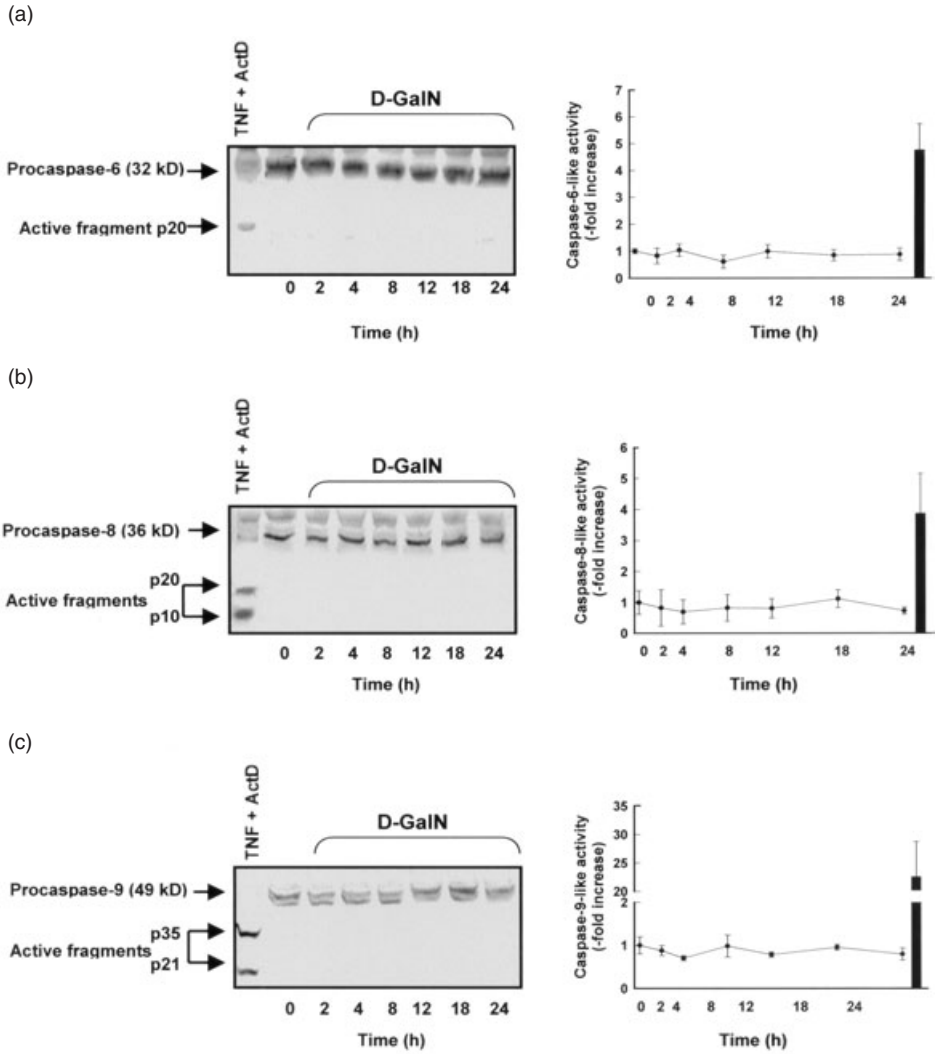

Figure 3 Kinetic study of (a) caspase-6, (b) caspase-8 and (c) caspase-9 processing (left panel) and activity (right panel) in (●) D-galactosamine (D-GalN)-treated hepatocytes. Hepatocytes treated with (■) tumor necrosis factor (TNF)-α (2000 U/mL) and actinomycin D (ActD) (0.2 μg/mL) were used as positive controls. Caspase-6, -8 and -9 activation were measured as described in the Methods section. D-GalN did not induce a significant activation of caspase-6, -8 and -9. The images of caspase processing are representative of five independent experiments. Caspase activity is presented as mean ± SE of six independent experiments.

observed in control hepatocytes. Nevertheless, D-GalN did not change these activities in cultured rat hepatocytes.

Role of PGE_1 in D-galactosamine-induced apoptosis in cultured hepatocytes

PGE_1 reduces apoptosis and necrosis induced by D-GalN in rat liver[20–22] and cultured hepatocytes.[23,24] In the present study, we also observed that PGE_1 reduced DNA fragmentation (Fig. 1a) and caspase-3 activation (Fig. 2). PGE_1 did not change cell necrosis induced by D-GalN (Fig. 1b). Interestingly, PGE_1 did not change the activation of caspase-6, -8 and -9, nor mitochondrial cytochrome c release or SM activities (data not shown).

DISCUSSION

The induction of hepatocyte damage by D-GalN is a suitable experimental model of human liver failure.[15] The administration of PGE_1 exerts cytoprotection against cell death induced *in vivo*[20–22] and *in vitro*[23,24] by D-GalN. The present study shows that the induction of apoptosis by D-GalN is not through the activation of caspase-8 or the mitochondrial-derived cell death pathway. PGE_1 cytoprotection is associated with the capacity of the prostanoid to regulate caspase-3 activation without interfering with the extrinsic and intrinsic cell death pathways.

D-GalN-induced cell death is related to its capacity to reduce the intracellular pool of uracil nucleotides in hepatocytes, thus inhibiting the synthesis of RNA and proteins.[15] D-GalN induces apoptosis and necrosis in primary culture of rat hepatocytes.[18,23,24] Cytotoxicity by D-GalN is associated with moderate intracellular free radical production.[29,30] Nevertheless, D-GalN (5 mM) was not able to induce any significant modification of mitochondrial transmembrane potential, glutathione reduced/glutathione oxidized (GSH/GSSG) ratio, or lipid peroxidation.[30] The present study was focused to elucidate whether D-GalN induces apoptosis through a death receptor- or mitochondria-mediated pathway. We were not able to demonstrate participation of caspase-8

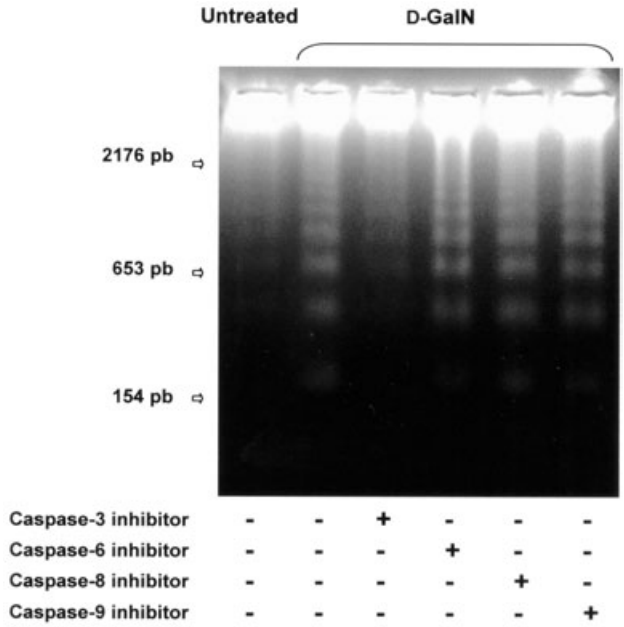

Figure 4 Effect of caspase-3, -6, -8 or -9 inhibitors on DNA fragmentation induced by D-galactosamine (D-GalN) in cultured hepatocytes. DNA fragmentation was measured following the procedure described in the Methods section. The addition of caspase-3, but not -6, -8 or -9, abolished DNA fragmentation induced by D-GalN. The image is representative of three independent experiments.

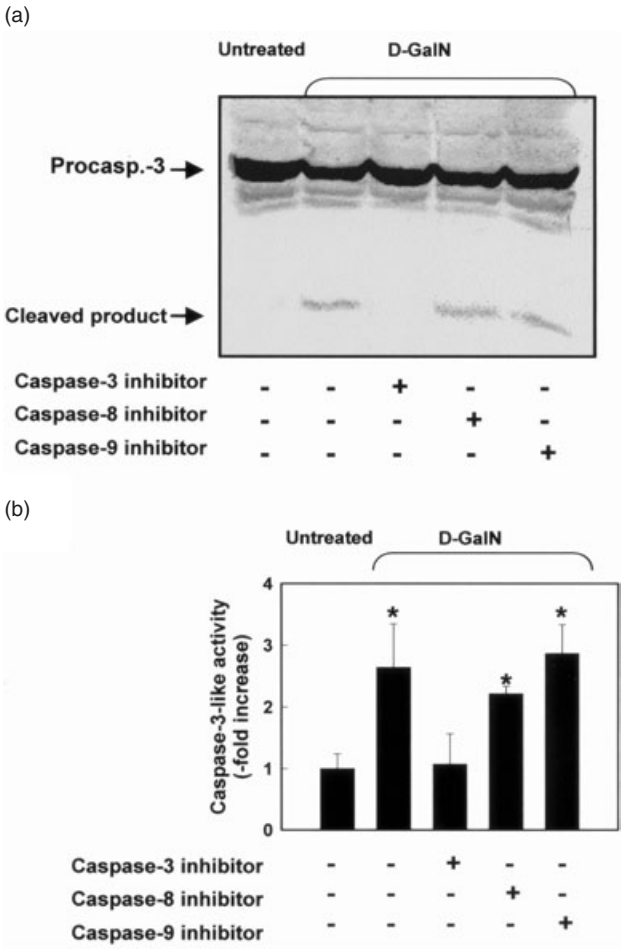

Figure 5 Effect of caspase-3, -8 or -9 inhibitors on the (a) processing and (b) activity of caspase-3 in D-galactosamine (D-GalN)-treated hepatocytes. Caspase-3 processing and activity were measured as described in the Methods section. Caspase-8 and -9 inhibitor did not change caspase-3 activation in D-GalN-treated hepatocytes. The image of caspase-3 processing is representative of three independent experiments. Caspase-3 activity is presented as mean ± SE of three independent experiments. $^*P < 0.05$ (D-GalN- and D-GalN + caspase-8 or -9 inhibitor-treated groups *vs* untreated and D-GalN + caspase-3 inhibitor-treated groups).

activation (Figs 3,4) characteristic of the extrinsic pathway, nor caspase-9 activation (Figs 3,4) and cytochrome c release (Fig. 6) characteristic of the intrinsic pathway. Conversely, the participation of caspase-3 was fully demonstrated by the activation of procaspase-3 in D-GalN-treated hepatocytes (Fig. 2) and the reduction of D-GalN-dependent apoptosis by a caspase-3 inhibitor (Ac-DMQD-CHO) (Fig. 4). In addition, the inefficacy of Ac-IEPD-CHO and Ac-LEHD-CHO in reducing DNA fragmentation (Fig. 4) and procaspase-3 processing (Fig. 5) induced by D-GalN showed that caspase-8 and -9 were not involved in this model of cell death. Our results suggest a novel pathway for caspase-3 activation by D-GalN in primary hepatocytes. Our data are in concordance with a recent report published by Feng and Kaplowitz showing a similar cell death pathway induced by staurosporine in murine hepatocytes.[31]

Ceramide generation has emerged as a key intracellular mediator related to the intrinsic cell death pathway.[13] The generation of ceramide has been shown in different experimental models of hepatocyte cell death.[28,32] Ceramide induces cell death by the disruption of mitochondrial function,[14] leading to the generation of reactive oxygen species in hepatocytes.[33] We have recently shown that nitric oxide mediates the induction of apoptosis by D-GalN in primary culture of rat hepatocytes.[24] In addition, ceramide has been shown to be generated during nitric oxide-induced apoptosis in human leukemia (HL) cells.[34] The relationship between ceramide generation during nitric oxide-dependent cell death, as well as the intracellular oxidative stress, encourage us to study the potential participation of ceramide during D-GalN-induced apoptosis. Ceramide is generated from sphingomyelin hydrolysis by nSMase and aSMase, that act as regulators of intracellular ceramide levels and consequently ceramide-mediated functions. D-GalN did not change the basal nSMase and aSMase activities observed in primary culture of rat hepatocytes (Fig. 7). This data supports previous results in which the mitochondrial potential was not disrupted during D-GalN (5 mM)-induced apoptosis in cultured rat hepatocytes.[30]

PGE_1 exerts cytoprotection in different experimental models of liver injury.[19] The reduction of D-GalN-induced apoptosis by the preadministration of pros-

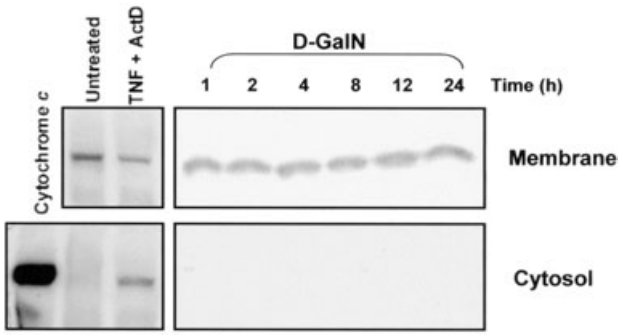

Figure 6 Kinetic study of mitochondrial cytochrome c release in D-galactosamine (D-GalN)-treated hepatocytes. Evaluation of cytochrome c content in cytosolic and mitochondria-enriched heavy-membrane fractions was carried out as described in the Methods section. Hepatocytes treated with tumor necrosis factor (TNF)-α (2000 U/mL) and actinomycin D (ActD) (0.2 μg/mL) were used as positive controls. D-GalN did not induce mitochondrial cytochrome c release in cultured hepatocytes. The image is representative of five independent experiments.

tanoid is related to its ability to induce inducible nitric oxide synthase (iNOS) expression in hepatocytes.[22,24] Several intracellular signal pathways have been involved in the anti-apoptotic effect of nitric oxide. It has been shown that nitric oxide prevents cell apoptosis through the cyclic guanosine monophosphate (cGMP)-dependent pathway,[35,36] heat shock protein expression[37] and S-nitrosylation.[38,39] In the present study, PGE_1 reduced DNA fragmentation (Fig. 1a) and caspase-3 activation (Fig. 2), but not cell necrosis (Fig. 1b) induced by D-GalN in primary culture of rat hepatocytes. PGE_1 did not change the basal activity of caspase-6, -8 and -9, or SM activities and mitochondrial cytochrome c release (data not shown). The inability of PGE_1 to reduce cell necrosis by D-GalN is probably related to its lack of efficiency to reduce intracellular oxidative stress induced by the hepatotoxin.[30] Different experiments carried out recently have suggested that rapid nitric oxide production by preadministration of PGE_1 is interfering with the further iNOS transcriptional activation induced by D-GalN, reducing the noxious effect of a high generation of nitric oxide by the hepatotoxin (data not shown). This observation may suggest a potential implication of nitric oxide generation by the prostanoid on the regulation of proapoptotic protein expression, leading to a reduction in caspase-3 activation.

In conclusion, our study showed that D-GalN induced apoptosis through a mechanism not related to caspase-8 activation (extrinsic pathway) or mitochondrial-derived factors (intrinsic pathway) in primary culture of rat hepatocytes. In this sense, D-GalN is able to stimulate DNA fragmentation through caspase-3 activation without involving any other caspase studied, such as caspase-8, -9 and -6. This novel apoptotic pathway induced by D-GalN is effectively reduced by PGE_1 administration in cultured hepatocytes.

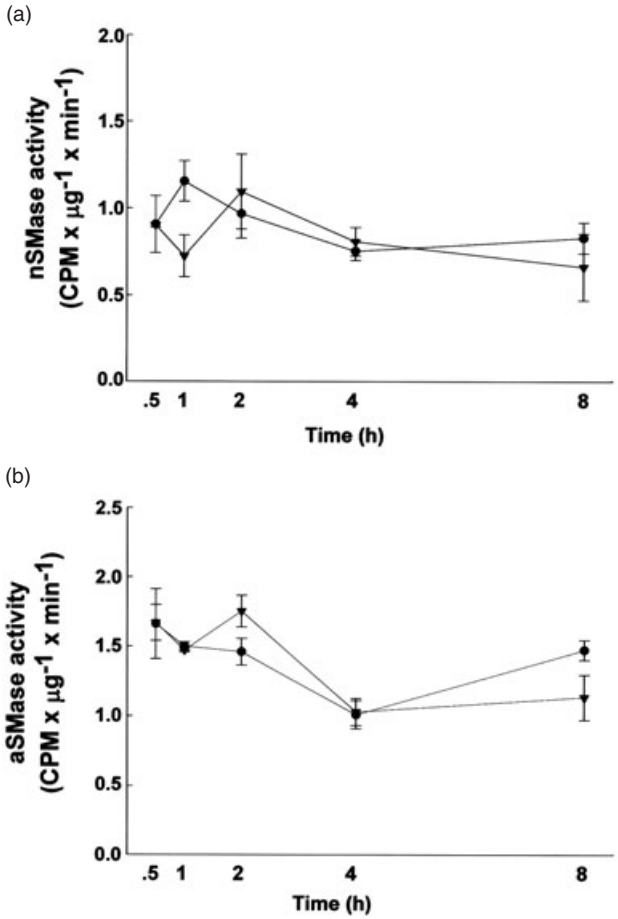

Figure 7 Kinetic study of (a) neutral sphingomyelinase (nSMase) and (b) acid sphingomyelinase (aSMase) activities in (▼) D-galactosamine (D-GalN)-treated hepatocytes. nSMase and aSMase activities were evaluated, as described in the Methods section. D-GalN did not modify basal SMase activities in cultured hepatocytes. SMase activity is presented as the mean ± SE of three independent experiments. (●) Untreated.

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