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PGE_1 Protection against Apoptosis Induced by D-galactosamine is Not Related to the Modulation of Intracellular Free Radical Production in Primary Culture of Rat Hepatocytes

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D-galactosamine (D-GalN) toxicity is a useful experimental model of liver failure in human. It has been previously observed that PGE_1 treatment reduced necrosis and apoptosis induced by D-GalN in rats. Primary cultured rat hepatocytes were used to evaluate if intracellular oxidative stress was involved during the induction of apoptosis and necrosis by D-GalN (0–40 mM). Also, the present study investigated if PGE_1 (1 μM) was equally potent reducing both types of cell death. The presence of hypodiploid cells, DNA fragmentation and caspase-3 activation were used as a marker of hepatocyte apoptosis. Necrosis was measured by lactate dehydrogenase (LDH) release. Oxidative stress was evaluated by the intracellular production of hydrogen peroxide (H_2O_2), the disturbances on the mitochondrial transmembrane potential (MTP), thiobarbituric-reacting substances (TBARS) release and the GSH/GSSG ratio. Data showed that intermediate range of D-GalN concentrations (2.5–10 mM) induced apoptosis in association with a moderate oxidative stress. High D-GalN concentration (40 mM) induced a reduction of all parameters associated with apoptosis and enhanced all those related to necrosis and intracellular oxidative stress, including a reduction of GSH/GSSG ratio and MTP in comparison with D-GalN (2.5–10 mM)-treated cells. Although PGE_1 reduced apoptosis induced by D-GalN, it was not able to reduce the oxidative stress and cell necrosis induced by the hepatotoxin in spite to its ability to abolish the GSH depletion.

Keywords: Apoptosis; D-Galactosamine; Liver injury; Necrosis; Oxidative stress; PGE_1

Abbreviations: DCFDA, 2′ 7′-dichlorofluorescindiacetate; D-GalN, D-galactosamine; H_2O_2, hydrogen peroxide; LDH, lactate dehydrogenase; MTP, mitochondrial transmembrane potential; PBS, phosphate buffer solution; PGE_1, prostaglandin E_1; TBARS, thiobarbituric-reacting substances; TMR, tetramethylrhodamine ethyl esterl

INTRODUCTION

Lethal cell injury occurs by either necrosis or apoptosis. Apoptosis is a type of cell death that is fundamentally different from necrosis in terms of its morphological appearance, biochemical mechanisms, and mode of initiation.[1] In apoptosis, the cell actively participates in its own death by the purposeful activation of a specific program of events. ATP generation and protein synthesis are inhibited in necrosis but are preserved during apoptosis.[1] In the liver, apoptosis is considered to be involved in the normal regulation of the organ size as well as the underlying mechanism of liver disease in different experimental models and clinical pathological states.[2] Toxic oxygen species are thought to cause cell necrosis *via* oxidation of critical cellular proteins, DNA, and lipids.[3] Although severe oxidative stress may result in cell necrosis, sublethal oxidative stress also has important implications for cell function and

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may lead to cell death by apoptosis. Sublethal oxidative stress has been associated with alterations in transcription and gene expression, ribosomal messenger RNA interactions, membrane receptor turnover, and membrane protein expression and distribution.[1] Mitochondria, as the most important cellular sources of free radicals, play an essential role in the development of cell death either by necrosis or apoptosis.

Hepatic injury induced by D-galactosamine (D-GalN) is a suitable experimental model of human liver failure.[4] D-GalN induces inhibition of RNA and protein synthesis, thus altering hepatocellular function.[5] Both effects may be consequences of a depleted intracellular pool of uracil nucleotides.[5] D-GalN has been shown to induce specific hepatocyte cell death *in vivo*[6–9] and *in vitro*.[10] Several studies have reported that prostaglandin E reduces the liver injury induced *in vivo* by D-GalN,[8,9,11] thioacetamide,[12] aflatoxin B_1,[13] CCl_4,[14] bile duct ligation,[15] fat-enriched and choline-deficient diet,[16] viral hepatitis[17] and complement-mediated hepatic necrosis.[18] Furthermore, prostaglandin E_1 (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.[19,20]

The aim of the study was to investigate the participation of mitochondria and intracellular free radical production during D-GalN-induced apoptosis and/or necrosis in primary culture of rat hepatocytes. Also, we would like to evaluate if PGE_1 was equally potent in reducing the induction of either apoptosis or necrosis by D-GalN.

MATERIALS AND METHODS

Materials

All chemical reagents were from Sigma Chemical (St Louis, MO, USA) unless otherwise indicated. William's medium E was obtained from Eurobio (Les Ulis, France). Antibiotics-antimycotic solution and fetal bovine serum were purchased from Life Technologies (Paisley, UK). 2′, 7′-dichlorofluoresceindiacetate (DCFDA) and tetramethylrhodamine ethyl ester (TMR) were from Molecular Probes Europe BV (Leiden, The Netherlands). Caspase-3 antibodies (H-277) were from Santa Cruz Biotechnology, (CA, USA). Caspase-3 substrate (Ac-DEVD-pNA) was purchased from Bachem AG (Bubendorf, Switzerland).

Preparation of Primary Hepatocytes and Cell Culture

Hepatocytes from anesthetized male Wistar rats (200–250 g) were isolated by a non-recirculating *in situ* collagenase perfusion of livers through portal vein following a modification of the procedure described by Seglen *et al*. [21] Livers were firstly perfused with solution I (10 mM HEPES, 6.7 mM KCl, 145 mM NaCl, 2.4 mM EGTA) pH 7.4 at 37°C at a flow of 40 ml/min for 10 min, and secondly with solution II (100 mM HEPES, 6.7 mM KCl, 67 mM NaCl, 10 g/l albumin, 4.8 mM $CaCl_2$, 0.05% collagenase A) pH 7.4 at 37°C at a flow of 20 ml/min for 10 min. The livers were then gently minced on a Petri dish and filtered through a nylon mesh. Hepatocytes were centrifuged and washed three times at 50 g for 5 min. Cell viability was consistently >85% as determined by trypan blue exclusion. Contamination of the cell cultures with kupffer cells was not detected morphologically, through latex beads ingestion (3 μm) or by fluorescein isothiocyanate-labelled ED-1 antibodies. Hepatocytes (150,000 cells/cm^2) were cultured on collagen-coated plates in William's E medium pH 7.4 containing fetal bovine serum (5%), insulin (1 μM), hydrocortisone (0.6 μM), HEPES (15 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), amphotericin (0.25 μg/ml), glutamine (2 mM), and sodium bicarbonate (26 mM). Two hours after seeding, the medium was removed and replaced with fresh medium without fetal bovine serum for 24 h. After culture stabilization, PGE_1 (1 μM) was added 2 h before D-GalN (0–40 mM) and the cultures were maintained for 24 h before sample collection.

Quantification of DNA Hypoploidy by Flow Cytometry Analysis

Apoptosis was followed by detection of hypodiploid cells with lower DNA content as described previously.[8] Hepatocytes were recovered using a non-enzymatic cell dissociation solution. The whole hepatocyte population, including the floating cells obtained from collected culture medium, was washed twice with phosphate buffer solution (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4) pH 7.4 at 284 *g* at 4°C for 5 min. The cell fraction was permeabilized and fixed in ethanol (70%) for 4 h at 4°C. Afterwards, hepatocytes were washed in PBS and incubated with RNAse A (5 U/ml) for 10 min at room temperature and with propidium iodide (20 μg/ml) for 10 min at room temperature. After incubation, the DNA content in hepatocytes was evaluated using a FACScan (Becton Dickinson Immunocytometry Systems, San Jose, USA). DNA fluorescence pulse processing was used to discriminate between single cells and aggregates of cells (doublet discrimination) by evaluating the FL2-width versus FL2-area scatter plot. Light scatter gating was used to eliminate smaller debris from analysis. DNA content was displayed on a 4-decade logarithmic scale. An analysis gate was set to limit

the measurement of hypoploidy to an area of 2-fold loss of DNA content.

DNA Fragmentation

The whole hepatocyte population, including the floating cells obtained from collected culture medium, was treated with 1 ml of lysis buffer I (100 mM Tris–HCl pH 8.00, 5 mM EDTA, 150 mM NaCl, 0.5% sarkosyl, 400 μg/ml proteinase K) at 55°C for 1 h. After incubation, the cell lysates were centrifuged at 20,800 *g* at 4°C for 10 min. DNA in the supernatants was precipitated with cold isopropanol (1:1) at −20°C for 12 h and recovered by centrifugation of the samples at 20,800 *g* at 4°C for 10 min. DNA was washed with ethanol (70%), dried and resuspended (5 μg/μl) in Tris–EDTA buffer (10 mM Tris, 50 mM EDTA) pH 7.4. After RNAse (40 μg/ml) incubation for 2 h at 37°C, the samples were applied and analyzed on agarose (1.5%) gel with ethidium bromide (0.5 μg/ml).

Evaluation of Caspase-3 Processing

The whole hepatocyte population, including the floating cells obtained from collected culture medium, was treated with 1 ml of lysis solution II (25 mM HEPES pH 7.5, 2 mM EDTA, 100 mM NaCl, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 5 μg/ml leupeptin and 5 μg/ml pepstatin A) for 10 min on ice. Afterwards, the cell lysates were centrifuged at 20,800*g* at 4°C for 10 min. Proteins (100 μg) were separated on 12% SDS-PAGE and transferred to nitrocellulose membrane. Immunobloting was carried out using anti-caspase-3 rabbit polyclonal antibodies as primary antibody and anti-rabbit-IgG-alkaline phosphatase as secondary antibody. Proteins were visualized incubating the membrane with alkaline phosphatase substrate, BCIP-NBT.

Assay for Caspase-3 Activity

The whole hepatocyte population, including the floating cells obtained from collected culture medium, was treated with 1 ml of lysis solution II as described above. The caspase-3-like activity in the cell extract (100 μg) diluted in incubating buffer (25 mM PIPES pH 7.2, 10% sucrose, 0.1% CHAPS, 1 mM EDTA and 5 mM DTT) was measured by colorimetric assay using the peptide-based substrate Ac-DEVD-p-NA. The enzymatically-released pNA is followed by the linear increase in the absorbance at 405 nm using a DU® 640 Spectrophotometer (Beckman Coulter, CA, USA).

Measurement of Cell Necrosis by Lactate Dehydrogenase Release

Lactate dehydrogenase (LDH) in the culture medium was measured by modification of a previous described method.[22] Briefly, a volume of medium from cell culture was incubated with β-NADH (0.2 mM) and pyruvic acid (0.4 mM) diluted in PBS pH 7.4. LDH concentration in the sample was proportional to the β-NADH consumption measured as the linear decrease in the absorbance at 334 nm using a DU® 640 Spectrophotometer (Beckman Coulter, CA, USA). LDH concentration was calculated using a commercial standard (Merck, Darmstadt, Germany).

Measurement of Cell Viability by Trypan Blue Exclusion

Hepatocytes were recovered using a non-enzymatic cell dissociation solution. The whole hepatocyte population, including the floating cells obtained from collected culture medium, was washed twice with PBS pH 7.4 by centrifugation at 284 *g* at 4°C for 5 min. Cells were resuspended in PBS and an aliquot was treated with trypan blue (0.2%) solution. After 5 min of incubation at room temperature, the non-viable cells showed an intense blue staining.

Determination of Reactive Oxygen Species

The production of reactive oxygen species was monitored using the DCFDA fluorescent probe, which oxidizes in the presence of hydrogen peroxide (H_2O_2).[23] Hepatocytes were incubated with DCFDA (2 μM) 30 min before collection of the cells. H_2O_2 production was determined as an enhancement on the FL1 fluorescence of hepatocyte population measured in a FACScan (Becton Dickinson Immunocytometry Systems, San Jose, USA).

Determination of Mitochondrial Transmembrane Potential

The mitochondrial transmembrane potential (MTP) was evaluated in cultured hepatocytes using the membrane potential-sensitive dye TMR. Hepatocytes were incubated with TMR (2 μM) 30 min before collection of the cells. Reduction on the mitochondrial potential resulted in a reduction on the FL2 fluorescence of hepatocyte population measured in a FACScan (Becton Dickinson Immunocytometry Systems, San Jose, USA).

Evaluation of Lipid Peroxidation

The presence of thiobarbituric acid-reactive substances (TBARS) in the culture medium was used as

an index of lipid peroxidation in the hepatocytes following a modification of the procedure described previously.[24] Briefly, the samples (100 μl) were treated with trichloroacetic acid (10%) and centrifuged at 20,800 *g* at 4°C for 5 min. EDTA (1.34 mM) and GSH (0.65 mM) were added to the supernatant to prevent further lipid peroxidation caused during the assay. The samples were treated with 1 ml of HCl (25%) and 1 ml of thiobarbituric acid (1% diluted in 50 mM NaOH), and the mixture was heated at 100°C for 1 h. TBARS were evaluated measuring the absorbance of the samples at 532 nm in a DU® 64 Spectrophotometer (Beckman Coulter, CA, USA). Standard curve was prepared daily using 1,1,3,3-tetraethoxypropane (Sigma Chemical, St Louis, MO, USA) diluted in ethanol as source of TBARS.

Quantification of GSH/GSSG Ratio

The GSH/GSSG ratio was evaluated in cell extracts obtained from cultured hepatocytes following the procedure described previously.[25] The whole hepatocyte population, including the floating cells obtained from collected culture medium, was treated with precipitating solution (12% perchloric acid, 40 mM *N*-ethylmaleimide and 2 mM bathophenanthrolinedisulfonic acid) at 4°C for 5 min. The samples were centrifuged at 20,800 *g* at 4°C for 5 min. Afterwards, 50 μl of glutamyl glutamate (1 mM) (Sigma Chemical, St Louis, MO, USA) as internal standard and 10 μl of *m*-cresol purple (1 mM) (Sigma Chemical, St Louis, MO, USA) as pH indicator were added to the samples (500 μl). The pH of the solution was adjusted to 8.0–8.5 with KOH (2 M) containing MOPS (0.3 M) to prevent excessive alkalization. After centrifugation of the samples at 20,800 *g* at 4°C for 5 min, a volume (25 μl) was derivatized with 50 μl of 1-fluoro-2, 4-dinitrobenzene (1%) (Sigma Chemical, St Louis, MO, USA) in a small glass tube. After 45 min incubation in the dark at room temperature, samples were desiccated under vacuum and stored at −20°C until injection. Afterwards, the samples were dissolved in 50 μl of methanol (80%) and injected (25 μl) into the HPLC system (Beckman Instruments, Palo Alto, CA, USA) equipped with a Sperisorb NH_2 column (20 × 04 cm, 5 μm particles) (Teknokroma, Barcelona, Spain). The flow rate was set at 1 ml/min. Two mobile phases were used: solvent A (80% methanol) and solvent B (0.5 M sodium acetate in 64% methanol). After injection of the sample, the mobile phase was held at 80% of solvent A and 20% of solvent B for 5 min followed by a 10 min linear gradient up to 1% solvent A and 99% solvent B. Later, the mobile phase was held at 99% solvent B until GSSG had eluted. The concentration of GSH and GSSG was quantified using the areas below the corresponding HPLC peaks of the sample. Standard curve was drawn using commercial GSH and GSSG (Sigma Chemical, St Louis, MO, USA).

Statistical Analysis

Results are expressed as mean ± SE of ten separate experiments. Comparisons were made using ANOVA with the Least Significant Difference test (LSD). Statistical significance was set at $p \leq 0.05$. The statistical significance between groups was indicated using superscript letters in the figures and table. In this way, the groups with different superscript letters are significantly different ($p \leq 0.05$).

RESULTS

PGE_1 Reduced D-galactosamine-induced Apoptosis

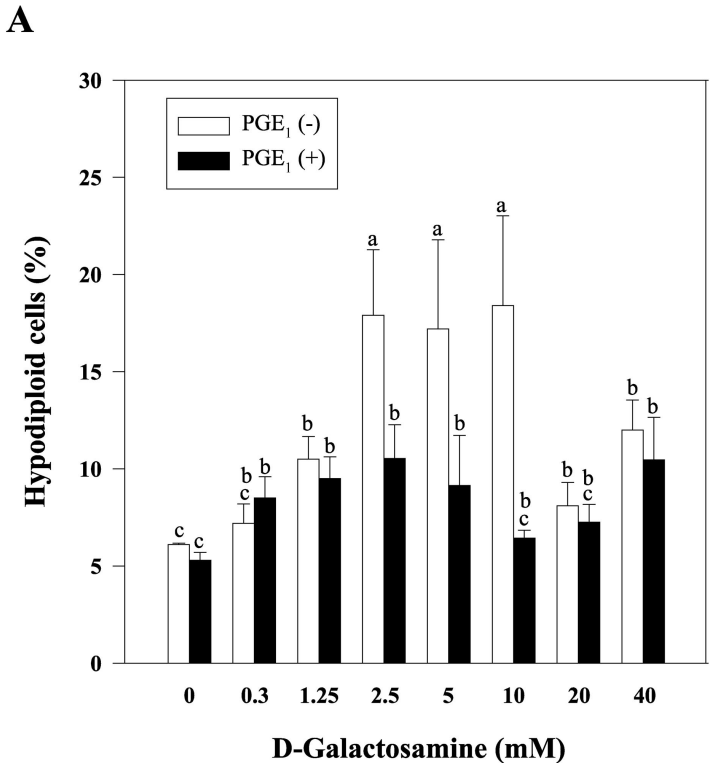
Consistent with a previous *in vivo* report,[8] PGE_1 was able to reduce the biochemical features of apoptosis induced by D-GalN. In the present *in vitro* study the apoptotic effect of D-GalN was reproduced but it was dependent on the concentration of the hepatotoxin used. The percentage of hypodiploid cells was significantly increased in the range between 2.5 and 10 mM D-GalN but it decreased at higher concentration (Fig. 1A) ($p \leq 0.05$). A similar profile of apoptotic response by D-GalN was observed when measuring DNA fragmentation in cell extract (Fig. 1B). In relation to the protective effect of PGE_1 on this experimental model, it was shown that the prostanoid was able to reduce significantly the percentage of hypodiploid cells (Fig. 1A) and DNA fragmentation (Fig. 1B) induced by D-GalN ($p \leq 0.05$).

Protection by PGE_1 of Caspase-3 Processing and Activation in D-galactosamine-treated Hepatocytes

Three concentrations of D-GalN (0.3, 5 and 40 mM) were selected to follow the induction of caspase-3 processing and activation. In these conditions, an intermediate concentration of D-GalN (5 mM) induced the highest p17 caspase-3 cleavage product (Fig. 2A) that correlated to the highest significant increase in caspase-3 activity (Fig. 2B) ($p \leq 0.05$). PGE_1 clearly reduced caspase-3 processing (Fig. 2A) and activation (Fig. 2B) induced by D-GalN.

Cell Necrosis

The necrotic cell death of hepatocytes was evaluated using the release of LDH into the culture medium and the cell viability measured by trypan blue



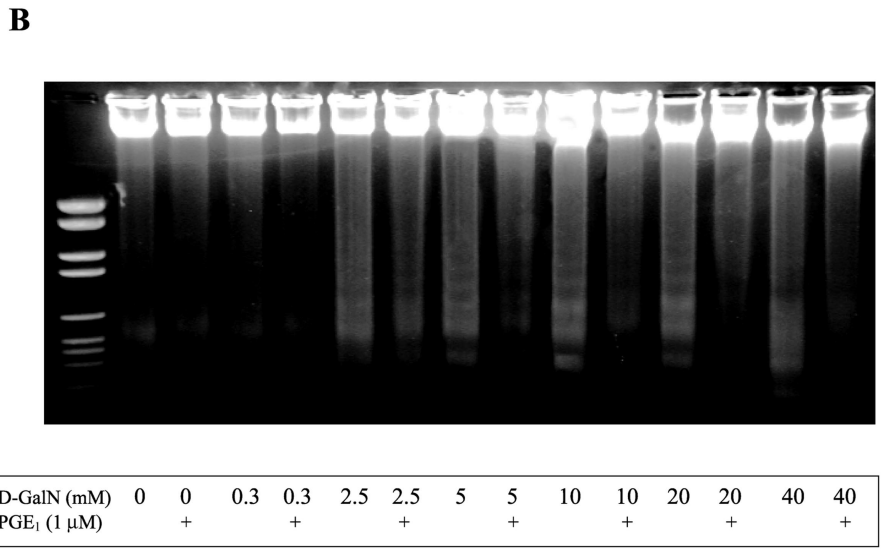


FIGURE 1 (A) Hypodiploid cells (%) and (B) DNA fragmentation in hepatocytes after D-galactosamine (D-GalN) and/or PGE$_1$ treatments. PGE$_1$ was pre-administered to D-GalN-treated hepatocytes as described in "Materials and Methods" section. PGE$_1$ reduced the increase on the percentage of hypodiploid cells induced by D-GalN (2.5–10 mM). Data are the mean ± SD of ten different experiments.[a,b,c]Groups with different superscript letters are significantly different ($p \leq 0.05$).

exclusion (Table I). There were observed no differences on the cell number in samples treated or not with D-GalN. D-GalN (5 mM) induced a significant moderate release of LDH without diminution of the cell viability compared to the values obtained in the control group ($p \leq 0.05$). The highest degree of cell necrosis was observed at 40 mM of D-GalN with an important release of LDH into the culture medium and a significant reduction (30%) of cell viability ($p \leq 0.05$). PGE$_1$ did modify neither the enhanced LDH release nor the loss of cell viability induced by D-GalN.

Reactive Oxygen Species Production in Hepatocytes Treated with D-galactosamine

The measurement of intracellular oxidative stress measured as H_2O_2 production is shown in Fig. 3. The production of H_2O_2 increased gradually with D-GalN concentration reaching a statistically significant value

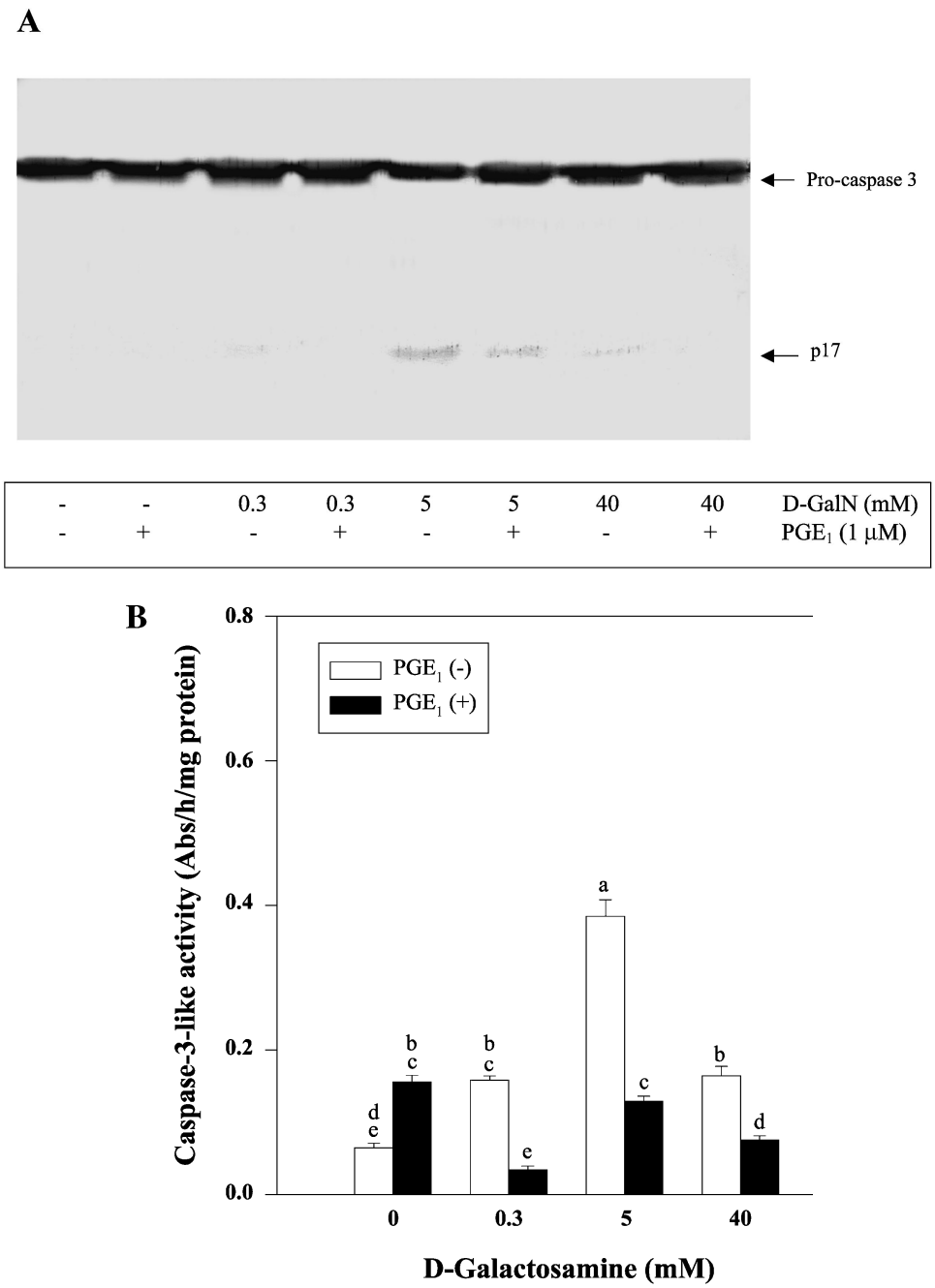


FIGURE 2 (A) Expression and (B) Associated-activity of caspase-3 in cell extracts after D-galactosamine (D-GalN) and/or PGE_1 treatments. PGE_1 was pre-administered to D-GalN-treated hepatocytes as described in "Materials and Methods" section. Maximum apoptosis was observed at intermediate D-GalN (5 mM) concentration. PGE_1 reduced the enhanced expression and activity of caspase-3 induced by D-GalN. Data are the mean ± SD of ten different experiments. [a,b,c,d,e]Groups with different superscript letters are significantly different ($p \leq 0.05$).

at a concentration higher than 5 mM of the hepatotoxin ($p \leq 0.05$). PGE_1 did not essentially modify the intracellular production of H_2O_2 induced by D-GalN.

Mitochondrial Transmembrane Potential Disturbances Induced by D-galactosamine

The MTP had a tendency to decline with D-GalN concentration (Fig. 4). Nevertheless, the reduction of MTP only reached a significant value in comparison with the control group at the highest D-GalN concentration (40 mM) ($p \leq 0.05$). PGE_1 did not essentially modify the decrease on MTP induced by D-GalN.

Thiobarbituric-reacting Substances Release Induced by D-galactosamine

The TBARS concentration in the culture medium, as a measurement of lipid peroxidation (Fig. 5), correlated

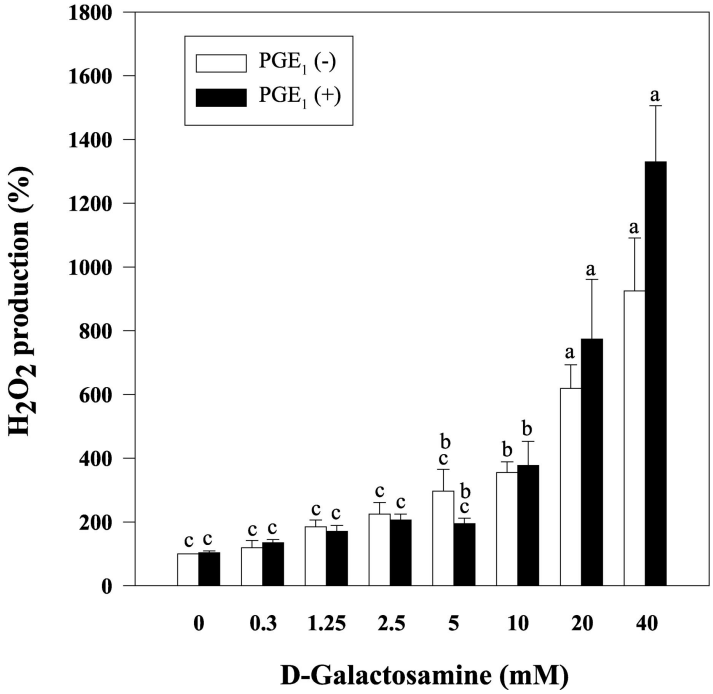


FIGURE 3 Intracellular hydrogen peroxide (H_2O_2) production observed after D-galactosamine (D-GalN) and/or PGE_1 treatments. PGE_1 was pre-administered to D-GalN-treated hepatocytes as described in "Materials and Methods" section. PGE_1 did not affect the gradual increase of H_2O_2 production induced by D-GalN. Data are the mean ± SD of ten different experiments. [a,b,c]Groups with different superscript letters are significantly different ($p \leq 0.05$).

to the intracellular production of H_2O_2 and the decline on MTP presented above. In this sense, the values of TBARS were only significantly higher than the control group at 40 mM of D-GalN concentration ($p \leq 0.05$). PGE_1 did not essentially modify the lipid peroxidation induced by D-GalN.

Modification of the GSH/GSSG Ratio Induced by D-galactosamine And/or PGE_1

The GSH/GSSG ratio was only significantly reduced at the highest D-GalN concentration (40 mM) (97 ± 4.1) compared to the control hepatocytes (179 ± 19.7) (Fig. 6) ($p \leq 0.05$). PGE_1 was able to recover the GSG/GSSG ratio to control level in D-GalN (40 mM)-treated hepatocytes (Fig. 6).

DISCUSSION

The present study shows that D-GalN induces apoptosis and necrosis in a concentration- and free radical-dependent fashion in primary culture of rat hepatocytes. Exacerbation of necrosis by D-GalN is associated with a decrease of all biochemical parameters related to apoptosis. PGE_1 treatment is able to reduce the induction of apoptosis, but not necrosis, induced by D-GalN in primary rat hepatocytes.

D-GalN has been shown to be a suitable experimental model of liver injury.[4] D-GalN reduces the intracellular pool of uracil nucleotides in hepatocytes, thus inhibiting the synthesis of RNA and proteins.[5] The *in vivo* administration of D-GalN causes apoptosis[7,8] and necrosis[6–9,26–28] in rat liver parenchymal cells. In the present study, D-GalN induced apoptosis of cultured hepatocytes at the lowest concentration used (0.3 mM) reaching the highest apoptotic response around 5–10 mM of the hepatotoxin (Figs. 1 and 2). As previously observed,[10] the necrotic response induced by D-GalN was already evident at 5 mM of D-GalN (Table I). Nevertheless, the cell survival was only reduced at the highest D-GalN concentration (40 mM) (Table I).

Free radical-dependent cell death has been demonstrated in different experimental models of hepatocyte cytotoxic *in vivo*[13,29,30] and *in vitro*.[31–36] The intracellular free radical production was gradually increasing with the D-GalN concentration (Fig. 3). It is interesting to observe that high induction of apoptosis (Figs. 1 and 2) was associated with a moderate increase of free radicals (Fig. 3). In concordance with a previous study,[37] the moderate intracellular oxidative stress may mediate the induction of apoptosis by D-GalN. The mitochondrial dysfunction is a potential main source of intracellular free radicals production during the induction of cell death.[31,35,38–40] In our conditions, a significant decline on the MTP (Fig. 4) induced by 40 mM D-GalN was associated with a high intracellular H_2O_2 production (Fig. 3), GSG/GSSG ratio

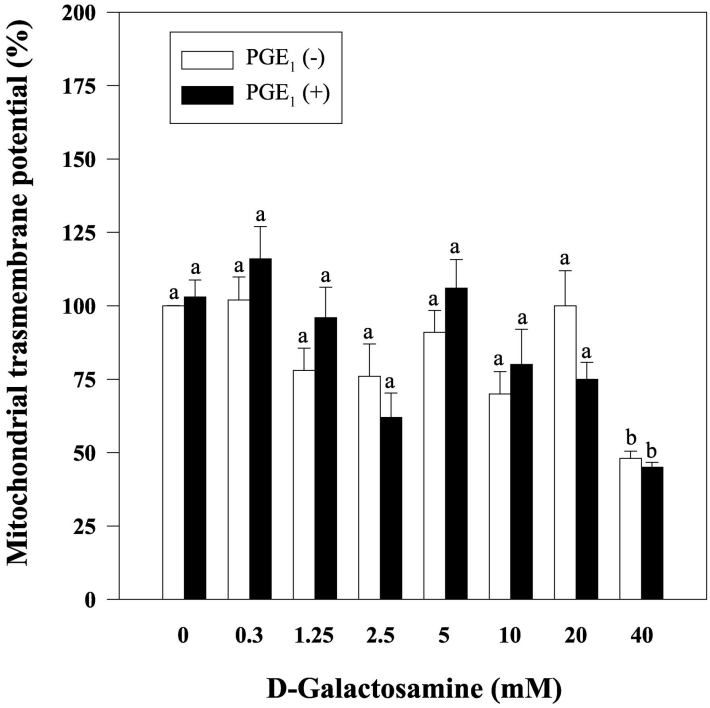


FIGURE 4 Mitochondrial transmembrane potential (MTP) disturbance in hepatocytes observed after D-galactosamine (D-GalN) and/or PGE_1 treatments. PGE_1 was pre-administered to D-GalN-treated hepatocytes as described in "Materials and Methods" section. PGE_1 did not affect the gradual decrease in MTP induced by D-GalN. Data are the mean ± SD of ten different experiments. [a,b]Groups with different superscript letters are significantly different ($p \leq 0.05$).

reduction (Fig. 5) and TBARS release (Fig. 6). In these conditions of high free radical production induced by D-GalN (40 mM), an important change in the cell death mechanism took place with a reduction of the apoptotic machinery (Figs. 1 and 2) and enhancement of the necrotic pathway (Table I). The opposite relationship between necrosis and apoptosis may be related to the recently described oxidant-induced caspase inactivation.[41] Nevertheless, it seems also feasible that the reduction of the MTP

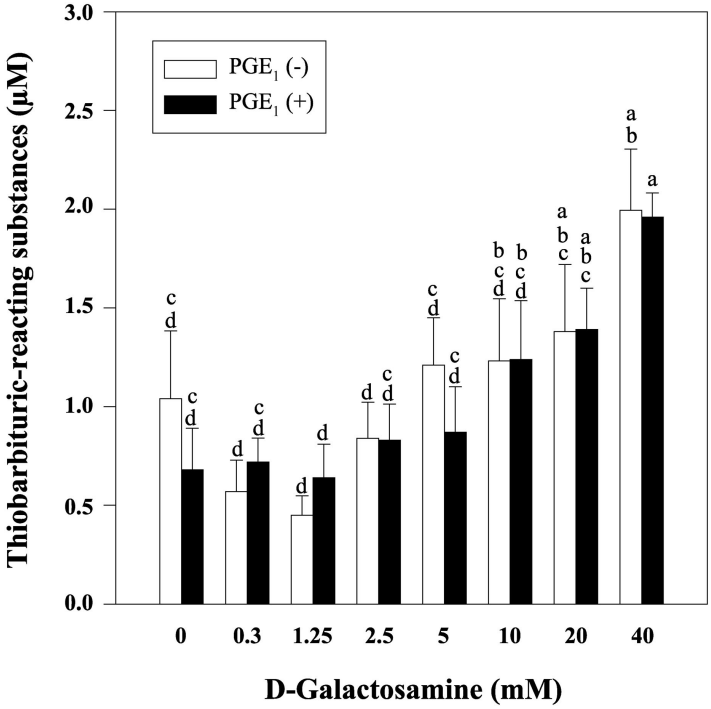


FIGURE 5 Thiobarbituric-reacting substances (TBARS) released after D-galactosamine (D-GalN) and/or PGE_1 treatments. PGE_1 was pre-administered to D-GalN-treated hepatocytes as described in "Materials and Methods" section. PGE_1 did not affect the gradual increase in TBARS release induced by D-GalN. Data are the mean ± SD of ten different experiments. [a,b,c,d]Groups with different superscript letters are significantly different ($p \leq 0.05$).

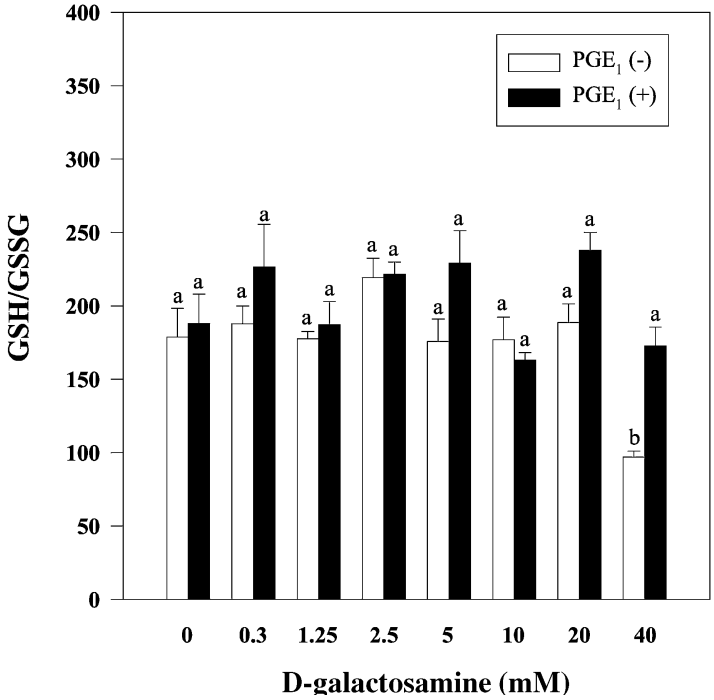


FIGURE 6 Intracellular GSH/GSSG ratio observed after D-galactosamine (D-GalN) and/or PGE$_1$ treatments. PGE$_1$ was pre-administered to D-GalN-treated hepatocytes as described in "Materials and Methods" section. PGE$_1$ blocked the reduction of GSH/GSSG ratio induced by D-GalN (40 mM). Data are the mean ± SD of ten different experiments. [a,b]Groups with different superscript letters are significantly different ($p \leq 0.05$).

induced by high D-GalN (40 mM) concentration may cause a failure on the ATP synthesis. The low ATP content has also shown to induce the switch from apoptosis to necrosis cell death pathway.[42]

Several reports have shown that prostaglandin E decrease apoptosis[8] and necrosis[8,9,11] of hepatocytes induced by intraperitoneal administration of D-GalN to rats. We have previously shown that the protective effect of PGE$_1$ against liver injury induced by D-GalN was related to its capacity to stimulate the expression of inducible nitric oxide synthase in hepatocytes.[43] In the present study, PGE$_1$ was able to reduce all the parameters associated with apoptosis (Figs. 1 and 2) but not those of necrosis (Table I) induced by D-GalN in primary culture of rat hepatocytes. This inability of the prostanoid to reduce cell necrosis was associated with no effect of PGE$_1$ on the enhanced oxidative stress induced by high concentration of the hepatotoxin (Figs. 3–5). Surprisingly, PGE$_1$ was able to abolish the GSH/GSSG ratio reduction observed in D-GalN (40 mM)-treated hepatocytes (Fig. 6) pointing out that the recovery of the GSH/GSSG ratio by the prostanoid is not sufficient to reduce the necrosis induced by D-GalN. The protection of PGE$_1$ against *in vitro* cell death without reduction of lipid peroxidation has also been observed during *tert*-butyl hydroperoxide toxicity in primary culture of rat hepatocytes.[34] In relation to the potential intracellular mechanism involved in the protection against D-GalN-induced apoptosis by PGE$_1$, preliminary data indicates that inhibition of inducible nitric oxide synthase by N_ω-nitro-L-arginine methyl ester abolished the protective effect of PGE$_1$ in this experimental model of cytotoxicity (data not shown).

In conclusion, D-GalN-dependent hepatocyte cell death through apoptosis or necrosis may be related to an impaired mitochondrial function associated with intracellular free radical generation and/or energy crisis induced by the hepatotoxin. More studies are necessary to determine the role of free radicals and/or ATP depletion during the change from apoptosis to necrosis in this experimental model. Our data presented herein strongly support

TABLE I LDH release and cell viability observed after D-galactosamine (D-GalN) and/or PGE$_1$ treatments. PGE$_1$ was pre-administered to D-GalN-treated hepatocytes as described in "Materials and Methods" section. PGE$_1$ did not affect the cell necrosis induced by D-GalN (40 mM). Data are the mean ± SD of ten different experiments. [a,b,c]Groups with different superscript letters are significantly different ($p \leq 0.05$)

Groups	LDH (mIU/ml)	Cell viability (%)
Control	0.020 ± 0.0053^c	100 ± 3.7^a
PGE$_1$	0.013 ± 0.0027^c	96 ± 5.3^a
D-GalN (0.3 mM)	0.016 ± 0.0031^c	88 ± 3.0^a
PGE$_1$+D-GalN (0.3 mM)	0.016 ± 0.0042^c	89 ± 0.9^a
D-GalN (5 mM)	0.030 ± 0.0073^b	98 ± 2.3^a
PGE$_1$+D-GalN (5 mM)	0.034 ± 0.0060^b	99 ± 0.7^a
D-GalN (40 mM)	0.246 ± 0.0335^a	67 ± 7.3^b
PGE$_1$+D-GalN (40 mM)	0.247 ± 0.0461^a	77 ± 7.5^b

the hypothesis that PGE_1 is able to regulate apoptosis induced by D-GalN by a mechanism probably downstream mitochondrial-dependent induction of cell death. Currently, several studies are being performed to elucidate the mechanism by which nitric oxide may be mediating the protective effect of PGE_1 against D-GalN-induced apoptosis in primary culture of rat hepatocytes.

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