Does prostaglandin-E1 modulate d-galactosamine induced cell death in primary culture of human hepatocytes?

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Abstract

Background: Cell death pathway can occur under physiological or pathological conditions. In vitro and in vivo studies have shown that d-galactosamine (DGA) induces hepatocyte damage. Objective: The present study aims to evaluate the protective effect of prostaglandin E1 (PGE1) on DGA-induced apoptosis, necrosis and oxidative stress in primary culture of human hepatocytes. Methods: Normal human hepatocytes were obtained from the safety margin of liver specimens, removed during hepatectomy operation to liver cancer patients, and isolated using the classical collagenase perfusion method. After culture stabilization, PGE1 (1 μM) was added 2 h before DGA (5 mM). Cultures were maintained for 24 h before the parameters for apoptosis, necrosis and oxidative stress were measured. Apoptosis was studied by DNA-fragmentation, neutral (nSMase) and acid (aSMase) sphingomyelinase and caspase-3 activity. Necrosis was investigated by lactate dehydrogenase (LDH) and transaminases (ALT & AST) enzymes. The oxidative stress was assessed by malondialdehyde (MDA), glutathione (GSH), oxidized glutathione (GSSH), glutathione S-transferase (GST), glutathione peroxidase (GSPx), catalase (CAT), superoxide dismutase (SOD) and nitric oxide (NO). Results: The hepatotoxin DGA induced apoptosis and enhanced all parameters related to necrosis and oxidative stress. In addition, PGE1 proved also to prevent GSH depletion. The obtained results provided evidences for the biochemical hepatotoxic effects of DGA (5 mM) especially through the induction of apoptosis.
1. Introduction

The liver is the principle organ involved in the metabolism and detoxification of biological toxins and medicinal agents. Such metabolism is always associated with the disturbance of hepatocyte biochemistry and generation of reactive oxygen species (ROS). The oxidative stress is an important mechanism responsible for dysfunction of liver. There is a wide range of liver pathologies ranging from sub-clinical hepatitis up to necro-inflammatory hepatitis, cirrhosis and carcinoma that have been proved to be associated with the redox imbalance and oxidative stress.

Apoptosis and necrosis are two different types of cell death. Necrosis is a non-controlled cellular disruption as a consequence of extreme noxious irreversible conditions. In contrast, apoptosis is a genetically controlled cell death pathway that can occur under physiological or pathological conditions. It plays an important role in removing redundant, damaged or infected cells and has a vital role in maintaining homeostasis of liver tissue as well as during the pathogenesis of liver diseases.

DGA is an amino-sugar with unique hepatotoxic properties in mammals. The mechanism for DGA-induced liver injury, although poorly understood, seems to be partly related to the immune system. Its toxic effects are connected with insufficiency of UDP (uracil nucleotides)-sugar “UDP-glucose and UDP-galactose”, release of pro-inflammatory mediators (such as TNF-α from Kupffer’s cells), loss of intracellular calcium homeostasis and altering uridine-pool in hepatocytes. These changes affect cell membranes, organelles and the synthesis of proteins and nucleic acids. DGA, at higher dose, inhibits the energy metabolism of hepatocytes, destroys the enzymes involved in the transport of substrates to the mitochondria and modifies the phospholipid composition of membranes.

DGA is commonly used in experimental model for induction of hepatocellular injury. A 5 mM dose of DGA has been established as the dose which potentially induces apoptosis, necrosis and alters cultured human hepatocytes functions.

Prostaglandins are biologically active polyunsaturated fatty acids derived from arachidonic acid present in most mammalian tissues. They have been suggested to be involved in membrane stabilization. In the liver, prostaglandins were found to be involved in hepatocyte proliferation and vital defence processes such as inflammation, tissue repair and immune responses. PGE1 was found to be effective as delivering drug selectively to hepatocytes. Several studies have been conducted to assess the ability of PGE1 to reduce in vivo and in vitro DGA-induced cell death in cultured rat hepatocytes. There were growing evidences that changes in intracellular redox state appear to regulate critical biological responses. ROS influence signals transduction and transcription-factors, and play a central role in the pathophysiology burden of liver injury in this sense. Propagation of the oxidative stress induced intracellular reactions further shifts cell death from apoptotic to necrotic pathways in DAG-treated rats’ hepatocytes.

The present study aimed at exploring the intracellular biochemical alterations in cultured human hepatocytes that are involved in the induction of apoptosis, necrosis and oxidative stress by 5 mM of the hepatotoxin DAG. It also aimed at assessing the possible protective effect of PGE1 against the progression of the DAG-induced damage in the cultured human hepatocytes.

2. Materials and methods

This study was approved by the Ethics Committee of Medical Research Institute, Alexandria University. All reagents used in this study were supplied by Sigma–Aldrich Chemical (St. Louis, MO, USA) unless otherwise stated.

Human hepatocytes were isolated from normal hepatic parenchyma at the apparently free margin around lesions obtained during hepatectomies done at the department of Surgery, Medical Research Institute, Alexandria University. A written informed consent was obtained from all participants before surgery including consent to hepatectomies and to use their resected specimen in this study. All patients were thoroughly investigated assessing the hepatic functions, abdominal ultrasound and triphasic multidetector C.T. for abdomen and pelvis were done. The included patients were six; three men and three women with 40 ± 8 years old. Hepatectomies were done in three cases of synchronous non-anatomical resection of colorectal liver metastasis (one case with cancer sigmoid and solitary lesion at segment IV b and the other two cases with cancer rectum and having metastasis at segments V and VIII) and the other cases; anatomical left lateral segmentectomy was done for two patients who had hepatocellular carcinoma (HCC) involving segments II and III and the last case had an adenoma at segment IV b. We excluded patients with Hepatitis-C virus, Hepatitis-B, patients with liver cirrhosis or periportal fibrosis.

A crush clamp technique using Kelly clamp was used for parenchymal transection to fracture the parenchyma and expose the vessels. Sealing of the vessels was done using monopolar diathermy, bipolar diathermy or harmonic scalpel (Ethicon EndoSurgery, Cincinnati, OH, USA) and wide caliber vessels were secured with ties or clipping. Bleeding from hepatic parenchyma was secured using monopolar diathermy and Argon Plasma Coagulation.

2.1. Preparation of primary hepatocytes and cell culture

The human hepatocytes were isolated (in immunology lab.) by in situ collagenase perfusion of liver samples according to the method described by Ferrini et al. Liver samples were first perfused with a non-recirculating washing solution I, pH 7.4 at a flow of 75 ml/min in order to remove blood cells. Afterward, they were perfused with a non-recirculating chelating solution II, pH 7.4 at a flow of 75 ml/min for 10 min then with recirculating isolation solution III at a flow of 75 ml/min. for
10 min. Cell suspension was filtered through Nylon mesh (250 μm) and washed three times at 50 g for 5 min at 4 °C in culture medium (DEM:Ham-F12 and Williams E medium (1:1) supplemented with 26 mM NaHCO3, 15 mM HEPES, 0.2 g/l glutamine, 50 mg/ml vitamin C, 0.04 mg/l dexta-methasone, 2 mg/l insulin, 200 mg/l glucagon, 50 mg/l transferrin, 4 ng/l ethanolamol).

Cell viability was consistently more than 85% as determined by trypsin blue dye exclusion technique, based on impermeability of viable cells to trypan blue. The number of viable lymphocytes/ml was calculated according to the following equation: % of viable cells = (no of viable cells/total no counted) × 100.

To determine the viability of hepatocytes, cultures were stained with the dye Hoechst 33342. The percentage of viable cells was calculated as described by Siendones et al.

2.2. DNA fragmentation in hepatocytes

The fragmentation of DNA in hepatocytes was evaluated using electrophoresis as described by Siendones et al. The whole hepatocyte population was treated with 1 ml of lysis buffer, pH 8.0, at 4 °C for 10 min. Samples were incubated with RNAse (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 Chemical Co.) extraction and precipitated with cold isopropanol (1:1) at 20 °C for 12 h. DNA was recovered by centrifugation of the sample at 10,000 g for 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.

2.3. Caspase-3-associated activity

Hepatocytes were treated with 1 ml of lysis solution at 4 °C for 10 min, transferred to eppendorf tubes, and centrifuged at 10,000g at 4 °C for 5 min. Caspase-3 associated activity was measured in samples (25 μg protein) by a fluorometric assay using the peptide-based substrate ac-N-acetyl-Asp-Glu-Val-Asp-AFC (Ac-DEVD-AFC) (Bachem, Bubendorf, Switzerland).

2.4. Assays for sphingomyelinase activities

The measurement of neutral (nSMase) and acid (aSMase) sphingomyelinase activities was carried out following the procedure described by Martin et al.

2.5. Measurement of LDH-release and transaminases (ALT & AST) activities

Lactate dehydrogenase (LDH) was measured by a colorimetric routine laboratory method. Volume of cell lyse ranging from 50 to 200 ml was incubated with 0.2 mM β-NADH and 0.4 mM pyruvic acid diluted in PBS, pH 7.4. LDH concentration in the sample was proportional to the linear decrease in the absorbance at 343 nm. LDH concentration was calculated using commercial standard. Alanine transaminase (ALT) and aspartate transaminase (AST) were estimated according to Bergmeyer and Bernt.

2.6. Evaluation of lipid peroxidation and quantification of glutathione (GSH) and oxidized glutathione (GSSG) longer

The presence of malondialdehyde (MDA) in culture medium was used as an index of lipid peroxidation in hepatocytes following TBARS “thio-barbituric acid reactive substances” assay. GSH, GSSG were quantified in hepatocytes following the procedure described by Teare et al. Liver samples (~100 μg) were homogenized in ice-cold 0.1 M phosphate buffer (pH 7.4). For GSH content, the homogenate was immediately mixed with sulfosalicylic acid, shaken well, centrifuged, and 100 μl of the supernatant was added to 9.9 ml water and spectrophotometric absorbance was recorded at 412 nm. For GSSG, 200 μl supernatant was added to 3.78 ml water to which 40 μl of acetylpyridine was mixed to mask the GSH and left at room temperature for 3 h before estimation.

2.7. Glutathione S transferase (GST) and glutathione peroxidase (GSPx) assays

GST and GSPx activities were measured as described by Habig et al. and Awasthi et al., respectively. GST activity was assayed, using 1-chloro-2, 4-dinitrobenzene (CDNB) and 3, 4-dichloronitrobenzene (DCNB), by spectrophotometer at room temperature by monitoring the change in absorbance at 344, 270, and 344 nm. The activity of GSPx was assayed in a 1-ml system containing 0.1 M potassium phosphate buffer, 0.2 mM NADPH, 1 i.u. glutathione reductase, 4 mM GSH, 4 mM EDTA, 4 mM sodium azide, and 0.02 ml glutathione peroxidase. The mixture was incubated at 37 °C for 10 min after which 10 μl of 10 mM t-butyl hydroperoxide was added and the rate of reaction was measured using spectrophotometer.

2.8. Catalase (CAT) and superoxide dismutase (SOD) activities

CAT was assayed colorimetrically at 620 nm as described by Tawfik et al. SOD activities were determined according to the methods of Pottathil et al.
2.9. Nitric oxide (NO) metabolite measurement

The release of NO was indirectly assessed by measuring the accumulated nitrite using the Griess reaction as described by Green et al.\textsuperscript{31} Nitrite was detected and analyzed by formation of a red pink color upon treatment of a NO$_2$-containing sample with the Griess reagent.

2.10. Protein assay

Total protein concentration was determined by the Bio-Rad\textsuperscript{32} assay, using bovine serum albumin as standard. Measurements were done on UV Spectrophotometer at 595 nm.

3. Statistical analysis

The Data were collected and entered into the personal computer. Statistical analysis was conducted using the Statistical Package for the Social Sciences (SPSS) version 20. Statistical significance was set at (P-value < 0.05). Results are expressed as means ± SD of duplicate experiments. Differences between groups were assessed by one-way analysis of variance (ANOVA) using the least significant differences (LSD) test as multiple comparison analysis according to Berenbaum.\textsuperscript{33}

4. Results

The pathological examination of the resected specimen revealed the same pathology expected preoperatively with free safety margin in all cases. The histopathological reports revealed; metastatic adenocarcinoma from colorectal cancer in three cases, Fibrolamellar variant of HCC in two cases and an adenoma in the last case.

![Figure 1](image1.png)

**Figure 1** 1.5% agarose gel showing the effect of PGE$_1$-treatment on DNA-fragmentation due to DGA-induced injury in cultured human hepatocytes. “C” refers to control.

![Figure 2](image2.png)

**Figure 2** Effect of PGE$_1$-treatment on caspase-3-associated activity (absorbance/h/mg protein) due to DGA-induced injury in cultured human hepatocytes (a significantly different among control group, b significantly different among PGE$_1$ group, c significantly different among DGA group).

![Figure 3](image3.png)

**Figure 3** Effect of PGE$_1$-treatment on nSMase and aSMase (cpm/μg protein/min) activities due to DGA-induced injury in cultured human hepatocytes (a significantly different among control group, b significantly different among PGE$_1$ group, c significantly different among DGA group).

The effect of DGA-treatment (5 mM) revealed a general increase in the biochemical quantitation of the parameters for apoptosis, in the cultured hepatocytes. DGA increased the measured arbitrary units from apoptosis expressing DNA fragmentation by gel electrophoresis (Fig. 1: 1232 ± 114, 1241 ± 117, 2689 ± 141\textsuperscript{a,b}, and 1312 ± 141\textsuperscript{c}, for control, PEG1 treatment, DGA treatment and PEG$_1$ treatment before DGA addition, respectively). Caspase-3-associated activity in cultured hepatocytes was expressed with the highest values in cultures treated with DGA. These values were reduced nearly by 50% when PGE$_1$ was added before treatment with DAG (Fig. 2).

Sphingomyelinase (nSMase and aSMase) activities denote cellular levels of ceramide at the indicated times. They decreased significantly in DGA-group. PGE$_1$-treatment restored sphingomyelinase activities within normal range (Fig. 3).

Levels of transaminases (ALT, AST) and LDH were used as indicators to evaluate the attribution of PGE$_1$ to the structure damage of the hepatocytes. In the present study, the enzyme assays of transaminases showed that a toxic dose of DGA significantly raised the both levels of ALT and AST. PGE$_1$ could inhibit the enzyme activities effectively. The
ALT and AST level decreased significantly compared to control group (Fig. 4).

Depletion of intracellular-GSH and the increase in lipid peroxidation as MDA accumulation and GSSH in cells (Fig. 5) were used to assess the onset of oxidative injury induced by DGA. PGE1 abolished GSH depletion and exhibited remarkable lessening of raised MDA and GSSH (Fig. 5).

As shown in Figs. 6 and 7, a single dose of DGA induced severe oxidative damage and the GST, GSPx, CAT and

**Figure 4** Effect of PGE1-treatment on LDH (mIU/L), ALT and AST (IU/L) activities due to DGA-induced injury in cultured human hepatocytes (\(^a\) significantly different among control group, \(^b\) significantly different among PGE1 group, \(^c\) significantly different among DGA group).

**Figure 5** Effect of PGE1-treatment on MDA, GSH and GSSH (nmol/μg protein) contents due to DGA-induced injury in cultured human hepatocytes (\(^a\) significantly different among control group, \(^b\) significantly different among PGE1 group, \(^c\) significantly different among DGA group).

**Figure 6** Effect of PGE1-treatment on total GST and GSPx (U/mg protein) activities due to DGA-induced injury in cultured human hepatocytes (\(^a\) significantly different among control group, \(^b\) significantly different among PGE1 group, \(^c\) significantly different among DGA group).

**Figure 7** Effect of PGE1-treatment on CAT (a) and SOD (b) (U/lng protein) activities due to DGA-induced injury in cultured human hepatocytes (\(^a\) significantly different among control group, \(^b\) significantly different among PGE1 group, \(^c\) significantly different among DGA group).

**Figure 8** Effect of PGE1-treatment on NO concentration (μmol/mg protein) due to DGA-induced injury in cultured human hepatocytes (a significantly different among control group, b significantly different among PGE1 group, c significantly different among DGA group).
SOD activities decreased markedly. PGE₁ proved to normalize the enzyme activities of the four indexes.

Similar results were obtained in case of the nitric oxide (NO) concentration (Fig. 8). PGE₁ restored nitric oxide depletion caused by DGA hepatotoxin.

5. Discussion

ROS, products of normal metabolism, cause oxidant injury if they accumulate in pathological amounts. Under normal conditions, where the levels of ROS are tightly regulated and play a role in normal metabolism, the body’s antioxidant defenses are able to successfully manage non-pathological amounts of exogenous or endogenous oxidants from reactive oxygen/nitrogen species. Among therapeutics for liver diseases, protective drugs have attracted more and more attention, such as antioxidant prevention approaches. The environment can be a source of excess oxidants, which can cause both acute and chronic cellular injury. Moreover, ROS were thought to be involved in several cellular mechanisms including apoptosis.

Hepatic injury induced by DGA is a suitable experimental model of human liver failure. It induces an intense oxidative stress in hepatocytes. The present work shows that DGA induces apoptosis followed by necrosis in cultured human hepatocytes. The identification of DNA fragmentation and caspase-3 activity modifications induced by DGA in human hepatocytes can help in the insight of the causal molecular mechanisms responsible for the induction of apoptosis and necrosis.

Apoptosis, known as programmed cell death, is a cellular self-destruction mechanism involved in a variety of biological events such as developmental remodeling, tissue homeostasis and the removal of unwanted cells in most living tissues, including the mammalian liver. Similar to other organs, it plays a role in the pathogenesis of hepatic failure and organ dysfunction after liver ischemia/reperfusion injury. Sienones et al. found that generation of ROS with perturbation of pro- and anti-oxidant ratio caused alteration in mitochondria structure and hepatocytes membrane potential.

Activation of caspases has been demonstrated to be involved during in vivo and in vitro hepatocytes’ apoptosis. Induction of apoptosis by DGA was related to a significant increase in caspase-3 processing and activity observed in hepatocytes. Genetic and biochemical studies have uncovered a functional network of cell death regulators. It has shown that mammals have two distinct apoptosis signaling pathways. One pathway is activated by “death receptors” (a subgroup of the tissue nuclear factors family (TNF)). The other is initiated by certain cytotoxic stress. Excessive generation of ROS and caspase activation have been shown to be essential in cell apoptosis.

Mammalian sphinomyelinase (SMase) hydrolyzes the phosphodiester bond in sphingomyelin (SM), yielding ceramide and phosphorylcholine. SMase is transiently activated in response to a variety of extracellular stimuli, which leads to increased cellular levels of ceramide. Ceramide is a member of a group of lipid signaling molecules and has a specific role in the onset of different stress responses, including cell growth arrest, inflammation, and apoptosis. In the present study, sphinomyelinase decreased markedly in DGA group. Garcia et al. have reported that GSH has a role in the regulation of nSMase so GSH depletion sensitizes hepatocytes to the aSMase-induced apoptosis. Osawa et al. have concluded that activation of aSMase contributed to hepatocyte apoptosis.

MDA level is a main marker of endogenous lipid peroxidation. Besides, successful protection of liver damage by efficiently inhibiting MDA formation and decreasing ALT and AST, PGE₁ could enhance the activities of antioxidant enzyme system of the hepatocyte cells, including LDH (Fig. 4).

The replenishment of GSH may occur via reduction of GSSG by glutathione reductase or by de novo synthesis of GSH from its constituent amino acids. It plays a key protective role against oxidant-induced cell death. There was a significant GSH-depletion and GSSG-formation after liver reperfusion injury. GSH exhibits a large panel of actions in controlling apoptosis mechanisms or membrane transport. Usually GSSG represents only 1–10% of the total liver GSH. In the present study, liver GSSG in the DGA treated hepatocytes culture plate accounted almost 50% of total GSH, suggesting that there was a significant intracellular oxidative stress during the DGA treatment.

SOD and GSPx are intracellular antioxidant enzymes that protect against oxidative stress. Hepatocytes showed susceptibility to oxidative injury with depletion of antioxidant defenses, exemplified by decreased GSH and CAT and raised MDA. It has been suggested that induction of GST enzyme was a major cell protective mechanism and accompanied by elevation of intracellular GSH level.

Our results indicate that PGE₁ has a positive hepatoprotective impact on the DGA induced lesion. Since antioxidant enzymes such as SOD and GSPX are considered to be a primary defense system for oxidative damage prevention. PGE₁ exerts antioxidant effect not only through its own radical scavenging activity but also, by boosting the host antioxidant enzyme system. On the other hand, we found that several indexes showed a significant change. GST, GSPX, CAT and SOD activities were raised significantly on addition of PGE₁ to the cultured hepatocytes.

Massive intervention into the redox state by pharmaceutical doses of exogenous antioxidants should be considered with caution due to the indefinite role of free radicals in regulation of apoptosis and cytotoxicity of injured cells. Our results showed that DGA could increase ROS generation, but PGE₁ could reverse such effect by lowering the level of intracellular ROS.

It has previously shown that NO mediates apoptosis by DGA in primary culture of rat hepatocytes and has been recognized as a critical mediator in numerous biological processes. Some chemical aspects of NO molecule determine its free radical capacity to mediate cell signaling. In addition, it has been shown to exert a noxious effect in the initiation and progression of cell death.

Similar studies have evidenced that PGE₁ pre-administration protects against NO-dependent cell death through a rapid increase of inducible-NO synthase (NOS-2) expression which protected against DGA-induced cell death. The factors that inhibit hepatic injury may be, those intensifying the hepatic antioxidants or free radical scavengers and those inducing hepatocyte regeneration.
6. Conclusion

PGE\textsubscript{1} can effectively protect the hepatocytes from oxidation induced death through scavenging intracellular oxidative damage induced by ROS.

Conflict of interest

There is no conflict of interest or financial ties to include.

References


