Expression of p53 during Apoptosis Induced by D-Galactosamine and the Protective Role of PGE1 in Cultured Rat Hepatocytes

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Abstract: p53 is a critical player in the prevention of tumor development. It can contribute directly to DNA repair and inhibition of angiogenesis and subsequently to the induction of apoptosis. The regulation of p53 expression is mediated by the transcription factor NF-κB. This includes regulation of p53 protein stability, control of its subcellular localization and conformational changes that allow activation of the DNA binding activity of p53. Rat hepatocytes were isolated from male Wistar rats following collagenase perfusion of liver. We examined the change in the expression level of p53 by western blotting in hepatocytes and its effect on apoptosis as a response of treatment with D-galactosamine, prostaglandin E1 and/or the Proteosome Inhibitor (PSI). A kinetic study of the extracellular lactate dehydrogenase activity, NF-κB activation, induced nitric oxide synthase expression and nitric oxide production was carried out in hepatocytes. The addition of prostaglandin E1 to control and D-galactosamine-treated hepatocytes increased p53 expression in the cytoplasm during 24 h. While the addition of PSI in the absence of prostaglandin E1 decreased p53 expression at 5 mM D-galactosamine. This inhibition is reversed in the presence of prostaglandin E1 at 5 and 40 mM D-galactosamine. The protective action of prostaglandin E1 against the apoptotic effect of D-galactosamine is mediated by NF-κB activation, induced nitric oxide synthase and p53 expression.

Key words: p53, prostaglandin E1, D-galactosamine, apoptosis and necrosis, primary culture of rat hepatocytes

INTRODUCTION

Apoptosis is the process of programmed cell death that may occur in multicellular organisms and is involved in the normal regulation of organ size. It is controlled by a diverse range of extracellular and/or intracellular cell signals that affect some biochemical reactions. The consequences of these reactions lead to the formation of blebs, cell shrinkage, nuclear fragmentation, chromatin condensation, DNA fragmentation and the formation of apoptotic bodies. D-galactosamine (D-GalN) is proved to be a cause of experimentally induced apoptosis in rat hepatocytes and PGE1 was found to have a protective role (Fouad et al., 2004; Siendones et al., 2004). Many transcription factors are involved in cellular responses, among of them are NF-κB and p53. Moreover, PGE1 reduces the experimentally-induced liver injury.

p53 is a transcription factor that play a critical role in signaling pathways controlling the cell cycle (Joerger and Fersht, 2010). It can contribute directly to DNA repair (Albrecht et al., 1999), inhibition of angiogenesis (Vogelstein et al., 2000) and maintain the integrity of the human genome (Vousden and Prives, 2009). The regulation of p53 expression is mediated by nuclear factor kappa-B (NF-κB) (Webster and Perkins, 1999). This includes regulation of p53 protein stability, control of its subcellular localization and conformational changes that allow activation of the DNA binding activity of p53 (Balint and Vousden, 2001).

The NF-κB is a transcriptional factor composed of two subunits and is involved in the prevention of apoptosis. In unstimulated cells, the NF-κB dimers are sequestered in the cytoplasm by a family of inhibitors, called IκBs (Inhibitor of κB) which mask the nuclear localization signals of NF-κB keeping them inactive in the cytoplasm (Jacobs and Harrison, 1998). The phosphorylation of IκBα by certain kinase leads to its degradation; hence NF-κB is then freed to enter the

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nucleus where it can turn on the expression of specific genes that have DNA-binding sites for NF-κB. Such activation of gene expression leads to some biochemical responses; some of them positively affect cellular proliferation causing protection from cell death (Mercurio et al., 1999).

Hepatic injury induced by D-GalN is a suitable experimental model of human liver failure (Quintero et al., 2002). D-GalN has been shown to induce specific hepatocyte cell death in vivo (El-Mofy et al., 1975; Tsutsui et al., 1997; Muntane et al., 1998, 2000a) and in vitro (Tran-Thi et al., 1985; Fouad et al., 2004).

Several studies have reported that PGE1 (Fouad et al., 2004), luteolin (Lee et al., 2011), genipin (Kim et al., 2010), diadzin (Kim et al., 2009) and etoposide (Nakama et al., 2001) have a protective role against apoptosis induced by D-galactosamin in hepatic failure in several experimental models. PGE1 is biologically active polyunsaturated fatty acids derived from arachidonic acid and presents in most mammalian tissues (Bergstrom et al., 1968). It reduces liver injury induced experimentally (Fouad et al., 2004) and fulminant viral hepatitis in humans (Sinelair et al., 1989). It has been suggested that membrane stabilization (Stachura et al., 1981), antifibrogenic (Miura and Fukui, 1979), hepatocyte proliferation (Greig et al., 1989), vasodilatory properties (Ruwart et al., 1988) and the induction of Inducible Nitric Oxide Synthase (iNOS) in hepatocytes (Muntane et al., 2000a) may be involved in their protective mechanism. Moreover, PGE1 reduces liver injury conducted by chemical other than D-GalN (Bergasa et al., 1992; Rush et al., 1989; Stachura et al., 1981).

The purpose of the present study was to evaluate the role of p53 in PGE1 protection against cell death induced by D-GalN in primary culture of rat hepatocytes. In addition, we will determine if the previously reported association between NF-κB activation and PGE1 protection against D-GalN apoptosis was mediated by p53 expression.

**MATERIALS AND METHODS**

**Materials:** All reagents were from Sigma Chemical Co. (St. Louis, MI) unless otherwise stated. William’s medium E was from AppliChem (Darmstadt, Germany). Antibiotics-antimycotic solution and fetal bovine serum were from Invitrogen Ltd. (Paisley, UK). The inhibitor of chymotrypsin-like activity of the proteasome (PSI), Cbz-Ile-Glu(Ot-Bu)-Ala-leucinal, was obtained from Calbiochem-Novabiochem Corporation (Darmstadt, Germany). Male Wistar rats (body weight 225-275 g) were used for the experiments. Animals were kept on standard laboratory conditions. The experiments were conducted in accordance with the Spanish legislation on protection of animals and the NIH Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council).

**Preparation of primary hepatocytes and cell culture:** Hepatocytes were isolated from male Wistar rats (225-275 g) by a non-recirculating in situ collagenase perfusion of liver cannulated through portal vein following a protocol described by Seglen et al. (1976). Hepatocytes were plated in a petri dish coated with collagen type I (Iwaki, Gyouda, Japan) at a density of 150000 cells cm⁻² and cultured in William’s medium E, pH 7.4, supplemented with 1 mM insulin, 0.6 mM hydrocortisone, 15 mM HEPES, 100 U mL⁻¹ penicillin, 100 mg mL⁻¹ streptomycin, 0.25 mg mL⁻¹ amphoteric 2 mM glutamine and 26 mM NaHCO₃. A kinetic study was first carried out on the effect of 2 h preadministered PGE1 (1 mM) (Alprostadil, Pharmacia and Upjohn, Puurs, Belgium) on cell cytotoxicity, p53 expression induced by D-GalN (5 or 40 mM). In particular, D-GalN-induced apoptosis and necrosis was assessed at 1, 3, 6, 12 and 24 h after hepatotoxin administration. The expression of p53 in cytoplasm was determined at 0, 1, 3, 6, 12 and 24 h after D-GalN administration. Afterwards, we studied the effect of NF-κB inhibition using a PSI on PGE1-related cytoprotection against D-GalN-induced cell death and iNOS expression in cultured rat hepatocytes. As previously described (Quintero et al., 2002), although D-GalN (5 mM) induced a strong apoptotic signal, cell necrosis is mild being only relevant at 24 h after D-GalN treatment. In this sense, PSI (5 mM) was administrated 2 h before PGE1 or 4 h before D-GalN collecting the samples 24 h after hepatotoxin administration.

**Measurement of lactate dehydrogenase (LDH) release:** Extracellular LDH, as index of necrosis, was measured in the culture medium according to the method of Tafiss and Sítkovsky (1991).

**DNA fragmentation:** DNA fragmentation was used as an index of apoptosis. The whole hepatocytes were treated for 10 min with 1 mL of cold lysis buffer (100 mM Tris-HCl, 5 mM EDTA, 150 mM NaCl and 0.5% sarkosyl, pH 8.0), incubated at 37°C for 2 h with RNase (50 mg mL⁻¹) and at 48°C for 45 min with protease K (100 mg mL⁻¹). DNA was extracted by phenol:chloroform:isoamyl alcohol (25:24:1). One hundred mg DNA were separated by electrophoresis in 1.5% agarose gel containing ethidium bromide and visualized in UV transilluminator.
Preparation of nuclear extracts and electrophoretic mobility shift assay: Nuclear extracts from hepatocytes were prepared according to Schreiber et al. (1989) with some modifications. Electrophoretic Mobility Shift Assay (EMSA) was performed using the NF-κB consensus oligonucleotides 5'-AGTTGAGGGGACTTCTCCAGGC-3' and 3'-TCAACTCCCTTGAAGGGTCCG-5' (Promega, Madison, WI) as previously described (Fouda et al., 2004).

p53 protein expression by western blotting: The whole hepatocyte population was recovered in 800 μL of lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 5 μg mL⁻¹ aprotinin, 10 μg mL⁻¹ leupeptin, 0.5 mM phenylmethylsulfonyl fluoride and 1 mM DTT) allowing to swell on ice for 15 min. Afterwards, 50 μL of 10% Nonidet NF-40 was added to samples, vigorously vortexed for 30 sec and centrifuged at 15 000 x g for 1 min at 4°C. The supernatant, as cytoplasmatic fraction, was collected and frozen at -80°C.

The expression of p53 in hepatocytes was monitored by western blotting. One hundred microgram protein of the cytoplasmatic fraction per lane were loaded on sodium dodecyl sulphate polyacrylamide gels (SDS-PAGE) according to Laemmli (1970). The separated proteins were transferred to nitrocellulose membrane (Amersham™ Hybond™-ECL) according to Towbin et al. (1979). After blockade of nonspecific binding sites, membranes were incubated for 2 h at room temperature with the primary anti-p53 (Santa Cruz Biotechnology) followed by anti-rabbit IgG-horseradish peroxidase as the secondary antibody (Santa Cruz Biotechnology). Protein expression was visualized by means of luminal enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech) and the membrane was exposed to autoradiography film (Hyperfilm ECL, Amersham).

NO metabolite measurement: The release of NO to the culture medium was indirectly assessed by quantification of its related metabolite, NOx. In the assay, nitrate was converted to nitrite by nitrate reductase and total nitrite was measured using the Griess reaction (Green et al., 1982).

Caspase-3 processing and associated activity: The caspase-3 processing was detected in the cytoplasmatic fraction by Western blot as previously described using polyclonal primary antibody (antisapase-3, Santa Cruz Biotechnology, California, USA). The caspase-3-associated activity was measured in the cytoplasmatic fraction colorimetrically using the peptide-based substrate Ac-N-acetyl-Asp-Glu-Val-Asp-p-nitroanilide (Bachem AG, Bubendorf, Switzerland). The activity was monitored spectrophotometrically at 405 nm by measuring the increase in absorbance due to the release of pNA for 10 min.

iNOS protein expression: The crude hepatocyte extract (100 mg) were separated by 5% SDS-PAGE and transferred to nitrocellulose. The iNOS was detected using mouse monoclonal primary antibodies (BD Transduction Laboratories, Erembodegem, Belgium) and anti-mouse-IgG-horseradish peroxidase (Santa Cruz Biotechnology) as secondary antibody revealing protein content by ECL.

Statistical analysis: Results are expressed as means with their corresponding standard errors. Differences between groups were assessed by the one-way Analysis of Variance (ANOVA) using the Least Significant Differences (LSD) test as multiple comparison analysis.

RESULTS

The effect of PGE1 on apoptosis and necrosis induced by D-GalN: The hepatotoxin D-GalN has been shown to induce cell death in primary culture of rat hepatocytes (Tran-Thi et al., 1985; Quintero et al., 2002). In the present study, the addition of 40 mM D-GalN caused DNA fragmentation on the primary culture of hepatocytes after 24 h compared to the control (Fig. 1a). The addition of the Proteosome Inhibitor (PSI) 4 h before or 2 h after treatment with D-GalN increased DNA fragmentation. The same experiment was repeated with the addition of PGE1 before any treatment. There is no clear change in DNA fragmentation with PGE1 comparing with the samples treated with 40 mM D-GalN. The DNA fragmentation was noticed also in samples supplemented with PSI 4 h before D-GalN addition.

It has been reported that apoptosis is induced by the inhibition of NF-κB activation. In this experiment we inhibited NF-κB activation by preventing the dissociation of its cytoplasmic coupled inhibitor IkBα. This inhibition is performed by the addition of PSI which results in the prevention of passage of NF-κB to the nucleus. The treatment with 40 mM D-GalN causes DNA fragmentation as an apoptotic marker. This fragmentation is increased in by the addition of PSI. This enhancement of DNA fragmentation was significantly associated with higher expression of caspase-3 active fragment (Fig. 1b) and caspase-3-associated activity (Fig. 1c).

LDH is an intracellular enzyme. The release of LDH to the culture media indicates cell necrosis. The effect of
Fig. 1(a-c): Effect of PSI in PGE1 protection against DNA fragmentation (a), procaspase-3 processing (b) and its activity (c) induced by D-GalN in cultured hepatocytes. *PSI administered 4 h before D-GalN, **PSI administered 2 h after D-GalN in cultured hepatocytes as described in Materials and methods. DNA fragmentation and procaspase-3 processing and its activity were used as indexes of apoptosis in cultured hepatocytes. The images are representative of five experiments. *(p ≤ 0.05 vs the corresponding non-PSI-treated group)

PGE1 addition on cell necrosis was monitored by measuring the extracellular LDH. The present study showed large increase in LDH which indicates that the addition of 40 mM D-GalN directed the cells to necrosis and hence nonsignificant protection by PGE1 was noticed (Fig. 2).

Effect of PGE1 and PSI on p53 expression: The addition of PGE1 increased the expression of p53 in all tested times (Fig. 3). This finding suggests the presence of some kinds of relationship between the effect of PGE1 on the reduction of apoptosis and the activation of the NF-κB through the expression of p53. Furthermore, PSI increased p53 expression in the presence of PGE1 and D-GalN (40 mM) (Fig. 4).

Effect of PSI on iNOS expression: The expression of iNOS in hepatocytes and NOx content in culture medium were evaluated after treatment with D-GalN and/or PGE1 (Fig. 5, 6). The addition of PSI to control and D-GalN-treated hepatocytes (lane 2 and 3) increased iNOS expression. PGE1 increased iNOS after treatment with D-GalN (lane 4). In particular, the expression of iNOS was higher in PGE1+D-GalN than D-GalN-treated hepatocytes. The enhancement of iNOS expression by PGE1 in D-GalN-treated hepatocytes was related to high concentration of NOx in culture medium in the first 3 h (Fig. 6).
Fig. 3: Effect of the treatment with PGE1 in the cytoplasmic expression of p53 in control and D-GaIN treated rat hepatocyte primary culture.

Fig. 4: Effect of NF-κB proteosome inhibitor, PSI on p53 expression in cultured hepatocytes treated with D-GaIN and/or PGE1. *PSI administrated 4 h before D-GaIN, **PSI administrated 2 h after D-GaIN.

Fig. 5: Effect of PSI on PGE1-induced inducible NOS protein expression in DGalN-treated hepatocytes. *PSI administered 4 h before **PSI administrated 2 h after D-Galn in cultured hepatocytes as described in Materials and methods.

Fig. 6: Kinetic study of NOx production in cultured hepatocytes treated with 40 mM D-GalN and/or PGE1. PGE1 (1 mM) was administrated 2 h before D-GalN in cultured hepatocytes as described in materials and methods. Data are the Mean±SD of 10 experiments.
**Fig. 7:** Kinetic study of NF-κB activation in cultured hepatocytes treated with 40 mM D-GaIN and/or PGE1. PGE1 (1 mM) was administered 2 h before D-GaIN in cultured hepatocytes as described in Materials and methods.

**Activation of NF-κB:** The administration of D-GaIN (40 mM) increased the activation of NF-κB at all times (Fig. 7). The administration of PGE1 increased the activation of NF-κB in the zero time and after 1 h of treatment with D-GaIN, then, PGE1 reduced NF-κB activation after 3, 6, 12 and 24 h.

**DISCUSSION**

We have previously shown that PGE1 protects cultured rat hepatocytes from the apoptotic effect of D-GaIN through NF-κB-mediated stimulation of NO production (Siendones et al., 2003; Fouad et al., 2004). In the present study, we have shown that this effect was associated with an increase in p53 expression as indicated by the kinetic study in the period from 0-24 h following PGE1 administration. It has been reported that there is a rapid expression of p53 after DNA damage (Emanuel and Scheinfeld, 2007). This increase in p53 expression may be explained by its role on regulation of cell cycle and DNA repair. p53 stimulates the transcription of several genes that mediate cell-cycle arrest and apoptosis causing a pause in cell cycling, giving enough time to the cells to repair the DNA damage. This p53 also helps in the DNA repair process. If the DNA damage cannot be successfully repaired during the pause in cell division, normal p53 induces apoptosis (Onel and Cordon-Cardo, 2004).

D-GaIN is a suitable experimental model of liver injury based on its capacity to reduce the intracellular pool of uracil nucleotides in hepatocytes, thus inhibiting the synthesis of RNA and proteins (Keppler et al., 1968). D-GaIN induces hepatocyte cell death in vivo (Muntane et al., 2000b; Vasanth Raj et al., 2010) and in vitro (Tran-Thi et al., 1985; Quintero et al., 2002). The large increase of LDH in the culture medium indicates that the addition of 40 mM D-GaIN directed the cells to necrosis and, hence, release of LDH out of the cells. This means that PGE1 nonsignificantly protected cells from death and hence it was directed to necrosis (Fig. 2). The results obtained in the kinetic study 0-24 h after D-GaIN cytotoxicity may be consequence of the moderate intracellular oxidative stress induced by D-GaIN which could cause a shift from an apoptotic to necrotic pathway as suggested in some models of cell death in cultured hepatocytes (Samali et al., 1999; Quintero et al., 2002). It has been reported that PGE1 does not exert cytoprotection against cell necrosis when the shift from apoptosis to necrosis occurred (Fouad et al., 2004). Other intracellular mediators, such as NO, play an important role during apoptosis in liver (Clemens, 1999). The present study indicated that the PSI increased the expression of p53. It is documented that PSI inhibits NF-κB activation and abolishes both PGE1-dependent iNOS expression and cell protection in D-GaIN treated hepatocytes (Fouad et al., 2004). The cytoprotective properties of PGE1 have been related to its capacity to enhance iNOS expression and NO production in hepatocytes (Kim et al., 2000; Siendones et al., 2003).

In summary, D-GaIN caused a significant increase in p53 levels in cultured hepatocytes when compared with the normal control. Such result coincides with the in vivo study performed by Vasanth Raj et al. (2010). p53 plays a key role in the process of apoptosis, p53-mediated apoptosis involves multiple mechanisms. D-GaIN intoxication up regulated the p53 levels and PGE1 may increase such upregulation. In conclusion, p53-dependent NF-κB activation and iNOS expression induced by PGE1 preadministration was responsible for the protective effect of the prostanoit against D-GaIN-induced apoptosis.

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