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Fructose 1,6-bisphosphate reduced TNF-α-induced apoptosis in galactosamine sensitized rat hepatocytes through activation of nitric oxide and cGMP production

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ABSTRACT

Fructose 1,6-P2 (F1,6BP) protects rat liver against experimental hepatitis induced by galactosamine (GalN) by means of two parallel effects: prevention of inflammation, and reduction of hepatocyte sensitization to tumour necrosis factor-alpha (TNF-α). In a previous paper we reported the underlying mechanism involved in the prevention of inflammation. In the present study, we examined the intracellular mechanisms involved in the F1,6BP inhibition of the apoptosis induced by TNF-α in parenchyma cells of GalN-sensitized rat liver. We hypothesized that the increased nitric oxide (NO) production in livers of F1,6BP-treated rats mediates the antiapoptotic effect. This hypothesis was evaluated in cultured primary rat hepatocytes challenged by GalN plus tumour necrosis factor-alpha (GalN + TNF-α), to reproduce *in vitro* the injury associated with experimental hepatitis. Our results show a reduction in apoptosis concomitant with an increase in NO production and with a reduction in oxidative stress. In such conditions, guanylyl cyclase is activated and the increase in cGMP reduces the TNF-α-induced apoptosis in hepatocytes. These results provide new insights in the protective mechanism activated by F1,6BP and confirm its interest as a hepatoprotective agent.

1. Introduction

The glycolytic intermediate fructose 1,6-bisphosphate (F1,6BP) protects multiple organs and tissues against injuries produced by a wide range of insults, of both physiological and chemical origin (Lazzarino et al., 1992; Roig et al., 1994; Sano et al., 1995; Ahn et al., 2002; Nunes et al., 2002; Vexler et al., 2003). This protection increases the interest of F1,6BP as a therapeutic agent and a component of graft-preservation solutions (Roig et al., 1994; Sano et al., 1995; Sola et al., 2001; Nunes et al., 2003; Bordignon et al., 2003; Sola et al., 2004; Alves Filho et al., 2004; Moresco et al., 2004; Genesca et al., 2005; Cuesta et al., 2006; Lopes et al., 2006; Gamez et al., 2008).

Empirical evidence indicates that the protective action of F1,6BP in stress situations is explained by its incorporation as an energy substrate and by the prevention of critical alterations in membrane function. The relative contribution of each factor depends on the cell type and the challenge. Results obtained with vascular smooth muscle (Hardin and Roberts, 1994) and myocardial cells (Hardin et al., 2001) indicate that F1,6BP is taken up and incorporated as a glycolytic substrate. On the other hand, F1,6BP does not contribute to glycolytic metabolism in brain cells, and its protective action is associated with its effect on the cell membrane (Donohoe et al., 2001; Fahlman et al., 2002). In accordance with these results, we and others have shown that the protective action of F1,6BP against hepatic injury involves both energy metabolism and cell-membrane function of liver parenchyma cells (De Oliveira et al., 1992), as well as of other cells involved in liver inflammation (Hirokawa et al., 2002; Nunes et al., 2003; Cuesta et al., 2006).

We have studied the mechanisms underlying the protective effects of F1,6BP in experimental hepatitis induced by galactosamine (GalN) in rats (Cuesta et al., 2006). The liver specificity of GalN is attributable to the high levels of galactokinase and UDP-glucose:galactose-1-P-uridyltransferase in hepatocytes (Keppler and Decker, 1969). GalN metabolism depletes the uridine pool of hepatocytes, which inhibits transcription and protein synthesis (Keppler et al., 1970). Transcriptionally arrested hepatocytes are highly sensitive to cytokines such as TNF-α (Leist et al., 1994). Moreover, GalN metabolism induces mitochondrial dysfunction (Quintero et al., 2002), increasing free-radical production and inducing caspase 3 activation (Siendones et al., 2005), which contributes to the sensitization of hepatocytes to pro-inflammatory cytokines. Additionally, GalN targets other cell types when administered to rats, such as mast cells and macrophages. The activation of these cells induces endotoxemia and inflammation, with the subsequent increase of cytokines in plasma and tissues (Cuesta et al., 2006). Thus, the main cause of liver injury in experimental hepatitis is the extensive apoptosis induced by TNF-α in the sensitized liver parenchyma cells (Leist et al., 1994; Li and Billiar, 1999).

In an earlier study we reported that F1,6BP reduced TNF-α-induced apoptosis in the livers of GalN-challenged rats (Cuesta et al., 2006). Both the protective effect of F1,6BP and the hepatotoxic action of GalN have been associated with the capacity of these agents to increase NO production (Mihas et al., 1997; Rao et al., 1998; Sola et al., 2001; Mihas

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et al., 2003; Siendones et al., 2003; Siendones et al., 2004). Therefore, the opposite effects of F1,6BP and GalN suggest other targets inside the cells for these compounds in addition to NO activation. It is known that GalN increases oxidative stress in hepatocytes and that TNF-α exacerbates this effect impairing mitochondrial function (Galanos et al., 1979; Chojkier and Fierer, 1985; Leist et al., 1995; Angermuller et al., 1999). In this paper, we hypothesized that the capacity of F1,6BP to increase nitric oxide (NO) production (Roig et al., 1994; Sano et al., 1995; Rao et al., 1998; Li and Billiar, 1999; Sola et al., 2001), to improve mitochondrial function (Sano et al., 1995), and to prevent oxidative stress (Gamez et al., 2008) mediates its antiapoptotic effect in the liver. We evaluated our hypothesis in cultured primary rat hepatocytes. Cultures of primary hepatocytes obtained by the collagenase or liberase perfusion method produce cells that exhibit a wide range of functions characteristic of hepatocytes in intact liver. Primary hepatocyte cultures have been accepted for many years as suitable models of liver parenchyma cells in ex-vivo studies (Bartrons et al., 1983).

2. Materials and methods

2.1. Animals and treatment protocols

Male Sprague–Dawley rats, weighing 150–250 g, were maintained under standard conditions and fed water and standard diet *ad libitum*. All animals were given humane care in compliance with the guidelines of the Experimental Animal Ethics Committee of the University of Barcelona.

2.2. Isolation and culture of primary hepatocytes

Animals were anesthetized with ketamine/xylazine i.p. Primary hepatocytes were obtained by a modification of the collagenase perfusion method (Bartrons et al., 1983), in which collagenase was replaced by liberase. Cell viability, as assessed by the trypan blue exclusion criterion, was always higher than 90%.

2.3. Culture of primary hepatocytes

The isolated cells were placed in collagen-precoated plates. The plates and cell densities were selected in accordance with the instructions of suppliers and authors. Culture medium was Williams' E medium supplemented with 1 nM dexamethasone, 1 nM insulin, 50 mg/ml gentamicin and 1 mM L-glutamine, containing 10% foetal calf serum to facilitate cell recovery and adhesion. Cultures were incubated in a humidified atmosphere with 5% CO_2 at 37 °C.

After 2 h, the medium was removed and replaced by fresh supplemented medium without foetal calf serum so as to avoid cell differentiation. After 16 h, primary hepatocytes plated at 10^5 cell/well (50,000 cell/cm^2) showed a mean of 450 ± 134 apoptotic cell/well, when evaluated by flow cytometry. This mean apoptotic value of controls corresponds to 0.30 ± 0.02 of mean absorbance at 405 nm, when determined apoptosis by DNA fragmentation kit.

2.4. Nitric oxide determination

Nitric oxide (NO) is a free radical with a short half life. NO_2^- and NO_3^- are the final products of its reduction. Accurate data for NO production associated with treatments must be assessed by quantification of its end products $NO_2^- + NO_3^-$ (NO_x) inside the medium. In the assay, nitrate was converted to nitrite by nitrate reductase and total nitrite was measured using the Griess reaction (Muntane et al., 2000; Siendones et al., 2003).

For NO_x determinations, primary hepatocytes were placed in 24-well plates at 80,000 cell/cm^2 and incubated with the corresponding treatment for 16 h. To inhibit NO synthesis, hepatocytes were incubated with *N^G-monomethyl L-arginine* (NMMA, Sigma, St. Louis, MO) 1.5 mM for 24 h. The NO donor, *S-nitroso-N-acetyl-penicillamine* (SNAP, Sigma, St. Louis, MO) was used at 1.2 nM. NO_x was measured in the culture medium with the Nitrate/Nitrite Fluorometric Assay kit (Cayman Chemical Company, Alexis, Switzerland). Lysate *L-Lactate Dehydrogenase* (LDH) content was determined using Cytotoxicity Detection Kit (Roche, Basel, Switzerland).

2.5. DNA fragmentation

Cells were placed in 24-well plates at 50,000 cell/cm^2. Following 16 h of challenge the supernatant was collected and centrifuged (200 ×*g*), and the pellet was washed in PBS. The cell pellet and washed cells remaining in the wells were lysed and added to microtiter plates as described in the ELISA kit. DNA fragmentation was quantified by Cell Death Detection ELISA^{PLUS} (Roche, Basel, Switzerland). The enrichment of mono- and oligonucleosomes (histone-associated-DNA-fragments) released into the cytoplasm was calculated as the ratio of the absorbance of the sample cells/absorbance of control cells. The enrichment factor was used as a parameter of apoptosis (an enrichment factor of 1 represents background or spontaneous apoptosis). The non-specific caspase inhibitor *Z-Val-Ala-DL-Asp-fluoromethylketone* (zVAD, Bachem AG, Bubendorg, Switzerland) (20 μM), and the apoptosis inductor *Transforming Growth Factor-beta* (TGF-β, Sigma, St. Louis, MO) (2 ng/ml) were used as controls.

2.6. Detection of reactive oxygen species

Primary hepatocytes placed in 12-well plates at 100,000 cell/cm^2 were challenged for 3 h. Cultures were then washed twice in Hanks' Balanced Salt Solution (HBSS: KCl 5 mM, $Na_2HPO_4 \cdot 2H_2O$ 0.3 mM, $NaHCO_3$ 3.2 mM, NaCl 0.14 M, D-glucose 5.5 mM, pH 7.4) and loaded with HBSS containing 5 μmol/l of *2′,7′-dichlorodihydrofluorescein diacetate* (H_2DCFDA, Molecular Probes, Invitrogen Ltd. Albany, NY) for 30 min at 37 °C with 5% CO_2. The oxidation of nonfluorescent H_2DCFDA to the highly fluorescent *2′,7′-dichlorofluorescein* is commonly used to detect reactive oxygen intermediates (Reinehr et al., 2005). The flavoprotein inhibitor diphenyleneiodonium (DPI), 5 μM, was used to inhibit NADPH oxidase-dependent fluorescence. After loading, cells were washed briefly in ice-cold HBSS, and then lysed in 0.1% Triton X-100 (v/v). Lysates were centrifuged immediately (10,000 ×*g*, 4 °C, 1 min) and the 440 nm/520 nm fluorescence was measured.

2.7. Quantification of GSH/GSSG

Primary hepatocytes placed in 10 cm diameter Petri dishes at 80,000 cell/cm^2 were treated for 3 h. They were then washed twice in PBS and lysed in 0.1% Triton x-100 (v/v). Lysates were centrifuged immediately and the GSH/GSSG content was measured following the *o-phthaldialdehyde fluorometric* (OPT, Sigma, St. Louis, MO) procedure described elsewhere (Senft et al., 2000).

2.8. cGMP enzyme immunoassay

Primary hepatocytes were placed in 12-well culture plates at 100,000 cell/cm^2. Following 3 h of treatment, cells were washed twice in PBS and lysed with 0.1 M HCl. After 20 min, HCl extracts were collected, centrifuged (1000 ×*g*) and analyzed for cyclic guanosine monophosphate (cGMP) using the cGMP Enzyme Immunoassay kit (Cayman Chemical Company, Alexis, Switzerland). The absorbance from lysates was measured at 405 nm. The guanylyl cyclase inhibitor ODQ (20 μM) and the cGMP analogue 8Br-cGMP (800 μM) were used as controls. Protein content from lysates was determined using *BCA Protein Assay* (Pierce, Switzerland).

2.9. Data analysis

Results are shown as means ± S.D. of the values obtained from the number of experiments indicated in each case. The differences between groups were tested by *t*-Student test and the appropriate a