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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

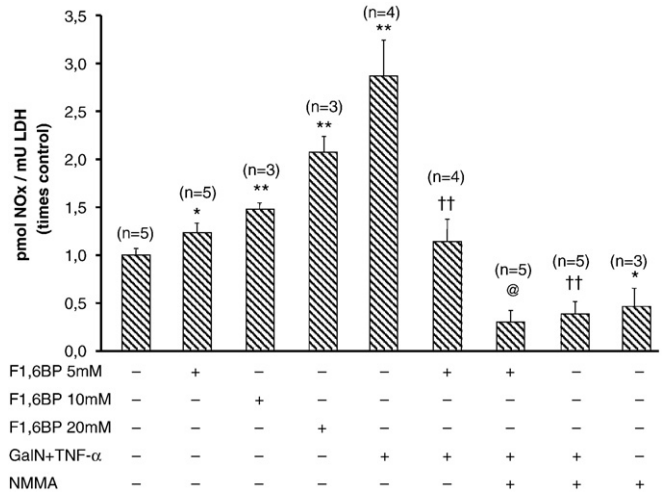

Fig. 1. F1,6BP activated NO production by cultured primary rat hepatocytes. F1,6BP (5 mM, 10 mM and 20 mM) increased NO production 1.25, 1.4 and 2.2-fold respectively, whereas GalN + TNFα increased NO production 2.7-fold. The GalN + TNFα-induced increase of NO production in hepatocytes was significantly reduced in the presence of F1,6BP. Results are means ± S.D. of the indicated number of experiments. *P<0.05 and **P<0.01 vs control; $^{\dagger\dagger}P$<0.01 vs GalN + TNFα; $^{@@}P$<0.01 vs F1,6BP + GalN + TNFα.

priori contrast methods. For the analysis of longitudinal data, repeated-measures ANOVA was used. Differences between groups were considered significant at P<0.05.

3. Results

3.1. F1,6BP enhanced NO production and inhibited apoptosis in hepatocytes

To evaluate the role of the F1,6BP-induced NO production in the inhibition of hepatocyte apoptosis observed in GalN-challenged rats (Cuesta et al., 2006), primary rat hepatocytes were treated with GalN (5 mM) and TNF-α (20 ng/ml) (GalN + TNF-α) either in the presence or in the absence of F1,6BP (5 mM). The resulting NO production and apoptosis were then compared. F1,6BP induced a dose-dependent increase in NO production in cultured primary rat hepatocytes (Fig. 1) and reduced the spontaneous apoptosis observed in controls (Fig. 2). In contrast, the increased NO production in GalN + TNF-α challenged hepatocytes (Fig. 1) correlated with an enhancement of apoptosis (Fig. 2). The non-specific caspase inhibitor zVAD abolished GalN + TNF-α-induced apoptosis, suggesting that caspase activation was the major effector of this apoptosis in hepatocytes (Fig. 2).

F1,6BP reduced 0.6 fold the GalN + TNF-α-induced apoptosis in hepatocytes (Fig. 2). To ascertain whether NO overproduction mediates the protective action of F1,6BP, we treated cultured primary hepatocytes with NMMA (1.5 mM) to avoid NOS activation and with the NO donor SNAP (1.2 nM) instead of F1,6BP. The inhibition of NO synthesis by NMMA reduced the antiapoptotic effect of F1,6BP, whereas SNAP mimicked the protective effect of F1,6BP against GalN + TNF-α-induced apoptosis (Fig. 2). Taken together, these data confirm that NO production mediates the effect of F1,6BP against GalN + TNF-α-induced apoptosis in hepatocytes.

Table 1
Effect of F1,6BP on radical production and on the redox status of cultured primary rat hepatocytes challenged by GalN + TNFα.

		Challenge			
		Control	F1,6BP	GalN + TNFα	F1,6BP + GalN + TNFα
Relative increase in H_2DCFDA fluorescence	− DPI	Set as 1 ± 0.08 (n = 20)	0.81 ± 0.11[b] (n = 5)	1.76 ± 0.45[c] (n = 10)	1.34 ± 0.32[d] (n = 11)
	+ DPI	1.01 ± 0.24 (n = 11)	0.96 ± 0.30 (n = 5)	1.12 ± 0.28 [g] (n = 11)	1.08 ± 0.34[f] (n = 11)
GSH(10^{-6} M)		42.5 ± 1.7 (n = 6)	63.3 ± 9.3[a] (n = 3)	18.3 ± 2.6[b] (n = 6)	43.5 ± 7.9[e] (n = 6)
GSSG(10^{-6} M)		0.85 ± 0.11 (n = 6)	0.64 ± 0.01 (n = 3)	1.96 ± 0.35[a] (n = 6)	1.26 ± 0.15[a] (n = 6)
GSH/GSSG		50.1 ± 1.8 (n = 6)	98.6 ± 9.3[b] (n = 3)	9.4 ± 3.0[b] (n = 6)	34.6 ± 8.1[e] (n = 6)

DPI-inhibitable free-radical production, as assessed by H_2DCFDA fluorescence 3 h following treatment, indicates free-radical production associated to flavoprotein activity, such as NADPH oxidase. The ratio GSH/GSSG, associated with changes in GSH and GSSG content, indicates the reducing power of cells.
([a]P<0.05, [b]P<0.01 and [c]P<0.01 vs. control; [d]P<0.05 and [e]P<0.01 vs. GalN + TNFα; [f]P<0.05 and [g]P<0.001 vs. challenge − DPI).

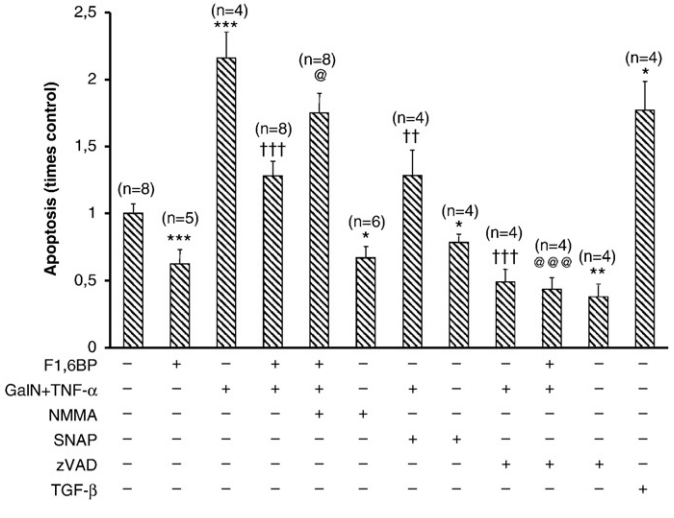

Fig. 2. Antiapoptotic effect of F1,6BP. F1,6BP (5 mM) reduced the spontaneous and the GalN + TNFα (5 mM and 20 ng/ml)-induced apoptosis in primary cultures of rat hepatocytes, as assessed by DNA fragmentation 16 h following challenge. NMMA (1.5 mM) reduced the antiapoptotic effect of F1,6BP, whereas SNAP (1.2 nM) mimicked the effect of F1,6BP. The caspase inhibitor zVAD (20 μM), and the apoptosis inductor TGF-β (2 ng/ml) were used as controls. Results are means ± S.D. of the indicated number of experiments. *P<0.05. **P<0.01 and ***P<0.001 vs control; $^{\dagger\dagger}P$<0.01 and $^{\dagger\dagger\dagger}P$<0.001 vs GalN + TNFα; $^{@}P$<0.05 and $^{@@@}P$<0.001 vs F1,6BP + GalN + TNFα.

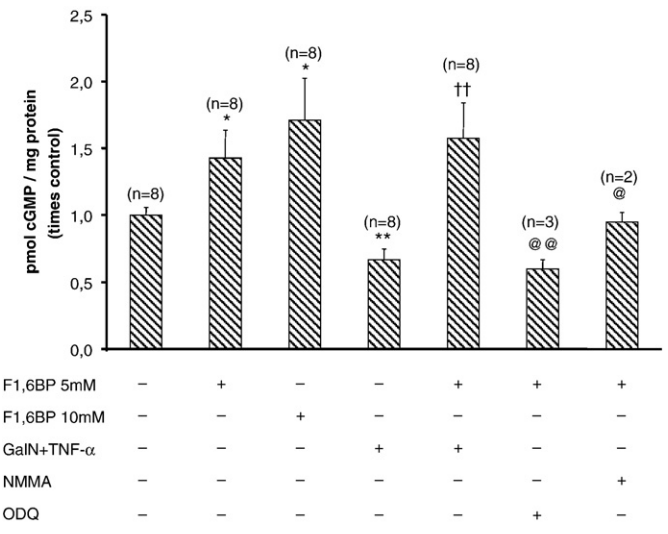

Fig. 3. F1,6BP increased cGMP in cultured primary rat hepatocytes. F1,6BP 5 mM and 10 mM increased cGMP pools 1.4 and 1.7-fold respectively. In addition, F1,6BP prevented the GalN + TNFα-induced reduction of cGMP in challenged hepatocytes. Moreover, F1,6BP did not increase cGMP in hepatocytes treated with the NO synthase inhibitor, NMMA (1.5 mM). The guanylyl cyclase inhibitor ODQ (20 μM) was used as control. Results are means ± S.D. of the indicated number of experiments, of cGMP measured 3 h after challenge. *P<0.05 and **P<0.01 vs. control; $^{\dagger\dagger}P$<0.01 vs. GalN + TNFα; $^{@}P$<0.05 and $^{@@}P$<0.01 vs. F1,6BP (5 mM).

Both F1,6BP and GalN + TNF-α treatments increased NO production in hepatocytes, with contrary effects. Therefore, the discriminatory target must be downstream of NOS activation. The beneficial effect of NO overproduction requires concomitant inhibition of free-radical production in the cells (Meurer et al., 2005; Gerassimou et al., 2007). In addition, an excess of NO in an oxidative milieu generates the hyper-reactive free-radical peroxynitrite, which increases oxidative stress and activates apoptosis pathways (Guzik et al., 2002). To evaluate the effect of F1,6BP and GalN + TNF-α on the redox status of hepatocytes we measured the production of reactive oxygen species, both in the presence and in the absence of the NADPH oxidase inhibitor diphenyleneiodonium (DPI), and the pools of reduced and oxidized glutathione (GSH and GSSG respectively). F1,6BP reduced free-radical production and increased the GSH/GSSG ratio (Table 1), both results indicating a reduction of oxidative stress in control and GalN + TNF-α-challenged hepatocytes. Moreover, most of the GalN + TNF-α-induced free-radical production was DPI-inhibitable (Table 1), suggesting an activation of NADPH oxidase and superoxide anion production in this oxidative stress.

3.2. cGMP mediated the antiapoptotic action of F1,6BP in primary hepatocytes

Under conditions of low oxidative stress, soluble guanylyl cyclase is the major target of the overproduced NO in the cell (Meurer et al., 2005; Gerassimou et al., 2007). To test the effect of F1,6BP on guanylyl cyclase activity, we measured the levels of cGMP in treated hepatocytes. F1,6BP dose-dependently increased the cGMP content in hepatocytes (Fig. 3). In contrast, GalN + TNF-α insult reduced the pools of cGMP to below control levels. To determine whether the protective effect of F1,6BP against the GalN + TNF-α-induced depletion of cGMP requires NOS activation, cultured primary hepatocytes were treated with NMMA (1,5 mM) to inhibit NO production. Fig. 3 shows that the protective effect of F1,6BP vanished when NO production was inhibited by NMMA-treatment.

Increased cGMP levels inhibited actinomycin D plus TNF-α-induced apoptosis in cultured primary rat hepatocytes, preventing cytochrome *c* release and caspase 3 activation (Li et al., 2000; Wang et al., 2006). To test whether cGMP mediated the protective effect of F1,6BP against the GalN + TNF-α-induced apoptosis, primary hepatocytes were treated with the inhibitor of guanylyl cyclase, ODQ, and the analogue of cGMP, 8Br-cGMP. Respectively, ODQ impaired and 8Br-cGMP mimicked the antiapoptotic effect of F1,6BP against the insult GalN + TNF-α in hepatocytes (Fig. 4).

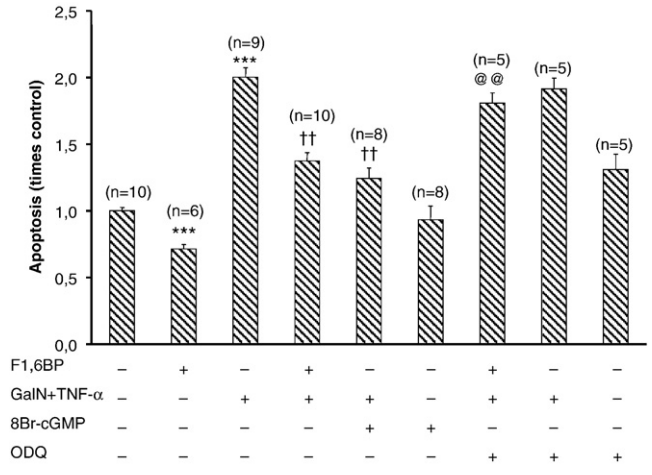

Fig. 4. cGMP mediated the antiapoptotic effect of F1,6BP in hepatocytes. The guanylyl cyclase inhibitor ODQ (20 μM), abolished the antiapoptotic effect of F1,6BP, whereas the cGMP analogue 8Br-cGMP, mimicked the effect of F1,6BP and reduced 0.6-fold the GalN + TNFα-induced apoptosis in primary hepatocytes. Results are means ± S.D. of the apoptosis, as assessed by DNA fragmentation, in cultured primary rat hepatocytes 16 h after challenge. ***$P<0.001$ vs. control; $^{\dagger\dagger}P<0.01$ vs. GalN + TNFα; $^{@@}P<0.01$ vs. F1,6BP (5 mM).

4. Discussion

GalN-induced hepatitis in rats involves multiple steps, such as macrophage activation and sensitization of parenchyma cells to cytokines, which lead to liver failure. F1,6BP protects rat liver against GalN-induced injury by preventing inflammation and reducing hepatocyte apoptosis (Roig et al., 1994; Roig et al., 1997). In a previous study, we described the physiological mechanisms involved in the anti-inflammatory action of F1,6BP (Cuesta et al., 2006). Here, we examined the cellular mechanisms underlying the antiapoptotic action of F1,6BP in cultured primary hepatocytes challenged by GalN + TNF-α.

The increase in TNF-α in plasma of rats challenged by GalN as a consequence of the inflammatory response plays a crucial role in GalN-induced experimental hepatitis. TNF-α produces two contrary effects in hepatocytes. First, TNF-α binding to membrane death receptors is followed by the recruitment of a series of intracellular proteins, activation of caspase 8, release of cytochrome *c* and subsequent activation of caspase 3 and apoptosis (Li et al., 1999). Second, TNF-α activates protective pathways via NF-κB, which reduces the effects of injury and makes the hepatocytes highly refractory to cytokines. Consequently, TNF-α does not injure hepatocytes unless they are sensitized by inhibition of the protective gene activation (Leist et al., 1994). GalN is one of the most widely used effectors of hepatitis, and it sensitizes hepatocytes to TNF-α via two complementary effects: (i) the arrest of transcription, by depletion of uridine pools (Keppler et al., 1970; Cuesta et al., 2006), and (ii) the increase of oxidative stress and GSH depletion, which exacerbates mitochondrial dysfunction (Quintero et al., 2002). Consequently, TNF-α activation of apoptosis is not compensated by the transcription of survival genes in GalN-sensitized hepatocytes, but rather enhanced by increased free-radical production and oxidative stress, as shown in GalN + TNF-α-challenged cells (Table 1 and Fig. 2).

F1,6BP inhibited the effects of GalN metabolism in hepatocytes (De Oliveira et al., 1992; Roig et al., 1994; Roig et al., 1997) and reduced the sensitization to TNF-α (Fig. 2). The protective effect of F1,6BP against the GalN + TNF-α insult cannot be attributed to TNF-α-induced activation of protective pathways via NF-κB, since F1,6BP does not restore GalN-induced arrest of transcription in hepatocytes (Cuesta et al., 2006). However, F1,6BP may prevent TNF-α-induced apoptosis, by reducing GalN-induced free-radical production and GSH depletion (Table 1) and subsequent mitochondrial dysfunction (Roig et al., 1994) and cytochrome *c* release, which would otherwise enhance TNF-α activation of apoptotic pathways (Quintero et al., 2002).

We hypothesized that the F1,6BP-induced increase of NO production in the liver mediates its antiapoptotic effect. This assumption is based on reports that related the increased NO production with the prevention of the GalN-induced apoptosis in rat hepatocytes (Muntane et al., 2000; Quintero et al., 2002; Siendones et al., 2003; Fouad et al., 2004; Siendones et al., 2004; Siendones et al., 2005) and with the F1,6BP-induced rat intestinal preconditioning (Sola et al., 2001).

An increase of NO production may produce a dual effect in the liver, either preventing (Mojena et al., 2001; Mihas et al., 2003; Siendones et al., 2003; Siendones et al., 2004) or improving (Mustafa et al., 1999) the injury produced by the inflammatory response. Accordingly, our results (Fig. 1 and in Fig. 2) show either a protective or a deleterious effect of NO overproduction in hepatocytes, depending on whether it was induced by F1,6BP or by GalN + TNF-α. The switch between the dual effect of NO overproduction is strongly dependent on the superoxide anion production and the redox state of the cell (Meurer et al., 2005; Brune, 2005; Gerassimou et al., 2007). In a reductive environment, NO inhibits apoptosis in hepatocytes by s-nitrosylation of caspases (Li et al., 1999; Kim et al., 2000; Vodovotz et al., 2004). Moreover, in these conditions, NO is also a potent activator of cytosolic guanylyl cyclase, which converts GTP into the second messenger cGMP (Cary et al., 2006). cGMP inhibits NADPH oxidase activity (Table 1), mitochondrial permeability transition and cytochrome *c*

release, through activation of cGMP-dependent protein kinase pathway, and thus reduces TNF-α-induced apoptosis in hepatocytes (Li et al., 2000; Muzaffar et al., 2004; Kim et al., 2004; Wang et al., 2006).

On the other hand, a concomitant increase of nitric oxide and superoxide anion produces peroxynitrite by direct combination of the two free radicals. Peroxynitrite is a highly toxic metabolite associated with organ damage in septic shock (Guzik et al., 2002). Additionally, reactive oxygen species down-regulate sGC expression and activity by tyrosine phosphorylation (Meurer et al., 2005; Gerassimou et al., 2007), reducing the apoptosis inhibition mediated by cGMP. The GalN + TNF-α-induced NO production in hepatocytes (Fig. 1) paralleled an increase of oxidative stress and superoxide production (Table 1), preventing cGMP enhancement (Fig. 3) and improving cytokine-induced apoptosis (Figs. 2 and 4). Treatment with F1,6BP attenuated the GalN + TNF-α-induced oxidative stress (Table 1), which reduced the sensitization of hepatocytes to cytokines and favoured the activation of cGC. Altogether, these F1,6BP-dependent effects attenuated apoptosis in challenged hepatocytes.

In conclusion, our results indicate that the elevation of cGMP levels via NO overproduction mediates the antiapoptotic effect of F1,6BP in hepatocytes. This protective effect of F1,6BP in the liver parenchyma cells parallels the *in vivo* inhibition of GalN-induced endotoxemia and inflammation observed in F1,6BP-treated rats (Cuesta et al., 2006). Taken together, these results indicate that F1,6BP activates similar protective mechanisms to preconditioning (Hotter et al., 1996) or prostaglandin E1 (Muntane et al., 2000; Siendones et al., 2004; Siendones et al., 2005), which enhances its potential application as a hepatoprotective compound.

Acknowledgments

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