

# TNF- $\alpha$ but not IL-1 $\alpha$ is correlated with PGE<sub>1</sub>-dependent protection against acute D-galactosamine-induced liver injury

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**BACKGROUND:** Prostaglandin E<sub>1</sub> (PGE<sub>1</sub>) treatment of humans and rodents during acute hepatic failure ameliorates different parameters of hepatic dysfunction.

**PURPOSE:** To investigate whether prevention of acute liver injury induced by D-galactosamine (D-GalN) with preadministration of PGE<sub>1</sub> is correlated with a change in the concentration of two proinflammatory cytokines, as tumour necrosis factor-alpha (TNF- $\alpha$ ) and interleukin (IL)-1 $\alpha$ , and/or nitrite+nitrate (NOx), as nitric oxide-related end products in serum.

**RESULTS:** D-GalN significantly increased alanine aminotransferase (ALT) and TNF- $\alpha$  concentration in serum 5 and 10 mins, respectively, after treatment compared with the control group (P $\leq$ 0.05). D-GalN did not change the IL-1 $\alpha$  concentration at any time during the study. Preadministration of PGE<sub>1</sub> to D-GalN-treated rats significantly reduced the ALT content and increased significantly the TNF- $\alpha$  concentration in serum 1, 2.5, 5 and 10 mins after D-GalN treatment compared with the D-GalN group (P $\leq$ 0.05). Nitric oxide was not involved in either the toxic effect due to D-GalN or the protection observed with PGE<sub>1</sub> against D-GalN toxicity.

**CONCLUSIONS:** Acute liver injury induced by D-GalN is cor-

related with an increased TNF- $\alpha$  release. Preadministration of PGE<sub>1</sub> to D-GalN-treated rats exerted a priming effect on inflammatory cells to release enhanced levels of TNF- $\alpha$  but not IL-1 $\alpha$ . These findings indicate that stimulation of TNF- $\alpha$  release may be involved in the acute D-GalN-induced liver injury and also in PGE<sub>1</sub> protection from hepatotoxicity in clinical and experimental studies.

**Key Words:** Cytokines; D-galactosamine; Hepatotoxicity; Interleukin-1alpha; Liver injury; Nitric oxide; Prostaglandin E<sub>1</sub>; Tumour necrosis factor-alpha

**Contrairement  $\alpha$  l'IL-1, le TNF- est en corrélation avec la protection conférée par la PGE<sub>1</sub> contre l'hépatotoxicité aiguë provoquée par la D-galactosamine**

**HISTORIQUE :** Le traitement à la prostaglandine E<sub>1</sub> (PGE<sub>1</sub>) chez l'être humain et le rongeur durant l'insuffisance hépatique aiguë améliore les différents paramètres de la dysfonction hépatique.

**BUT :** Vérifier si la prévention de l'atteinte hépatique aiguë causée par la D-galactosamine en administrant préalablement un traitement à la PGE<sub>1</sub> est en corrélation avec un changement de concentration de deux cytokines pro-inflammatoires, soit le TNF- $\alpha$  (pour *tumour necrosis factor- $\alpha$* ) et l'interleukine (IL)-1 $\alpha$ , et (ou) du nitrite + nitrate (NOx) comme

voir page suivante

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Received for publication March 13, 1998. Accepted January 13, 1999

métabolites finaux liés à l'oxyde nitrique dans le sérum.

**RÉSULTATS :** La D-GalN a fait significativement augmenter les concentrations sériques d'alanine aminotransférase (ALT) et de TNF- $\alpha$ , respectivement 5 et 10 minutes respectivement après le traitement en comparaison avec le groupe témoin ( $P \leq 0,05$ ). La D-GalN n'a modifié la concentration d'IL-1 à aucun moment durant l'étude. L'administration préalable de PGE<sub>1</sub> à des rats traités à la D-GalN a significativement réduit la teneur en ALT et a fait significativement augmenter la concentration sérique de TNF- $\alpha$  1, 2,5, 5 et 10 minutes après le traitement à la D-GalN, comparativement au groupe D-GalN ( $P \leq 0,05$ ). L'oxyde nitrique n'a pas

été associé à l'effet de la D-GalN ni à la protection observée avec la PGE<sub>1</sub> contre la toxicité de la D-GalN.

**CONCLUSIONS :** L'atteinte hépatique aiguë induite par la D-GalN est en corrélation avec une libération accrue de TNF- $\alpha$ . L'administration préalable de PGE<sub>1</sub> à des rats traités à la D-GalN a exercé un effet stimulant les cellules inflammatoires à libérer des taux accrus de TNF- $\alpha$ , mais non d'IL-1 $\alpha$ . Si l'on se fie à ces résultats, la stimulation de la libération de TNF- $\alpha$  peut participer à l'atteinte hépatique aiguë induite par la D-GalN et aussi à la protection conférée contre l'hépatotoxicité par la PGE<sub>1</sub> dans le cadre d'études cliniques et expérimentales.

Experimental studies have shown that prostaglandins have protective properties against different models of liver injury. Prostaglandin E (PGE) reduces liver toxicity induced by D-galactosamine (D-GalN) (1), thioacetamide (2), aflatoxin B<sub>1</sub> (3), carbon tetrachloride (4), bile duct ligation (5), fat-enriched and choline-deficient diet (6), viral hepatitis (7) and complement-mediated hepatic necrosis (8). Furthermore, PGE has a beneficial effect on fulminant viral hepatitis in humans, reducing serum transaminase levels and improving encephalopathy and coagulation factors (9,10). Prostacyclin also reduces transaminase levels during D-GalN-induced hepatic necrosis (11).

Different hypotheses have been suggested to explain the protective effect of prostaglandins in liver injury according to the different experimental models used. Among those cited are increased vascularization (5,12), reduced plasma membrane microviscosity (13), modification of the arachidonic acid cascade profile (14), antifibrotic activity (6) and immunomodulatory activity (15).

Cytokines and nitric oxide have also been shown to prevent liver damage induced by noxious agents. In this sense, D-GalN-treated animals can be protected to an otherwise lethal challenge of either lipopolysaccharide (LPS) or tumour necrosis factor-alpha (TNF- $\alpha$ ) toxicity by pretreatment with TNF- $\alpha$  or interleukin (IL)-1 $\alpha$  (16). Nitric oxide has been implicated in the development of tolerance to LPS- or cytokine-induced toxicity (17). The present article focuses on the potential involvement of TNF- $\alpha$ , IL-1 $\alpha$  and nitric oxide in the protection obtained with PGE<sub>1</sub> against D-GalN-induced injury in liver.

#### ANIMALS AND METHODS

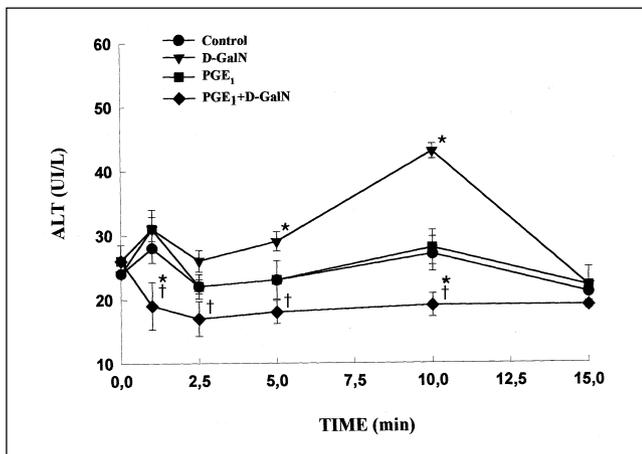
**Reagents:** All chemicals were obtained from Sigma Chemical Co (St Louis, Missouri). D-GalN was obtained from Sigma Chemical Co. PGE<sub>1</sub> (alprostadil) was purchased from The Upjohn Co (Kalamazoo, Michigan). Antigen and polyclonal antibody obtained from Genzyme Diagnostics (Cambridge, Massachusetts) were used for quantification of TNF- $\alpha$  and IL-1 $\alpha$  by ELISA. Nitrate reductase and lactate dehydrogenase (LDH) were obtained from Boehringer Mannheim (Mannheim, Germany).

**Animals and treatments:** Male Wistar rats (175 to 225 g) were housed in a climate-controlled (21°C) room under a 12 h light-dark cycle and were given tap water and standard

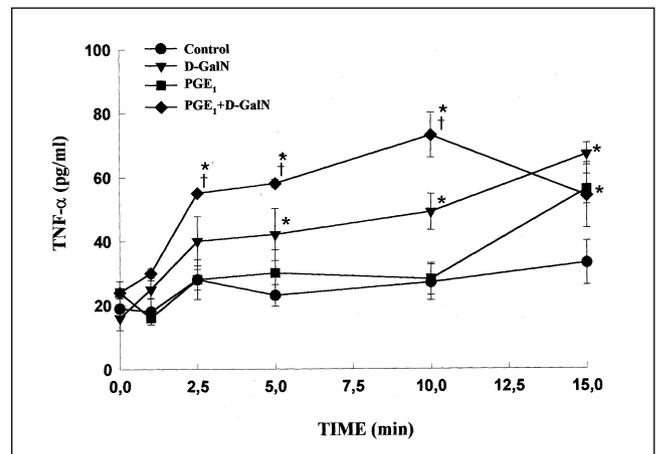
laboratory rat chow ad libitum. Animal care standards and experimental protocols were according to the *Guide for the Care and Use of Laboratory Animals* (18). Rats were divided into four groups of 30 according to treatment: control, D-GalN, PGE<sub>1</sub>, and PGE<sub>1</sub>+D-GalN. Each group was further divided into another six groups according to the time point of the kinetics (0, 1, 2.5, 5, 10 and 15 mins) in relation to the last treatment. All operations were performed under general (pentobarbital) anesthesia. The abdomen was entered through a midline incision to observe clearly the hepatic lobules and portal vein. To restrain and accelerate liver response, treatments (in a volume of 500  $\mu$ L) were infused as a bolus through the cannulated portal vein instead of intraperitoneally. Control and PGE<sub>1</sub>-treated animals received the corresponding volume of 0.9% sodium chloride or PGE<sub>1</sub> (250  $\mu$ g/kg) dissolved in 20% ethanol. D-GalN was also dissolved in 0.9% sodium chloride, and the dose (200 mg/kg) administered was adjusted according to the mode of administration compared with higher doses used in intraperitoneal administration. In the PGE<sub>1</sub>+D-GalN group, PGE<sub>1</sub> was infused 10 mins before D-GalN infusion. The dose of PGE<sub>1</sub> was in the range used in several experimental studies (2,3,6) and the total infusion dose used during clinical trials (9). To check the effect of 20% ethanol as a solvent of PGE<sub>1</sub>, an equal amount of ethanol to that used in the PGE<sub>1</sub>-treated groups was infused to another group of rats 10 mins before infusion of the volume of 0.9% sodium chloride. Serum was obtained from blood collected after puncturing the suprahepatic vein. Alanine aminotransferase (ALT) was assayed immediately, but TNF- $\alpha$ , IL-1 $\alpha$  and nitrite/nitrate (NOx) were measured afterwards in frozen serum samples. No differences in ALT, TNF- $\alpha$ , IL-1 $\alpha$  and NOx concentrations were observed between the control group and the group treated with 20% ethanol in the time intervals studied.

**ALT quantification in rat serum:** ALT was assayed with a commercial kit obtained from Merck (Darmstadt, Germany) based on a routine method (19).

**TNF- $\alpha$  quantification in rat serum:** TNF- $\alpha$  was measured in serum by competitive ELISA. The standards used were calibrated against the National Institute for Biological Standards and Control (NIBSC) reference standard. Wells of ELISA plates were coated with 200  $\mu$ L of TNF- $\alpha$  (2.5 ng/mL) for 1 h at 37°C. Afterwards, wells were blocked with a solution containing 2% bovine serum albumin in 10 mM phosphate



**Figure 1** Alanine aminotransferase (ALT) content in serum from rats treated with D-galactosamine (D-GalN) and/or prostaglandin E<sub>1</sub> (PGE<sub>1</sub>). ALT was measured at 0, 1, 2.5, 5, 10 and 15 mins after D-GalN (200 mg/kg) or 0.9% sodium chloride infusion. PGE<sub>1</sub> (250 µg/kg) was administered 10 mins before D-GalN or 0.9% sodium chloride infusion. Samples were recovered from the suprahepatic vein. Data are presented as means ± SEM; \*P≤0.05 compared with the corresponding value of the control group; †P≤0.05 compared with the corresponding value of the D-GalN group



**Figure 2** Tumour necrosis factor alpha (TNF-α) content in serum from rats treated with D-galactosamine (D-GalN) and/or prostaglandin E<sub>1</sub> (PGE<sub>1</sub>). TNF-α was measured 0, 1, 2.5, 5, 10 and 15 mins after D-GalN (200 mg/kg) or 0.9% sodium chloride infusion. PGE<sub>1</sub> (250 µg/kg) was administered 10 mins before D-GalN or 0.9% sodium chloride infusion. Samples were recovered from the suprahepatic vein. Data are presented as means ± SEM. \*P≤0.05 compared with the corresponding value of control group; †P≤0.05 compared with the corresponding value of D-GalN group

buffer solution comprising 0.05% Tween in phosphate buffered saline (PBS-T) pH 7.4. At the same time, samples and polyclonal antibodies (32.5 ng/mL) were incubated for 2 h at 37°C, transferred to wells and incubated for 2 h at 37°C. Afterwards, wells were washed with PBS-T and incubated with biotinylated secondary antibody (125 µg/mL) for 1 h at 37°C. Furthermore, wells were washed with tris buffer (100 mM) pH 7.6 and incubated with commercial streptavidin-alkaline phosphatase (MD, Granada, Spain) solution for 30 mins at 37°C. Finally, the plates were washed and incubated with the alkaline phosphatase substrate (Sigma 104, Sigma Chemical Co) and read after 1 h with the 405 nm filter in a titrated Organon Teknika 510 ELISA reader (Organon Teknika, Turnhout, Belgium).

**IL-1α quantification in rat serum:** IL-1α was measured in serum by competitive ELISA. The standards used were calibrated against the NIBSC reference standard. Wells of ELISA plates were coated with 200 µL of IL-1-α (5 ng/mL) for 1 h at 37°C. Afterwards, wells were blocked with a solution containing 2% bovine serum albumin in PBS-T pH 7.4. At the same time, samples and polyclonal antibodies (2 ng/mL) were incubated for 2 h at 37°C, transferred to wells and incubated for 2 h at 37°C. Afterwards, wells were washed with PBS-T and incubated with biotinylated secondary antibody (17.5 ng/mL) for 1 h at 37°C. Furthermore, wells were washed with tris buffer (100 mM) pH 7.6 and incubated with commercial streptavidin-alkaline phosphatase (MD, Granada, Spain) solution for 30 mins at 37°C. Finally, the plates were washed and incubated with the alkaline phosphatase substrate (Sigma 104) and read after 1 h with the 405 nm filter in a titrated Organon Teknika 510 ELISA reader.

**NOx quantification in rat serum:** NOx measurements were used as an index of nitric oxide production. Nitrate was re-

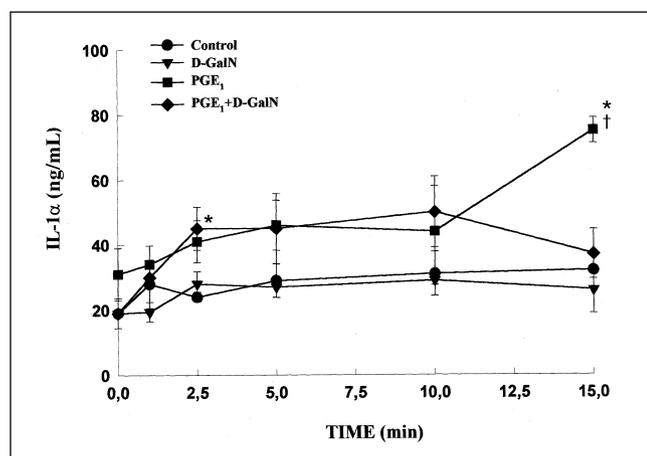
duced to nitrite after incubation of samples with nitrate reductase (0.2 U/mL), flavin adenine dinucleotide (5 mM) and reduced nicotinic adenine dinucleotide phosphate (50 mM) for 20 mins at 37°C. The reaction was stopped by adding sodium pyruvate (10 mM) and LDH (24 mg/mL) for 5 mins at 37°C, and precipitated with 1.4% zinc sulphate. Total nitrite was reacted with Griess reagent (1% sulphanilamide, 2.5% phosphoric acid, 0.1% N-naphthyl-ethylene-diamine) for 10 mins at 37°C and read with the 540 nm filter in a titrated Organon Teknika 510 ELISA reader.

**Statistical analysis:** Results are expressed as means ± standard errors. Data were evaluated by one-way ANOVA, with four groups per variable. Because the homogeneity of variances assessed by the Bartlett test was sufficient, groups were subjected to the multiple comparison least significant differences test. Statistical significance was set at P≤0.05.

## RESULTS

**Effect of PGE<sub>1</sub> and D-GalN on serum ALT activity:** The normal range of ALT activity in rat serum is 18 to 20 U/L (20). In the present study, D-GalN infusion induced a significant increase 5 and 10 mins after treatment in ALT (29±1.5 and 43±1.2 U/L, respectively) activity compared with the control group (23±0.3 and 27±2.7 U/L, respectively) (P≤0.05) (Figure 1). PGE<sub>1</sub> did not significantly change ALT activity. Preadministration of PGE<sub>1</sub> to D-GalN-treated rats significantly reduced ALT levels to values below those obtained in the control group (P≤0.05) (Figure 1).

**Effect of PGE<sub>1</sub> and D-GalN on serum TNF-α concentration:** The normal range of TNF-α concentration in rat serum is 11 to 18 pg/mL (20). TNF-α release increased significantly 5 to 15 mins after D-GalN infusion compared with the control group (P≤0.05) (Figure 2). PGE<sub>1</sub> resulted in a significant



**Figure 3** Interleukin (IL)-1 $\alpha$  content in serum from rats treated with D-galactosamine (D-GalN) and/or prostaglandin E<sub>1</sub> (PGE<sub>1</sub>). IL-1 $\alpha$  was measured 0, 1, 2.5, 5, 10 and 15 mins after D-GalN (200 mg/kg) or 0.9% sodium chloride infusion. PGE<sub>1</sub> (250  $\mu$ g/kg) was administered 10 mins before D-GalN or 0.9% sodium chloride infusion. Samples were recovered from the suprahepatic vein. Data are presented as means  $\pm$  SEM. \*P $\leq$ 0.05 compared with the corresponding value of the control group; †P $\leq$ 0.05 compared with the corresponding value of D-GalN group

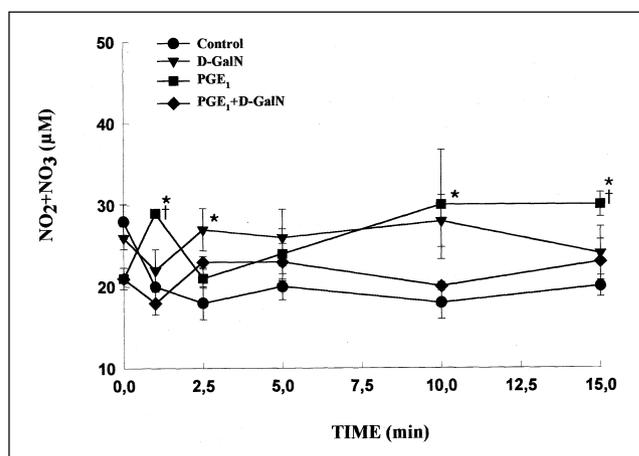
increase in TNF- $\alpha$  (56 $\pm$ 4.7 pg/mL) 15 mins after 0.9% sodium chloride infusion compared with the control group (33 $\pm$ 6.9 pg/mL) (P $\leq$ 0.05) (Figure 2). Preadministration of PGE<sub>1</sub> to D-GalN-treated rats caused an additional significant increase in TNF- $\alpha$  concentration from 1 to 10 mins after D-GalN infusion compared with the D-GalN group (P $\leq$ 0.05) (Figure 2).

**Effect of PGE<sub>1</sub> and D-GalN on serum IL-1 $\alpha$  concentration:** D-GalN infusion did not change IL-1 $\alpha$  concentration in serum (Figure 3). PGE<sub>1</sub> significantly increased IL-1 $\alpha$  (75 $\pm$ 3.9 ng/mL) concentration in serum 15 mins after 0.9% sodium chloride infusion compared with the control group (32 $\pm$ 5.7 ng/mL) (P $\leq$ 0.05) (Figure 3). Nevertheless, there were no differences between the D-GalN and PGE<sub>1</sub>+D-GalN groups (Figure 3).

**Effect of PGE<sub>1</sub> and D-GalN on the concentration of NOx in serum:** D-GalN did not essentially modify the concentration of NOx over the time points of the study (Figure 4). Only PGE<sub>1</sub> by itself significantly increased the level of NOx 1, 10 and 15 mins after treatment compared with the control values (P $\leq$ 0.05) (Figure 4). Nevertheless, preadministration of PGE<sub>1</sub> did not modify the concentration of NOx in the PGE<sub>1</sub>+D-GalN group compared with the D-GalN group (Figure 4).

## DISCUSSION

D-GalN administration is a model of hepatotoxicity used extensively in experimental studies. D-GalN depletes the intracellular uracil nucleotides in hepatocytes that lead to inhibition of RNA and protein synthesis (21-24). D-GalN increases serum transaminase levels, hepatic necrosis and coma (1,20,25,26). In the present study and in another (20), D-GalN induced liver injury and increased TNF- $\alpha$  concen-



**Figure 4** The contents of nitric oxide products (NOx) in serum from rats treated with D-galactosamine (D-GalN) and/or prostaglandin E<sub>1</sub> (PGE<sub>1</sub>). NOx were measured 0, 1, 2.5, 5, 10 and 15 mins after D-GalN (200 mg/kg) or 0.9% sodium chloride infusion. PGE<sub>1</sub> (250  $\mu$ g/kg) was administered 10 mins before D-GalN or 0.9% sodium chloride infusion. Samples were recovered from the suprahepatic vein. Data are presented as means  $\pm$  SEM. \*P $\leq$ 0.05 compared with the corresponding value of the control group; †P $\leq$ 0.05 compared with the corresponding value of the D-GalN group

tration in rat serum. TNF- $\alpha$  is essential to induce cell death in unviable hepatocytes and exacerbates D-GalN-induced damage. In this sense, TNF- $\alpha$  treatment induces apoptosis *in vivo* and *in vitro* when D-GalN is also administered (27-30). Under our conditions, a short time after D-GalN portal infusion, a significant increase in serum ALT (Figure 1) and TNF- $\alpha$  (Figure 2) was observed compared with the control group. Nevertheless, the serum concentrations of IL-1 $\alpha$  (Figure 3) and NOx (Figure 4) did not change with D-GalN administration. Such inability of D-GalN to stimulate IL-1 $\alpha$  release has also been pointed out using IL-1 receptor antagonist to prevent the lethal combination of TNF- $\alpha$  and D-GalN treatment (31). Because the manufacturer indicated that the D-GalN had no trace amount of endotoxin, our results indicate that D-GalN is able to stimulate inflammatory cells to release inflammatory mediators. In our experimental conditions, the toxicity induced by D-GalN was low, and ALT levels (Figure 1) decreased 15 mins after infusion. This may be due to the low dose of D-GalN used or some other extrahepatic changes induced by D-GalN (32).

Several reports have shown that PGE<sub>1</sub> decreases both *in vivo* and *in vitro* the liver damage induced by D-GalN (1,13,20). In a previous study (20), intraperitoneal preadministration of PGE<sub>1</sub> 30 mins before D-GalN reduced ALT release and enhanced TNF- $\alpha$  concentration in serum. In the present study, protection by PGE<sub>1</sub> against D-GalN was also obtained when PGE<sub>1</sub> was infused 10 mins before D-GalN through the portal vein, indicating that the different effects may be restricted to the liver and/or systemic circulation. Such stimulation of TNF- $\alpha$  release induced by PGE<sub>1</sub> differs from the results of some other experiments. In these studies, it has been observed that PGE reduces TNF- $\alpha$  release *in vitro* from LPS-stimulated Kupffer cells (33,34)

and peritoneal macrophages (35,36). The authors pointed out that this effect of PGE<sub>1</sub> was related to cAMP formation. Nevertheless, in agreement with our data, other authors have reported that low PGE concentration stimulates cGMP and TNF- $\alpha$  release from peritoneal macrophages (37,38). In vivo experiments have also given controversial results. In this sense, administration of indomethacin, as an inhibitor of prostaglandin synthesis, increased serum TNF- $\alpha$  concentration and exacerbated gastric mucosal damage in rats (39). Other authors indicated that indomethacin did not modify LPS-induced TNF- $\alpha$  release in mice (40). In the present study and in another (20), the effect of PGE<sub>1</sub> administered before D-GalN may have been different from that found in other studies in which it was administered after the induction of liver injury. Additionally, controversial results about the effect of PGE<sub>1</sub> on TNF- $\alpha$  release may also be related to the model of liver injury (toxic, hepatitis, endotoxemia, etc).

The potential role of TNF- $\alpha$  in preventing liver injury is under investigation. In this sense, it has also been shown that preadministration of low doses of TNF- $\alpha$  is able to desensitize against murine endotoxic shock (41). TNF- $\alpha$  stimulates the secretion of several acute phase proteins in the liver (42). Also, preadministration of IL-1 or the nitric oxide donor sodium nitroprusside protects mice against endotoxic shock (43,44). In these studies, protection was also related to a stimulation of TNF- $\alpha$  release from peritoneal macrophages. Our data show that IL-1 $\alpha$  and nitric oxide do not

seem to be directly involved in the protection by PGE<sub>1</sub> during D-GalN liver injury. Nevertheless, it cannot be excluded that IL-1 $\alpha$  and/or nitric oxide may play a protective role during a more prolonged time (12 h) of D-GalN liver toxicity. Different studies are underway in which antibodies against TNF- $\alpha$  or inhibitors of inducible nitric oxide synthase are administered to elucidate the role of these inflammatory mediators during protection by PGE<sub>1</sub> on D-GalN liver injury.

## CONCLUSIONS

Stimulation of TNF- $\alpha$  but not IL-1 $\alpha$  release from inflammatory cells induced by D-GalN was correlated with an increase in hepatic damage. Protection against D-GalN-induced liver injury by preadministration of PGE<sub>1</sub> was correlated with an additional increase of serum TNF- $\alpha$  but not IL-1 $\alpha$  concentration in serum. Further studies are required to confirm whether the priming effect of PGE<sub>1</sub> on TNF- $\alpha$  release is responsible for prostanoid protection during experimental and therapeutic treatment of liver dysfunction.

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**ACKNOWLEDGEMENTS:** This work was supported by the Programa de Promoción de la Investigación en Salud del Ministerio de Sanidad y Consumo (FIS) 97/1300. We thank the Schering-Plough Ltd laboratories and the Fundación Hospital Reina Sofia-CajaSur in Spain for their financial support.

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