Gastrointestinal, Hepatobiliary and Pancreatic Pathology

Intestinal Glucose Uptake Protects Liver from Lipopolysaccharide and D-Galactosamine, Acetaminophen, and Alpha-Amanitin in Mice

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We have recently observed that oral administration of D-glucose saves animals from lipopolysaccharide (LPS)-induced death. This effect is the likely consequence of glucose-induced activation of the sodium-dependent glucose transporter-1. In this study, we investigated possible hepatoprotective effects of glucose-induced, sodium-dependent, glucose transporter-1 activation. We show that oral administration of D-glucose, but not of either D-fructose or sucrose, prevents LPS-induced liver injury, as well as liver injury and death induced by an overdose of acetaminophen. In both of these models, physiological liver morphology is maintained and organ protection is confirmed by unchanged levels of the circulating markers of hepatotoxicity, such as alanine transaminase or lactate dehydrogenase. In addition, D-glucose was found to protect the liver from α -amanitin-induced liver injury. In this case, in contrast to the previously described models, a second signal had to be present in addition to glucose to achieve protective efficacy. Toll-like receptor 4 stimulation that was induced by low doses of LPS was identified as such a second signal. Eventually, the protective effect of orally administered glucose on liver injury induced by LPS, overdose of acetaminophen, or α -amanitin was shown to be mediated by the anti-inflammatory cytokine interleukin-10. These findings, showing glucose-induced protective effects in several animal models of liver injury, might be relevant in view of possible

therapeutic interventions against different forms of acute hepatic injury. (Am J Pathol 2009, 175:1066–1076; DOI: 10.2353/ajpath.2009.090071)

Liver failure is one of the most devastating syndromes observed in clinical practice. It is associated with high overall mortality, ranging from 30% to 80%, depending on the underlying etiology.^{1,2} The most common etiologies are acute viral hepatitis, drug overdose, idiosyncratic drug reactions, and ingestion of other toxins.³ Insulting agents can cause hepatocyte death through different mechanisms of action, and when the amount of functioning cells decreases to a level at which the organ is no longer capable of fulfilling its metabolic and synthetic tasks, hepatic failure takes place.⁴

Recently we demonstrated, in a murine model of septic shock, that oral administration of D-glucose saves mice from death, most likely as a result of glucose-induced activation of the intestinal sodium-dependent glucose transporter-1 (SGLT-1).⁵ Using this model, we made preliminary observations that the liver, one of the organs most severely affected by lipopolysaccharide and D-galactosamine (LPS/D-GalN) treatment, was protected by oral administration of D-glucose. The expression of SGLT-1 on the apical membrane of enterocytes and the observation that protection from LPS shock was observed only on oral administration of D-glucose, but not on i.p. administration, suggested an important role of intestinal epithelial cells in protection from LPS-induced injury to the liver and other organs.

Here, we report on a more extensive investigation on the hepatoprotective effect of orally administered D-glucose. Glucose was evaluated in LPS-induced shock, as well as in two other models of acute liver failure, ie,

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acetaminophen (APAP)- and α -amanitin-induced hepatic toxicity. APAP overdose is a common cause of drug-induced liver injury⁶ since this drug, when administered at very elevated doses, generates the highly reactive intermediate, *N*-para-aminoquinonimine,⁷ which covalently binds to hepatocyte macromolecules, leading to cellular death.⁸ This can lead to a potentially fatal centrilobular hepatic necrosis.⁹ On the other hand, α -amanitin toxin, which provide the major morbidity and mortality associated with toxic mushroom ingestion, interact with RNA polymerase II and affected cells undergo apoptosis due to insufficient protein synthesis. Organs mainly affected by α -amanitin include the intestinal mucosa, hepatocytes, and proximal tubules of kidney. The most severely affected organ is the liver, and hepatic toxicity is the actual cause of α -amanitin-induced lethality.

In the herein-described experiments, we show that in all three models it is possible to achieve liver protection by D-glucose-induced intestinal SGLT-1 activation. However, while in models of LPS shock and APAP overdosing, D-glucose is therapeutically active when administered alone, in the α -amanitin model the concomitant administration of a low dose of LPS was required to achieve protection.

Materials and Methods

Reagents

D-glucose was from Merck (Darmstadt, Germany) and sucrose from BDH (Poole, UK). D-fructose, LPS from *Salmonella enterica* (serotype *abortus equi*), LPS from *Escherichia coli*, D-GalN, APAP, α -amanitin, and phloridzin were from Sigma-Aldrich (St. Louis, MO); neutralizing rat anti-mouse interleukin 10 (IL-10) monoclonal antibody (Ab) was from BD Pharmingen (San Jose, CA).

Animals and Animal Experiments

C57BL/6 mice were purchased from Charles River, (Calco, Italy); TLR4-deficient mice were provided by Dr. Besusso, Istituto Nazionale Tumori, Milan, Italy. Experimental protocols were approved by the Ethics Committee for Animal Experimentation of Istituto Nazionale Tumori, Milano. To study the percentage of survival in sepsis model, mice ($n = 10$) were injected i.p., with 250 μ g/kg of LPS from *S. enterica* and 1 g/kg of D-GalN, with or without D-glucose, D-fructose, or sucrose treatment (500 μ l, 0.56 M/L administered per os with a stomach tube), 1 hour before endotoxin administration. Control groups were treated per os with sterile water or D-glucose alone. To analyze histology, five mice for each group were treated as above reported and sacrificed 6 hours after treatment, and livers were collected and fixed in neutral buffered formalin. For experiments on APAP overdose, mice (10/group) were fasted overnight (16 to 18 hours) before i.p. administration of the drug with or without per os pretreatment with D-glucose. APAP was administered at 300 mg/kg or 1125 mg/kg in 400 μ l of saline and glycol (1:1). For IL-10 neutralization *in vivo*, mice ($n = 5$) were treated

i.p. with 100 μ g/mouse of rat anti-mouse IL-10 Ab, 1 hour before per os administration of D-glucose (2.5 g/kg), followed by APAP (1125 mg/kg) administration, and survival was compared with that of mice treated with D-glucose without the neutralizing Ab. For the experiments on α -amanitin intoxication, a group of mice ($n = 10$) was treated with α -amanitin (3.5 mg/kg administered i.p.), another group received α -amanitin plus a sublethal dose of LPS from *E. Coli* (250 μ g/kg). Both groups had been pretreated with D-glucose and control groups were treated per os with sterile water or D-glucose alone. Blood was collected 6 hours after LPS challenge, 8 and 24 hours after sublethal APAP treatment and 8 hours after α -amanitin administration.

Cell Treatments

Primary mouse cell cultures were prepared by standard two-step collagenase perfusions of livers as described above.¹⁰ Cells were cultured for 18 hours in William's E medium, or in medium with high glucose concentration (10 g/L = 5 $\times$ standard concentration), and then incubated for 24 hours with APAP (10 mmol/L). Cytotoxicity was measured by the release of lactate dehydrogenase (LDH) into the culture medium.

Measurement of Enzymes, Cytokines, and LPS

Alanine transaminase (ALT) and (LDH) in plasma samples or supernatants were determined using commercial kits, and performed according to the manufacturer's instructions (FAR srl, Verona, Italy).

Liver level of glutathione (GSH) was measured using the GSH assay kit from Cayman (Ann Arbor, MI). Concentrations of IL-10 and tumor necrosis factor- α were evaluated using enzyme-linked immunosorbent assay kits from Endogen (Woburn, MA) and Pierce Biotechnology (Rockford, IL), respectively. Plasma samples were analyzed for LPS content using E-TOXATE KIT (Sigma-Aldrich), following the manufacturer's instructions and using sterile, pyrogen-free, plasticware.

H&E Staining and Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling Assay

Liver sections were deparaffinized, rehydrated, and stained with H&E, and were mounted in Entellan (Merck). Samples were observed with a Nikon Eclipse 80i microscope equipped with a digital Nikon DS-L1 camera. Apoptotic hepatic cells were detected by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay (In Situ Cell Death Detection Kit, Roche Diagnostics GmbH, Mannheim, Germany), performed according to the manufacturer's instructions.

Figure 1. Plot of the percentage of survivors over time among mice injected i.p. with lipopolysaccharide and D-galactosamine (LPS/D-GalN). Mice were pretreated with D-glucose (closed square), D-fructose (open square), sucrose (open circle), or saline (closed triangle) 1 hour before LPS/D-GalN administration. Experimental group consisted of 10 mice.

Intracellular Adhesion Molecule-1 Immunohistochemical Staining and Western Blot Analysis

Paraffin specimens were deparaffinized and rinsed in PBS. After antigen retrieval by autoclaving in 0.01 M/L (pH 6) sodium citrate, and after quenching of endogenous peroxidase in 0.3% H₂O₂ in PBS, sections were incubated with anti-mouse intracellular adhesion molecule (ICAM-1, 1:10 in PBS, 1% bovine serum albumin

[BSA]) for 1 hour at 37°C. After washing in PBS/3%BSA, samples were incubated for 30 minutes at room temperature with secondary Ab, rabbit-anti-goat (DAKO, Denmark) Ab in PBS/3%BSA, washed again, and incubated with goat peroxidase anti-peroxidase (Jackson ImmunoResearch Laboratories, West Grove, PA) in PBS/3%BSA for 30 minutes at room temperature. The staining was finally detected by peroxidase substrate kit diaminobenzidine (Vector Laboratories, Burlingame, CA). For Western blot, proteins (15 µg) were extracted from liver samples and quantitatively estimated using the BCA Protein Assay kit (Pierce Biotechnology, Rockford, IL), and fractionated on an 8% acrylamide slab gel containing 0.1% SDS and transferred onto a nitrocellulose filter (Amersham Biosciences, Uppsala, Sweden) by electroblotting. After incubation for 1 hour in TBS with 0.1% Tween 20 and 5% milk powder to block nonspecific binding sites, the filter was incubated with anti-ICAM-1 Ab overnight at 4°C. After washing in TBS, 1% Tween 20, and 5% milk powder, the filter was incubated with secondary biotinylated Ab (Vector Laboratories), washed in TBS, 1% Tween 20 (Sigma), and examined using the ABC kit (Vector Laboratories). Bands were visualized using enhanced chemiluminescence Western blotting detection (Amersham Biosciences).

Statistical Analysis

Student's *t*-test (paired two-tailed) and GraphPad Prism software (GraphPad Prism Software Inc., San Diego, CA) were used for comparisons between groups. *P* values less than 0.05 were considered significant.

Figure 2. Histology of liver tissue after lipopolysaccharide and D-galactosamine (LPS/D-GalN) administration. Liver sections were stained with H&E. Liver sections from LPS/D-GalN-treated animals show acute hepatitis (A) with important hemorrhagic necrosis. In the box are shown apoptotic bodies (arrow) and infiltration of inflammatory cells in the portal area (arrowhead). The histological changes caused by LPS/D-GalN are still present in fructose- (B) or sucrose- (C) treated mice, but absent in D-glucose-treated animals (D). Scale bar = 40 µm; box = 15 µm. E-F: TUNEL assay. In the liver of mice receiving LPS/D-GalN numerous apoptotic cells are detected (arrows) (E), whereas in animals treated with D-glucose before LPS/D-GalN administration the staining for apoptotic nuclei is negative (F). Scale bar = 20 µm.

Results

Oral Administration of D-Glucose, but Not Sucrose or D-Fructose, Has a Hepatoprotective Effect in Mice Injected with LPS/ D-GalN

Our observation that oral administration of D-glucose (500 μ l, 0.56 M/L) saved mice from LPS/D-GalN-induced death, likely via activation of SGLT-1 expressed on the apical membrane of intestinal enterocytes,⁵ prompted us to investigate in this model, the effect of the disaccharide sucrose, formed by D-glucose and D-fructose, or of the monosaccharide D-fructose.

C57BL/6 mice (10 mice/group) were treated per os with sucrose (500 μ l, 0.56 M/L), D-fructose (500 μ l, 0.56 M/L), D-glucose (500 μ l, 0.56 M/L), or saline. One hour later, they were injected i.p. with LPS (250 μ g/kg) plus D-GalN (1 g/kg) (LPS/D-GalN). LPS/D-GalN induced death within 36 hours in 100% of saline-, D-fructose-, and sucrose-treated mice, whereas all mice treated with D-glucose survived (Figure 1).

Histological analysis of liver tissues recovered 6 hours after LPS/D-GalN treatment, from mice that had been pretreated with saline, sucrose, or D-fructose, showed signs of acute hepatitis (Figure 2, A–C). Thus, blood cells were seen invading the hepatocyte organization and important liver hemorrhage was observed. At high magnification, apoptotic bodies with condensed chromatin and infiltration of inflammatory cells in the portal area were evidenced. These histological alterations were almost absent in liver tissues from mice that had been pretreated with D-glucose (Figure 2D). In the liver of LPS/D-GalN-treated mice, numerous apoptotic cells were detected by TUNEL assay (Figure 2E), while in animals treated with D-glucose, the staining for apoptotic nuclei is negative (Figure 2F). Analysis of two markers of liver injury, ie, ALT and LDH^{11,12} in the sera of the different groups of mice, measured 6 hours after LPS/D-GalN administration, further confirmed the protective effect of D-glucose; in fact, the strong increase of plasma ALT and LDH levels observed after LPS/D-GalN administration was significantly reduced in D-glucose pretreated mice (ALT: LPS/D-GalN alone = 1009 ± 121 U/L versus glucose+LPS/D-GalN = 489 ± 48 U/L, $P = 0.0075$; LDH: LPS/D-GalN alone = 3076 ± 109 U/L versus glucose+LPS/D-GalN = 815 ± 100 U/L, $P = 0.001$. Untreated mice: ALT = 172 ± 38 U/L, LDH = 890 ± 193 U/L) (Figure 3, A and B).

During LPS/D-GalN intoxication, ICAM-1 expression in hepatocytes and nonparenchymal cells is enhanced. Up-regulation of this molecule is necessary for extravasation, into the liver parenchyma, of neutrophils, which produce cytotoxic damage to hepatocytes.^{13,14} To determine whether D-glucose oral administration interferes with the cytotoxic injury mediated by adhesion molecules, ICAM-1 expression was examined (Figure 4). Immunohistochemistry shows a faint constitutive ICAM-1 expression in a small number of endothelial cells, restricted to hepatic sinusoids (Figure 4A); LPS/ D-GalN injection results in a massive induction of ICAM-1, mainly distributed along the wall of sinusoids and central veins (Figure 4B),

Figure 3. ALT (A) and LDH (B) plasma levels induced by lipopolysaccharide and D-galactosamine (LPS/D-GalN) at 6 hours after treatment. Data are the means ($\pm$ SD) of two independent experiments (3 mice/group). Untr = untreated mice; glu = mice treated with D-glucose; LPS/D-GalN = mice treated only with LPS/D-GalN and glu+LPS/D-GalN = mice treated with D-glucose and after 1 hour with LPS/D-GalN (* $P < 0.05$, ** $P < 0.005$ glu+LPS/D-GalN versus LPS/D-GalN).

while D-glucose oral administration reduced the toxin-induced up-regulation of the protein (Figure 4C). We have previously shown that the administration of IL-10 neutralizing antibodies nullified the protective activity of D-glucose against LPS/D-GalN.⁵ Accordingly with this observation, in the presence of anti-IL-10 antibodies, SGLT-1 fails to reduce ICAM-1 expression (Figure 4D). These results were confirmed by Western blot analysis on protein extracts from the liver of same animals (Figure 4E).

Pretreatment with Oral D-Glucose Prevents Liver Injury and Lethality from APAP Overdosing

The protective effect of D-glucose pretreatment was then evaluated in a model of liver injury induced by APAP overdosing. C57BL/6 mice (10/group) were treated per os with D-glucose or saline, and after 1 hour they were injected with a sublethal dose of APAP (300 mg/kg i.p.). Histological analysis of sections from livers collected 24 hours after APAP treatment showed, in APAP-treated mice, cytoplasmic vacuolization and parenchyma disorganization (Figure 5, A and B), while liver sections from mice that had been pretreated with D-glucose presented an almost normal morphology (Figure 5C). Moreover, plasma levels of ALT and LDH, which were greatly in-

Figure 4. ICAM-1 liver expression. Expression of ICAM-1 was examined by immunohistochemistry (A–D) and by Western blot analysis (E). Immunohistochemistry shows a faint constitutive ICAM-1 expression, restricted to hepatic sinusoids in control animals (A), LPS/D-GalN injection results in a massive induction of ICAM-1 (B) while oral D-glucose administration reduced the toxin-induced up-regulation of the protein (C), but fails when administered together with IL-10-neutralizing antibodies (D). Scale bar = 40 μ m. Western blot analysis on protein extracts from the liver of same animals confirms the results (E).

creased 24 hours after APAP treatment, were similar to those of controls in mice pretreated with D-glucose (ALT: APAP = 2303 ± 670 U/L versus glucose+APAP = 126 ± 100 , $P = 0.038$. LDH: APAP = 3293 ± 446 U/L versus glucose+APAP = 1126 ± 118 U/L at 24 hours, $P = 0.022$) (Figure 6, A–B). These findings show that D-glucose exerts a protective effect also in APAP-induced liver injury.

Several experiments were then performed to clarify the mechanism of action underlying the protective effect of D-glucose in this model of liver injury. Our previous studies in LPS-induced shock had shown that D-glucose afforded protection through activation of SGLT-1.

In the present setting, we first investigated whether inhibition of APAP-induced liver injury by D-glucose pretreatment is related to alterations of the APAP metabolism. In fact, APAP is metabolized in the liver by cytochrome P450 enzymes to the highly reactive metabolite *N*-para-aminoquinonimine that reacts rapidly with GSH, thereby depleting the intracellular pool of enterocytes. For this reason, we measured the level of hepatic GSH in animals treated with APAP with or without per os pretreatment with D-glucose. In animals treated with APAP alone,

the levels of hepatic GSH are strongly decreased. Pretreatment with D-glucose does not modify the levels of this scavenger, neither at 1 hour or 4 hours after APAP administration (Figure 7).

The protective effect of D-glucose was shown also to be unrelated to a direct effect of D-glucose on hepatocytes. For this purpose we performed experiments with primary murine hepatocytes, widely used to study *in vitro* APAP cytotoxicity.^{15,16} Supernatants of such cells treated with APAP (10 mmol/L) in a control medium with 2 g/L of D-glucose or high D-glucose medium (10 g/L) contain markedly higher levels of LDH (used as a marker of cytotoxicity) than cells not treated with APAP (APAP = 737 ± 70 U/L versus untreated = 109 ± 4 , $P = 0.0037$. LDH: glu+APAP = 741 ± 47 U/L versus untreated, $P = 0.0016$) (Figure 8). The results of these experiments allow us to conclude that the protective effect of D-glucose is not the consequence of alterations of the APAP metabolism.

In another experiment aimed at elucidating the mechanism of action of D-glucose, we treated mice with a lethal dose of APAP (1125 mg/kg)¹⁷ with or without per os pretreatment with D-glucose. All mice treated with APAP

Figure 5. Histology of liver tissue after acetaminophen (APAP) treatment with or without pretreatment with per os D-glucose. Liver sections, stained with H&E, from animals treated with APAP (**B**) show hepatocyte cytoplasmic vacuolization (**arrow**), fatty degeneration and constipation (**arrowhead**) in comparison with untreated mice (**A**). Liver of animals treated with D-glucose (**C**) maintains its physiological morphology, while pathological alterations remain evident when D-glucose is administered with IL-10 neutralizing antibodies (**D**). Scale bar = 40 μ m.

alone died within 6 hours, whereas all 10 mice pretreated with per os D-glucose (2.5 g/kg) survived. On the other hand, when D-glucose was administered i.p., a route of administration that does not induce activation of SGLT-1 expressed by intestinal epithelial cells, no protection from APAP induced mortality was observed (Figure 9A). The orally administered D-glucose is absorbed in the gut, across the brush border of enterocytes, by the sodium dependent transporter SGLT-1.¹⁸ To study the involvement of SGLT-1, in the observed protection, mice pretreated with D-glucose were treated also with the competitive inhibitor of SGLT-1 phloridzin (30 to 300 mg/kg) before administration of APAP (1125 mg/kg). Phloridzin completely nullified the protective effect of D-glucose, since all mice treated with phloridzin, D-glucose, and APAP died within 8 hours (Figure 9A). These results suggest that D-glucose, to exert its protective efficacy against acute liver injuries, requires interaction with and activation of SGLT-1 expressed on intestinal epithelial cells, similar to what we had previously observed in an animal model of LPS-induced shock.⁵

We have previously described that D-glucose pretreatment induces an increase in circulating levels of IL-10 in LPS/D-GalN-treated mice and that systemic administration of a neutralizing anti-IL-10 antibody removes the protective effect exerted by per os administered D-glucose on LPS/D-GalN-treated animals.⁵ In the present setting, circulating IL-10 levels in mice treated with a lethal

dose of APAP, with or without the administration (per os or i.p.) of D-glucose, reveal that D-glucose, if administered per os, strongly increased IL-10 levels also in this model of acute liver failure (APAP = 223 ± 167 vs glucose p.o. + APAP = 2457 ± 445 , $P = 0021$) (Figure 9B). In accordance with previous findings in LPS-induced shock, pretreatment with D-glucose did not protect from death induced by APAP in mice ($n = 5$) pretreated with neutralizing anti-IL-10 antibodies (Figure 9A), and the neutralization of endogenous IL-10 eliminates the protective effect of D-glucose on liver tissue (Figure 5D), resulting moreover in high ALT and LDH plasma levels (Figure 6).

Pretreatment with Per Os D-Glucose Does Not Protect from α -Amanitin-Induced Toxicity

Next, we evaluated the effect of D-glucose in an animal model of mushroom intoxication. For this purpose, C57BL/6 mice were treated per os with D-glucose or saline, and after 1 hour they were injected with 3.5 mg/kg of α -amanitin. Pretreatment with D-glucose almost completely failed to protect from amanitin toxicity, since all D-glucose-pretreated mice died within 14 hours showing only a small delay with respect to mice treated only with amanitin (Figure 10A). In accordance with the results from the survival experiment, profound morphological al-

Figure 6. Determination of ALT (A) and LDH (B) levels in acetaminophen (APAP)-treated mice. Data are the means ($\pm$ SD) of two independent experiments (3 mice/group). Untr = untreated mice; APAP = mice treated only with APAP, glu p.o.+APAP = mice orally treated with D-glucose and after 1 hour with APAP, glu i.p.+APAP = mice intraperitoneally treated with D-glucose plus APAP; abIL10 glu p.o.+APAP = mice treated with IL-10 neutralizing antibodies and D-glucose before APAP administration (* P < 0.05 glu+APAP versus APAP).

terations, dominated by extended fatty degeneration and necrosis (Figure 11A), were detected in liver samples from amanitin-treated mice, whether or not pretreated with per os D-glucose (Figure 11B). Moreover, the lack of protective activity by D-glucose was accompanied by a lack of increase of circulating levels of IL-10 (Figure 9B).

Figure 7. Determination of hepatic GSH concentration in APAP-challenge mice. Untreated mice (untr) or D-glucose-treated mice (glu) were challenged with APAP. Liver tissues from groups of three mice at the indicated times after APAP challenge were harvested, and hepatic GSH levels were measured.

Figure 8. Cytotoxicity of APAP in primary mouse hepatocytes. Mouse primary hepatocytes were treated with APAP (10 mmol/L) with or without D-glucose (10 g/L) for 24 hours. Treatment was started after 24 hours of seeding and LDH release in culture medium is measured 24 hours after APAP-treatment. Mean $\pm$ SD (n = 3). All experiments were performed with hepatocytes from three independent preparations (** P < 0.005 APAP and glu+APAP versus untr).

Protection by Per Os D-Glucose against APAP-Induced Liver Toxicity Requires the Contribution of a Second Signal

Our previously published data,⁵ together with the finding that i.p. administered D-glucose does not protect from APAP toxicity, strongly suggest that the D-glucose protection is mediated by the activation of SGLT-1 located in the apical membrane of enterocytes. These cells express also members of the TLR family and recognize LPS.¹⁹ In the LPS/D-GalN model, the increase of LPS plasma concentration that occurs during sepsis is induced by i.p. administration and it has been previously postulated an APAP-related LPS translocation from the gut caused by a secondary injury to the intestinal epithelium.²⁰ To explain the differences in the protective effect afforded by per os administered D-glucose against LPS- or APAP-induced liver injury on one hand, and amanitin-induced liver injury on the other hand, we reasoned that there might be differences in the activation of TLR expressed on enterocytes via LPS in the two different settings. Interestingly, no evidence about LPS was found in α -amanitin toxic mechanism in accord with the lack of systemic elevation of inflammatory cytokines.²¹

In accordance with this possibility, we found that plasma samples from APAP- but not amanitin-treated mice were positive at the Endotoxin test (ETOXATE kit), thereby suggesting the presence of circulating LPS in the first, but not the second liver injury model. To further confirm our hypothesis, we performed experiments with APAP $\pm$ D-glucose in TLR4 knock-out mice (TLR4^{-/-}). Wild-type mice treated with D-glucose before APAP challenge had low levels of circulating ALT, which were comparable with those of untreated mice. On the other hand, TLR4^{-/-} mice had high ALT levels, thereby revealing a failure of per os D-glucose to protect from liver damage in these mice (TLR4^{-/-} mice: glucose $\pm$ APAP = 1803 $\pm$ 168 versus wild-type mice: glucose + APAP = 126 $\pm$ 99

Figure 9. A: Plot of the percentage of survivors over time among mice injected i.p. with APAP. Mice were pretreated with saline (closed triangle), D-glucose orally (closed square) or intraperitoneally (open square) administered, phloridzin plus D-glucose (half-filled circle) or IL-10 neutralizing antibodies plus D-glucose (asterisk), 1 hour before APAP administration. Experimental group consisted of 10 mice. **B:** Determination of IL-10 plasma levels. Plasma samples were assayed for IL-10 levels by enzyme-linked immunosorbent assay. Values represent the mean $\pm$ SD (5 mice) (* $P < 0.05$ glu p.o.+APAP versus APAP and glu+LPS E amanitin versus amanitin).

after 24 hours; $P = 0.007$) (Figure 12A). Moreover, in TLR4^{-/-} mice, per os D-glucose administered before APAP failed to induce an increase in circulating levels of IL-10 like that observed in wild-type (TLR4^{-/-} mice: glucose + APAP = 728 ± 219 versus wild-type mice: glucose + APAP = 2457 ± 445 after 24 hours, $P = 0.039$) (Figure 12B).

Pretreatment with Per Os D-Glucose in the Presence of LPS Protects Mice from α -Amanitin Toxicity

The role of a second signal in D-glucose-mediated hepatic protection was then evaluated in α -amanitin induced toxicity. Groups of C57BL/6 mice were orally treated with D-glucose or saline; after 1 hour the mice were injected i.p. with amanitin or with amanitin plus a low, nonlethal dose of *E. Coli* LPS (250 μ g/kg); mice treated with 100% D-glucose plus amanitin died within 14 hours, showing a small delay with respect to only-aman-

Figure 10. Plot of the percentage of survivors over time among mice injected i.p. with α -amanitin. A: Mice were pretreated with saline and injected with α -amanitin (closed triangle) or with α -amanitin plus LPS (open triangle) and pretreated with D-glucose before α -amanitin (open square) or α -amanitin plus LPS administration (wild-type mice: closed square). Mice treated with phloridzin before α -amanitin plus LPS administration (split circle). **B:** TLR4^{-/-} mice; asterisk; open diamond: pretreatment of mice with neutralizing IL-10 antibodies. Experimental group consisted of 10 mice.

itin-treated mice, with death induced within 8 hours, while all D-glucose- and LPS-plus amanitin-treated mice survived. The protective effect of D-glucose was nullified in mice treated with phloridzin before α -amanitin plus LPS administration suggesting the involvement of SGLT-1 (Figure 10A). A strong increase in IL-10 plasma level was found in mice treated with α -amanitin plus LPS after D-glucose administration versus α -amanitin treated mice, while no significant differences are observed in mice treated with α -amanitin plus LPS or with D-glucose and α amanitin versus the group of α -amanitin treated mice (Figure 9B). The pretreatment of animals ($n = 5$) with neutralizing anti-IL-10 antibodies eliminates the protective effect of D-glucose before α -amanitin plus LPS administration, since all mice died within 10 hours (Figure 10B). In accordance with the survival pattern, in mice treated with amanitin plus LPS after D-glucose administration, a normal liver morphology was observed (Figure 11C) and the protective effect on liver tissue is abolished by the administration of anti-IL-10 antibodies (Figure 11D). The same experiment was performed also in TLR4^{-/-} mice. In this case, 100% mortality was observed also in mice treated with D-glucose and amanitin plus LPS (Figure 12B).

Figure 11. Histology of liver tissue after α -amanitin treatment. Sections from livers of α -amanitin treated mice show extended fatty degeneration (arrowhead) and necrosis (arrow) (A) and the same hepatic pathological alterations affect tissues from mice pretreated with D-glucose (B). When D-glucose treatment is followed by administration of α -amanitin plus a sublethal LPS dose, the liver maintains its normal morphology (C), whereas pathological alterations remain evident when D-glucose is administered with IL-10 neutralizing antibodies (D). Scale bar = 40 μ m.

Discussion

In the present study, we provide evidence that oral administration of D-glucose protects mice from hepatic injuries due to LPS shock or APAP overdose. Protection is afforded also against α -amanitin intoxication, but only when a second, inflammatory signal, such as a sublethal dose of LPS is co-administered with amanitin.

No protection from LPS-induced liver injury was observed on oral administration of D-fructose, a six-carbon monosaccharide that is absorbed in the gut by the facultative transporter GLUT5, expressed on the apical membrane of enterocytes.²² This is at variance to D-glucose, which uses SGLT-1 for absorption from the gut.²³ This finding supports the hypothesis that also in the present setting, as we had observed previously for LPS-induced shock, a specific D-glucose–SGLT-1 interaction is required to achieve protection from hepatic injury. Similarly, no protection from LPS/ D-GalN-induced liver injury is observed on oral administration of the disaccharide sucrose. Sucrose is hydrolyzed in the gut by the brush border enzyme sucrase²⁴ to yield D-glucose and D-fructose that were consequently absorbed in a longer time range, in comparison with what happens to the same monosaccharides administered alone.²⁵ The difference in the protective efficacy between D-glucose and sucrose, suggests the requirement for a copious availability of D-glucose in its monomeric form to trigger the protec-

tive response, most likely via a prompt activation of SGLT-1. During LPS/D-GalN intoxication an ICAM-1 overexpression¹⁴ has been observed in hepatocytes and nonparenchymal liver cells and was related to neutrophils extravasation,²⁶ the early apoptosis of hepatocytes and the amplification of necrotic liver injury.¹⁴ Our findings show a reduction of the endotoxin-caused overexpression of hepatic ICAM-1, consequent to intestinal SGLT-1 activation, that could explain the observed reduction in hepatocytes apoptosis.

Pretreatment was found to protect also from APAP-induced hepatic damage. Liver toxicity from APAP overdosing ensues when the drug is metabolized by P-450 enzymes to produce the highly toxic metabolite, *N*-acetyl-p-benzo-quinone imine. Under normal circumstances *N*-acetyl-p-benzo-quinone imine is inactivated through conjugation with GSH,⁷ but on APAP overdose, GSH is depleted and the formation of *N*-para-aminoquinonimine is strongly increased. As a result, this reactive metabolite binds covalently to cellular macromolecules⁸ leading to a potentially fatal centrilobular hepatic necrosis.⁹ Pretreatment with per os D-glucose in mice treated with a sublethal dose of APAP (300 mg/kg) succeeded in preserving a normal liver morphology and maintaining circulating ALT and LDH levels comparable with control mice. The same treatment rescued from death 100% of mice treated with a lethal dose of APAP (1125 mg/kg). The

Figure 12. **A:** ALT levels in TLR4^{-/-} mice. Wild-type (wt) or TLR4^{-/-} (KO) mice were pretreated with per os D-glucose and after 1 hour with acetaminophen (APAP). Plasma samples were collected after 8 and 24 hours and assayed for ALT activity. Data are the means ($\pm$ SD) of two independent experiments performed in triplicate. **B:** IL-10 serum levels. Mice were pretreated with per os D-glucose and after 1 hour with APAP. Samples were collected after 8 and 24 hours and were assayed for IL-10 levels by enzyme-linked immunosorbent assay. Values represent the mean $\pm$ SD (* P < 0.05 and ** P < 0.01 KO glu+APAP versus wt glu+APAP).

protection afforded by D-glucose in this model is not the result of an alteration of APAP metabolism, since reduction of tissue levels of GSH that takes place in the liver of APAP-treated mice remained unchanged also in those mice that were pretreated with D-glucose. Moreover, the failure of D-glucose to reduce cytotoxic effects caused by APAP treatment on murine primary hepatocytes, allows to exclude also any direct effect on hepatocytes. Eventually, the lack of protection by D-glucose administered i.p. confirms the essential role of intestinal epithelial cells in affording D-glucose-induced liver protection. This result is fully consistent with a requirement for SGLT-1-mediated glucose uptake, since hepatocytes, in contrast to intestinal epithelial cells, lack SGLT-1 on their plasma membrane.

When D-glucose was tested in a model of hepatonecrosis induced by amanitin intoxication, no protection was observed a part a delay in the dead onset. The former is probably due to an increase of liquid and metabolic increase. Hepatic failure may then ensue. α -amanitin, a typical mushroom poison,²⁷ is highly toxic for the liver due to high uptake and consequent fatty degeneration and necrosis of the affected hepatocytes.^{21,28}

We reasoned that the lack of protection by D-glucose in this setting might be due to the lack of a second, inflammatory signal like LPS that was present in the other

models that were investigated. In fact, studies from others had shown a significant involvement of TLR4-mediated response in APAP toxicity.²⁹ This led us to postulate an APAP-induced LPS translocation²⁰ from the gut to the bloodstream as a hallmark of APAP-induced toxicity. In accordance with these data and hypothesis, we detected LPS in plasma samples from APAP- but not amanitin-treated mice. Moreover, it is known from the literature that APAP overdosing induces an elevation of systemic inflammatory cytokines,³⁰ while in amanitin-induced toxicity, no such increase has been reported.²¹ In further support of our two-signal hypothesis, we observed that pretreatment with per os D-glucose of animals that are co-treated with α -amanitin and LPS prevents amanitin-induced liver injury. On the other hand, pretreatment with D-glucose failed to protect TLR4^{-/-} mice from APAP- and amanitin and LPS-induced liver toxicity. These results are highly suggestive of a mandatory role of a TLR4-mediated second signal in the protective effects of D-glucose in the herein investigated models of hepatotoxicity.

Protection from APAP and α -amanitin toxicity by pretreatment with per os D-glucose was found to correlate with the circulating levels of IL-10. The source of the anti-inflammatory cytokine IL-10 is widespread and unrelated to a single cell type. In response to a noxious stimulus, at the intestinal level, IL-10 might be directly synthesized by epithelial gut cells,³¹ but the prevailing concept in the literature is that IL-10 is synthesized, mainly by immune cells, including regulatory T cells, B cells, macrophages, and dendritic cells.^{32,33} Further studies are needed, aimed to identify the immunological players involved and to investigate on the mechanism triggered by D-glucose administration, leading to IL-10 production.

In accordance with the present results of ours, previous results had shown that administration of this cytokine preserves liver from endotoxemia,³⁰ APAP,³⁴ and carbon tetrachloride-induced injury, as well as concanavalin A-induced acute T cell-mediated hepatitis.³⁵ IL-10 neutralizing antibodies, inhibiting the observed protective effects in APAP and α -amanitin models, prove that IL-10 is necessary for the protection, while not excluding the involvement of other factors.

Overall, these results show that per os administered D-glucose exerts protective effects in different models of acute liver injury and that these effect results, among others, by the increase of the anti-inflammatory cytokine IL-10. This protective activity requires the contribution of a secondary, inflammatory signal, which may be generated in the course of the disease process or needs to be administered deliberately. These findings might in view of possible therapeutic interventions in settings of acute hepatic injury.

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