Glycine and Uridine Prevent D-Galactosamine Hepatotoxicity in the Rat: Role of Kupffer Cells

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Extrahepatic factors, such as increased gut permeability and bacteria from the gut, have been shown to play a role in D-galactosamine toxicity in rats. Because bacterial endotoxin activates Kupffer cells, the purpose of this study was to clarify the role of Kupffer cells in the mechanism of D-galactosamine hepatotoxicity in rats and determine whether uridine, a compound that rescues animals from D-galactosamine toxicity, affects Kupffer cells. Rats were fed control or glycine (5%) containing diets to prevent Kupffer cell activation or treated with gadolinium chloride ($GdCl_3$, 20 mg/kg) to destroy Kupffer cells selectively before injection of D-galactosamine (500 mg/kg, intraperitoneally). D-galactosamine caused panlobular focal hepatocellular necrosis, polymorphonuclear cell infiltration, and increased serum transaminases significantly at 24 hours. Dietary glycine or pretreatment with $GdCl_3$ prevented these effects. D-galactosamine caused a transient increase in circulating endotoxin that was maximal at 1 hour and was blunted significantly by dietary glycine. Additionally, antisera to tumor necrosis factor-α (TNF-α) prevented hepatotoxicity caused by D-galactosamine. Moreover, apoptosis in hepatocytes caused by D-galactosamine occurred before necrosis (6 hours) and was prevented by glycine, $GdCl_3$, TNF-α antiserum, and uridine. Thus, it was hypothesized that TNF-α from Kupffer cells causes apoptosis after D-galactosamine administration in the rat. Indeed, increases in TNF-α messenger RNA (mRNA) were detected as early as 2.5 hours after D-galactosamine treatment. Previous work proposed that uridine blocks D-galactosamine toxicity by preventing inhibition of mRNA synthesis. In view of these results, the possibility that uridine might affect Kupffer cells was investigated. Uridine significantly blunted the increase in $[Ca^{2+}]_i$ and release of TNF-α caused by endotoxin in isolated Kupffer cells and prevented apoptosis caused by D-galactosamine treatment *in vivo*. These data support the hypothesis that uridine prevents D-galactosamine hepatotoxicity not only by rescuing the hepatocyte in the late phases of the injury but also preventing TNF-α release from Kupffer cells thereby blocking apoptosis that occurs early after D-galactosamine treatment. Taken together, these data strongly support the role of Kupffer cell activation by endotoxin early after D-galactosamine treatment as an important event in the mechanism of hepatotoxicity in the rat. (Hepatology 1999;29:737-745.)

D-galactosamine causes panlobular focal hepatocyte necrosis, infiltration of polymorphonuclear cells, and enlargement of macrophages resembling drug-induced hepatitis.[1-3] In recent years, the mechanism by which D-galactosamine causes liver injury has been a subject of debate. Early biochemical studies postulated that D-galactosamine caused liver injury by depleting the uridine pool in hepatocytes, resulting in inhibition of messenger RNA (mRNA) and protein synthesis.[4-6] It was concluded that the toxicity of D-galactosamine was caused by the inability of the cell to synthesize critical cell membrane components, leading to hepatocyte death.[5,7] In support of this conclusion, uridine supplementation prevented D-galactosamine toxicity *in vivo* and *in vitro*.[8,9]

However, more recent studies suggest that extrahepatic factors such as increased gut permeability, bacterial translocation, and endotoxemia are involved in D-galactosamine hepatotoxicity.[10-13] Indeed, in the mouse, liver injury after D-galactosamine is observed only when a low dose of endotoxin is also administered,[10] causing activation of Kupffer cells and tumor necrosis factor-α (TNF-α) release.[14] On the other hand, D-galactosamine causes hepatotoxicity in the rat without additional endotoxin.[1] This difference may be because of the fact that D-galactosamine causes endotoxemia in rats but not in mice.[15] The role of endotoxin in the rat has been postulated, because monoclonal antibodies to endotoxin, colectomy, and sterilization of the gut block D-galactosamine-mediated liver injury.[11,12] However, the role of Kupffer cells in the mechanism of D-galactosamine toxicity is less clear. Shiratori et al.[12] showed that modifying macrophage activation with injection of latex beads also modulates D-galactosamine hepatotoxicity, but the role of Kupffer cells has not been tested directly. Similarly, Chojkier and Fierer[16] showed that the lymphoreticular endothelial system and sensitivity to endotoxin in the mouse played roles in the toxicity of D-galactosamine in the mouse. Based on these studies, it is plausible that gut-derived endotoxin released

Abbreviations: mRNA, messenger RNA; TNF-α, tumor necrosis factor-α; $[Ca^{2+}]_i$, intracellular calcium; cDNA, complementary DNA; PCR, polymerase chain reaction; G_3PDH, glyceraldehyde-3-phosphate dehydrogenase; LPS, lipopolysaccharide.

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after D-galactosamine treatment activates Kupffer cells leading to the production of cytokines, such as TNF-α, resulting in liver injury.

The purpose of this study was to test the hypothesis that activated Kupffer cells and TNF-α are involved in the mechanism of D-galactosamine–mediated liver injury in the rat using glycine and gadolinium chloride to block Kupffer cells or antisera to TNF-α. Dietary glycine prevents mortality caused by intravenous endotoxin administration in the rat by blocking Kupffer cell activation and blunting increases in serum TNF-α.[17] The mechanism of glycine-mediated inhibition involves activation of a chloride channel on the Kupffer cell membrane resulting in chloride influx and hyperpolarization of the cell membrane, which blocks agonist-induced increases in intracellular calcium ($[Ca^{2+}]_i$).[18] Furthermore, based on the results from these studies and the fact that uridine protects against D-galactosamine hepatotoxicity, the hypothesis that uridine alters Kupffer cell function was also tested. Preliminary accounts of these studies have appeared elsewhere.[19]

MATERIALS AND METHODS

Animals, Diet, and Treatment Protocols. Male Sprague-Dawley rats (175-225 g) were fed a diet containing 5% glycine in powdered rat chow or 5% valine (control diet) in powdered rat chow for 3 days before administration of D-galactosamine.[17] Supplementing the diet with valine balances nitrogen and does not affect the response of Kupffer cells to endotoxin.[17,18] Rats were given 500 mg/kg D-galactosamine, (Sigma Chemical Company, St. Louis, MO; intraperitoneally, 50 mg/mL dissolved in pyrogen-free saline, pH 7.4) or a comparable amount of saline vehicle and allowed free access to powdered diets and water. This preparation of D-galactosamine contained no detectable levels of endotoxin. In some experiments, animals were given gadolinium chloride ($GdCl_3$, 20 mg/kg intravenously, dissolved in acidic saline) 24 hours before, TNF-α antisera (2,500,000 U/kg, intravenously) 1 hour before, or uridine (1 mmol/kg) immediately preceding and every 3 hours after the injection of D-galactosamine. All animals were given humane care in compliance with institutional guidelines according to "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health.

Collection of Liver Sections and Blood. After D-galactosamine or saline injection, rats were anesthetized with methoxyflurane, and 3 mL of blood were taken from the vena cava and allowed to clot. Serum was collected and stored at −20°C for analysis of aspartate transaminase and alanine transaminase activity by standard enzymatic methods.[20] The organ was flushed via the portal vein with 30 mL of phosphate buffered saline (pH 7.4) followed by 30 mL of 4% phosphate-buffered formalin, sectioned, and prepared for light microscopy. One thousand cells were counted per section by a reviewer blinded to the treatment groups to determine the frequency of apoptosis in the liver.[21]

Determination of Blood Endotoxin. Endotoxin was measured essentially as described by Iimuro et al.[22] Briefly, the rat was anesthetized and blood was collected from the portal vein in pyrogen-free sterile syringes after a sterile laparotomy. Blood was dispensed into pyrogen-free glass tubes and centrifuged at 1,000*g* for 10 minutes. Plasma was collected and stored in pyrogen-free glass tubes until measurement of endotoxin with a Limulus Amebocyte Lysate test kit (Kinetic-QCL; BioWhittaker, Walkersville, MD).

Determination of TNF-α mRNA in the Liver by Reverse Transcription-Polymerase Chain Reaction. Complementary DNA (cDNA) synthesis and polymerase chain reaction (PCR) amplification of mRNA isolated from livers were performed essentially as described elsewhere.[23] Approximately 100-mg samples of liver tissue were homogenized in 2 mL of RNAzol B solution (Biotex Laboratories, Inc., Houston, TX), and total cellular RNA was extracted according to the manufacturer's instructions. RNA was dissolved in Tris-ethylenediaminetetraacetic acid buffer (10 mmol/L Tris-HCl, 1 mmol/L ethylenediaminetetraacetic acid, pH 8.0). For the synthesis of cDNA, 3 μg total RNA from each sample was resuspended in a 20 μL final volume of a reaction buffer consisting of 25 mmol/L Tris-HCl, pH 8.3, 37.5 mmol/L KCl, 10 mmol/L dithiotreitol, 1.5 mmol/L $MgCl_2$, 10 mmol/L of each dNTP (Perkin Elmer Cetus, Norwalk, CT), and 0.5 ng oligo d(T) 12-18 primer (Gibco-BRL, Gaithersburg, MD). After the reaction mixture reached 42°C, 400 U SuperScript reverse transcriptase (Gibco-BRL) was added to each tube, incubated for 35 minutes at 42°C, and stopped by denaturing the enzyme at 99°C for 5 minutes. The reaction mixture was diluted with distilled water to a volume of 50 μL. PCR primers for rat TNF-α and glyceraldehyde-3-phosphate dehydrogenase (G_3PDH) were purchased from Clontech Laboratories, Inc. (Palo Alto, CA) and contained the following sequences: sense, 5′-TACTGAACTTCGGGGTGATTGGTCC-3′; antisense 5′-CAGCCTTGTCCCTTGAAGAGAACC-3′ for TNF-α and sense, 5′-TGAAGGTCGGTGTCAACGGATTTGGC-3′; anti-sense, 5′-CATGTAGGCATGAGGTCCACCAC-3′ for G_3PDH. The amplified products contained 692 bp for TNF-α and 983 for G_3PDH.

Five-microliter aliquots of the synthesized cDNA (corresponding to 10 ng of mRNA) were added to 45 μL of mixture containing 5 μL of 10 × PCR buffer, 1 μL deoxynucleotides (1 mmol/L each), 0.5 μL of sense and antisense primers (0.15 mmol/L), and 0.25 μL DNA polymerase (GeneAmp PCR kit; Perkin Elmer Cetus). The reaction mixture was covered with a wax tablet (Perkin Elmer Cetus), and amplification was initiated by 1 minute of denaturation at 94°C for 1 cycle, then by 25, 30, or 35 cycles at 94°C for 15 seconds, 55°C for 30 seconds, and 72°C for 30 seconds using a DNA thermal cycler (Perkin Elmer Cetus). The samples were incubated for 7 minutes at 72°C after the last cycle of amplification. When necessary, the concentrations of cDNA were normalized to G_3PDH and the PCR process repeated. For each set of primers, dilutions of cDNA were amplified for 20, 23, 25, 28, 30, 33, and 35 cycles to define optimal conditions for linearity and to permit semiquantitative analysis of signal strength. When appropriate, the specificity of the PCR bands was confirmed by restriction site analysis of the amplified cDNA, which generated restriction fragments of the expected size.

The amplified PCR products were separated by electrophoresis through 2.0% agarose gel (UltraPure; Gibco-BRL) at 60 V for 90 minutes. Bands of cDNA were visualized by ultraviolet illumination after staining with 0.5 g/mL ethidium bromide. Gels were photographed with type-55 positive/negative film (Polaroid, Cambridge, MA), and films were analyzed using the Eagle Eye II Image Analysis System (Stratagene, La Jolla, CA) and NIH Image 1.54 software (National Institutes of Health, Bethesda, MD).

Toxicity of D-Galactosamine in Cultured Hepatocytes. Hepatocytes were isolated by enzymatic digestion and plated at 1×10^5 cells/well in glycine-free Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin and the medium was changed 3 hours later.[24] The plated cells contained less than 0.1% Kupffer cells as determined by phagocytosis of latex beads. Hepatocytes were cultured for 24 hours in the presence of D-galactosamine (0.5 mmol/L), glycine (0 to 10 mmol/L), or uridine (3 mmol/L). Release of lactate dehydrogenase was determined by standard enzymatic methods and was used as a marker of cytotoxicity.[20]

Isolation and Culture of Kupffer Cells and Measurement of $[Ca^{2+}]_i$. Kupffer cells were isolated by enzymatic digestion and density centrifugation as described by Smedsrod et al.[25] Cells were cultured on glass coverslips for 1 hour and the medium was changed to remove nonadherent cells. Twenty-four hours after cell isolation, cytosolic free Ca^{2+} in individual Kupffer cells was assessed fluorometrically using the fluorescent calcium indicator fura-2 and a microspectrofluorometer as described elsewhere.[18] Kupffer cells were incubated in modified Hanks' balanced salt solution containing 5 μmol/L fura-2/acetoxymethyl ester (Molecular Probes Inc., Eugene, OR) at 25°C for 30 to 40 minutes. Cells were stimulated with endotoxin (lipopolysaccharide [LPS]; *Escherichia coli*, serotype

026:B6; Sigma Chemical Co.) containing 5% rat serum in modified Hank's balanced salt solution. Changes in fluorescence intensity of fura-2 at excitation wavelengths of 340 nm and 380 nm were monitored in individual cells using a microspectrofluorometer (PTI, South Brunswick, NJ) interfaced with an inverted microscope (Nikon Ltd., Tokyo, Japan). Each value was corrected by subtracting the system dark noise and autofluorescence was assessed by quenching fura-2 fluorescence with Mn^{2+}. Intracellular free calcium was calculated from the equation:

$$[Ca^{2+}]_i = K_d([R - R_{min}]/[R_{max} - R])(Fo/Fs),$$

where Fo/Fs is the ratio of fluorescent intensities evoked by 380 nm light from fura-2 pentapotassium salt in buffered salt solutions containing nanomolar Ca^{2+} ($[Ca^{2+}]_{min}$) and millimolar Ca^{2+} ($[Ca^{2+}]_{max}$); R is the ratio of fluorescent intensities at excitation wavelengths of 340 nm and 380 nm; R_{max} and R_{min} are values of R at $[Ca^{2+}]_{max}$ and $[Ca^{2+}]_{min}$, respectively. The values of these constants were determined at the end of each experiment, and a K_d value of 135 nmol/L was used.[26]

Measurement of TNF-α in Isolated Kupffer Cells. TNF-α was measured from isolated Kupffer cells cultured in 24-well plates essentially as described by Ikejima et al.[18] Briefly, cells were plated at 5×10^5 cells/mL for 24 hours before experiments. Endotoxin (10 μg/mL) was added to the wells in the presence of 5% rat serum and the medium was harvested 4 hours later. TNF-α was determined using an enzyme-linked immunosorbent assay kit (Genzyme, Cambridge, MA).

Statistics. Data are presented as mean ± SEM. Comparisons were made using ANOVA with Bonferroni's post-hoc test or Student's *t* test, where appropriate, as indicated in the figure legends. The criterion for significance of $P < .05$ was selected before the study.

RESULTS

The Effect of Glycine and Gadolinium Chloride on D-Galactosamine Hepatotoxicity. To determine if compounds that block Kupffer cell function would prevent the toxicity of D-galactosamine, animals were fed a diet containing 5% glycine or control diet for 3 days before treatment with 500 mg/kg D-galactosamine. In some experiments, animals fed control diet were treated with $GdCl_3$ (20 mg/kg, intravenously) 24 hours before D-galactosamine. Blood samples for serum aminotransferase activity and liver histology were collected at 24 hours, the peak of injury after a single dose of D-galactosamine.[3] As shown in Fig. 1, D-galactosamine increased serum aspartate aminotransferase (Fig. 1A) and alanine aminotransferase (Fig. 1B) significantly, as expected. However, dietary glycine or $GdCl_3$ pretreatment prevented the increase of transaminases caused by D-galactosamine almost completely.

Representative histology of liver from the different treatment groups is shown in Fig. 2. A liver section from a control animal is shown in Fig. 2A for comparison. D-galactosamine (Fig. 2B) caused panlobular focal necrosis and periportal

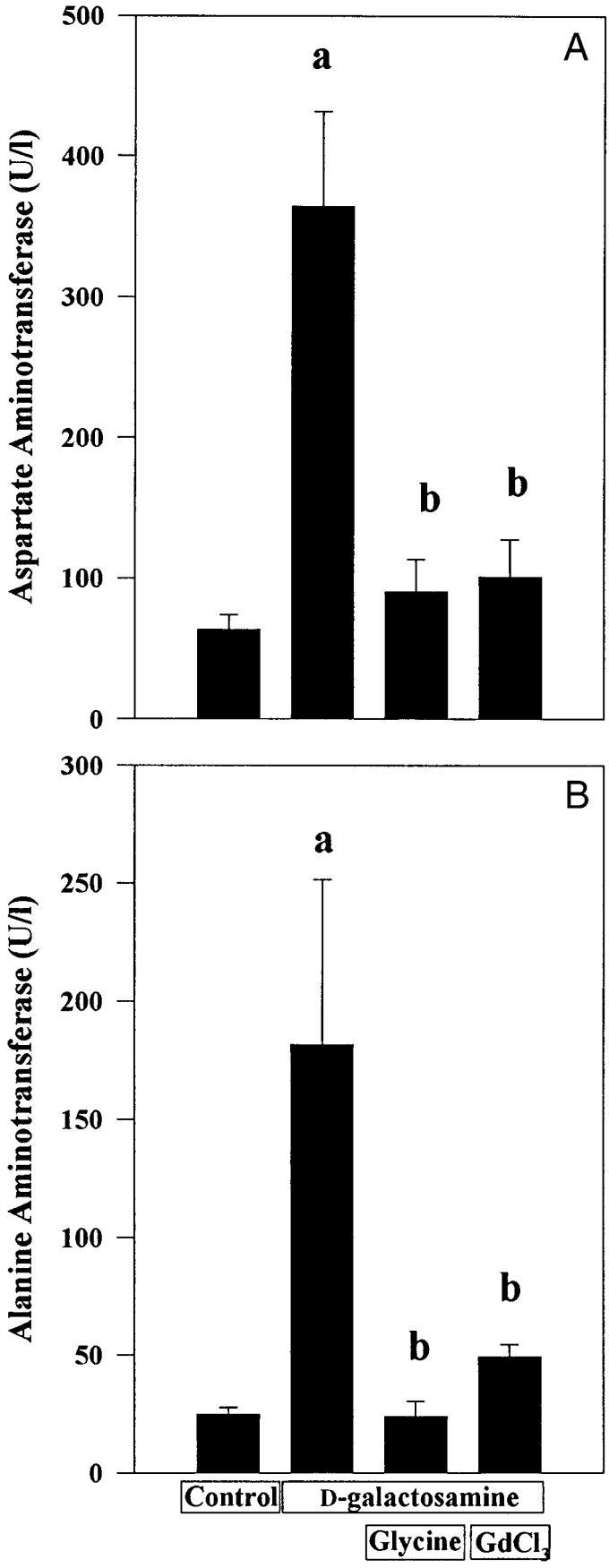


FIG. 1. The effect of dietary glycine or gadolinium chloride on serum transaminases after D-galactosamine treatment. Animals were fed glycine or control diet for 3 days before injection of D-galactosamine as described in Materials and Methods. In some experiments, rats fed control diet were treated with $GdCl_3$ (20 mg/kg) intravenously 24 hours before D-galactosamine. Values are expressed as mean ± SEM (n = 4). (A) Aspartate aminotransferase. (B) Alanine aminotransferase. (a) Significantly different from saline-treated animals fed control diet. (b) Significantly different from animals treated with D-galactosamine. $P < .05$ by two-way ANOVA with Bonferroni's post-hoc test.

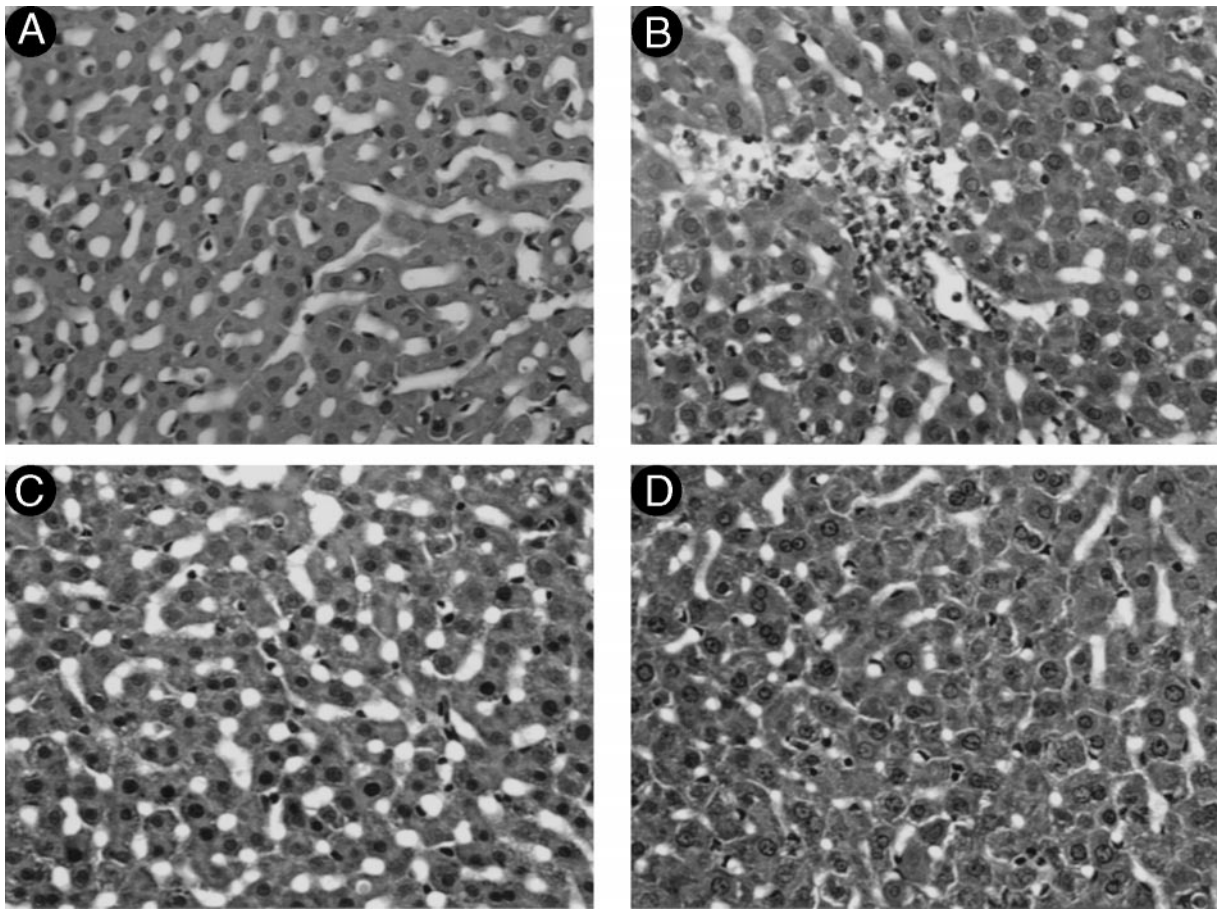


FIG. 2. The effect of dietary glycine or $GdCl_3$ on histology of liver from D-galactosamine-treated rats. Livers were perfused and prepared for light microscopy as described in Materials and Methods. (A) Control liver, (B) 500 mg/kg D-galactosamine for 24 hours, (C) dietary glycine (5%) + D-galactosamine, (D) $GdCl_3$ (20 mg/kg) + D-galactosamine. Figure shows typical liver histology at original magnification ×100.

inflammation, which was accompanied by an inflammatory infiltrate of predominantly polymorphonuclear cells with a few lymphocytes and swollen macrophages, as expected.[2] Feeding glycine for 3 days (Fig. 2C) or treating with $GdCl_3$ (Fig. 2D) before D-galactosamine injection prevented these changes nearly completely.

Inflammation was quantified by counting infiltrating polymorphonuclear cells 24 hours after D-galactosamine. Few polymorphonuclear cells were observed in livers of control animals (Fig. 3); however, D-galactosamine treatment caused more than a fivefold increase compared with controls. Feeding dietary glycine or destroying Kupffer cells with $GdCl_3$ before D-galactosamine reduced infiltrating polymorphonuclear cells significantly nearly to basal values.

The Effect of Glycine on D-Galactosamine Injury to Cultured Hepatocytes. Because it is possible that glycine could act *in vivo* by protecting hepatocytes from the toxic effects of D-galactosamine directly like uridine,[27,28] freshly isolated hepatocytes were cultured and treated with 0.5 mmol/L D-galactosamine or a comparable amount of medium (control) in the presence of glycine (0-10 mmol/L) or 3 mmol/L uridine. Cell viability was assessed 24 hours later. D-galactosamine caused death of hepatocytes in culture (69.7 ± 2.2% viability compared with control). Glycine (10 mmol/L) had no effect on D-galactosamine toxicity to hepatocytes *in vitro* (71.3 ± 4.6% viability compared with control); however, as expected,[27] uridine (3 mmol/L) provided complete protection (102.6 ± 4.7% viable compared with control).

The Effect of Dietary Glycine on Blood Endotoxin Levels After D-Galactosamine. D-galactosamine increases the translocation of bacteria to the spleen,[29] and glycine decreases injury after hypoxia-reoxygenation of the gut.[30] Furthermore, in models where Kupffer cell activation and increased gut permeability are implicated in the mechanism of toxicity, such as ethanol-induced liver injury,[31] it has been shown that blood endotoxin is increased and serves as a stimulus to macrophages. In pilot studies it was shown that endotoxin levels in blood increased from barely detectable levels to near 60 pg/mL 1 hour after D-galactosamine (Fig. 4). Within 2.5 hours, blood endotoxin levels were back to control values (data not shown). As shown in Fig. 4, dietary glycine decreased peak blood endotoxin levels significantly compared with controls.

The Effect of TNF-α Antiserum on D-Galactosamine Hepatotoxicity. D-galactosamine potentiation of LPS-induced hepatotoxicity in the mouse is blocked by antiserum to TNF-α,[14] but the role that TNF-α plays in D-galactosamine–mediated necrosis in the rat is unclear. Accordingly, rats were treated with TNF-α antiserum 1 hour before administration of D-galactosamine and sacrificed 24 hours later. As before, D-galactosamine increased serum aminotransferases significantly; however, levels were lower than in previous experi-

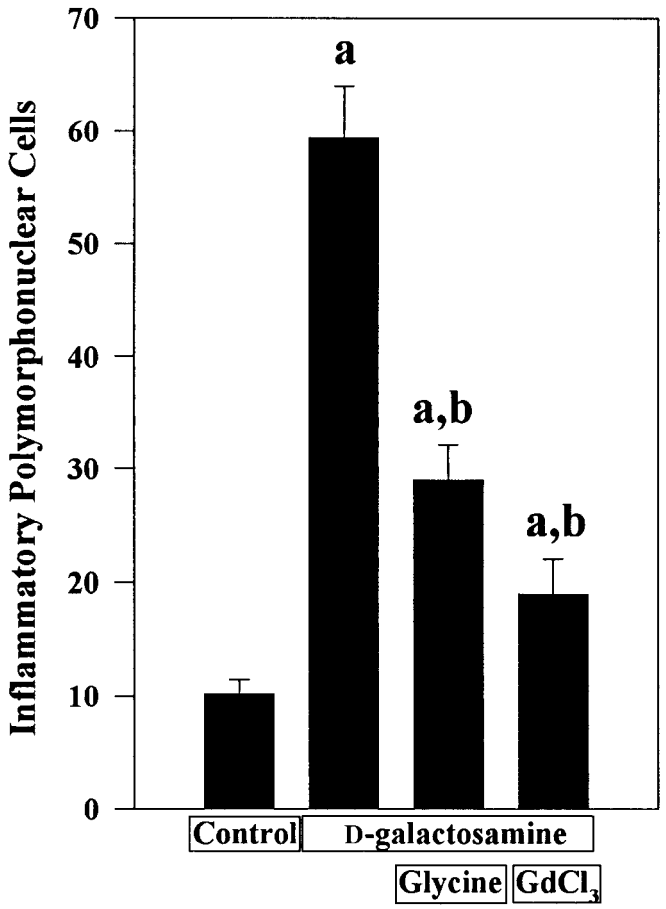


FIG. 3. The effect of dietary glycine or $GdCl_3$ treatment on polymorphonuclear infiltrate due to D-galactosamine treatment. Polymorphonuclear cells were counted in 10 random histological fields 24 hours after D-galactosamine. Data are expressed as mean of cells per 10 fields ± SEM (n = 4). (a) Significantly different from saline-treated animals fed control diet. (b) Significantly different from animals treated with D-galactosamine. $P < .05$ by two-way ANOVA with Bonferroni's post-hoc test.

ments. This may be because of the fact that a different lot of D-galactosamine was used in these experiments. In any event, TNF-α antiserum prevented the increase in serum transaminases caused by D-galactosamine (Fig. 5) and also decreased necrosis and infiltration of polymorphonuclear cells.

Apoptosis of Hepatocytes After D-Galactosamine. TNF-α production has also been associated with apoptosis of hepatocytes, which precedes necrosis in many models[32,33]; yet apoptosis was not observed 24 hours after D-galactosamine in this study. TNF-α message was increased as early as 2.5 hours after D-galactosamine (data not shown). Therefore, to determine if D-galactosamine caused apoptosis before increases in serum transaminases (*i.e.*, necrosis), rats were sacrificed 6 hours after D-galactosamine. Indeed, nearly 2% of hepatocytes exhibited apoptosis at 6 hours (Fig. 6). Inhibiting Kupffer cell function with $GdCl_3$ or glycine, treatment with TNF-α antibody, and uridine blunted the increase in apoptosis caused by D-galactosamine significantly.

The Effect of Uridine on Kupffer Cell Activation. Uridine has been shown to prevent D-galactosamine hepatotoxicity *in vivo*[5] and in isolated hepatocytes[27,28]; therefore, the hypothesis that uridine also affects Kupffer cells was tested. Kupffer cells were isolated and plated, and endotoxin-mediated increases in $[Ca^{2+}]_i$ were measured fluorometrically after fura-2 loading in the presence or absence of uridine (1 mmol/L) (Fig. 7A and B). Endotoxin treatment caused a transient increase in $[Ca^{2+}]_i$ to more than 250 nmol/L above basal values, which was blunted over 60% by uridine. Furthermore, uridine (1 mmol/L) prevented LPS-induced production of TNF-α in isolated Kupffer cells (Fig. 8).

DISCUSSION

Dietary Glycine Prevents D-Galactosamine Toxicity in the Rat. One major finding of this study is that feeding glycine to rats blocks D-galactosamine hepatotoxicity as evidenced by the prevention of increases in serum transaminases (Fig. 1), decreased histological changes (Fig. 2), and blunted infiltration of polymorphonuclear cells (Fig. 3). However, glycine does not protect hepatocytes *per se*. The precise mechanism by which glycine prevents injury is not entirely clear; however, two actions could be involved and are summarized in Fig. 9. First, glycine prevents increases in blood endotoxin levels caused by D-galactosamine (Fig. 4). This could decrease the activation of Kupffer cells by endotoxin, which has

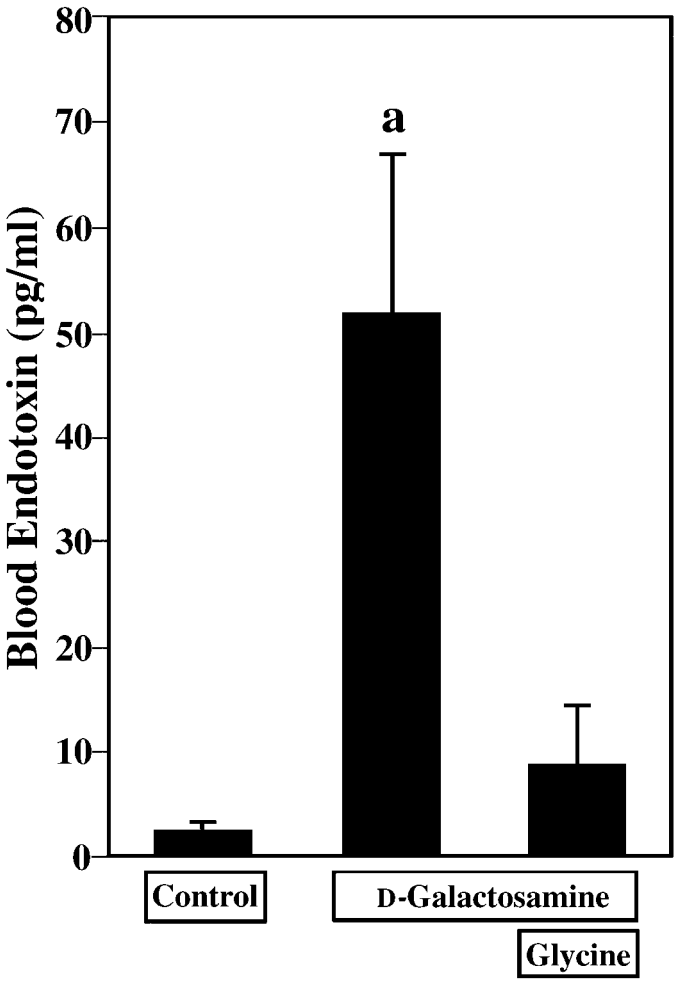


FIG. 4. The effect of glycine on blood endotoxin levels after D-galactosamine. Animals were fed glycine or control diet for 3 days before injection of D-galactosamine as described in Materials and Methods. Blood was collected from the portal vein 1 hour after D-galactosamine treatment. Data are expressed as mean ± SEM (n = 4). (a) Significantly different from control animals. $P < .05$ by one-way ANOVA with Bonferroni's post-hoc test.

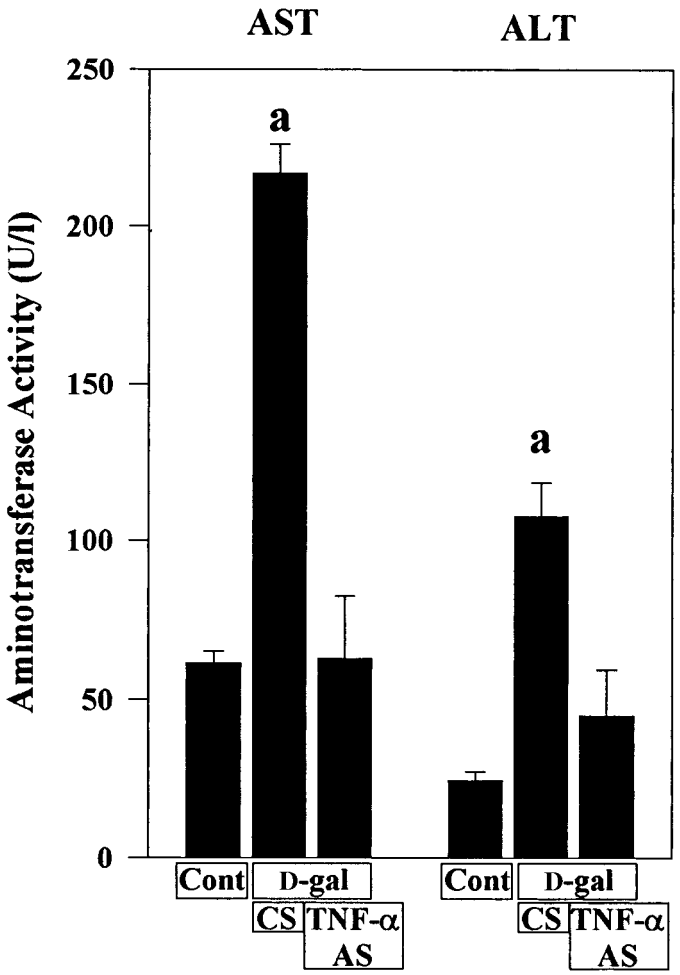


FIG. 5. The effect of TNF-α antiserum on increases in serum transaminases due to D-galactosamine. Animals were treated with control rabbit immunoglobulin G or TNF-α antiserum (2,500,000 U/mL) intravenously 1 hour before injection of D-galactosamine or a comparable amount of saline in controls. Values are expressed as mean ± SEM (n = 4). D-gal, D-galactosamine; CS, control serum; AS, antiserum. (a) Significantly different from control animals treated with rabbit immuoglobulin G. $P < .05$ by two-way ANOVA with Bonferroni's post-hoc test.

been implicated in the mechanism of D-galactosamine toxicity.[10-14] How D-galactosamine increases endotoxin levels in the blood is not understood, but overt gut pathology was not observed. However, D-galactosamine treatment decreases blood flow in the gut, which may allow bacteria or endotoxin to enter the blood through the intestinal wall.[14] D-galactosamine also activates mast cells directly to release histamine, which increases gut permeability.[11] Preliminary data from this laboratory suggest that glycine blunts mast cell degranulation.

Second, glycine may protect by blocking Kupffer cell function. $GdCl_3$, a specific Kupffer cell toxicant, blocked D-galactosamine toxicity (Figs. 1-3), clearly illustrating the importance of Kupffer cells in the mechanism of injury in the rat. Glycine blunts activation of isolated Kupffer cells by endotoxin[18] and prevents production of TNF-α both *in vitro* and *in vivo*.[17,18] This occurs through activation of a glycine-gated chloride channel on the Kupffer cell,[18] resulting in hyperpolarization, thereby preventing opening of voltage-dependent calcium channels found on the plasma membrane and blunting increases in $[Ca^{2+}]_i$.

TNF-α Plays a Role in D-Galactosamine Hepatotoxicity in the Rat. It has been shown previously that administration of small amounts of endotoxin to D-galactosamine–treated mice caused apoptosis and necrosis that were blocked by TNF-α antiserum.[15] Furthermore, inhibition of RNA or protein synthesis in hepatocytes with D-galactosamine, actinomycin D, or α-amanitin sensitized hepatocytes to the toxic effect of TNF-α both *in vivo* and *in vitro*.[34] Accordingly, because Kupffer cells participate in D-galactosamine hepatotoxicity and activated Kupffer cells are a major source of TNF-α in the liver,[35] it was hypothesized that TNF-α plays a role in D-galactosamine–mediated hepatotoxicity in the rat. Indeed, treatment of rats with TNF-α antiserum decreased liver injury caused by D-galactosamine in this study (Fig. 5). Furthermore, TNF-α message was increased before apoptosis after D-galactosamine. Therefore, it is concluded that TNF-α, which is released primarily from activated Kupffer cells, is

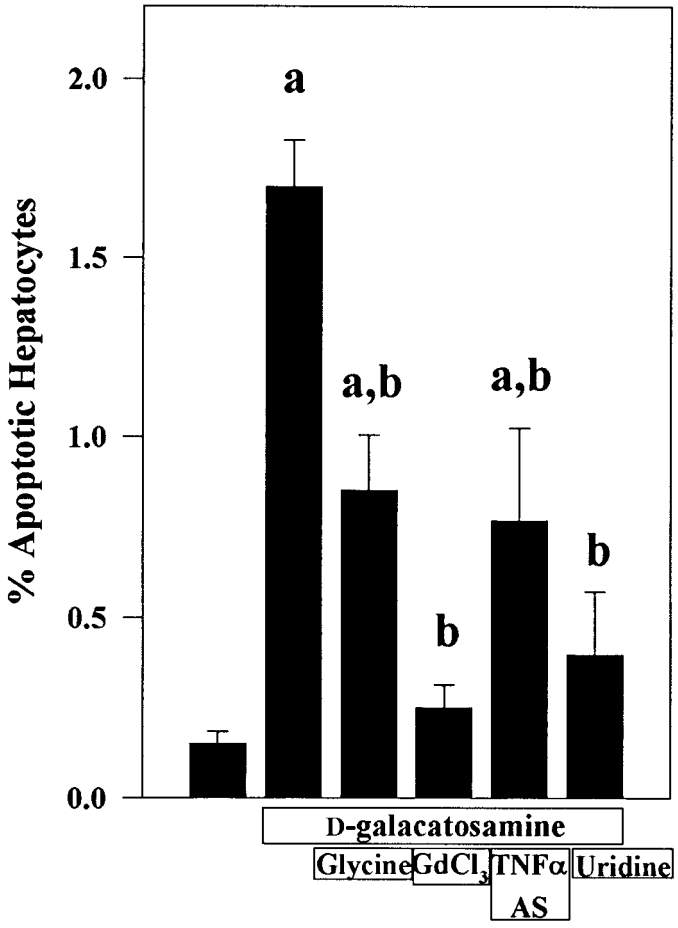
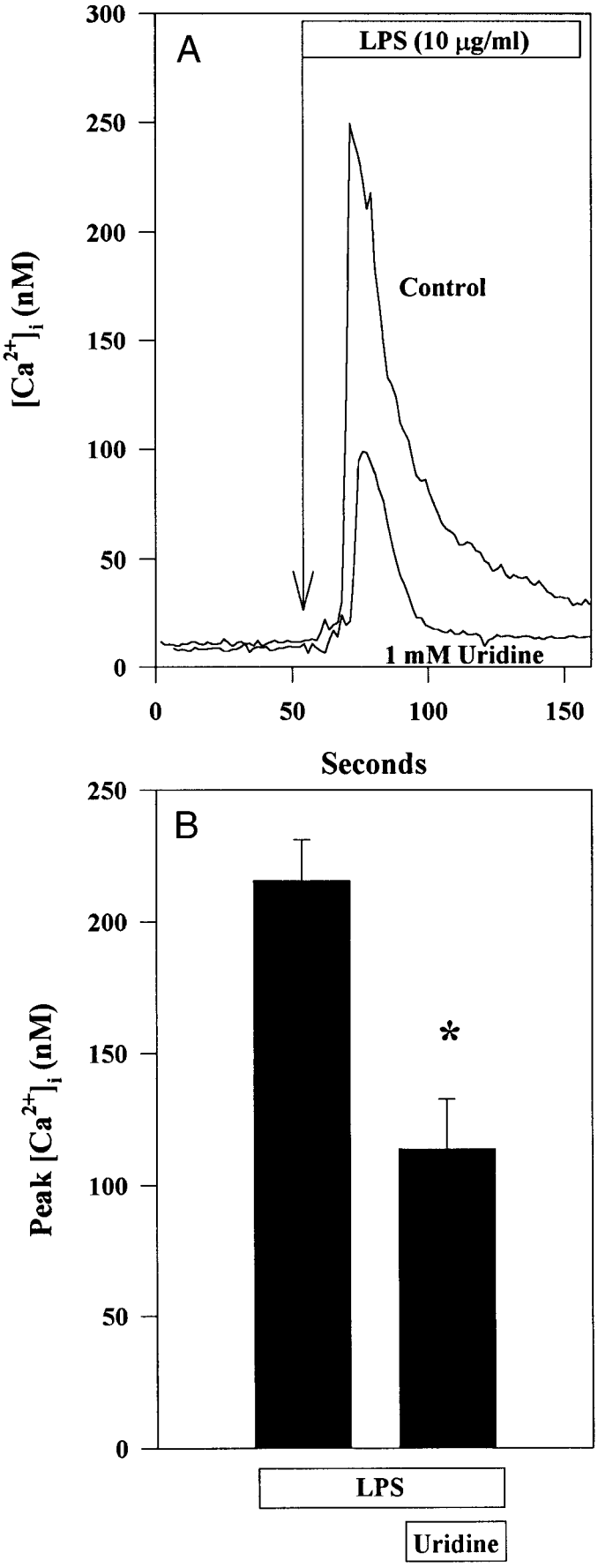


FIG. 6. The effect of dietary glycine, $GdCl_3$, TNF-α antiserum, or uridine on apoptosis after D-galactosamine. Animals were fed glycine or control diet for 3 days. In some experiments rats were treated with $GdCl_3$ (20 mg/kg) or TNF-α antiserum (2,500,000 U/mL) intravenously 1 hour before injection of D-galactosamine or uridine (1 mmol/kg every 3 hours) immediately before D-galactosamine as described in Materials and Methods. Rats were sacrificed 6 hours later and liver sections were prepared for light microscopy. One thousand hepatocytes were counted and the percentage of hepatocytes undergoing apoptosis was determined in slides blinded to the reviewer. Values are expressed as mean ± SEM (n = 4). AS, antiserum. (a) Significantly different from control. (b) Significantly different from animals treated with D-galactosamine alone. $P < .05$ by two-way ANOVA with Bonferroni's post-hoc test.



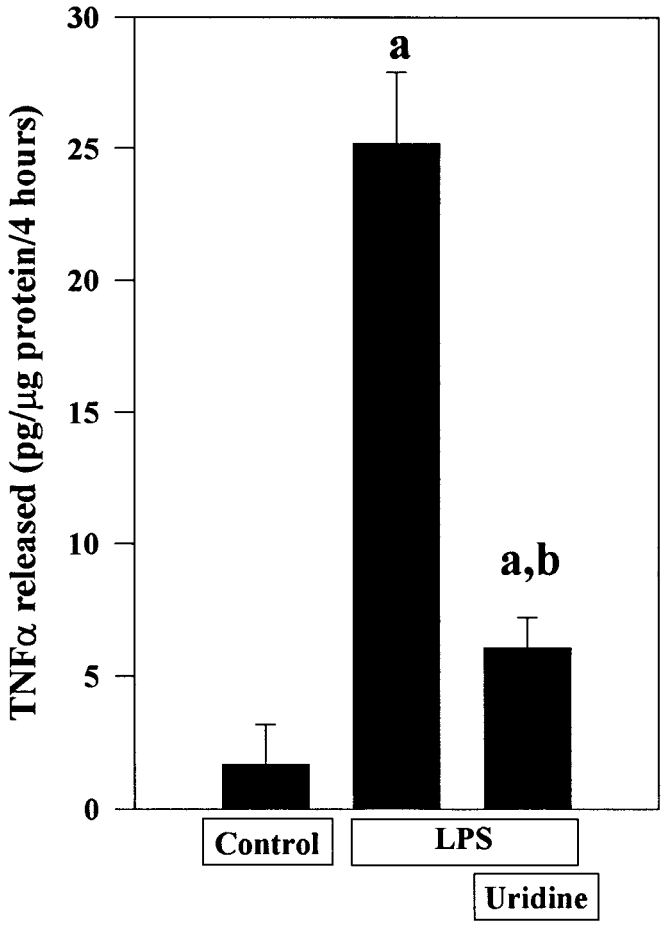


FIG. 8. The effect of uridine on TNF-α production by isolated Kupffer cells. Isolated Kupffer cells were cultured at 5×10^5 cells/mL for 24 hours before treatment with uridine (1 mmol/L) for 1 hour. LPS (final concentration 100 ng/mL in 5% rat serum) was added to the media and the incubation was continued for 4 hours. TNF-α was measured by enzyme-linked immunosorbent assay. Control treatment was rat serum (5%) without endotoxin. Data are presented as mean ± SEM (n = 4). (a) Significantly different from control. (b) Significantly different from endotoxin treatment. $P < .05$ by one-way ANOVA with Bonferroni's post-hoc test.

involved in hepatocyte death after D-galactosamine treatment.

Uridine Prevents Activation of Kupffer Cells. It is well known that D-galactosamine treatment depletes the uridine pool via formation of Uridine 5′-Diphosphate-galactosamine.[5] Uridine depletion occurs much faster than the *de novo* synthesis of pyrimidine nucleotides resulting in an arrest of RNA and protein synthesis. Furthermore, supplementing with uridine or compounds that increase uridine synthesis *in vitro* and *in vivo* protects the liver from D-galactosamine toxicity.[8] Thus, it has been concluded that the mechanism of protection by uridine is repletion of the uridine pool in the hepatocyte.[8]

FIG. 7. The effect of uridine on LPS-mediated increases in $[Ca^{2+}]_i$ in Kupffer cells. Intracellular calcium was measured in individual cultured Kupffer cells using the fluorescent calcium indicator fura-2 as described in Materials and Methods. Cells were incubated for 3 minutes with 1 mmol/L uridine (1 mM) or with no additions (control). The solution was then replaced with the same buffer containing LPS (final concentration 10 μg/mL) in 5% rat serum. (A) Representative data. (B) Mean ± SEM (n = 6-8). *Statistically different from LPS-treated cells. $P < .05$ by Student's *t* test.

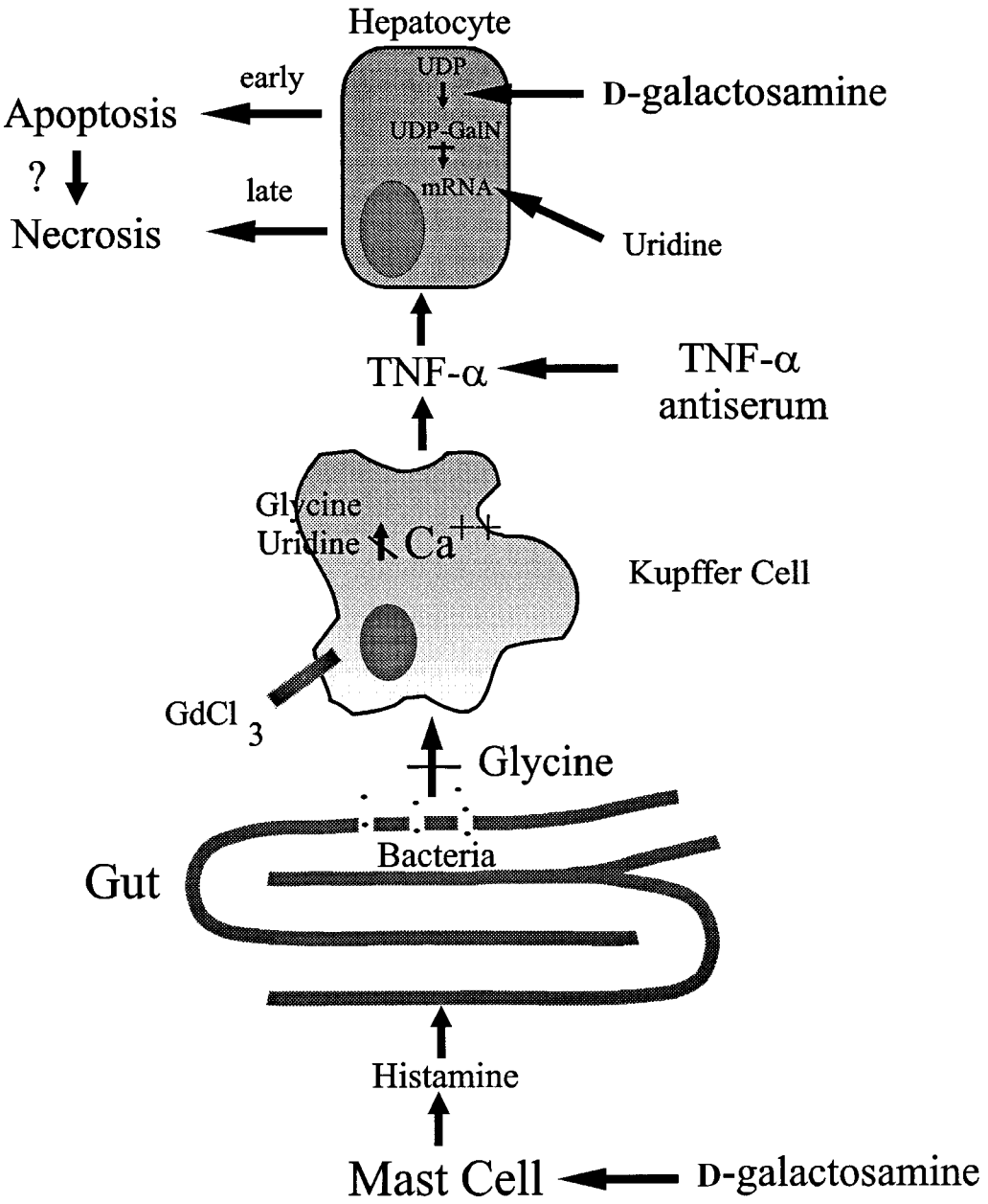


FIG. 9. The proposed mechanism of D-galactosamine hepatotoxicity in the rat. D-galactosamine treatment directly activates mast cells to release histamine, which increases gut permeability and release of bacterial products from the gut. Kupffer cells are activated, which is a pivotal event in the toxicity of D-galactosamine *in vivo* in the rat because compounds that prevent Kupffer cell activation ($GdCl_3$, glycine, and uridine) block the injury. D-galactosamine inhibits mRNA and protein synthesis in hepatocytes increasing the sensitivity to TNF-α released from Kupffer cells, which activate signaling pathways leading to cell death. Cells first begin to die via apoptosis as soon as 6 hours. Subsequently, inflammatory cells enter the liver parenchyma and areas of necrosis develop.

The results presented here show clearly that Kupffer cell activation plays an integral role in the toxicity of D-galactosamine and that uridine decreases TNF-α production after D-galactosamine. Accordingly, the hypothesis that uridine prevents Kupffer cell activation was tested. Indeed, uridine inhibited Kupffer cells, as evidenced by blunted endotoxin-induced increases in $[Ca^{2+}]_i$ (Fig. 7) and prevention of TNF-α release (Fig. 8). The mechanism by which uridine prevents stimulation of Kupffer cells by endotoxin is not clear; however, it is taken up by cells,[36] and in neurons it causes membrane hyperpolarization.[37] It is possible that uridine hyperpolarizes the Kupffer cell membrane decreasing activation of voltage-gated calcium channels analogous to the influx of chloride ions caused by the glycine-gated chloride channel.[18] It is concluded that uridine not only prevents D-galactosamine toxicity *in vivo* by increasing uridine pools in hepatocytes, but it also prevents activation of Kupffer cells and diminishes TNF-α production, which are critical for toxicity.

Apoptosis After D-Galactosamine Treatment Is Dependent on Kupffer Cells in the Rat. Apoptosis precedes or accompanies the development of necrosis after endotoxin administration to galactosamine-sensitized mice and is dependent on TNF-α.[15] However, apoptosis could not be detected at 24 hours after D-galactosamine treatment in this study (data not shown). Therefore, it was hypothesized that apoptosis occurs early after D-galactosamine administration before necrosis, which is reflected by release of serum transaminases. Indeed, a significant increase in apoptotic hepatocytes was detected 6 hours after D-galactosamine (Fig. 6), well before necrosis, and increases in serum transaminases were detected. Furthermore, dietary glycine, uridine, destruction of Kupffer cells with $GdCl_3$, or pretreatment with TNF-α antiserum prevented this early apoptosis. It is concluded that both apopto-

sis and necrosis caused by D-galactosamine are dependent on Kupffer cell activation and the production of TNF-α. Moreover, apoptosis and necrosis occur at discrete time points in this model of D-galactosamine hepatotoxicity in the rat. Therefore, the model described here could be useful in studying factors responsible for these two types of cell death. The data presented here are consistent with the hypothesis that apoptosis and necrosis are linked because they are both prevented by interventions that block Kupffer cell function.

A Plausible Mechanism of D-Galactosamine Toxicity in the Rat. Based on previous work and data presented here, the following model for the mechanism for D-galactosamine toxicity in the rat is proposed (Fig. 9). D-galactosamine directly activates mast cells to release histamine, which increases gut permeability.[11] Bacterial products released from the gut[13] activate Kupffer cells to release cytokines, such as TNF-α, which is a pivotal event in the toxicity of D-galactosamine *in vivo*. D-galactosamine inhibits mRNA and protein synthesis in hepatocytes[9] increasing the sensitivity of these cells to TNF-α–mediated events that activate signaling pathways leading to cell death.[38] Cells first begin to die via apoptosis as soon as 6 hours. Subsequently, inflammatory cells enter the liver parenchyma, and areas of necrosis develop later. Data from these studies suggest that the subsequent development of necrosis is triggered by similar pathways that activate apoptosis because both mechanisms are dependent on activation of the Kupffer cell and production of TNF-α.

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