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Galactose protects hepatocytes against TNF-α-induced apoptosis by promoting activation of the NF-κB signaling pathway in acute liver failure

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Saccharides are reported to protect hepatocytes from acute liver injury through distinct mechanisms. To date, the protective role of galactose against acute liver injury induced by lipopolysaccharide (LPS) and D-galactosamine (D-GalN) has been attributed to competition with D-GalN. Here, we showed that in addition to its effects on LPS/D-GalN and tumor necrosis factor alpha (TNF-α)/D-GalN models, galactose improves hepatic injury in mice challenged with LPS alone or TNF-α/actinomycin D. Consistent with this result, galactose enhanced the viability of TNF-α-stimulated Chang Liver and Hu7.5 hepatic cell lines. Specifically, galactose prevented TNF-α-induced apoptosis of hepatocytes through promoting phosphorylation of nuclear factor kappa B (NF-κB) p65. Additionally, galactose enhanced expression of the anti-apoptotic genes, c-IAP1 and A20, and inhibited cleavage of caspase-8 and caspase-3. These findings collectively suggest that galactose prevents TNF-α-induced liver injury through activation of the NF-κB signaling pathway. Considering that monosaccharides protect against liver injury via distinct mechanisms, these compounds may represent a promising clinical approach to treat acute liver failure.

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Acute liver failure (ALF), defined as rapid onset of severe hepatic dysfunction with poor prognosis, is a serious problem in clinical practice.[1] A model of hepatic injury induced by lipopolysaccharide (LPS) and D-galactosamine (D-GalN) has been widely used to explore the mechanisms of ALF and screen for potential hepatoprotective drugs.[2] In this model, LPS triggers innate immune responses and induces TNF-α, which subsequently activates the pro-apoptotic caspase reaction directly or indirectly via mitogen-activated protein (MAP) kinases, including p38, JNK1/2 and ERK1/2, as well as an anti-apoptotic signal via the transcription factor nuclear factor kappa B (NF-κB).[3–5] D-GalN, which acts as a sensitizing agent, amplifies the toxicity of LPS and TNF-α to liver.[6]

Since liver has a central role in human metabolism, ALF may cause metabolic defects and energy imbalance.[7] As the main source of energy, saccharides have an important role in maintaining hepatocyte survival and physiological processes. A recent study revealed that oral administration of glucose prevents endotoxin- and drug-induced liver failure by promoting secretion of IL-10 to repress liver inflammation in LPS/D-GalN and acetaminophen-treated mice.[8] Another monosaccharide, fructose, prevents TNF-α/ actinomycin D (ActD)-induced apoptosis of hepatocytes through inhibiting JNK signaling in a PKA-dependent manner.[9]

Earlier studies have reported that galactose prevents LPS/D-GalN-induced liver damage in mice.[10] A recent metabolic profiling study revealed a significant decrease in galactose in the plasma of LPS/D-GalN-treated mice.[7] For decades, the protective role of galactose has been attributed to competition with D-GalN.[11] However, recent evidence has challenged this notion. First, galactose and glucose share the same enzyme machinery for conversion to UDP-galactose and UDP-glucose, respectively, as well as D-GalN to UDP-GalN.[12] Since glucose can prevent liver damage without competing

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with D-GalN, the same strategy may also be feasible for galactose. Second, galactose specifically binds to the asialoglycoprotein receptor 1 (ASGR1) on the hepatocyte surface.[13–15] Notably, ASGR1-deficient mice exhibit more serious liver damage after CCl_4 challenge.[16] Thus, the precise mechanisms underlying the protective role of galactose against liver failure require further elucidation.

Data from the present study showed that galactose enhances the expression of anti-apoptotic genes via activating the NF-κB signaling pathway and inhibits caspase-3-mediated apoptosis in hepatocytes. Our results collectively provide molecular support for the application of galactose as a feasible strategy to protect hepatocytes against TNF-α-induced cell death.

MATERIALS AND METHODS

Reagents

LPS (from *E. coli*, O111:B4), D-galactosamine, galactose, fructose, glucose, lactose and ActD (from *Streptomyces* sp.) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Recombinant TNF-α (rTNF-α) was obtained from Sino Bio Biotech Co. (Shanghai, China). Complete protease inhibitor cocktail tablet (Cat. 4693116001) and phosphatase inhibitor cocktail tablet (Cat.04906837001) were acquired from Roche Applied Science (Mannheim, Germany).

Animals and Treatments

Male C57BL/6J mice (6–8 weeks old) were provided by the Institute of Laboratory Animal Sciences, Chinese Academy of Medical Sciences (Beijing, China), and housed under a 12 h light-dark cycle. All research animals were used in compliance with the guidelines of Institutional Animal Care and Use Committee of Capital Medical University.

To induce ALF, three mouse models of LPS/D-GalN, TNF-α/D-GalN and TNF-α/ActD were used. All reagents were injected intraperitoneally (i.p.). For LPS-induced ALF, mice were injected with LPS (0.25 mg/kg) plus D-GalN (400 mg/kg). In the TNF-α/D-GalN model, 30 μg/kg TNF-α and 700 mg/kg D-GalN were co-injected into mice, unless otherwise specified. In the TNF-α/ActD model, ActD (2 mg/kg) was injected 15 min before TNF-α (0.8 mg/kg) challenge. The total injection volume was 500 μl per mouse.

For oral administration, galactose, glucose, fructose or lactose was dissolved in 500 μl double-distilled water. For i.p. and intravenous (i.v.) administration, galactose was dissolved in 100 μl physiological saline and injected into mice 5 min after toxin challenge.

Mice were observed for up to 24 h to determine survival rates. To establish the effects of saccharides *in vivo*, mice were killed at 3 or 5 h after toxin challenge, and serum collected for detection of cytokine, chemokine and transaminase activities. A portion of liver was allocated for histologic analysis and the remaining portion stored for mRNA and protein detection.

Transaminase and Lactate Dehydrogenase Activities

Activities of serum alanine transaminase (ALT), aspartate transaminase (AST) and lactate dehydrogenase (LDH) were determined using an automatic analyzer (Model 7600 Series, Hitachi, Japan).

Multiplex Cytokine Assay

For serum cytokine measurement, the BD CBA Mouse Inflammation Kit (BD Biosciences, San Jose, CA, USA) was used to determine IL-12p70, TNF-α, IFN-γ, MCP-1, IL-10 and IL-6 levels. Data were acquired on a BD™ FACS Calibur flow cytometer from measurements performed using the manufacturer's instructions.

Histology and TUNEL Assay

Livers were excised and immediately fixed with 10% buffered formalin. Samples were embedded in paraffin and cut into 5 μm sections. Liver sections were deparaffinized, rehydrated and stained with hematoxylin and eosin (H&E) following the standard protocol for histological analysis. Apoptotic hepatocytes were detected with the terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assay using the *In Situ* Cell Death Detection kit (Merck, Darmstadt, Germany), according to the manufacturer's instructions.

Primary Hepatocyte Isolation and Apoptosis Detection

For hepatocyte preparation, mice were anesthetized with an i.p. injection of bromethol. Livers were subjected to two-step collagenase perfusion through the portal vein. First, liver was perfused with DPBS containing 0.02% EDTA at a flow rate of 5 ml/min for 10–15 min. When the liver swelled and began to turn white, the vena cava was quickly cut. This was followed by perfusion of liver with HBSS supplemented with 0.05% collagenase type I (Sigma-Aldrich Co.) at a flow rate of 3 ml/min for 8–10 min. Perfusion buffers were preheated to 37 °C. After two-step perfusion, liver was excised and hepatocytes flushed out and filtered through a 250-μm pore size mesh nylon filter. Cells were washed three times with cold DMEM/F12 and centrifuged at 500 r.p.m. for 3 min. Cell viability was $>80\%$, as evaluated with trypan blue exclusion. Hepatocytes were seeded on collagen-coated 24-well plates at a concentration of 1×10^5/well and cultured with DMEM/F12 containing 10% FBS. Two hours later, unattached cells were washed away with PBS and fresh medium with supplemental factors (5 μg/ml insulin, 0.1 μM dexamethasone, 25 ng/ml transferrin, 20 ng/ml epidermal growth factor, 100 μg/ml penicillin and 100 U/ml streptomycin) added for overnight culture.

To examine apoptosis, cells were pretreated with 20 μM BAY-11-7082 and 1 mg/ml galactose for 1 h, subsequently challenged with 0.5 μg/ml TNF-α and 50 ng/ml ActD for 12 h, and subjected to the Annexin V-FITC assay (BioLegend, San Diego, CA, USA) with flow cytometry.

Cell Culture and Treatment

Human hepatic Chang Liver and human hepatocarcinoma Hu 7.5 cells were maintained in RPMI-1640 (PAA, Pasching, Austria) supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a 5% CO_2 humidified incubator. For determination of protein expression, Chang Liver cells were pretreated with 1 μg/ml galactose for 1 h and stimulated with 200 ng/ml TNF-α for 5, 15 and 30 min, followed by washing with ice-cold PBS and homogenization with RIPA lysis buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS and 1 mM EDTA) containing proteinase and phosphatase inhibitors, and 1% phenylmethanesulfonyl fluoride (PMSF).

Cell Viability Assay

Chang Liver and Hu 7.5 cells were cultured with RPMI-1640 in the presence or absence of 10 or 100 μg/ml galactose. After 1 h, 50 ng/ml TNF-α and 0.5 μg/ml ActD were added and incubated for a further 24 h, followed by washing with PBS. Cell viability was evaluated using Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan). The absorbance of each well at 450 nm was measured using a microplate reader (Varioskan Flash; Thermo Fisher Scientific, Waltham, MA, USA).

Western Blot

Whole-cell protein and liver tissue homogenates were extracted for western blot analysis. The BCA Protein Assay Kit (Pierce, Rockford, IL, USA) was used to determine concentrations. Protein was denaturated with 5 × loading buffer at 95 °C for 5 min. Aliquots of protein (60 μg) were loaded in each lane, separated via SDS-PAGE, and transferred onto PVDF membrane. Membranes were incubated with primary antibodies against IKK-α, IKK-β, p-IKK-α/β, IκBα, p-IκBα, NF-κB p65, p-NF-κB p65, JNK1/2, p-JNK1/2, p38, p-p38, caspase-8, cleaved caspase-8, c-IAP1 and β-actin (Cell Signaling Technology, Beverly, MA, USA), antibody against caspase-3 (Santa Cruz Biotechnology, Inc., Texas, CA, USA), and antibodies against A20, Gadd45β and c-Flip (Abcam, Cambridge, England) overnight at 4 °C. All images are representative of at least two independent experiments.

Real-Time PCR

Total RNA from mouse liver was isolated with TRIzol reagent (Life Technology, Carlsbad, CA, USA). RNA (2 μg) was reverse-transcribed into cDNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA), and specific transcripts quantified with real-time PCR using the Applied Biosystems 7500 Real-Time PCR System. Quantitative real-time PCR was performed using Fast Start Universal SYBR Green Master (Roche Applied Science). The primers used in the study were as follows: c-Flip, GCGACCGCCCGGTAGTGTCT (forward) and CCACAGCA GCCAGGTTCTCGT (reverse); c-IAP1, TGTGGCCTGATG TTGGATAAC (forward) and GGTGACGAATGTGCAAATC TACT (reverse); Gadd45b, CAACGCGGTTCAGAAGATGC (forward) and GGTCCACATTCATCAGTTTGGC (reverse); A20, CCCCTGGTGACCCTGAAGGACA (forward) and CCGTGGTCCCAGCCTTGCAC (reverse); beta-actin, GGCT GTATTCCCCTCCATCG (forward) and CCAGTTGGTAA CAATGCCATGT (reverse). The relative mRNA levels of specific genes were normalized to that of β-actin.

Electrophoretic mobility shift assay

Nuclear extracts were prepared from 100 mg liver tissue using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Pierce). Electrophoretic mobility shift assay (EMSA) was conducted with the Light Shift Chemiluminescent EMSA Kit (Pierce), according to the manufacturer's protocol. Oligonucleotide probes were labeled with biotin. The sequence of the NF-κB response element probe was 5′-GAGAGTCACGTGACTTT GGAAAGTCCCGTGGAAATC-3′. In competitive binding assays, unlabeled oligonucleotides were added at 200-fold molar excess.

Statistical Analysis

Log-rank test (for survival) and one-way ANOVA (for cytokines, transaminase activity, cell viability and mRNA) were employed for statistical analyses. The Bonferroni method was used for comparison between two groups after one-way ANOVA. *P*-values less than 0.05 were considered as statistically significant.

RESULTS

Galactose Prevents LPS/D-GalN-Induced Liver Injury Without Affecting TNF-α Production

To investigate the effects of monosaccharides on LPS-induced ALF, mice were orally treated with galactose, glucose or fructose (1250 mg/kg body weight) 5 min after LPS/D-GalN challenge. Similar to previous findings, LPS/D-GalN challenge led to ALF, and mice died within 8 h.[17–19] Galactose induced a significant increase in the survival rates of LPS/D-GalN mice (Figure 1a), along with a decrease in transaminase activity in serum (Figure 1b). Histological examination of liver tissue via H&E staining revealed significant protection of the liver structures of galactose-treated mice (Figure 1c, top panel). The *in situ* TUNEL assay was further used to evaluate cell apoptosis in liver. As shown in Figure 1c (middle and bottom panels), we observed a significant increase in the number of TUNEL-positive cells indicative of apoptosis after LPS/D-GalN challenge, which was markedly reduced after galactose treatment. However, glucose and fructose at the same dose failed to protect mice from LPS/D-GalN-induced liver failure and apoptosis (Figures 1a–c).

Considering that abundant secretion of TNF-α induced by LPS appears to be the major cause of lethality in LPS/D-GalN mice, we investigated whether galactose affects the production of pro-inflammatory and anti-inflammatory cytokines. Notably, neither galactose nor glucose affected cytokine production in serum and liver (including TNF-α, IL-6,

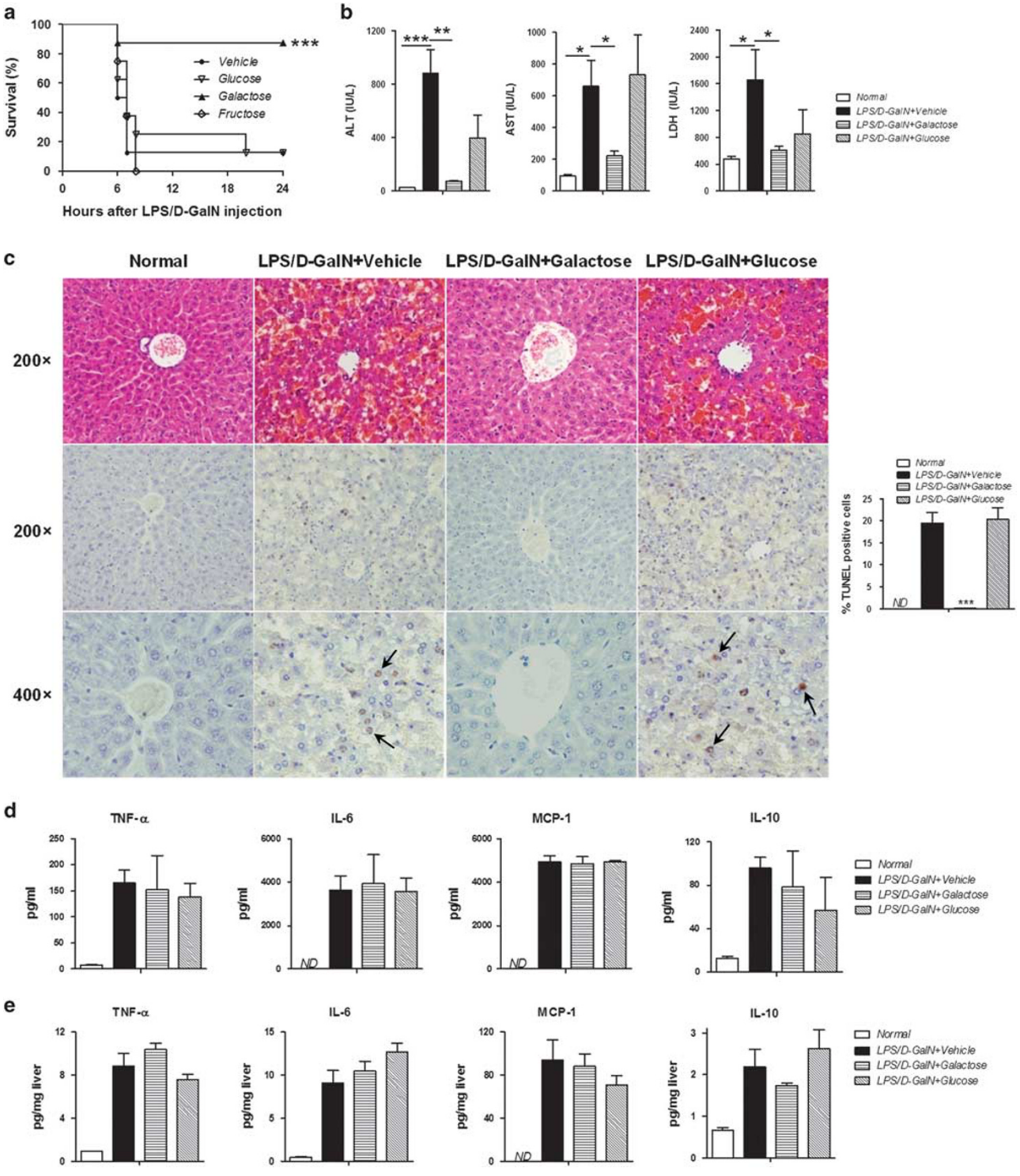

Figure 1 Galactose protects mice from LPS/D-GalN-induced ALF without affecting cytokine production in serum and liver. Mice were i.p. injected with LPS (0.25 mg/kg) plus D-GalN (400 mg/kg) to induce ALF. After 5 min, galactose, glucose or fructose (1250 mg/kg) was orally administered. Mice given the same volume of double-distilled water were used as the vehicle control. (**a**) Survival curves of LPS/D-GalN mice treated with galactose, glucose or fructose ($n=8$). Survival rates were analyzed using the log-rank test. (**b**) Activities of ALT, AST and LDH in serum were measured 5 h after LPS/D-GalN injection. Data represent means ± s.e.m. of three independent experiments ($n=4$). (**c**) Representative liver histopathology of mice treated 5 h after LPS/D-GalN injection with H&E (top) and TUNEL staining (middle and bottom). Arrows indicate TUNEL-positive hepatocytes induced by LPS/D-GalN challenge. Original magnification is ×200 or ×400. Percentage of TUNEL-positive hepatocytes summarized from the TUNEL assay. Values represent percentages from at least 1000 counted cells ($n=4$). Cytokine levels in serum (**d**) and liver (**e**) 3 h after LPS/D-GalN challenge, detected with CBA technology. Data represent means ± s.e.m. of three independent experiments ($n=4$). $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

MCP-1 and IL-10) at 3 h after LPS/D-GalN challenge (Figures 1d and e).

In view of our finding that the protective effect of galactose is not attributable to impaired TNF-α production, we speculated that galactose affects TNF-α-induced hepatic failure. To test this hypothesis, we investigated the effects of galactose on livers of mice treated with TNF-α (30 μg/kg) and D-GalN (700 mg/kg). In challenged mice, galactose protected against TNF-α/D-GalN-induced liver injury (Figures 2a and e, top panel). The protective effect of galactose on liver was further confirmed by treatment with different doses of TNF-α (Figure 2b). Additionally, examination of the survival rate and serum transaminase activities consistently showed that galactose exerts a protective effect in a dose-dependent manner (Figures 2c and d). The TUNEL assay further revealed that galactose reduces the frequency of apoptosis induced by TNF-α/D-GalN to a significant extent (Figure 2e, middle and bottom panels). Moreover, this protective effect could be achieved by oral administration, i.v. injection, as well as i.p. injection (Figure 2f).

The Protective Effect of Galactose Is Not Attributed to Competition with D-GalN

To further clarify whether galactose exerts a protective effect via competing with GalN, we examined the serum ALT level in LPS-injected mice. Consistent with data from LPS/D-GalN and TNF-α/D-GalN models, galactose triggered a significant decrease in ALT activity (Figure 3a).

In combination with TNF-α, the sensitizing agent ActD, a transcription inhibitor, was employed to induce liver injury instead of D-GalN.[9,20] As shown in Figure 3b, serum transaminase activities in TNF-α/ActD mice were reduced upon galactose treatment via oral administration, while i.v. injection led to reduction of ALT and LDH activities, but not AST. We additionally cultured two hepatic cell lines, Chang Liver and Hu 7.5, in the presence or absence of TNF-α (50 ng/ml) and ActD (0.5 μg/ml) for 24 h. In keeping with *in vivo* data, galactose impaired TNF-α/ActD-induced cell death in a dose-dependent manner (Figures 3c and d). Thus, the protective effect of galactose appears independent of competition with D-GalN. To further evaluate the mechanism underlying the anti-apoptotic effect of galactose, we performed Annexin-V staining on primary hepatocytes from mice. As shown in Figure 3e, TNF-α/ActD induced evident apoptosis, which was compromised upon galactose administration. However, treatment with BAY-11-7082, a potential and irreversible inhibitor of IκBα phosphorylation, resulted in an antagonized effect of galactose. These findings indicate that galactose protects hepatocytes against apoptosis via the IκBα/NF-κB signaling pathway.

Galactose Activates the NF-κB Signaling Pathway

In hepatocytes, TNF-α-induced apoptosis is mainly regulated by the NF-κB and MAP kinase pathways. To further clarify the mechanism by which galactose protects hepatocytes from apoptosis, Chang Liver cells were pretreated with or without galactose for 12 h and stimulated with 200 ng/ml TNF-α for the indicated times. As shown in Figure 4a and Supplementary Figure 1a, galactose had no effect on TNF-α-induced phosphorylation of MAP kinases, including p38, JNK1/2 and ERK1/2. On the other hand, despite the finding that upstream signals of the NF-κB pathway, such as IKK-α and IKK-β, are not influenced by galactose, phosphorylation and degradation of IκBα and phosphorylation of NF-κB p65 were visibly enhanced in galactose-pretreated cells between 15 and 30 min after TNF-α stimulation (Figure 4b; Supplementary Figure 1b). Similarly, galactose enhanced phosphorylation of NF-κB p65 in livers of mice treated with TNF-α/D-GalN (Figure 4c; Supplementary Figure 1c) or TNF-α/ActD (Figure 4d; Supplementary Figure 1d). Our results collectively suggest that galactose promotes TNF-α-induced activation of the NF-κB signaling pathway.

Galactose Promotes Expression of c-IAP1 and A20 to Inhibit Caspase Activation

To further determine the effects of galactose on the DNA binding activity of NF-κB, EMSA was performed with mouse liver nuclear extracts. As shown in Figure 5a, we observed clear induction of NF-κB-DNA complex formation in nuclear extracts of TNF-α (lane 3) and TNF-α/D-GalN mouse liver (lane 4). The amounts of complex detected in liver nuclear extracts of galactose-treated mice were significantly higher (lanes 5 and 6), suggesting that galactose further enhances NF-κB activation in a dose-dependent manner.

The NF-κB pathway has a pivotal role in cell survival by regulating an array of genes, such as cellular FLICE-like inhibitory protein (c-Flip), cellular inhibitor of apoptosis protein 1 (c-IAP1), growth arrest and DNA-damage-inducible 45 beta (GADD45β), and A20.[21] To further investigate the anti-apoptotic mechanism of galactose, we determined the expression levels of NF-κB target genes in livers of TNF-α/D-GalN mice with the real-time PCR assay. Consistent with activation of the NF-κB pathway, enhanced expression of anti-apoptotic genes was observed upon galactose treatment in a dose-dependent manner, compared with control mice (Figure 5b). In addition, our western blot results confirmed that galactose induces expression of c-IAP1 and A20 proteins in the liver (Figure 5c).

Given that both c-IAP1 and A20 inhibit apoptosis by interfering with activation of caspase-8,[22–27] the key initiator protease in the cell extrinsic pathway, we further examined whether galactose affects caspase-8 activity. As shown in Figure 6 and Supplementary Figure 5, 18 and 43 kDa cleaved caspase-8 fragments were released 5 h after TNF-α stimulation. Notably, caspase-8 activation was effectively inhibited upon treatment with galactose. In keeping with this finding, cleavage of caspase-3, which is a major executioner of apoptosis and essential for DNA fragmentation, was also prevented by galactose. Our results confirm that galactose acts upstream

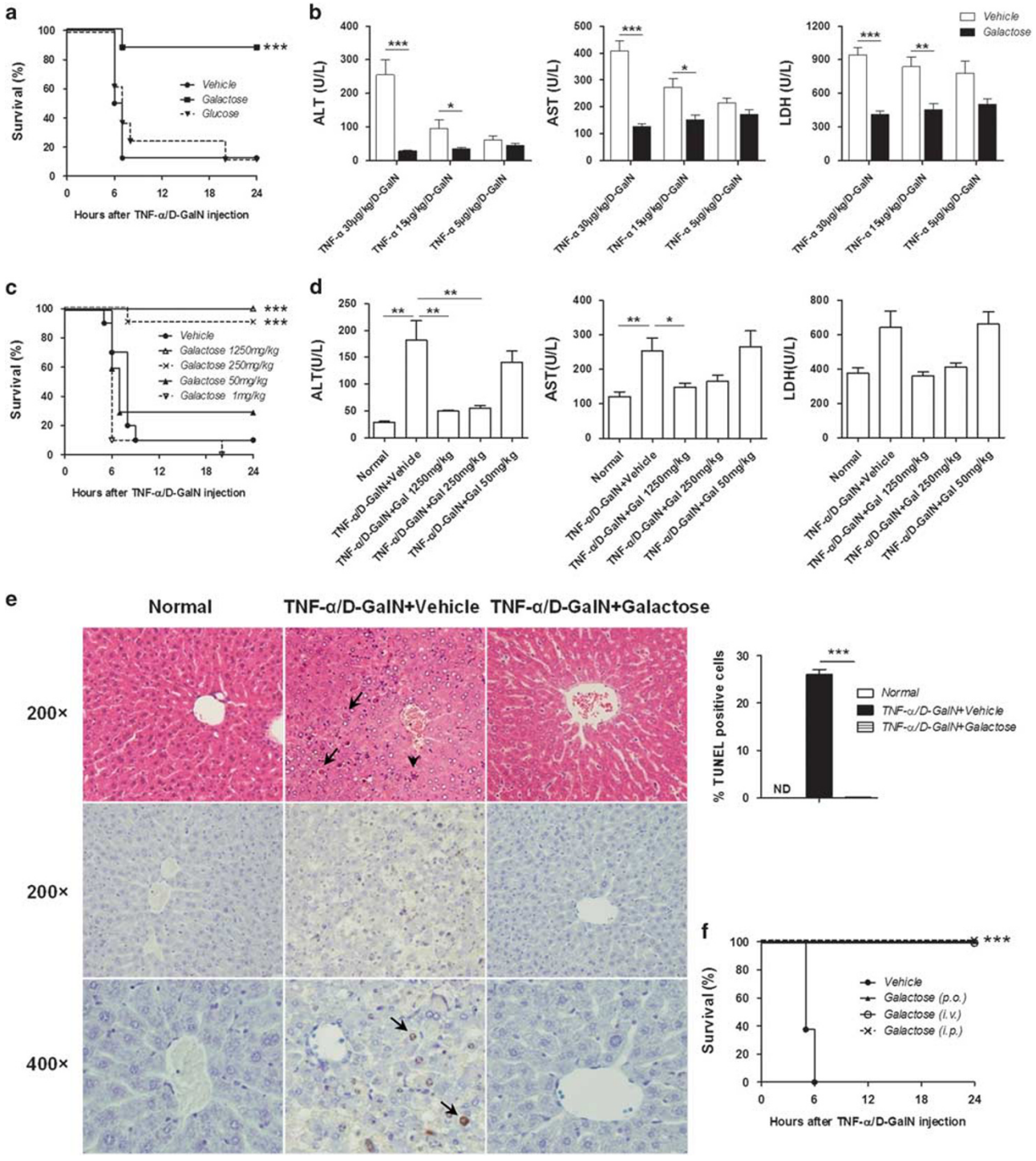

Figure 2 Protective effects of galactose on the TNF-α/D-GalN mouse model. (**a**) Survival curves of TNF-α/D-GalN model mice treated with 1250 mg/kg galactose or glucose ($n=8$). ***$P<0.001$. (**b**) Different doses of TNF-α were co-injected with D-GalN into mice. After 5 h, ALT, AST and LDH activities in serum were measured. Data represent means ± s.e.m. of two independent experiments ($n=8$). *$P<0.05$; **$P<0.01$; ***$P<0.001$. (**c**) Survival curves of TNF-α/D-GalN mice treated with different doses of galactose ($n=10$). ***$P<0.001$. (**d**) TNF-α/D-GalN mice were treated with 1250, 250 or 50 mg/kg galactose. After 5 h, ALT, AST and LDH levels in serum were measured. Values represent means ± s.e.m. of three experiments ($n=6$). *$P<0.05$; **$P<0.01$. (**e**) Representative liver histopathology with H&E staining (top) and apoptosis detection with the TUNEL assay (middle and bottom). Mice were treated as for (**c**). H&E staining showed councilman bodies (arrows) and infiltration of inflammatory cells in the portal area (arrowheads). The representative image of one experiment is shown. Original magnification: × 200 or × 400. Percentage of apoptotic cells determined from TUNEL assay data. Values were calculated from at least 1000 counted cells ($n=4$). Original magnification: × 200 or × 400. (**f**) Survival curves of TNF-α/D-GalN mice administered galactose via different routes ($n=8$). Vehicle control mice were injected i.v. with physiological saline at a volume equal to galactose.

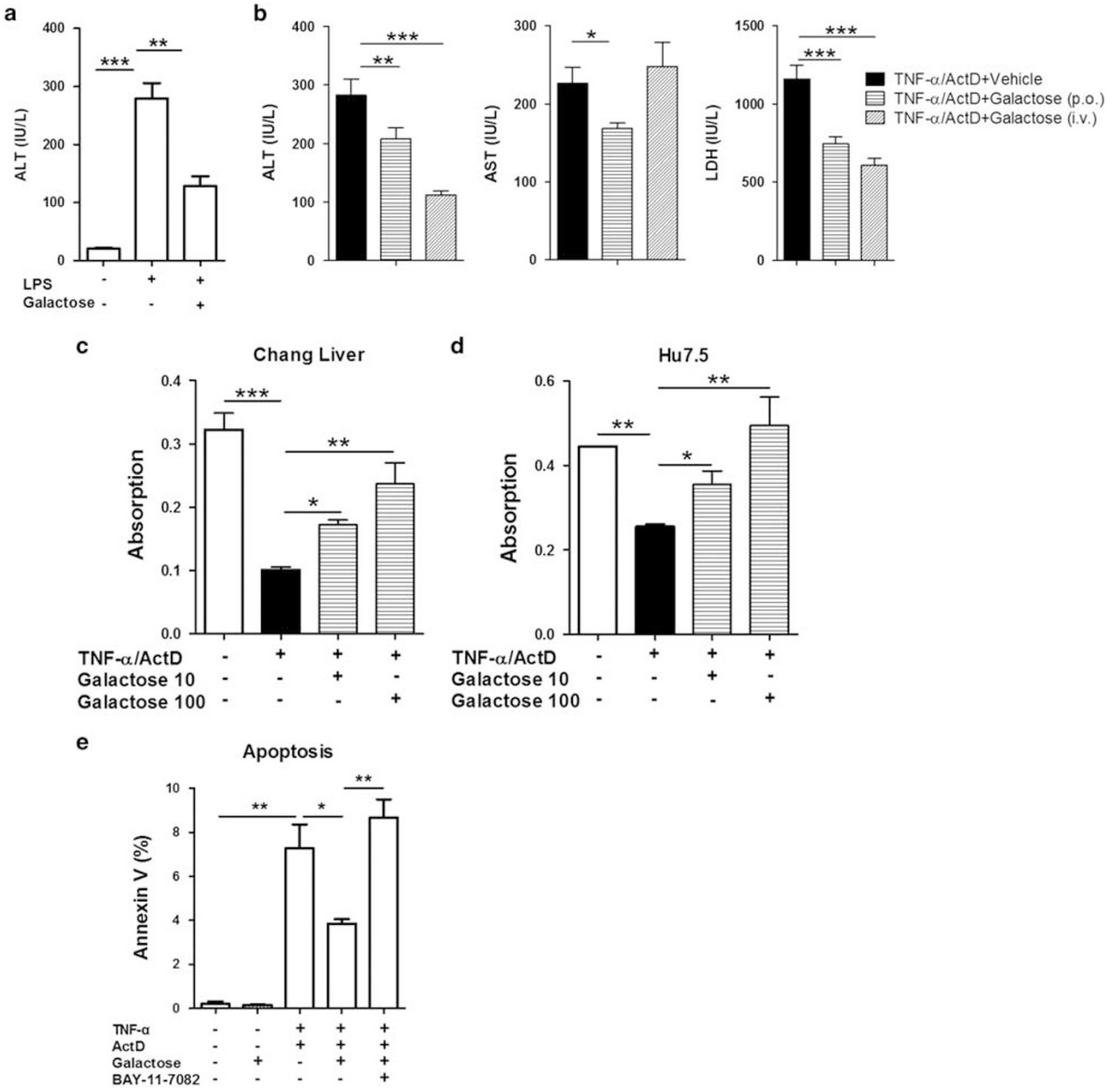

Figure 3 Galactose prevents hepatocytes from TNF-*α* and ActD-induced death. (**a**) Serum ALT levels of LPS-challenged mice. Mice were injected i.p. with 10 mg/kg LPS, and subsequently treated with 1250 mg/kg galactose or left untreated. After 24 h, serum ALT activity was detected. Data represent means ± s.e.m. from three independent experiments ($n = 6$). $^{**}P < 0.01$. (**b**) Mice were pretreated with 1250 mg/kg galactose orally or i.v. for 30 min, followed by i.p. injection with 0.8 mg/kg ActD, and subsequently challenged with 2 *μ*g/kg of TNF-*α* after 15 min. At 12 h after TNF-*α* injection, serum ALT, AST and LDH activities were measured. Data represent means ± s.e.m. ($n = 10$). Vehicle control mice were injected i.v. with physiological saline at a volume equal to galactose. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (**c** and **d**) Chang Liver and Hu7.5 cells were pretreated with 10 *μ*g/ml or 100 *μ*g/ml galactose for 1 h, and stimulated with 50 ng/ml TNF-*α* and 0.5 *μ*g/ml ActD for a further 24 h. Absorption values at 450 nm were measured with the CCK-8 assay. (**e**) Apoptosis of mouse primary hepatocytes was identified with the Annexin V assay. The number of annexin V-positive cells was decreased after galactose treatment, which was reversed in the presence of the NF-κB inhibitor, BAY-11-7082. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

of mitochondria during the process of TNF-*α*-induced apoptosis.

DISCUSSION

For decades, the protective effect of galactose supplementation on LPS/D-GalN-induced acute liver injury has been attributed to competition with D-GalN activity. However, data from the current study have provided new evidence showing that the anti-apoptotic effect of galactose in hepatocytes is independent of competition with D-GalN. Our main findings are as follows: (1) serum ALT is decreased by galactose in LPS-induced systemic inflammation (Figure 3a) and (2)

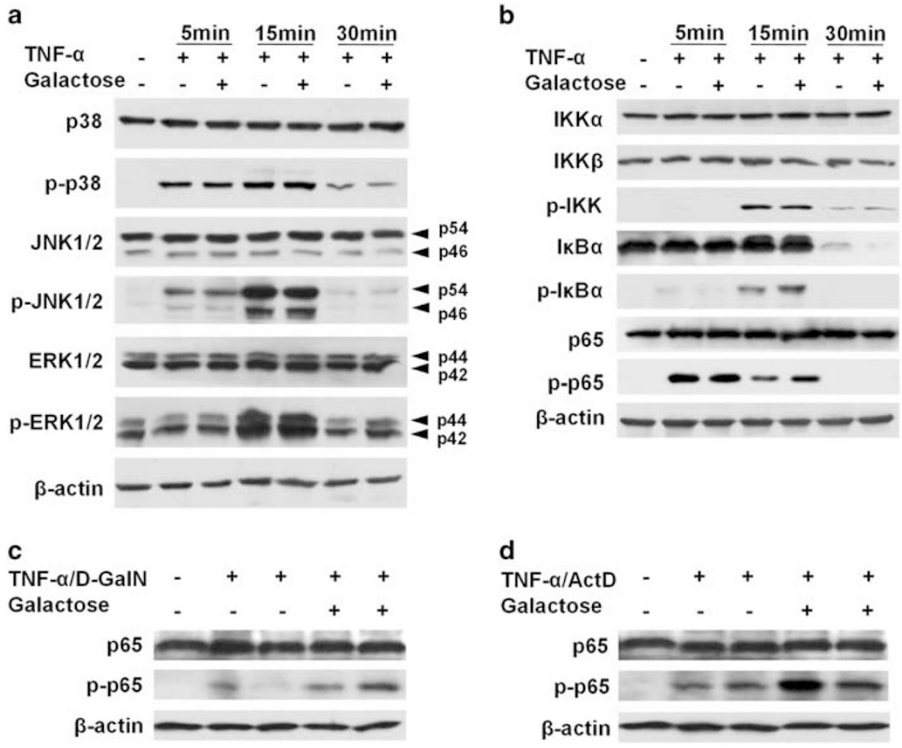

Figure 4 Galactose promotes phosphorylation of IκB*α* and NF-*κ*B p65 in hepatocytes without affecting the MAPK signaling pathway. (**a** and **b**) Western blot analysis of expression and phosphorylation of p38, JNK1/2, ERK1/2, IKK*α*, IKK*β*, IκB*α* and NF-*κ*B p65 in Chang liver cells. Cells were pretreated with 1 *μ*g/ml galactose or left untreated for 12 h, followed by treatment with 200 ng/ml TNF-*α* for the indicated times. Expression and phosphorylation of NF-*κ*B p65 in livers of TNF-*α*/D-GalN mice (**c**) and TNF-*α*/ActD mice (**d**) were detected via western blot. TNF-*α*/D-GalN and TNF-*α*/ActD mice were orally administered 1250 mg/kg galactose or left untreated. All mice were killed at 3 h. Data are representative of three independent experiments.

galactose evidently inhibits TNF-*α*/ActD-induced liver injury *in vivo* (Figure 3b) and promotes hepatocyte survival *in vitro* (Figures 3c–e).

The general mechanism underlying hepatocyte apoptosis among the ALF models employed in our present study is activation of the TNF-*α*/TNFR1 pathway. Analysis of the downstream signals of TNFR1 revealed that galactose exerts an anti-apoptotic effect by enhancing the transcriptional activity of NF-*κ*B. This theory was supported by a number of results. (1) Galactose did not inhibit MAP kinase signaling pathways that primarily mediate the pro-apoptotic response (Figure 4a; Supplementary Figure 1a). (2) Prevention of primary hepatocyte apoptosis by galactose was compromised by blocking the IκB*α*/NF-*κ*B pathway with BAY-11-7082 (Figure 3e). (3) Galactose promoted NF-*κ*B p65 phosphorylation, both *in vivo* and *in vitro* (Figures 4b–d; Supplementary Figure 1b–1d). (4) Galactose upregulated both c-IAP1 and A20 (Figure 5c), which act as potent inhibitors of death receptor-mediated apoptosis by interfering with caspase-8 activation.[23,24,26,27] (5) Galactose suppressed activation of caspase-8 and caspase-3 induced by TNF-*α*/D-GalN (Figure 6b; Supplementary Figure 5).

NF-*κ*B activation is a central event leading to multiple cell processes. Accumulating data in the area of TNF-*α*-induced hepatocyte death indicate that NF-*κ*B mainly has a beneficial role by inducing expression of a series of anti-apoptotic genes, such as A20, c-IAP1, c-IAP2, c-Flip, Xiap, Gadd45*β*, Traf1 and Traf2.[28–30] Among these factors, only A20, c-IAP1, c-Flip, Xiap and Gadd45*β* were induced in livers of TNF-*α*/D-GalN mice, while expression of Xiap was not affected by galactose (Figure 5b; Supplementary Figure 4). In particular, A20 and c-IAP1 were upregulated by galactose at both mRNA and protein levels (Figures 5b and c), suggesting that galactose prevents apoptosis in hepatocytes mainly through induction of these two factors. To investigate the effect of galactose on oxidant stress, we assessed the antioxidants, glutathione peroxidase (GSH-Px) and total superoxide dismutase (T-SOD), and the superoxides, reactive oxygen species (ROS), xanthine oxidase (XOD) and malondialdehyde (MDA), in livers of TNF-*α*/D-GalN mice. Among five tested parameters, we observed that galactose only significantly affected T-SOD level, which indicated that the antioxidant role of galactose is rather limited. Moreover, the effect of galactose on T-SOD was only observed at a lower dose of galactose (50 and 250 mg/kg), but not at a higher dose of 1250 mg/kg (Supplementary Figure 6), which was not consistent with the protective effect of galactose against TNF-*α*/D-GalN-induced damage at this dose.

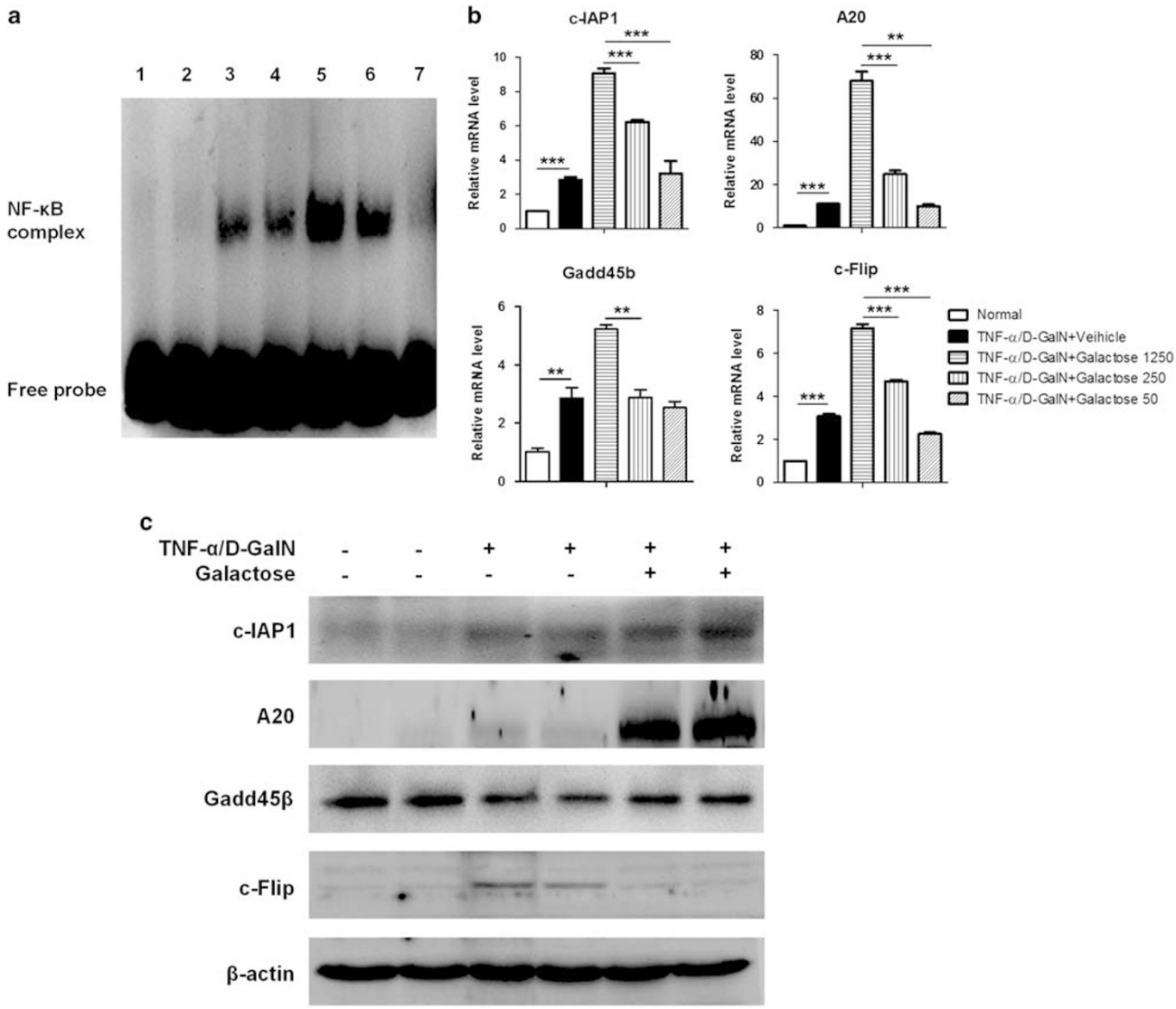

Figure 5 Galactose enhances the expression of NF-κB-induced anti-apoptotic genes. (**a**) EMSA was performed with mouse liver nuclear extracts (NE) and a biotin-labeled probe containing the NF-κB binding sequence. Lane 1, without NE; lane 2, untreated mice; lane 3, TNF-*α*; lane 4, TNF-*α*/D-GalN; lane 5, TNF-*α*/D-GalN and 1250 mg/kg galactose; lane 6, TNF-*α*/D-GalN and 250 mg/kg galactose; lane 7, TNF-*α*/D-GalN mouse liver NE and 200-fold excess cold competing probe. The position of the NF-κB-DNA complex is indicated with arrows. (**b**) mRNA levels of c-Flip, c-IAP1, GADD45*β* and A20 in liver were examined with real-time PCR. Mice were challenged with TNF-*α*/D-GalN and treated with different doses of galactose. Data are representative of mean values ± s.e.m. from three independent experiments. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. (**c**) Total lysates of liver (60 *μ*g) were subjected to western blotting with anti-c-Flip, c-IAP1, GADD45*β* and A20 antibodies.

Thus, antioxidant role might not be the key mechanism of galactose in preventing ALF.

Galactose recognizes and binds ASGR1 specifically on the surface of hepatocytes.[13] A previous study with ASGR1-deficient mice suggested that ASGR1 exerts a protective effect against CCl_4-mediated hepatic toxicity.[16] Recent research documented that ASGR1 activates the epidermal growth factor receptor (EGFR)-ERK pathway and induces matrix metalloproteinase-9 (MMP-9) expression.[31] In the present study, we observed short-term phosphorylation of ERK1/2 within 30 min after TNF-*α* stimulation, which peaked at 15 min, consistent with previous findings.[8] Although galactose enhanced phosphorylation of ERK1/2 slightly at 30 min, we speculate that this effect is abated. On the other hand, activity of NF-κB was significantly affected by galactose in hepatocytes. Further studies with ASGR1-deficient mice to establish whether galactose influences the NF-κB pathway via ASGR1 are warranted.

In accordance with the protective role of galactose, we found that lactose (composed of one galactose and one glucose molecule) also enhances the survival rate of TNF-*α*/D-GalN-treated mice (Supplementary Figure 2). Furthermore, oral administration of glucose induced a mild protective effect on ALT and LDH (Figure 1b), consistent with the findings of

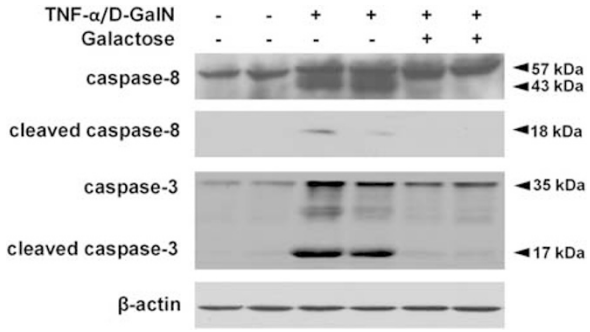

Figure 6 Caspase-8 and caspase-3 activities are suppressed by galactose. Expression of caspase-8 and caspase-3 and their cleavage in liver were detected using western blot. Total proteins were extracted 5 h after TNF-α/D-GalN injection. Data are representative of three independent experiments.

Rumio *et al.*[8] However, we observed no improvements in survival rate or pathologic damage (Figures 1a and c), even when glucose was injected 1 h before LPS/D-GalN administration, as described by Rumio *et al*[8] (Supplementary Figure 3). These inconsistent results may be attributed to the different survival periods of model mice. In keeping with earlier reports,[17–19] mice began to die at about 5 h after LPS/D-GalN or TNF-α/D-GalN challenge. Moreover, galactose enhanced NF-κB activity within 30 min of TNF-α injection, and subsequently, increased the expression of anti-apoptotic genes within 3 h. It is conceivable that galactose functions at the early stages after liver injury. However, mice died between 10 and 40 h after LPS/D-GalN challenge in the study of Rumio *et al.*[8] In view of the finding that glucose prevents ALF via activation of sodium-dependent glucose transporter-1 (SGLT-1), which is induced 6 h after LPS stimulation,[8,32] we prefer the hypothesis that glucose acts at a later stage of liver injury.

Galactose has been widely used at a dose of 3 g/kg daily for 8 weeks to induce neuronal apoptosis in an aging mouse model.[33] In the present study, the efficacious single dose for inhibition of ALF was as low as 250 mg/kg. We confirmed the safety of single treatment at doses between 250 and 2500 mg/kg to mice (data not shown). To conform to the guidelines of animal ethics, no more galactose-treated normal mice were employed as the negative control for each experiment. Considering that monosaccharides utilize distinct mechanisms to protect against acute liver injury, we propose that a combination of monosaccharides presents a promising approach for clinical treatment of ALF.

Supplementary Information accompanies the paper on the Laboratory Investigation website (http://www.laboratoryinvestigation.org)

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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