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Differential effects of pyrrolidine dithiocarbamate on TNF-α-mediated liver injury in two different models of fulminant hepatitis☆

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***Background/Aims*: Pyrrolidine dithiocarbamate (PDTC) is an inhibitor of nuclear factor kappa B (NF-κB) activation. The present study aimed to investigate the effects of PDTC on lipopolysaccharide (LPS)-induced liver injury in two different models of fulminant hepatitis.**

***Methods*: Mice infected with Bacillus Calmette Guerin (BCG) were challenged with LPS (0.2 mg/kg) to induce the model of inflammatory liver injury. Mice were injected with D-galactosamine (GalN, 600 mg/kg) and LPS (20 μg/kg) to induce the model of apoptotic liver injury. In the treatment groups, mice were pre-treated with PDTC (100 mg/kg), initiated 24 h prior to LPS.**

***Results*: PDTC pretreatment reduced the infiltration of inflammatory cells, inhibited NF-κB activation and the expression of tumor necrosis factor alpha (TNF-α), attenuated nitric oxide production, and alleviated hepatic glutathione depletion. Correspondingly, PDTC reduced serum alanine aminotransferase, improved hepatic necrosis, and prolonged the survival in the BCG/LPS model. Conversely, PDTC accelerated death and aggravated liver apoptosis in the GalN/LPS model, although it reduced nitric oxide production, attenuated glutathione depletion, and inhibited the expression of TNF-α in liver.**

***Conclusions*: PDTC protects mice against BCG/LPS-induced inflammatory liver injury through the repression of NF-κB-mediated TNF-α release, while it seems to be detrimental in GalN/LPS-induced apoptotic liver damage.**
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***Keywords*: Pyrrolidine dithiocarbamate; Lipopolysaccharide; Fulminant hepatitis; Nuclear factor kappa B; Tumor necrosis factor alpha**

1. Introduction

Lipopolysaccharide (LPS) is a toxic component of the cell walls of gram-negative bacteria and is widely present in the digestive tracts of humans and animals [1]. Humans are constantly exposed to low levels of LPS through infection. Gastrointestinal distress and alcohol consumption often increase permeability of LPS from gastrointestinal tract into the blood [2]. In humans, nanograms of LPS injected into the bloodstream can result in all the physiological manifestations of septic shock [3,4]. Hepatic dysfunction after sepsis is a frequent event, characterized by loss of synthetic function and hepatocellular necrosis [5,6].

Many studies have demonstrated that mice primed with Bacillus Calmette Guerin (BCG) are highly sensitive to LPS-induced liver injury [7–9]. BCG priming induces mononuclear cell infiltration into the liver lobules and granuloma formation [10]. BCG-activated

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macrophages (such as Kupffer cells) and T lymphocytes within granuloma are highly responsive to further stimuli such as LPS, resulting in a massive release of pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β, IL-6 and interferon (IFN)-γ. Thus, a subsequent LPS challenge in mice primed with BCG elicits acute and massive hepatic injury with marked mononuclear cell infiltration [11–13]. In the BCG/LPS model, pro-inflammatory cytokines are the major mediators leading to liver injury [14,15].

D-galactosamine (GalN) is an amino sugar selectively metabolized by the hepatocyte, which induces a depletion of the uridine triphosphate pool and thereby an inhibition of RNA synthesis [16]. When given together with a low dose of LPS, GalN highly sensitizes animals to develop lethal liver injury mimicking fulminant hepatitis [17]. Although TNF-α is the major mediator leading to liver injury [18], nitric oxide (NO) also plays an important role in GalN/LPS-induced apoptotic liver injury [19,20]. Recent studies have shown that hydrogen peroxide and glutathione (GSH) depletion sensitized hepatocytes to TNF-α-mediated apoptosis [21–23]. Conversely, antioxidants, such as rosmarinic acid and melatonin, protected against GalN/LPS-induced apoptotic liver injury [24,25].

Nuclear factor kappa B (NF-κB) activation is a common pathway that mediates LPS-induced up-regulation of gene encoding for pro-inflammatory cytokines. Pyrrolidine dithiocarbamate (PDTC) is an antioxidant and an inhibitor of NF-κB activity. An earlier study showed that PDTC protected against thioacetamide-induced fulminant liver failure [26]. The present study aimed to investigate the effects of PDTC on LPS-induced liver damage in two different models of fulminant hepatitis.

2. Materials and methods

2.1. Chemicals

Lipopolysaccharide (*Escherichia coli* LPS, serotype 0127:B8), pyrrolidine dithiocarbamate (PDTC) and D-galactosamine (GalN) were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO) if not otherwise stated.

2.2. Animals and treatments

Female CD-1 mice (6–8 weeks old, 22–24 g) were purchased from Beijing Vital River (Beijing, China). The animals were allowed free access to food and water at all times and were maintained on a 12-h light/dark cycle in a controlled temperature (20–25 °C) and humidity (50 ± 5%) environment for a period of 1 week before use.

Mice infected intravenously (i.v.) with BCG (2.5 mg, suspended in 0.2 mL saline) were intraperitoneally (i.p.) injected with LPS (0.2 mg/kg) to induce the model of inflammatory liver injury. Mice were injected with GalN (600 mg/kg, i.p.) and LPS (20 μg/kg, i.p.) to induce the model of apoptotic liver injury. In the treatment groups, mice were pre-treated with PDTC (100 mg/kg, i.p.), initiated 24 h prior to LPS. All procedures on animals followed the guidelines for human treatment set by the Association of Laboratory Animal Sciences and the Center for Laboratory Animal Sciences at Anhui Medical University.

2.3. Evaluation of liver injury

Serum ALT was colorimetrically measured using a commercially available kit. Liver specimen was fixed in 4% formaldehyde phosphate buffer. Liver sections were stained with hematoxylin and eosin and scored by two pathologists who were not aware of sample assignment to experimental groups. The degree of necrosis was expressed as the mean of twelve different fields within each slide classified on a scale of 0–3 (normal-0, mild-1, moderate-2, severe-3). The number of inflammatory cells was counted in twelve randomly selected fields from each slide at a magnification of ×400.

2.4. Measurement of hepatic GSH

Hepatic GSH content was measured by the method of Griffith [27]. GSH was expressed as nmol mg^{-1} protein.

2.5. Reverse transcription polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RT and PCR were performed as described previously [28]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal positive control standard. The primers were synthesized by Sangon Biological Technology (Shanghai, China), according to sequence designs previously described [28]. The number of cycles, annealing temperature and the size of the amplified fragments are given in Table 1. The amplified PCR products were electrophoresed at 75 v through 1.5% agarose gels (Sigma, St. Louis, MO) for 45 min. The pBR322 DNA digested with AluI was used for molecular markers (MBI Fermentas). Agarose gels were stained with 0.5 μg/ml ethidium bromide (Sigma, St. Louis, MO) TBE buffer.

2.6. Nitrate plus nitrite assay

Nitrate plus nitrite, the stable end products of L-arginine-dependent NO synthesis, were measured using a colorimetric method based on the Griess reaction [29].

Table 1
The number of cycles, annealing temperature and the size of the amplified fragments

Names	Denaturation (°C)	Annealing (°C)	Extension (°C)	The number of cycles (*n*)	The size of fragments (bp)
GAPDH	94	56	72	25	340
TNF-α	94	60	72	34	307
IL-1β	94	60	72	32	502
IL-6	94	60	72	35	600

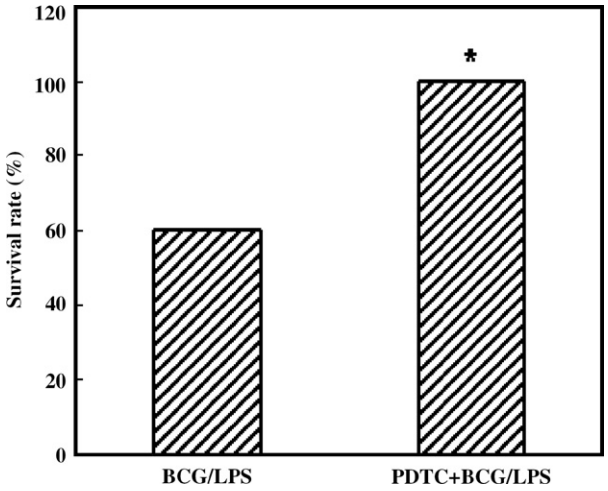

Fig. 1. PDTC protects mice against BCG/LPS-induced death and prolongs survival. Mice were infected intravenously (i.v.) with BCG (2.5 mg) and challenged 10 d later with LPS (0.2 mg/kg). In PDTC group, mice were i.p. injected with PDTC (100 mg/kg), initiated 24 h prior to LPS. Survival was monitored for 72 h (n = 15 for each group).

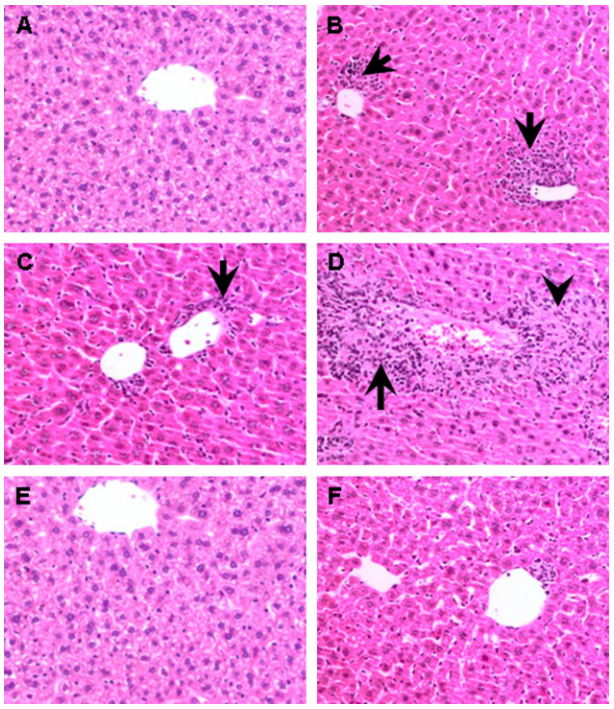

Fig. 2. Liver histology in BCG/LPS-induced acute liver failure. (A) The liver section from mice treated with saline. (B) The liver section from mice treated with BCG alone shows an infiltration of numerous inflammatory cells (arrow). (C) The liver section from mice treated with LPS alone displays the infiltration of a few inflammatory cells (arrow). (D) The liver section from mice treated with BCG/LPS shows numerous inflammatory cells (arrow) around the necrotic tissue (arrowhead). (E) The liver section of mice treated with PDTC alone. (F) Mice primed with BCG were i.p. injected with PDTC, initiated 24 h prior to LPS. The liver section shows minimal inflammation and necrosis. H & E, magnification: 200×. [This figure appears in colour on the web].

2.7. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from liver tissue by the method of Deryckere and Gannon [30]. For EMSA, a biotin-labeled double-strand DNA probe containing the consensus DNA-binding sequence for NF-κB (5′-AGT TGA GGG GAC TTT CCC AGG C-3′ and 5′-GCC TGG GAA AGT CCC CTC AAC T-3′) was synthesized by Sangon Biological Technology (Shanghai, China). EMSA was performed with a LightShift Chemiluminescence electrophoretic mobility shift assay kit (Pierce Biotechnology, Inc., Rockford, IL). For competition assays, unlabeled NF-κB consensus oligonucleotides were used. For supershift assays, 2 μg anti-p65 or anti-p50 subunit antibody (Santa Cruz Biotechnology) in each reaction was added.

2.8. DNA fragmentation analysis

The liver tissues were homogenized and incubated in 100 mM Tris–HCl (pH 8.0), 25 mM EDTA, 0.5% SDS, and 0.1 μg/ml proteinase K at 60 °C for 3 h. DNA was extracted with phenol/chloroform and electrophoresed on 1.5% agarose in TBE buffer.

2.9. Determination of caspase-3 activity

Hepatic caspase-3 activity was measured by a colorimetric method used previously [25] in a microplate reader at 405 nm.

2.10. Terminal dUTP nick-end labeling (TUNEL) staining

For the detection of apoptosis, paraffin-embedded sections were stained with the TUNEL technique using an in situ apoptosis detection kit (Promega) according to the manufacturer's instructions. The number of TUNEL-positive cells was counted in 12 randomly selected fields from each slide at a magnification of ×400.

2.11. Enzyme-linked immunosorbent assay (ELISA)

Serum TNF-α concentration was measured with an ELISA kit (R & D, Minneapolis, MN), following the manufacturer's instructions.

Table 2
The effects of PDTC on serum ALT, NO and hepatic GSH in BCG/LPS-treated mice

Groups	Animals (n)	ALT (IU/L, $\bar{x} \pm$ SE)	Nitrate/nitrite (μmol/L, $\bar{x} \pm$ SE)	GSH (nmol/mg protein, $\bar{x} \pm$ SE)
Saline	12	12.06 ± 1.36	10.40 ± 1.06	40.35 ± 2.41
BCG	12	$43.43 \pm 5.09^{\dagger\dagger}$	17.71 ± 0.24	$28.13 \pm 3.08^{\dagger}$
LPS	12	17.25 ± 1.96	$111.37 \pm 12.66^{\dagger\dagger}$	$26.67 \pm 0.46^{\dagger\dagger}$
BCG/LPS	12	$237.69 \pm 48.30^{\dagger\dagger}$	$1157.78 \pm 183.40^{\dagger\dagger}$	$13.48 \pm 2.20^{\dagger\dagger}$
PDTC	12	19.83 ± 0.93	6.39 ± 1.31	42.86 ± 4.64
PDTC + BCG/LPS	12	$59.50 \pm 8.81^{**}$	$516.61 \pm 121.90^{*}$	$26.59 \pm 1.25^{**}$

$^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ as compared with saline group.
$^{*}P < 0.05$, $^{**}P < 0.01$ as compared with BCG/LPS group.

2.12. Statistical analysis

The levels of TNF-α, IL-1β and IL-6 mRNA were normalized to GAPDH mRNA level in the same samples. The levels of TNF-α, IL-1β and IL-6 mRNA in the control were assigned as 100%. All quantified data were expressed as means ± SE at each point. ANOVA and the Student–Newmann–Keuls post-hoc test were used to determine differences among different groups. Percentages of surviving animals were compared with Fisher's exact test.

3. Results

3.1. PDTC protects against BCG/LPS-induced liver injury

Neither BCG nor LPS alone is lethal at low doses (data not shown). BCG plus LPS resulted in 40% (6/15) mortality in 72 h after LPS (Fig. 1). As shown in Table 2, LPS alone is not hepatotoxic at a low dose. BCG alone increased, to a lesser extent, serum ALT. BCG plus LPS dramatically increased serum ALT. Histopathologic examination showed an infiltration of numerous inflammatory cells into liver lobules in liver sections from mice primed with BCG (Fig. 2B). Numerous inflammatory cells around the necrotic tissue were observed in liver sections from mice treated by BCG and LPS (Fig. 2D). PDTC pretreatment improved the survival (Fig. 1), reduced serum ALT (Table 2), and alleviated BCG/LPS-induced inflammation and necrosis in liver (Fig. 2F and Table 3).

Table 3
The effects of PDTC on BCG/LPS-induced histopathologic damage

Groups	Animals (n)	The number of inflammatory cells (cells/field, $\bar{x} \pm SE$)	Necrosis (scale, $\bar{x} \pm SE$)
Saline	12	72.1 ± 2.2	0 ± 0
BCG	12	$146.0 \pm 21.1^{\dagger\dagger}$	$0.60 \pm 0.16^{\dagger\dagger}$
LPS	12	$129.6 \pm 5.4^{\dagger\dagger}$	$0.80 \pm 0.13^{\dagger\dagger}$
BCG/LPS	12	$248.2 \pm 23.5^{\dagger\dagger}$	$2.10 \pm 0.10^{\dagger\dagger}$
PDTC	12	66.7 ± 1.4	0.30 ± 0.15
PDTC + BCG/LPS	12	$10.6 \pm 4.7^{**}$	$1.00 \pm 0.00^{**}$

** $P < 0.01$ as compared with BCG/LPS group.
†† $P < 0.01$ as compared with saline group.

3.2. PDTC inhibits BCG/LPS-induced NF-κB activation

As shown in Fig. 3, BCG alone increased, to a lesser extent, NF-κB binding activity. LPS aggravated NF-κB activation in mice primed with BCG. The positive band disappeared in competition assay with unlabeled consensus oligonucleotides. When the conjugate of nuclear extract with DNA probes was preincubated with antibody against the p50 subunit of NF-κB, electrophoretic mobility decreased and the band shifted to a higher position, whereas the conjugate did not react with p65 antibody. As shown in Fig. 3A, PDTC pretreatment completely inhibited BCG/LPS-induced hepatic NF-κB activation.

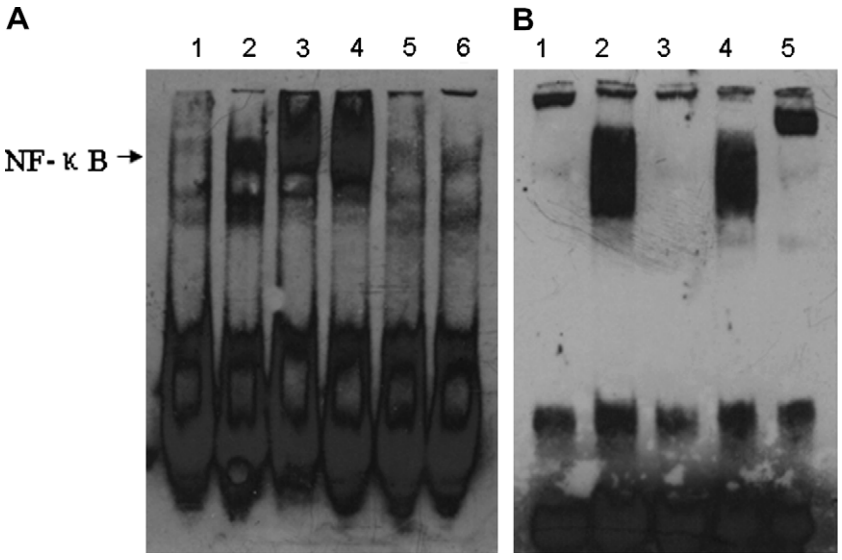

Fig. 3. The effects of PDTC pretreatment on BCG/LPS-induced NF-κB activation. Mice were infected intravenously (i.v.) with BCG (2.5 mg) and challenged 10 d later with LPS (0.2 mg/kg, i.p.). In PDTC group, mice were i.p. injected with PDTC (100 mg/kg), initiated 24 h prior to LPS. Nuclear extracts were isolated from liver at 1.5 h after LPS. (A) NF-κB activity was assessed by EMSA. Control (lane 1); BCG (lane 2); LPS (lane 3); BCG/LPS (lane 4); PDTC (lane 5); PDTC + BCG/LPS (lane 6). (B) For competition analysis, 500-fold excess of unlabeled consensus oligonucleotides was added to the reaction mixtures. For supershift assays, nuclear extracts were incubated with either p50 or p65 antibody. Control (lane 1); BCG/LPS (lane 2); Unlabeled consensus oligonucleotides competed the positive reaction (lane 3); Antibodies against the p50 subunit (lane 5) of NF-κB shifted an electric mobility of the band to the upper position, whereas anti-p65 antibody did not alter the mobility (lane 4).

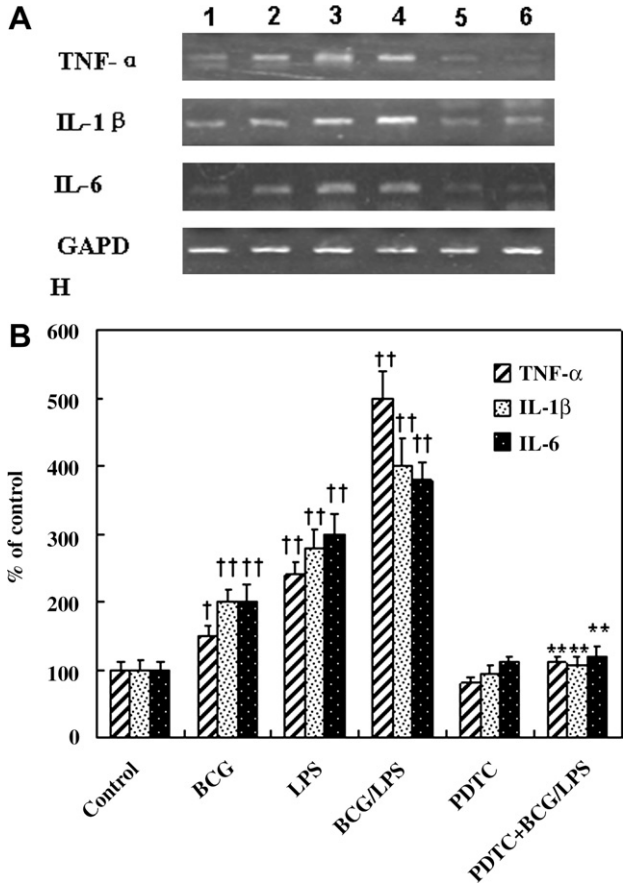

Fig. 4. The effects of PDTC pretreatment on BCG/LPS-induced proinflammatory cytokines. Mice were infected with BCG (2.5 mg, i.v.) and challenged 10 d later with LPS (0.2 mg/kg, i.p.). Some mice were pretreated with two doses of PDTC, one (100 mg/kg, i.p.) administered 24 h prior to LPS and the other (100 mg/kg, i.p.) administered 2 h before LPS. Total RNA was extracted from liver at 1.5 h after LPS. TNF-α, IL-1β and IL-6 mRNA was determined using RT-PCR. (A) A representative for TNF-α, IL-1β, IL-6 and GAPDH was shown. Control (lane 1); BCG (lane 2); LPS (lane 3); BCG/LPS (lane 4); PDTC (lane 5); PDTC + BCG/LPS (lane 6). (B) The TNF-α, IL-1β and IL-6 mRNA was normalized to GAPDH mRNA level in the same samples. The TNF-α, IL-1β and IL-6 mRNA level of the control was assigned as 100%. Data were expressed as means ± SE ($n = 6$). $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ as compared with control group. $^{**}P < 0.01$ as compared with BCG/LPS group.

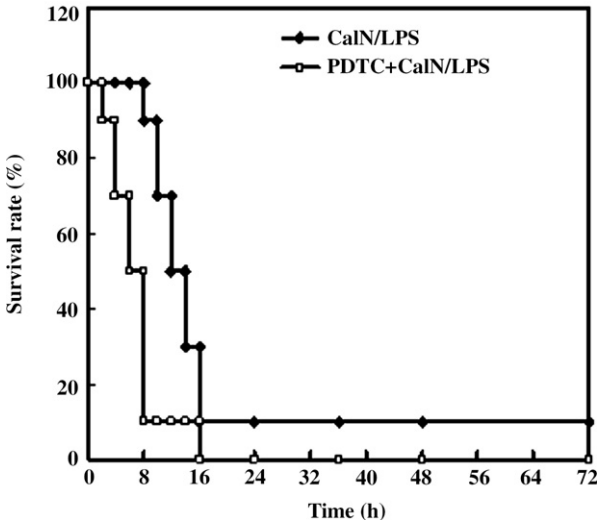

Fig. 5. PDTC accelerates GalN/LPS-induced death. Mice were i.p. injected with GalN and LPS. In PDTC group, mice were i.p. injected with PDTC (100 mg/kg), initiated 24 h prior to GalN/LPS. Survival was monitored for 72 h ($n = 10$ for each group).

3.3. PDTC inhibits BCG/LPS-induced expression of proinflammatory cytokines

As shown in Fig. 4, BCG alone up-regulated the expressions of TNF-α, IL-1β and IL-6 mRNA in liver. LPS aggravated the up-regulation of TNF-α, IL-1β and IL-6 mRNA, which was completely inhibited by PDTC pretreatment.

3.4. PDTC attenuates BCG/LPS-induced hepatic GSH depletion and NO production

The effects of PDTC on BCG/LPS-induced hepatic GSH depletion and NO production are shown in Table 2. Either BCG or LPS reduced, to a lesser extent, the level of hepatic GSH and serum NO. BCG plus LPS significantly decreased hepatic GSH content and serum NO level, which was significantly alleviated by PDTC pretreatment.

Table 4
The effects of PDTC on serum ALT, NO and hepatic GSH in GalN/LPS-treated mice

Groups	Animals (n)	ALT (IU/L, $\bar{x} \pm$ SE)	Nitrate/nitrite (μmol/L, $\bar{x} \pm$ SE)	GSH (nmol/mg protein, $\bar{x} \pm$ SE)
Saline	12	10.50 ± 1.95	9.36 ± 0.87	39.96 ± 1.69
GalN	12	32.01 ± 8.03††	5.28 ± 0.90	43.23 ± 3.11
LPS	12	8.37 ± 0.16	13.19 ± 1.16	40.55 ± 3.41
GalN/LPS	12	1048.63 ± 117.46††	14.82 ± 1.00†	10.42 ± 1.78††
PDTC	12	15.52 ± 3.03	6.99 ± 1.47	42.86 ± 4.64
PDTC + GalN/LPS	12	3246.16 ± 297.64**	2.91 ± 0.84**	24.47 ± 1.04**

$^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ as compared with saline group.
** $P < 0.01$ as compared with GalN/LPS group.

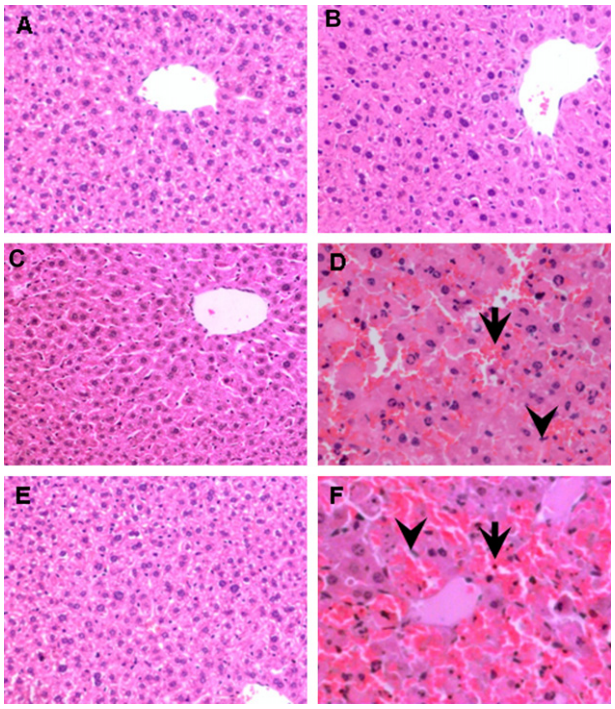

Fig. 6. Liver histology in GalN/LPS-induced acute liver failure. The liver section from mice treated with GalN and LPS shows massive infiltration of inflammatory cells (arrowhead), necrosis and erythrocyte agglutination (arrow) (D). Mice were i.p. injected with PDTC, initiated 24 h prior to GalN and LPS. The liver section displays more necrosis and erythrocyte agglutination (arrow) than in mice treated with GalN/LPS (F). Only minimal inflammation and necrosis are observed in the liver section from control mice treated with either saline (A) or GalN (B) or LPS (C) or PDTC (E). H & E, magnification: 200×. [This figure appears in colour on the web].

3.5. Effects of PDTC on GalN/LPS-induced liver injury

Neither GalN nor LPS alone is lethal at low doses (data not shown). Co-injection with GalN and LPS into mice resulted in rapid death within 16 h with a mortality of 90%, with severe congestion in livers of all dead mice. Although it did not increase mortality, PDTC accelerated death, in which 90% of mice died in 8 h after GalN/LPS (Fig. 5). As shown in Table 4, LPS alone is not hepatotoxic at a low dose. GalN alone increased, to a lesser extent, serum ALT. GalN and LPS dramatically increased serum ALT level. Histopathologic examination showed a widespread destruction of liver architecture characterized by massive panlobular hemorrhage and necrosis as well as an infiltration of inflammatory cells in liver sections from mice treated by GalN and LPS (Fig. 6D). PDTC pretreatment aggravated the elevation of serum ALT (Table 4) and worsened hepatic hemorrhage and necrosis (Fig. 6F and Table 5).

3.6. Effects of PDTC on GalN/LPS-induced liver apoptosis

A clear DNA laddering was shown in liver of mice treated by GalN and LPS (Fig. 7A). GalN and LPS greatly increased hepatic caspase-3 activity (Fig. 7B). As shown in Fig. 7C, numerous apoptotic cells were evidenced by the TUNEL assay. PDTC pretreatments increased the number of apoptotic cells and caspase-3 activity in livers of mice treated by GalN and LPS (Table 5 and Fig. 7).

3.7. PDTC inhibits GalN/LPS-induced NF-κB activation

As shown in Fig. 8, NF-κB was activated in liver of mice treated by GalN and LPS. The positive band disappeared in competition assay with unlabeled consensus oligonucleotides. When the conjugate of nuclear extract with DNA probes was preincubated with antibody against the p50 subunit of NF-κB, electrophoretic mobility decreased and the band shifted to a higher position, whereas the conjugate did not react with p65 antibody. The effects of PDTC on GalN/LPS-induced NF-κB activation are evaluated. Results showed that PDTC pretreatment significantly inhibited the activation of NF-κB in mouse liver (Fig. 8A).

3.8. PDTC inhibits GalN/LPS-induced expression of proinflammatory cytokines

As shown in Fig. 9, GalN and LPS up-regulated, to a lesser extent, the expressions of hepatic TNF-α, IL-β and IL-6 mRNA, which was inhibited by PDTC pretreatment.

Table 5
The effects of PDTC on GalN/LPS-induced histopathologic damage

Groups	Animals (n)	The number of inflammatory cells (cells/field, $\bar{x} \pm$ SE)	Necrosis (scale, $\bar{x} \pm$ SE)	The number of apoptotic cells (cells/fields, $\bar{x} \pm$ SE)
Saline	12	72.1 ± 2.2	0 ± 0	0 ± 0
GalN	12	91.6 ± 3.7††	0.80 ± 0.13†	0 ± 0
LPS	12	85.2 ± 4.7†	0.20 ± 0.13	0 ± 0
GalN/LPS	12	119.0 ± 7.6††	2.50 ± 0.17††	13.4 ± 1.1††
PDTC	12	66.7 ± 1.4	0.30 ± 0.15	0 ± 0
PDTC + GalN/LPS	12	130.2 ± 5.7	2.90 ± 0.10*	17.7 ± 1.2*

†$P < 0.05$, ††$P < 0.01$ as compared with saline group.
* $P < 0.05$ as compared with GalN/LPS group.

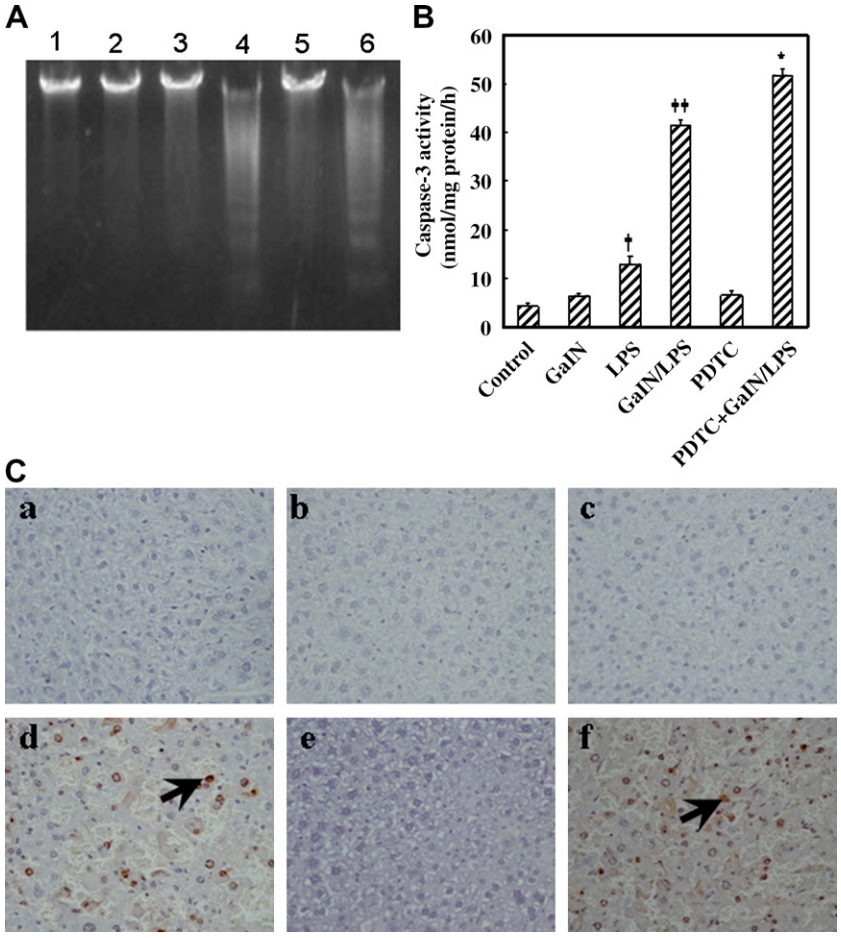

Fig. 7. The effects of PDTC on GalN/LPS-induced hepatic apoptosis. Mice were i.p. injected with GalN and LPS. In PDTC group, mice were i.p. injected with PDTC (100 mg/kg), initiated 24 h prior to GalN/LPS. (A) Hepatic DNA fragmentation. The results are a representative of four independent experiments. Control (lane 1); GalN (lane 2); LPS (lane 3); GalN/LPS (lane 4); PDTC (lane 5); PDTC + GalN/LPS (lane 6). (B) Hepatic caspase-3 activity. Data were expressed as means ± SEM ($n = 12$). $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ as compared with control group. $^{**}P < 0.01$ as compared with GalN/LPS group. (C) TUNEL staining (arrow) of liver sections from mice treated with GalN/LPS or PDTC plus GalN/LPS. The results are a representative of four independent experiments. Magnification: 200×. (a) Control; (b) GalN; (c) LPS; (d) GalN/LPS; (e) PDTC; (f) PDTC + GalN/LPS. [This figure appears in colour on the web].

3.9. PDTC attenuates GalN/LPS-induced hepatic GSH depletion and NO production

GalN plus LPS increased, to a lesser extent, serum NO level. GalN plus LPS dramatically decreased hepatic GSH level. PDTC significantly attenuated GalN/LPS-induced hepatic GSH depletion and NO production (Table 4).

3.10. LPS-induced TNF-α in two models of acute liver injury

Serum TNF-α was measured using ELISA. Results showed that BCG/LPS greatly increased serum TNF-α level. However, GalN/LPS increased, to a lesser extent, the level of serum TNF-α (Fig. 10).

4. Discussion

The present study showed that administration of a sub-lethal dose of LPS to mice primed with BCG resulted in 40% mortality. BCG plus LPS significantly increased serum ALT, with an infiltration of numerous inflammatory cells into liver lobules and massive hepatic necrosis. Numerous studies have demonstrated that TNF-α plays an important role in BCG/LPS-induced acute liver injury [12–15,31]. NF-κB activation is a common pathway that mediates LPS-induced up-regulation of gene encoding for pro-inflammatory cytokines. The present study showed that BCG plus LPS markedly activated hepatic NF-κB and up-regulated the expression of hepatic TNF-α, IL-1β and IL-6 mRNA. These results suggest that NF-κB-mediated up-regulation of pro-inflammatory cytokines may be involved in the pathogenesis of liver injury in model of BCG/LPS-induced fulminant hepatitis. PDTC is an inhibitor of NF-κB activation [32]. Several studies showed that PDTC protected against LPS-induced lethal shock and thioacetamide-induced fulminant liver failure through the repression of NF-κB activation [26,33]. In the present study, we found that PDTC pretreatments reduced

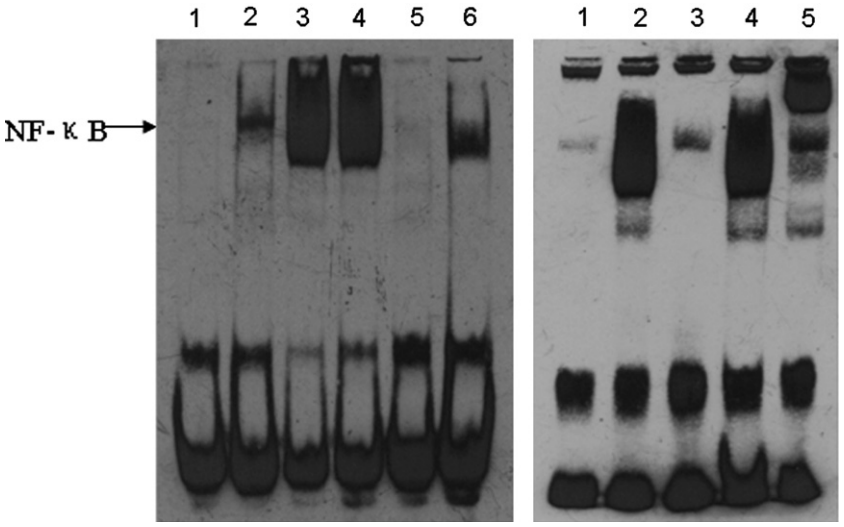

Fig. 8. The effects of PDTC on GalN/LPS-induced NF-κB activation. Mice were i.p. injected with GalN and LPS. In PDTC group, mice were i.p. injected with PDTC (100 mg/kg), initiated 24 h prior to GalN/LPS. Nuclear extracts were isolated from liver at 1.5 h after GalN/LPS. (A) NF-κB activity was evaluated by EMSA. Control (lane 1); GalN (lane 2); LPS (lane 3); GalN/LPS (lane 4); PDTC (lane 5); PDTC + GalN/LPS (lane 6). (B) For competition analysis, 500-fold excess of unlabeled NF-κB probes was added to the reaction mixtures. For supershift assays, nuclear extracts were incubated with either p50 or p65 antibody.

serum ALT, improved inflammation and necrosis in liver, and prolonged the survival in the model of BCG/LPS-induced acute liver injury. Correspondingly, PDTC completely inhibited NF-κB activation and down-regulated the expression of pro-inflammatory cytokines in liver of mice treated by BCG and LPS. These results suggest that the protection of PDTC against BCG/LPS-induced liver injury might be, at least partially, associated with the repression of NF-κB activation.

There is now substantial evidence that reactive oxygen species (ROS) contribute to the pathogenesis of various acute and chronic liver injury induced by viral, LPS, carbon tetrachloride, alcohol, etc. [34–37]. Numerous studies have focused on the pathogenic significance of ROS in experimental models of liver damage. The present study showed that hepatic GSH was decreased in the BCG/LPS model. PDTC is an antioxidant and exerts its direct antioxidant effect via scavenging free radicals and inhibiting free radical generation via its chelating activity for metal ions that may catalyze formation of free radicals [38]. The present study showed that PDTC significantly attenuated BCG/LPS-induced hepatic GSH depletion. These results indicate that PDTC might protect against BCG/LPS-induced inflammatory liver injury via its antioxidant effect.

The role of NO in the mechanisms of LPS-triggered hepatocellular necrosis in mice primed with BCG remains controversial. Some studies demonstrated that NO played a protective role against BCG/LPS-induced liver injury [39]. Conversely, other studies found that inhibition of inducible nitric oxide synthase (iNOS) also protected against BCG/LPS-induced liver damage [40]. The present study showed that BCG plus LPS greatly increased serum NO level. Moreover, PDTC pretreatment significantly attenuated the elevation of serum NO level, suggesting that the PDTC-mediated protection against BCG/LPS-induced inflammatory liver injury might be associated with the inhibition of NO.

In the present study, we investigated the effects of PDTC on GalN/LPS-induced apoptotic liver injury. As expected, co-injection of GalN and LPS into mice resulted in rapid death within 16 h with a mortality of 90%, with severe congestion in the liver of all the dead mice. Interestingly, PDTC pretreatment accelerated GalN/LPS-induced death, in which 90% of mice died in 8 h after GalN/LPS. PDTC aggravated serum ALT and worsened the hemorrhage and necrosis in livers of mice treated by GalN and LPS. GalN/LPS-induced liver injury is characterized by massive liver apoptosis. PDTC aggravated liver apoptosis in the GalN/LPS model, as shown by the higher level of caspase-3 activity and more apoptotic cells, although it significantly inhibited hepatic TNF-α expression and attenuated hepatic GSH depletion and NO production.

In the GalN/LPS model, TNF-α is the major mediator leading to apoptotic liver injury and acute liver failure [16]. However, TNF-a binding to the TNF receptor (TNFR) initiates apoptosis and simultaneously activates NF-κB, which suppresses apoptosis [41,42]. Thus, either recombinant TNF-α, at a dose as low as 10 μg/kg, or LPS, at a dose as low as 20 μg/kg, is not hepatotoxic. Actually, either LPS or TNF-α, at a low dose, initiates a lethal apoptotic process only under transcriptional inhibition. Administration of GalN induces a selective

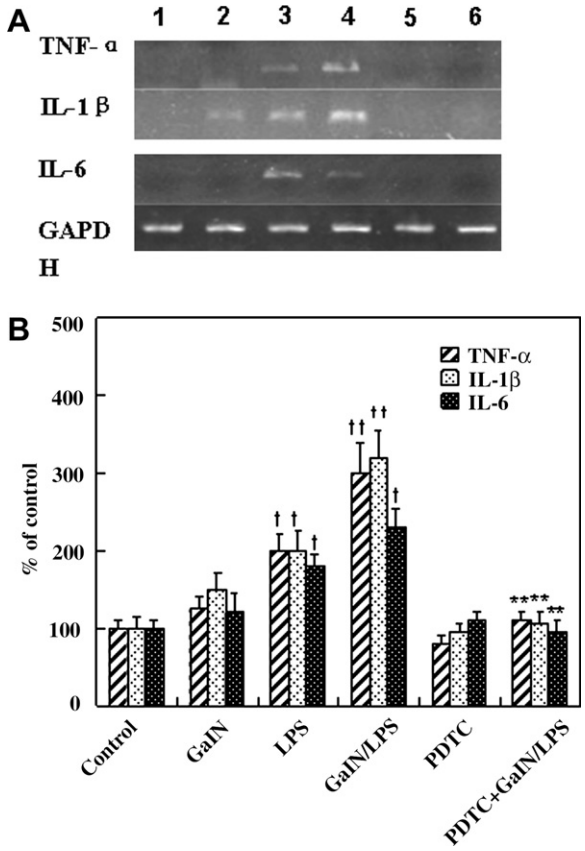

Fig. 9. The effects of PDTC pretreatment on GalN/LPS-induced expression of hepatic pro-inflammatory cytokines. Mice were i.p. injected with GalN and LPS. In PDTC group, mice were i.p. injected with PDTC (100 mg/kg), initiated 24 h prior to GalN/LPS. Total RNA was extracted from liver at 1.5 h after LPS. TNF-α, IL-1β and IL-6 mRNA was determined using RT-PCR. (A) A representative for TNF-α, IL-1β, IL-6 and GAPDH was shown. Control (lane 1); GalN (lane 2); LPS (lane 3); GalN/LPS (lane 4); PDTC (lane 5); PDTC + GalN/LPS (lane 6). (B) TNF-α, IL-1β, IL-6 mRNA was normalized to GAPDH mRNA level in the same samples. The TNF-α, IL-1β, IL-6 mRNA level of the control was assigned as 100%. Data were expressed as means ± SE ($n = 6$). $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ as compared with control group. $^{**}P < 0.01$ as compared with GalN/LPS-treated group.

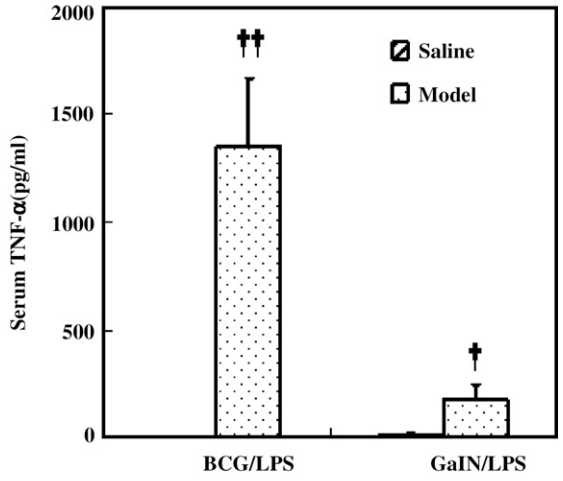

Fig. 10. LPS-induced TNF-α in two models of acute liver injury. In the BCG/LPS model, mice were infected intravenously (i.v.) with BCG (2.5 mg) and challenged 10 d later with LPS (0.2 mg/kg). In the GalN/LPS model, mice were i.p. injected with GalN and LPS. Serum TNF-α was measured 1.5 h after LPS using ELISA. Data were expressed as means ± SE ($n = 12$). $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ as compared with control group.

transcriptional inhibition in hepatocytes and thus increases the sensitivity of animals to LPS or TNF-α up to as much as a 1000-fold. On the other hand, NF-κB activation is the critical intracellular signal that determines whether TNF-α stimulates hepatocyte proliferation or apoptosis. An earlier study showed that TNF-α resulted in apoptosis in hepatocytes sensitized with actinomycin D that specifically blocked NF-κB-dependent genes, which counteracted the pro-apoptotic effects of TNFR1 activation [43]. Moreover, pretreatment with TNF-α or IL-1β, which activated NF-κB, dramatically protected mice against GalN/TNF-α-mediated liver apoptosis [44]. In the present study, PDTC pretreatment significantly inhibited NF-κB activation and thus aggravated GalN/LPS-induced liver apoptosis. However, as shown above, PDTC completely inhibited hepatic NF-κB activity, but it protected the acute liver injury in the BCG/LPS model. Indeed, LPS is a potent activator of NF-κB not only in Kupffer cells but also in hepatocytes [45,46]. LPS activates NF-κB in Kupffer cells and stimulates the release of TNF-α. Conversely, LPS-induced NF-κB activation in hepatocytes may play an anti-apoptotic effect. The present study showed that BCG/LPS produced far higher levels of TNF-α than GalN/LPS. Thus, inhibition of NF-κB in Kupffer cells may be more important in the BCG/LPS model thereby limiting the release of TNF-α and hepatocyte death, whilst in the GalN/LPS model hepatocyte NF-κB activation is more important for survival. The beneficial effects of PDTC might result from the inhibition of NF-κB-mediated TNF-α release in Kupffer cells in the BCG/LPS model, whilst the harmful effects might be attributed to the repression of NF-κB-mediated anti-apoptotic effect in the GalN/LPS model.

In the present study, we only investigated LPS-induced liver damage and the protective effects of PDTC in female mice. A recent study found that male mice were more susceptible to acute liver inflammation and damage [47]. Thus, additional work is required to determine the effects of PDTC on LPS-induced liver injury in male animals.

In summary, the present results indicate that PDTC has differential effects on LPS-induced liver damage in two different models of fulminant hepatitis. PDTC protects mice against BCG/LPS-induced inflammatory liver injury through the repression of NF-κB-mediated TNF-α release, while it seems to be detrimental in GalN/LPS-induced apoptotic liver injury.

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