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Protective effect of chloral hydrate against lipopolysaccharide/D-galactosamine-induced acute lethal liver injury and zymosan-induced peritonitis in mice

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ABSTRACT

In recent years, certain anesthetics have been shown to have protective effects against acute inflammation in experimental animals, an observation that may yield new options for adjunctive treatment of acute inflammation. In this study, we investigated the effects of chloral hydrate (CH) on the acute inflammatory response in BALB/c mice using lipopolysaccharide/D-galactosamine (LPS/D-GalN)-induced acute lethal liver injury and zymosan A-induced peritonitis models. The survival of mice following LPS/D-GalN treatment was significantly improved by a single injection with chloral hydrate, which could be administered simultaneously or as late as 3 h after challenge with LPS/D-GalN; liver injury was also attenuated. A sharp rise in serum levels of MCP-1, IL-6 and TNF-α was attenuated or delayed after chloral hydrate treatment. Furthermore, the mechanism by which chlorate hydrate inhibits inflammation was associated with an attenuated increase in nuclear factor κappaB (*NF-κB*) activity in *NF-κB*-RE-luc mice upon LPS/D-GalN treatment. In mice with acute peritonitis, leukocyte number and protein concentration in peritoneal exudates peaked with a 16 h lag, and serum levels of MCP-1, IL-6 and TNF-α were significantly lower at certain time points in the chloral hydrate-treated group compared to those in the normal saline (NS)-treated control group. In addition, chloral hydrate treatment *in vitro* attenuated the upregulation of TNF-α and IL-6 by peritoneal macrophages and *NF-κB* activity in RAW264.7 cells stimulated with LPS, suggesting that monocytes/macrophages may be a target of chloral hydrate. These results indicate that chloral hydrate has a protective effect against LPS/D-GalN-induced acute lethal liver injury in mice, which may be associated with an inhibition of *NF-κB* activity and delays in proinflammatory cytokine production. However, this phenomenon was not associated with levels of serum corticosterone. Chloral hydrate also attenuated the inflammatory response in zymosan A-induced acute peritonitis, a model of mild inflammation. In conclusion, treatment with only a single injection of chloral hydrate could significantly attenuate acute inflammation in mice treated with LPS/D-GalN and zymosan A. These effects are also likely associated with the inhibition of *NF-κB* activity.

1. Introduction

Protecting patients and experimental animals from acute or serious inflammation has been a very important issue not only in clinical settings but also in laboratories investigating the pathogenesis of infectious or noninfectious inflammation, developing new medicines, etc. Recently, several anesthetics have been found to possess anti-inflammatory and anti-infective effects apart from their uses for pain relief and muscle relaxation. Lidocaine, ketamine, isoflurane and pentobarbital can significantly improve the survival of mice and rats suffering from endotoxic shock [1,2] and protect them against liver and renal injury resulting from cecal ligation and puncture (CLP)-induced septic peritonitis [3,4]. However, there are no reports regarding the effect of chloral hydrate (CH) on acute inflammation. We became interested in CH because it has been used as a traditional sedative and anesthetic in medicine for over one hundred years and is currently used in pediatric operations including magnetic resonance imaging (MRI) and echocardiograms on children [5–9] and in animal experiments [10–13] in many countries. Furthermore, evaluation of the pharmacologic mechanism and safety of CH in humans has been emphasized in recent years [14–16].

In the present study, we sought to investigate the effects of CH on acute lethal liver injury induced by lipopolysaccharide/D-galactosamine (LPS/D-GalN) and on acute peritonitis, a mild inflammatory response induced by zymosan A, both of which are well defined animal models [17–21]. LPS/D-GalN-induced acute lethal liver injury has been a widely used experimental animal model for more than 20 years, allowing both investigation of the mechanisms of lethal hepatic failure and development of effective therapeutic strategies against endotoxin challenge and sepsis in humans [22,23]. Moreover, as reviewed by Johnson et al., manifestation of systemic inflammatory response syndrome (SIRS) in the absence of infection may occur in a manner similar to sepsis [24]. Therefore, our work could provide clues to improve treatment of acute lethal liver injury and sepsis.

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We also explored the mechanisms underpinning the protective effects of CH against LPS/D-GalN-induced acute lethal liver injury and peritonitis, including observation proinflammatory cytokine profiles and nuclear factor κappaB (*NF-κB*) activities *in vivo* and *in vitro*. Assessment and selection of the parameters for present work, including levels of IL-6, TNF-α and MCP-1, were done according to work on prognosis-related cytokines in patients with septic shock as reported by Bozza [25]. Among them, TNF has been proven to be closely associated with the survival of mice challenged with LPS alone or LPS/GalN [26]; MCP-1 is an important cytokine that can balance the proinflammatory and anti-inflammatory reactions in acute lethal inflammatory responses, such as sepsis [27].

2. Materials and methods

2.1. Materials

Iscove's modified Dulbecco's medium(IMDM) was purchased from Gibco (Grand Island, USA); LPS (*Escherichia coli* serotype 2630), D-GalN, zymosan A, D-Luciferin and Evans blue were purchased from Sigma (St. Louis, MO, USA); Chloral hydrate was from Nanfang hospital (Guangzhou, China); Mouse ALT/AST kits were from Jiancheng Bioengineering Institute (Nanjing, China) and corticosterone ELISA kit was from Cusabio biotech (Wuhan, China); Mouse cytokine CBA kit was from Bender Medsystems (Burlington, CA); Fetal bovine serum (FBS) was from Sijiqing Biological Engineering Materials (Hangzhou, China); Luciferase Assay System kit was purchased from Promega (Madison, MI, USA).

2.2. Animal models

Six-week-old BALB/c mice were purchased from Experimental Animal Center of Southern Medical University and *NF-κB*-RE-lu (Oslo) transgenic BALB/c mice from Guangzhou Institute of Biomedicine and Health (Guangzhou, China). The animals were maintained at 25 °C in a 12 h light:dark cycle with 50% humidity and were fed with commercial feed and sterile water. Acute lethal liver injury in BALB/c or *NF-κB*-RE-luc (Oslo) transgenic mice was induced by intraperitoneal (*i.p.*) injection of 10 μg LPS-2630 plus 800 mg of D-GalN × kg^{-1} of body weight [1]. Peritonitis in BALB/c mice was induced by *i.p* injection of 1 mg zymosan A [20].

The animal experiments were approved by the Ethics Committee for Experimental Animals at Southern Medical University and were performed according to the national guidelines for animal welfare.

2.3. Survival observation in LPS/D-GalN-induced acute lethal liver injury mice

LPS/D-GalN-induced acute lethal liver injury was induced in BALB/c mice as mentioned above, and the mice were slowly intravenously (*i.v.*) injected (within 5 min) with 0 [normal saline (NS)], 80, 160 or 320 mg/kg chloral hydrate (CH) via the tail vein. The survival times of these mice were recorded every 30 min. Additionally, the mice were slowly injected *i.v.* with 320 mg/kg CH at 0 (CH was given immediately after challenge with LPS/D-GalN), 1, 3 or 5 h after challenge with LPS/D-GalN and survival times were recorded every 30 min.

2.4. Measurement of serum levels of alanine aminotransferase, aspartate aminotransferase and corticosterone

Mice treated with LPS/D-GalN with or without CH treatment were sacrificed 0, 3, 6 or 9 h thereafter. Sera were isolated by centrifugation and frozen at −40 °C prior to alanine aminotransferase (ALT), aspartate aminotransferase (AST) and corticosterone analyses. Levels of serum ALT and AST were measured using mouse ALT/AST kits according to the manufacturer's instructions. Levels of serum corticosterone were measured using a mouse corticosterone ELISA kit according to the manufacturer's instructions.

2.5. Observation of histological changes in liver tissue

Mice treated with LPS/D-GalN with or without CH were sacrificed at 0, 3, 6 or 9 h after treatment. Livers were harvested, photographed and then quickly fixed with formalin followed by paraffin embedding, preparation of sections and staining with hematoxylin & eosin.

2.6. Measurement of serum levels of MCP-1, IL-6 and TNF-α

Immediately after the production of LPS/D-GalN-induced acute lethal liver injury or peritonitis in BALB/c mice, CH was administered *i.v* at a concentration of 320 mg/kg of body weight. Mice were sacrificed at each indicated time point, and sera were isolated by centrifugation and frozen at −40 °C until cytokine analysis. A mouse cytokine CBA kit was used and performed by flow cytometry (BD FACSCalibur™, USA) to measure serum IL-6, MCP-1 and TNF-α levels according to the manufacturer's instructions. Briefly, a mixture with 3 kinds of capture beads coated with antibodies specific for the above cytokines/chemokines (25 μl) with distinct fluorescence intensities (detected in FL3) was mixed with each serum sample or standard (25 μl). Then, PE-conjugated detection antibodies (detected in FL-2; 50 μl) were added to form sandwich complexes. After a 2 h incubation (in the dark at 18–25 °C), the samples were washed twice by centrifugation and resuspended in 300 μl of washing buffer followed by running on FACS Calibur TM. The two-color cytometric data for the samples were analyzed using CBA software (Bender Medsystems, Burlington, CA), and standard curves were generated for each cytokine using the mixed cytokine/chemokine standard provided.

2.7. Measurement of NF-κB activities in NF-κB-RE-luc (Oslo) transgenic mice with LPS/D-GalN-induced acute lethal liver injury

Imaging of *NF-κB*-RE-luc (Oslo) transgenic mice was performed using the IVIS Imaging System 200 (XENOGEN Corporation, Alameda, CA, USA). D-Luciferin (150 mg/kg) was dissolved in 250 μl sterile PBS (pH 7.8) followed by *i.p.* injection. After mice were placed in a dark chamber, color bar images were immediately obtained for reference before luminescence imaging. Luminescence emitted from the mice was integrated for 5 min starting at 3 min after luciferin injection ($T = 0$ h). Immediately thereafter, LPS/D-GalN-induced acute lethal liver injury was induced as described above, and CH (320 mg/kg) or NS was administered *i.v*. Images were taken at 0.5, 3, 4, 5, 6 and 10 h after challenge with LPS/D-GalN. Individual organs were also excised from mice at 10 h after the induction of LPS/D-GalN-induced acute lethal liver injury, placed on a heated (37 °C) stage and immediately imaged. Relative photon counts (RLU) from intact organs were normalized to the weight of individual organs. Data are presented as the mean fold induction over 0 h.

2.8. Determination of leukocyte counts and protein concentrations in peritoneal exudates

Immediately after zymosan A injection, NS or CH (320 mg/kg) was administered *i.v*. Leukocytes were isolated from the peritoneal exudates as previously described by Luo and Dorf [28] . Specifically, 0.5% Evans blue (150 μl/mouse) was administered via the tail vein and the animals were sacrificed at the indicated time points after treatment with NS or CH. The peritoneal cavity was irrigated, exudative fluid was collected and cells were separated by centrifugation. Cell precipitates were counted using Turk's solution containing 0.01% crystal violet and 3% acetic acid. The absorption value of the supernatant was measured at $O.D_{650\ nm}$ using a Beckmann 520 spectrophotometer.

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2.9. The effect of CH on murine peritoneal macrophages and RAW264.7 cells transfected with an NF-κB luciferase reporter plasmid

Separation, cultivation and identification of mouse peritoneal macrophages were all performed as preciously described by Yin et al. [29]. Cells were seeded at 10^6 cell/well in six-well cell culture clusters (Corning Inc. USA). The cells were grown in IMDM supplemented with 5% heat-inactivated FBS. Supernatants were collected at the indicated time points after stimulation with LPS (10 μg/ml) or LPS (10 μg/ml) plus CH (0.5 and 1 mg/ml) and frozen at −40 °C before TNF-α and IL-6 levels were measured using a mouse CBA kit.

Nuclear factor-κB (*NF-κB*) activity in the murine macrophage cell line RAW 264.7 cells was detected with an *NF-κB*-luciferase reporter vector (pSV40κB-luc) [30]. 5×10^6 RAW 264.7 cells were transiently transfected with 5 μg pSV40κB-luc (stored at laboratory) in a 400 μl final volume (IMDM/25% FBS) by electroporation (960 μF, 250 V, Gene Pulser Xcell, Bio-Rad) as a preciously described protocol with modifications [31] and subsequently washed and split into 50 wells (Corning Inc. USA), then cultured for 24 h in IMDM supplemented with 5% heat-inactivated FBS before stimulation with LPS (10 μg/ml) in the presence or absence of CH (0.5 and 1 mg/ml). For luciferase assays of transfected cells after stimulation for 6 and 12 h, harvested, lysed, and luciferase activity in extracts was measured with the Luciferase Assay System kit according to manufacturer's instruction. Data are presented as the mean fold of induction over the values obtained in unstimulated samples.

2.10. Statistical analysis

Data are presented as means ± S.D. Survival rates were estimated using the Kaplan–Meier method and tested for statistical significance using the Breslow–Gehan–Wilcoxon test. Comparisons between groups were performed using an unpaired Student's *t*-test and $P<0.05$ was considered to be significant.

3. Results

3.1. CH improved the survival of mice with LPS/D-GalN-induced acute lethal liver injury

We examined the effects of CH on the survival of mice with acute lethal liver injury induced by LPS/D-GalN. We found that CH treatment improved the survival of the mice in a dose-dependent manner, and the survival times in groups receiving 160 and 320 mg/kg were

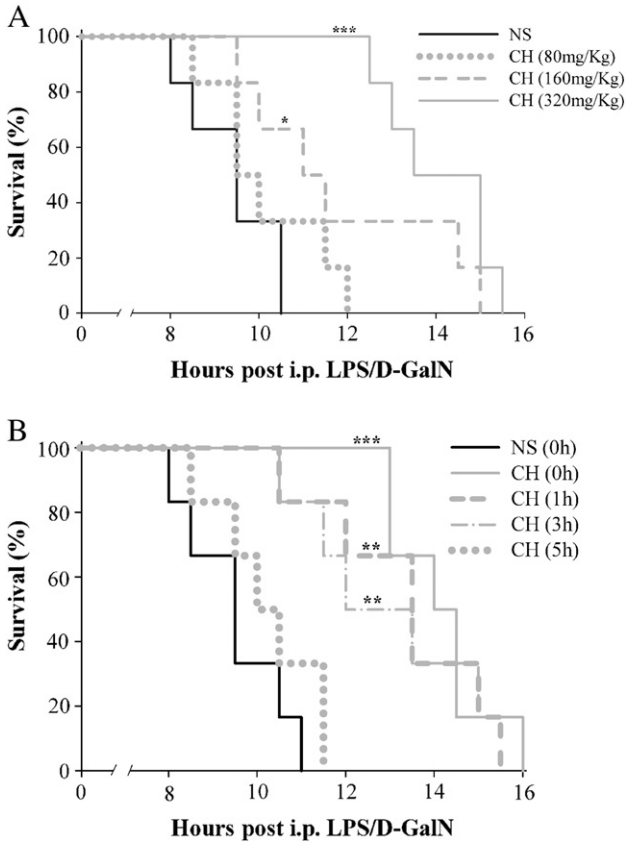

Fig. 1. CH improved the survival of mice with LPS/D-GalN-induced acute lethal liver injury. (A) Dose-dependent effect of CH on survival. Acute lethal liver injury was induced in BALB/c mice by *i.p.* injection of LPS/D-GalN and was immediately followed by *i.v.* injection with different doses of CH 0 [normal saline (NS)], 80, 160 or 320 mg/kg via the tail vein ($n=6$, 6 mice in each group). * $P<0.05$ and *** $P<0.001$ for the 160 and 320 mg/kg CH-treated group *vs.* the NS treated group (B) Time-dependent effect of CH on survival. Mice were also given CH (320 mg/kg) at the 0, 1, 3 or 5 h after challenge with LPS/D-GalN ($n=6$, 6 mice in each group). ** $P<0.01$ and *** $P<0.001$ for the CH (320 mg/kg)-treated group *vs.* the NS treated group as the control at 0, 1 and 3 h. Each experiment was independently repeated three times. Survival rates were recorded every 30 min, estimated using the Kaplan–Meier method and tested for statistical significance using the Breslow–Gehan–Wilcoxon test.

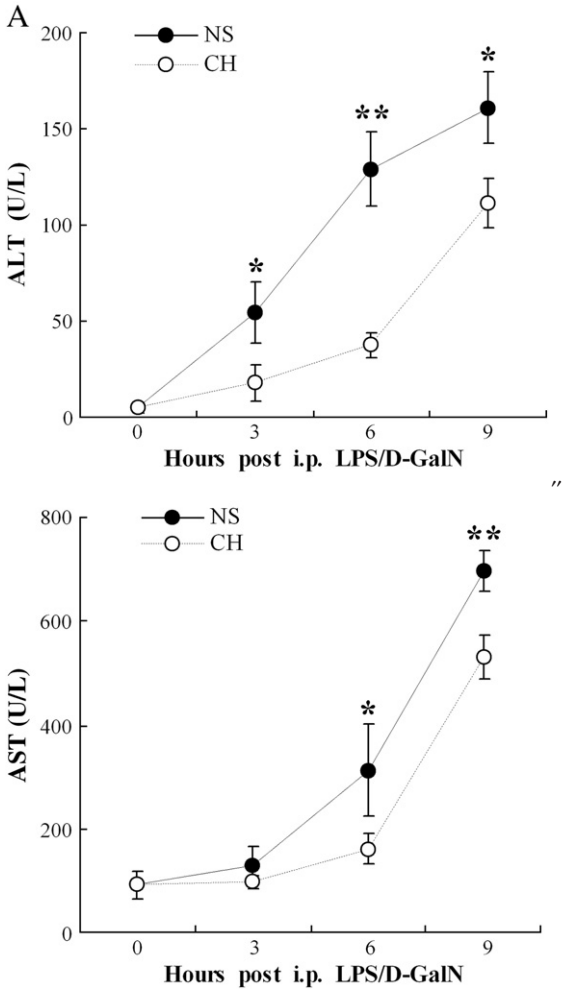

Fig. 2. CH attenuated LPS/D-GalN-induced liver injury in mice. Mice treated with LPS/D-GalN were treated immediately with CH (320 mg/kg) or NS as a control and were sacrificed at 0, 3, 6 or 9 h thereafter. Sera and liver tissues were collected for assessment of ALT/AST and pathologic changes in injured tissues. (A) Effects of CH on ALT and AST levels in mice with LPS/D-GalN-induced acute lethal liver injury. ALT and AST were measured and the data were expressed as means ± SD of three independent experiments ($n=3$). The experiment was independently repeated three times. * $P<0.05$, ** $P<0.01$ for the CH-treated group *vs.* the NS-treated group at the same time point (B) Gross morphological changes in livers of LPS/D-GalN-challenged mice treated with or without CH. (C) The pathological changes in liver sections observed using H&E staining in mice treated with or without CH (320 mg/kg).

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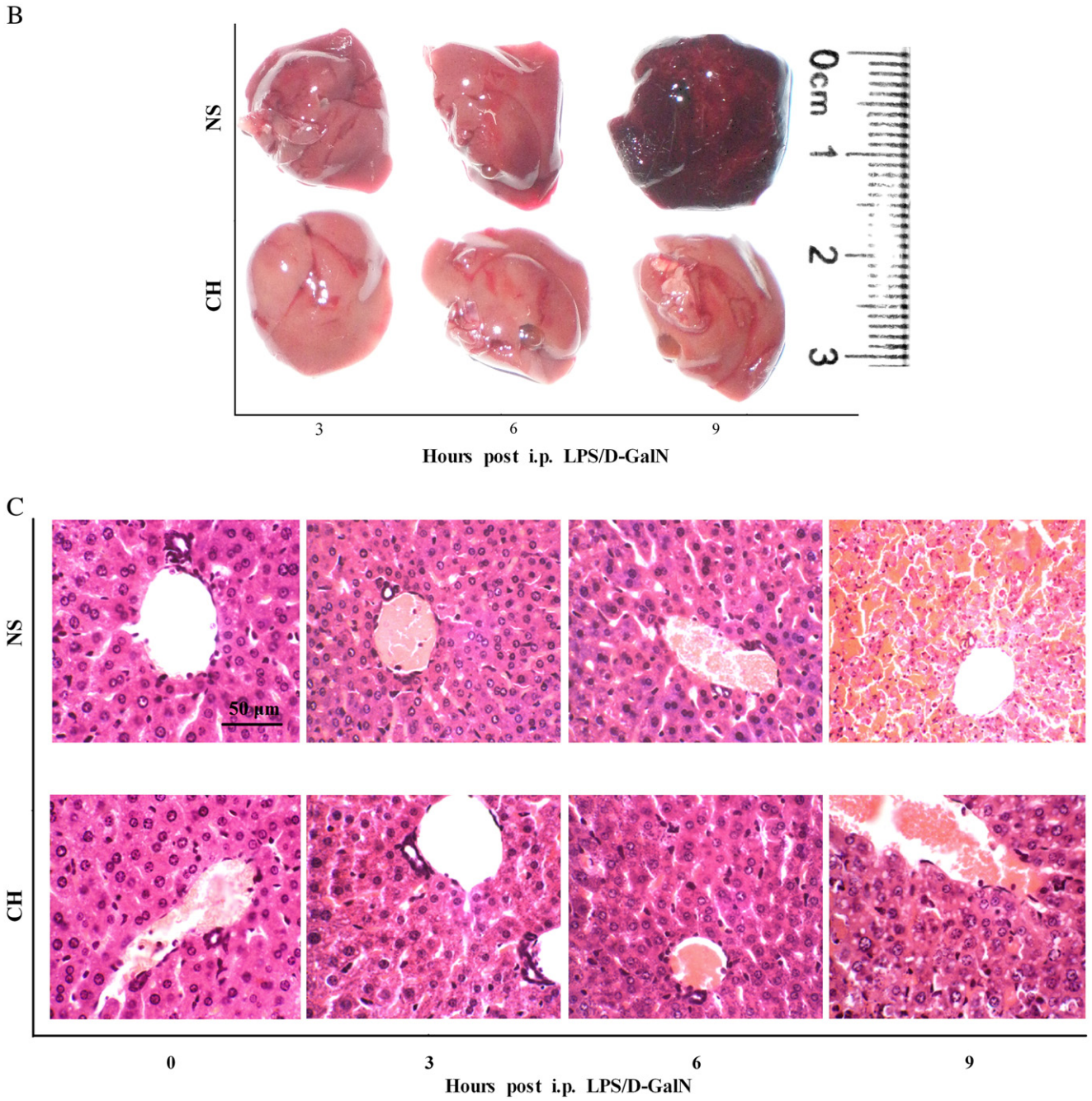

Fig. 2 (*continued*).

significantly improved compared with controls (11.68 ± 2.62 h, 13.78 ± 1.21 h *vs.* 9.17 ± 1.10 h, $P<0.05$ and $P<0.001$, respectively) (Fig. 1A). The time-dependent effect of CH was further examined by administering 320 mg/kg at various time periods after challenge with LPS/D-GalN. We found that CH administered at 0, 1 and 3 h, but not 5 h, after LPS/D-GalN challenge significantly improved the survival of these mice compared with controls ($P<0.001$, $P<0.01$, $P<0.01$ and $P>0.05$, respectively) (Fig. 1B) .

3.2. CH alleviated liver injury in mice with LPS/D-GalN-induced acute lethal liver injury

Levels of ALT and AST were further examined in mice treated with LPS/D-GalN. LPS/D-GalN treatment was found to induce a sharp rise in serum levels of ALT and AST in control mice. However, CH attenuated the rise of ALT and AST. Levels of ALT remained significantly lower in CH-treated mice than in controls at 3, 6 and 9 h after LPS/D-GalN challenge, while AST levels were significantly lower than controls at 6 and 9 h (All $P<0.05$) (Fig. 2A). Gross examination of the liver showed marked swelling, congestion and hemorrhage in the control group at 9 h after challenge, while the liver had only mild congestion in the CH-treated mice (Fig. 2B). Furthermore, liver H&E sections showed LPS/D-GalN-induced necrosis of hepatocytes in both the control and CH-treated groups; however, more severe liver pathology was induced by LPS/D-GalN in the control group than in the CH-treated group. Furthermore, obvious differences appeared at 9 h after challenge, including destruction of liver structure and marked necrosis of hepatocytes and hemorrhage in the control group, while only mild pathological changes were observed in the CH-treated group (Fig. 2C).

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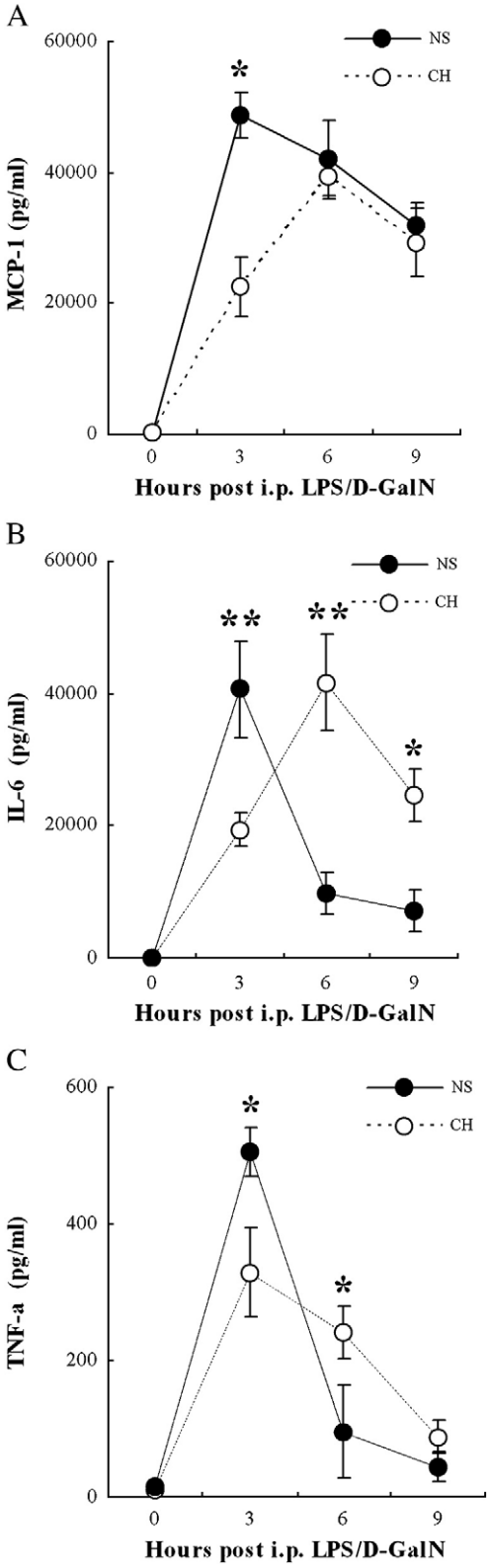

Fig. 3. CH attenuated and delayed increases in serum MCP-1, IL-6 and TNF-α levels in mice following LPS/D-GalN challenge. Mice treated with LPS/D-GalN with or without CH (320 mg/kg) were sacrificed 0, 3, 6 or 9 h thereafter. Sera were collected to measure levels of MCP-1, IL-6 and TNF-α using a specific CBA kit. Data are expressed as means ± SD ($n=3$). * $P<0.05$ and ** $P<0.01$ for the CH (320 mg/kg)-treated *vs.* the NS-treated animals at the same time point.

3.3. CH caused an attenuated and delayed rise of serum levels of MCP-1, IL-6 and TNF-α in mice treated with LPS/D-GalN

In LPS/D-GalN-treated mice, marked increases in serum levels of MCP-1, IL-6 and TNF-α were noted at 3 h after challenge. CH treatment, however, attenuated the increase in serum MCP-1, IL-6 and TNF-α levels at this time point compared with control mice ($P<0.01$, $P<0.01$ and $P<0.05$, respectively), while this attenuation did not appear at other time periods (Fig. 3). Serum levels of IL-6 and TNF-α underwent a sharp decline at 6 h after LPS/D-GalN challenge in the control group, while levels of MCP-1 gradually tapered off. In contrast to the control group, however, serum IL-6 levels underwent a sharp increase in the treatment group at 6 h after LPS/D-GalN challenge, which was significantly higher than the control group ($P<0.01$). Serum TNF-α levels in the CH-treated group peaked at 3 h and gradually declined thereafter, but they remained significantly higher than the control group at 6 h after LPS/D-GalN challenge. Serum levels of MCP-1 at 6 and 9 h after LPS/D-GalN challenge did not differ significantly between the CH-treated and control groups (Fig. 3).

3.4. CH attenuated the rise in NF-κB activity in NF-κB-RE-luc (Oslo) luciferase reporter transgenic mice challenged with LPS/D-GalN

LPS/D-GalN was injected to induce acute lethal liver injury in *NF-κB*-RE-luc (Oslo) luciferase reporter transgenic mice and the effect of CH on *NF-κB* activation was examined using *in vivo* imaging. The results suggested that LPS/D-GalN treatment can increase *NF-κB* activity in control mice treated with NS. This activity peaked at around 4 h after LPS/D-GalN challenge. CH, however, attenuated this increase in *NF-κB* activity in all *NF-κB*-RE-luc mice. *In vivo* imaging photos are shown in Fig. 4A and a. Examination of individual organs for *NF-κB* activity further revealed that the highest *NF-κB* activity occurred in the liver followed the intestines and lungs. Additionally, *NF-κB* activities in these three organs in control mice were significantly higher than those in the CH treatment group ($P<0.05$) (Fig. 4B and b).

3.5. CH did not impact serum corticosterone levels in mice with LPS/D-GalN-induced acute lethal liver injury

Corticosterone level is a major indicator for response to stress. Our results showed that there was no significant difference ($p>0.05$) in serum corticosterone levels between CH-treated and control mice. Also, corticosterone levels in the CH-treated group were a little lower than in the control group all time points (Fig. 5).

3.6. CH delayed influx of leukocytes and proteins into the peritoneal exudates in mice with zymosan A-induced peritonitis

We investigated the effect of CH on the inflammatory response by examining the number of leukocytes in the peritoneal exudates of mice following zymosan A-induced peritonitis. Zymosan A was found to cause a marked rise in leukocyte counts in peritoneal exudates, reaching $25.56 \pm 3.32 \times 10^6$ cells per mouse at 8 h after zymosan A challenge, while in the CH group, leukocyte counts peaked at 24 h after challenge. Leukocyte counts in the CH-treated group were significantly lower than in the control group at 5 and 8 h (both $P<0.05$) (Fig. 6A). The examination of proteins in the peritoneal exudates indicated a peak of protein exudation (expressed as a O. $D_{650\ nm}$ value of (1.69 ± 0.20)) at 1 h after zymosan A challenge in the control group, while the peak in the CH-treated group was decreased ($P<0.05$) and delayed 4 h. Protein exudates also differed between the two groups at 0.5, 1 and 8 h after zymosan A challenge ($P<0.05$, $P<0.01$ and $P<0.05$, respectively) (Fig. 6B).

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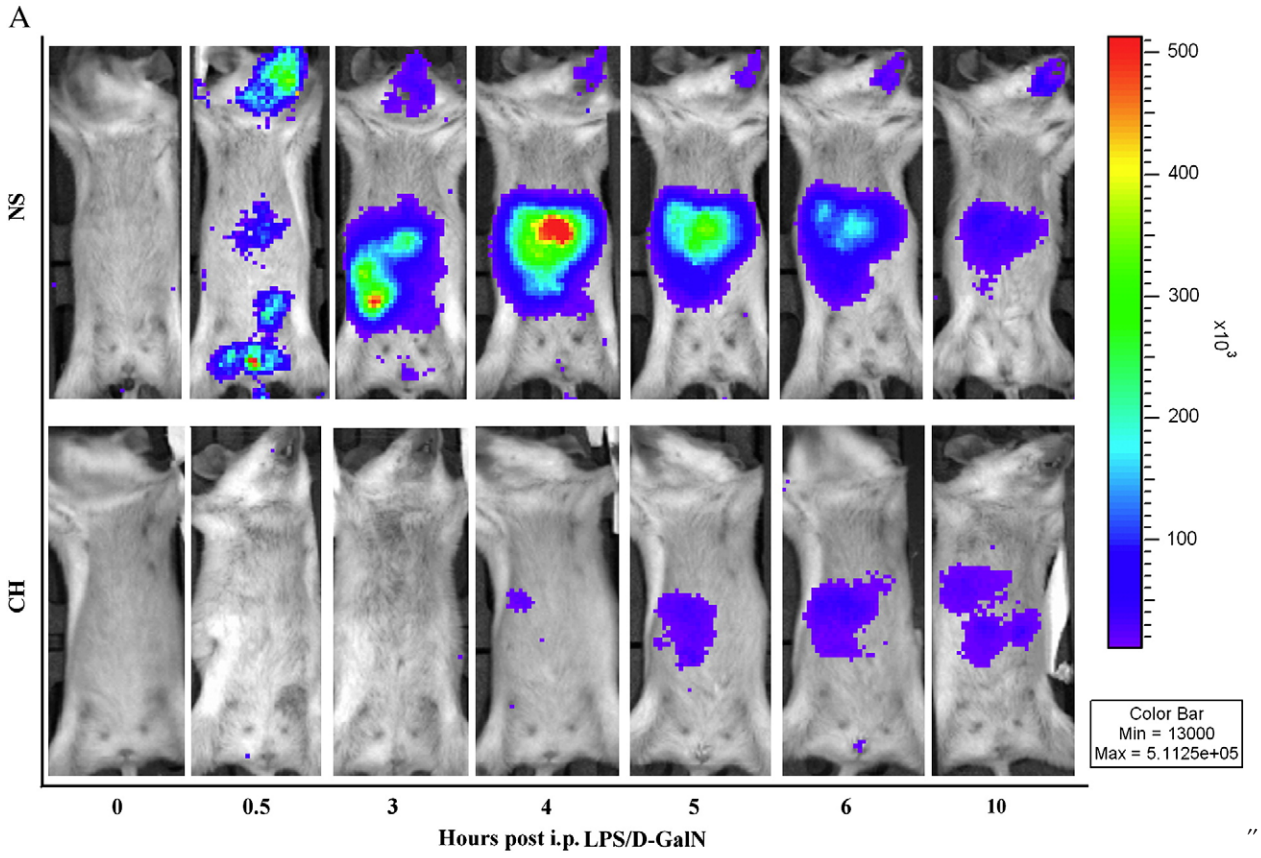

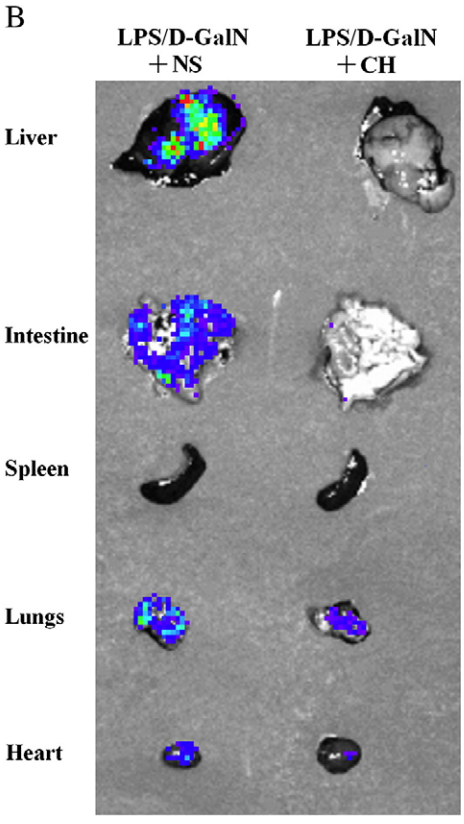

Fig. 4. CH attenuated and delayed upregulation of *NF-kB* activity during LPS/D-GalN-induced acute lethal liver injury. *NF-kB*-RE-luc (Oslo) luciferase reporter transgenic mice treated with LPS/D-GalN were immediately treated with CH (320 mg/kg) or with NS as a control. *In vivo* images were taken at 0.5, 3, 4, 5, 6 and 10 h after challenge with LPS/D-GalN. (A) (B) Representative samples showing *NF-κB* activities measured as described in Section 2.7. Data in (a) and (b) are expressed as means ± SD ($n = 3$). * $P<0.05$ and ** $P<0.01$ for the CH (320 mg/kg)-treated *vs.* the NS-treated animals at the same time point.

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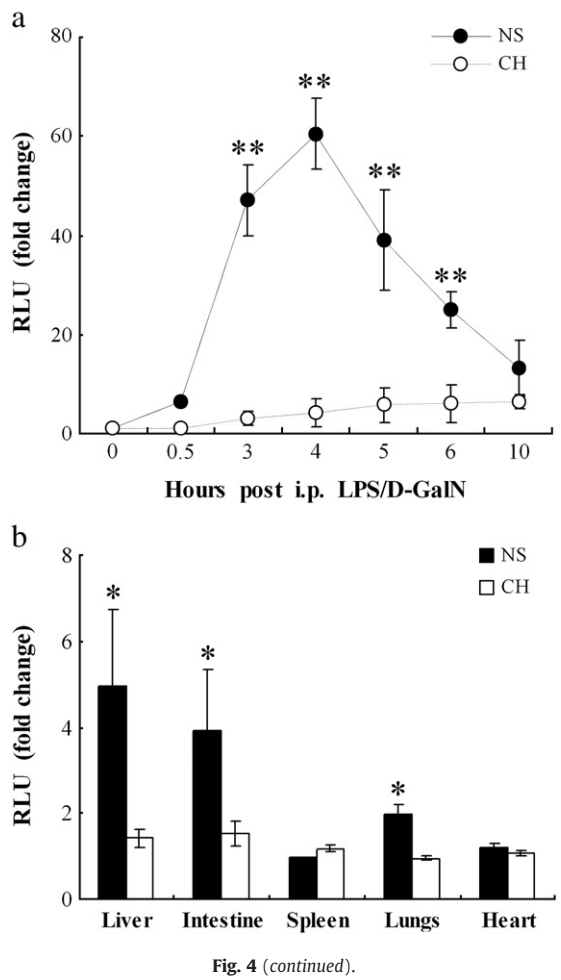

Fig. 4 (*continued*).

3.7. CH attenuated serum levels of MCP-1, IL-6 and TNF-α in mice with zymosan A-induced peritonitis

Noticeable increases in serum levels of MCP-1, IL-6 and TNF-α were found in both groups at 1 h after zymosan A challenge (Fig. 7). Specifically, serum levels of MCP-1 peaked at 8 h after zymosan A challenge and were significantly higher than in mice treated with CH at 2 and 8 h after challenge (both $P<0.05$). These results suggested that CH treatment attenuated levels of serum MCP-1. Similarly, serum levels of IL-6 were also significantly higher in the control group than in the CH-treated group at 1, 2 and 8 h after zymosan A challenge ($P<0.05$, $P<0.01$ and $P<0.01$, respectively). Serum levels of TNF-α increased sharply after zymosan A challenge in the control group and were significantly higher than in CH-treated mice at 1 and 2 h ($P<0.001$ and $P<0.01$, respectively). CH treatment appeared to delay zymosan A-induced increases in TNF-α levels, which peaked at 8 h after challenge (Fig. 7).

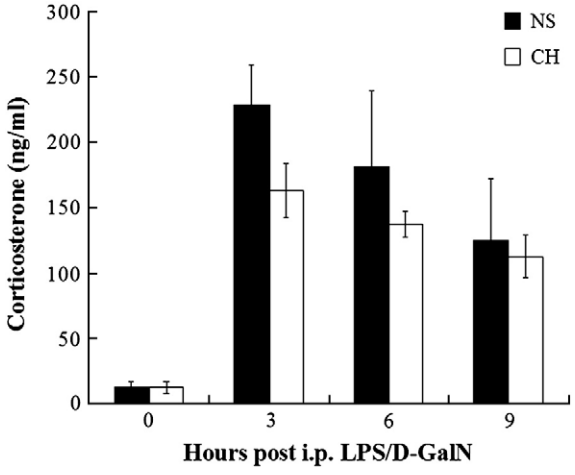

Fig. 5. The effect of CH on serum corticosterone levels in mice treated with LPS/D-GalN. Levels of serum corticosterone were measured using a mouse corticosterone ELISA kit as per the manufacturer's instructions. Corticosterone were measured and the data are expressed as means ± SD of three independent experiments ($n=3$). The experiment was independently repeated three times.

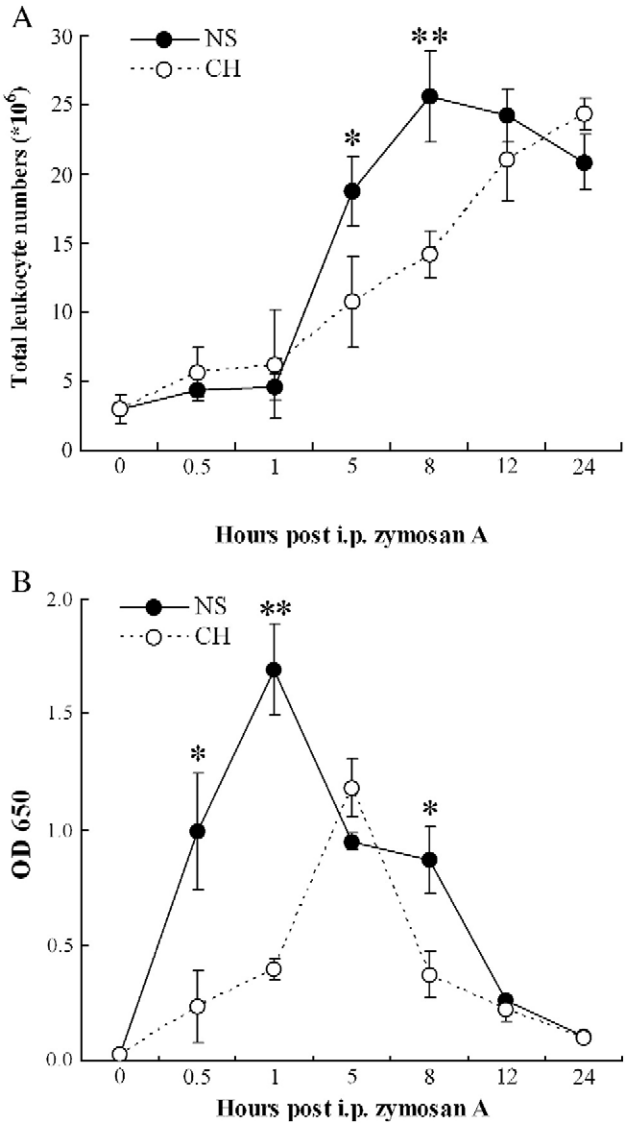

Fig. 6. CH delayed increases leukocyte number and protein content in peritoneal exudates following peritonitis induction. Peritonitis was induced by *i.p.* injection of 1 mg zymosan A in 0.5 ml normal saline and was followed immediately by treatment with CH (320 mg/kg) or NS as a control. Peritoneal exudates from mice were collected at 0, 0.5, 1, 5, 8, 12 and 24 h after zymosan A challenge to measure total leukocyte count and protein content. (A) Leukocyte counts in peritoneal exudates. (B) Protein content in peritoneal exudates. Data are expressed as means ± SD of three independent experiments ($n=3$). * $P<0.05$ and ** $P<0.01$ for the CH (320 mg/kg)-treated *vs.* the NS-treated animals at the same time point.

3.8. CH treatment attenuated levels of IL-6 and TNF-α produced by peritoneal macrophages and NF-κB activity in RAW264.7 cells stimulated with LPS

Our results showed that production of IL-6 and TNF-α increased sharply after stimulation with LPS (10 μg/ml) for 6, 12 and 24 h and

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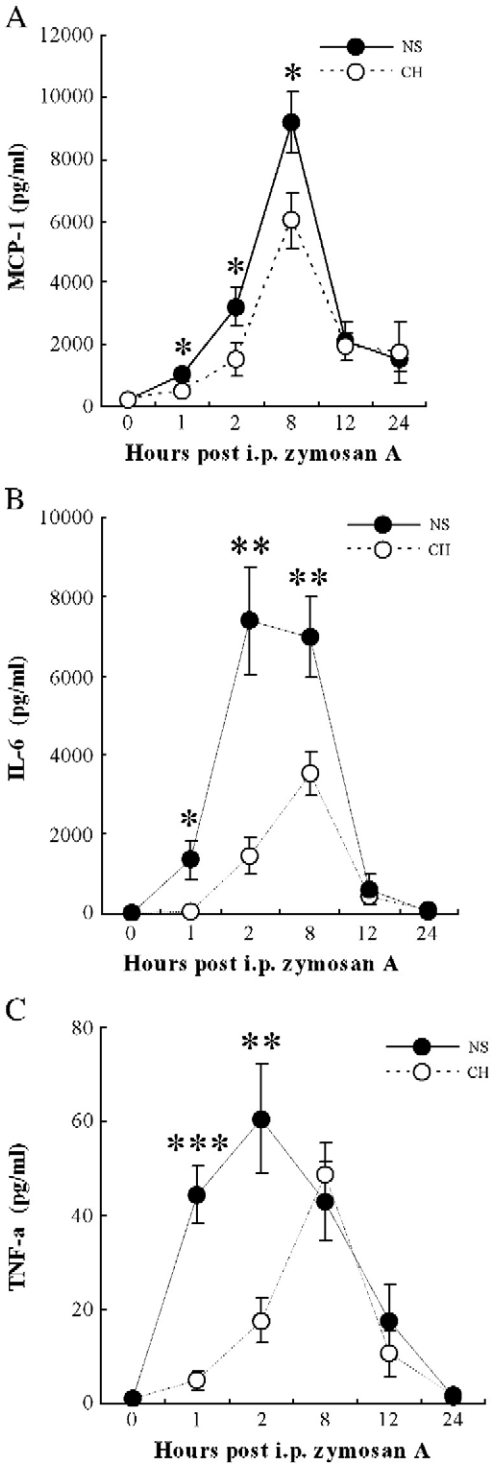

Fig. 7. CH attenuated and delayed increases in serum MCP-1, IL-6 and TNF-α levels in mice with peritonitis. Peritonitis was induced by *i.p.* injection of 1 mg zymosan A in 0.5 ml normal saline and was followed immediately by treatment with CH (320 mg/g) or NS as a control. Sera were collected to measure levels of MCP-1, IL-6 and TNF-α using a specific CBA kit. Data are expressed as means ± SD ($n = 3$). * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ for CH (320 mg/kg)-treated *vs.* NS-treated animals at the same time point.

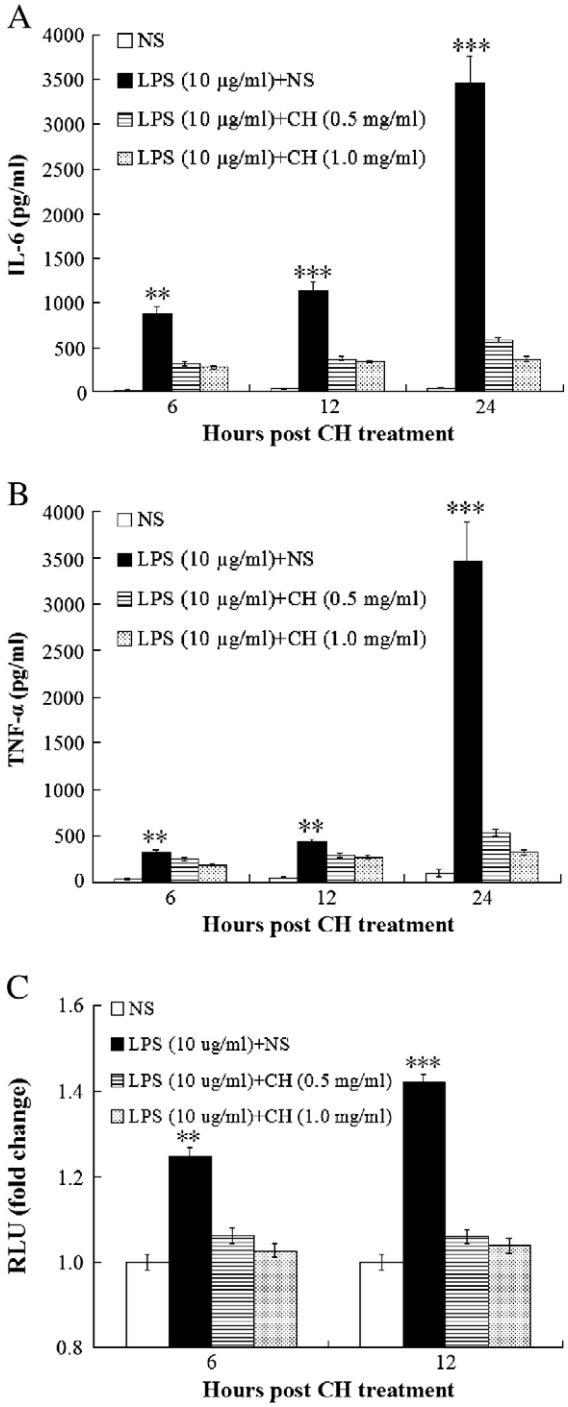

Fig. 8. CH attenuated upregulation of IL-6 and TNF-α by peritoneal macrophages and *NF-κB* activity in RAW264.7 cells following LPS stimulation. (A) CH treatment attenuated upregulation of inflammatory cytokines by LPS-stimulated peritoneal macrophages. The effect of CH (0.5 and 1 mg/ml) on levels of TNF-α and IL-6 produced by peritoneal macrophage (10^6 cell/well) after LPS stimulation for 6, 12 and 24 h is shown. (B) CH treatment inhibited LPS-induced *NF-κB* transcriptional activity in RAW264.7 cells. To analyze *NF-κB* transcriptional activity, pSV40κB-luc was transiently transfected into RAW264.7 cells by electroporation. One day after transfection, LPS (10 μg/ml) or LPS (10 μg/ml) plus CH (0.5 or 1 mg/ml) was added to the medium and cells were incubated for 6 and 12 h. Cell extracts were prepared and luciferase activities were determined. Data are expressed as means ± SD of three independent experiments ($n = 3$). ** $P<0.01$ and *** $P<0.001$ for CH (0.5 and 1 mg/ml)-treated *vs.* the NS-treated cells after LPS (10 μg/ml) stimulation at the same time point.

decreased significantly upon CH (0.5 and 1 mg/ml) treatment (all $P<0.01$) (Fig. 8A–B).

Luciferase activity in RAW264.7 transfected with pSV40κB-luc was measured at 6 and 12 h after LPS stimulation. Results are presented as the mean fold of induction over the values obtained in unstimulated samples. As shown in Fig. 8C, a roughly 0.5-fold increase in luciferase activity was observed after LPS stimulation for 12 h. This increase was significantly decreased by CH (0.5 and 1 mg/ml) treatment (all $P<0.01$).

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4. Discussion

The potentially fatal pathological conditions involved in LPS/D-GalN-induced acute liver injury in animal models and sepsis in human patients have been generally considered to be associated with cytokines such as TNF and IL-1. However, in recent years, the mechanisms behind LPS- or bacteria-induced acute inflammatory responses and therapeutic strategy with anti-cytokine have been re-evaluated. Most anti-cytokine therapeutic projects are directed against autoimmune diseases rather than sepsis because most sepsis-related anti-cytokine clinical trials exhibited unsatisfying or disappointing results [32–34]. Therefore, the development of new drugs and new therapeutic programs for patients with sepsis or other acute inflammations have been intensely researched recently.

It is well known that the development and approval of a new drug or therapy program requires a significant amount of time and funding. However, newly-discovered functions of older drugs may accelerate the development or application of new therapy programs. For example, the bisphosphonate zoledronic acid has been proven to attenuate the growth of breast cancer metastasis [35], while thalomide has been used to treat multiple myeloma [36]. The discovery of the protective effects of some anesthetics against infectious and inflammatory responses in animal models, especially in endotoxic shock in mice or rats [1–4], may yield new insight into anti-inflammation therapy. Compared with other anti-inflammatory anesthetics, CH is convenient for use in clinical and animal models because it can be administered by mouth, injection or enteroclysm and is effective with just a single dose, unlike isoflurane, which must be inhaled continuously. In addition, CH can be used without limitation in many countries, while the use of ketamine is heavily regulated in China, except as a surgical anesthetic [37], and is not approved for patients under 16 years old in the US [5].

In the present study, we used two animal models: 1) LPS/D-GalN-induced liver injury, a model of serious and lethal inflammation; and 2) zymosan A-induced peritonitis, a mild or non-fatal inflammation model. CH was found to attenuate or delay inflammatory responses in both models. Most impressive is the fact that only one injection of CH given as late as 3 h after LPS/D-GalN challenge improved the survival of mice and alleviated liver injury.

The administration of CH by i.v injection in this work is to avoid the possible interaction with LPS/D-GalN by i.p. and the injection should be given slowly (within 5 min). Moreover, the safety for normal mice ($n=12$) injected i.v slowly with 320 mg/kg of CH was confirmed by observation of survival, time of analepsia, retrieve of body temperature, in which the mice only treated with CH appeared lower body temperature last for about 3–4 h followed by to recover to normal, and there were no mice died at the end of one week.

Furthermore, treatment with 160 mg/kg CH was also protective in this model (11.68 ± 2.62 h *vs.* 9.17 ± 1.10 h, $P=0.024$). Such a dose will be more efficient if the dose of LPS is decreased to make the subsequent inflammation more moderate. For instance, Fuentes et al. [1] found that continuous infusion of isoflurane for 1 h significantly improved the survival using a rat endotoxic shock model with a survival period of 72 h after *i.p.* injection of LPS. Suliburk et al. [38] demonstrated that ketamine exerted a protective effect on liver injury in LPS-challenged mice, with AST levels in treated mice being significantly lower than in non-treated mice, while isoflurane did not show such protective effects. In the current study, we found that CH noticeably reduced levels of AST and ALT in the serum and alleviated the necrosis of hepatocytes and liver hemorrhage observed in mice after LPS/D-GalN challenge. Fuentes et al. [1] also demonstrated that administration of isoflurane, pentobarbital and ketamine plus xylazine immediately after LPS injection could significantly protect animals from endotoxic shock and reduce plasma levels of IL-6, TNF-a and IL-10. Interestingly, serum profiles of proinflammatory cytokines MCP-1, IL-6 and TNF-α seem to be attenuated by CH treatment at only 3 h after challenge, with an increase at 6 h after challenge with LPS/D-GalN. These results indicate that CH delays the production of proinflammatory cytokines rather than merely attenuating LPS/D-GalN-induced acute lethal liver injury in mice.

The results of *in vivo* imaging studies in *NF-κB*-RE-luc (Oslo) transgenic mice further revealed that CH also attenuates *NF-κB* activity, playing a critical role in the transcriptional regulation of proinflammatory gene expressions in various organs. This result indicates that the protective effects of CH could be attributed to its effects on *NF-κB* activity, resulting in attenuation of the inflammatory response by reducing production of proinflammatory cytokines. In this study, *NF-κB* activity in *NF-κB*-RE-luc (Oslo) transgenic mice peaked at 3 to 5 h and then declined gradually until 10 h after challenge with LPS/D-GalN. Similar studies using LPS (50 μg or 2 mg/kg) alone were performed by Carlsen et al. [39] and Austenaa et al. [40] in which peak *NF-κB* activity occurred at 3.5 to 5 h after LPS challenge, although they only observed 4 and 6 h after challenge, respectively. This peak of *NF-κB* activity at about 3 to 4 h after challenge seems to be consistent with cytokine production dynamics, particularly TNF-α and IL-6. However, the molecular mechanism behind CH-mediated cytokine production inhibition remains to be elucidated.

One of the mechanisms behind inhibition of acute inflammation could be changes in corticosterone levels. Corticosterone is one of the most important hormones in the pituitary-adrenergic axis produced during the stress response induced by endotoxins. Production of corticosterone occurs 30 min after *i.p.* injection of 100 μg LPS in mice [41] and higher levels of corticosterone can be detected 2 weeks after *i.p.* injection of 10 μg LPS [42]. In our study, the chosen time points for determination of corticosterone levels were 0, 3, 6, 9 h after CH treatment, which were matched with other parameters. A dose of 10 μg LPS was administered *i.p.*, which induced lethal liver injury synergistically with D-GalN. The results presented in Fig. 5 show no significant difference in corticosterone levels between CH-treated and control animals. Corticosterone levels in CH-treated animals were lower than those of control animals at all time points. This finding indicates that the effect of CH may not occur via the pituitary-adrenergic axis, suggesting that the effect of CH on LPS/D-GalN induced lethal liver injury is not mediated by hormones.

We also assessed the effect of CH on zymosan A-induced peritonitis, a very rapid and non-fatal inflammatory response that can be detected within 30 min after induction [43]. It has been noted that mast cells and macrophages are the most susceptible cells in zymosan A-induced peritonitis in the early stages of the inflammatory response, while neutrophils and macrophages are sensitive in later stages. We noticed that the increases in serum pro-inflammatory cytokine levels after zymosan A challenge were similar to findings reported by Kolaczkowska et al. [20]. The inflammatory response in mice with experimental peritonitis was apparently different from mice with acute lethal liver injury induced by LPS/D-GalN. The former presents serum levels of proinflammatory cytokines that are markedly lower than in the latter, suggestive of a more moderate inflammatory response. CH, however, could still attenuate this inflammatory response, demonstrating its protective effect in both mild and severe inflammation models.

In this study, one critical question was the cell type targeted by CH. As TNF-α, IL-6 and MCP-1 have been considered to be very important cytokines in LPS/D-GalN-induced acute liver injury and zymosan A-induced peritonitis and are produced by monocytes/macrophages, we chose to study murine peritoneal macrophages and RAW264.7 cells, a murine macrophage cell line. Cells were transfected with an *NF-κB*-luciferase reporter vector to observe the effect of CH. The results indicated that CH attenuated the rise in inflammatory cytokine levels produced by peritoneal macrophages and *NF-κB* activity in RAW264.7 cells stimulated with LPS, which was consistent with the results of our *in vivo* imaging experiments. These results suggest that the effect of CH on inflammation may be attributed to inhibition of the function of monocytes/macrophages. Moreover, LPS is a thymus-independent antigen (TI Ag) that minimally induces T cell-mediated immune responses, especially in such low doses (only 10 μg for each mouse in our work), although one report

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exists regarding recognition of zwitterionic polysaccharides by T cells [44] . Recently, T cell mediated inhibition of LPS-induced inflammation was reported [45] under very strict conditions including the following: 1) a longer survival in a mild inflammation model; 2) a longer co-culture time (20 h in presence of 2×10^6 pan-T cells and only 100 ng/ml LPS); 3) T cell deletion with anti-CD8 and CD4 antibodies 5 days before LPS challenge. Therefore, we can exclude the possibility of T cell effects on LPS/D-GalN-induced acute liver injury in our work.

It is also noteworthy that the anti-inflammatory effect of CH can be observed even when its administration at 1 to 3 h after LPS/D-GalN challenge, while most of other agents for the treatment of LPS or LPS/D-GalN induced acute lethal liver injury models were used prior to LPS challenge. For example, Austenaa et al. reported that retinoic acid suppresses inflammation at 3 h prior to the LPS (50 μg) challenge [40]. And Tetrandrine [46] and Asiaticoside [47] as suppressors were used at 1 h or 3 days before challenge, only p-methoxyldiphenyl diselenide [48] was used at 1 h after LPS/ D-GalN challenge, in which LPS in dose of 50 μg/kg and D-GalN in dose of 500 ~ 800 mg/kg were used, while LPS in dose of approximate 500 μg/kg and D-GalN in dose of 800 mg/kg were used in our present study. These findings suggest that CH or its derivatives might be further explored as therapeutic options for the treatment of acute inflammatory conditions, such as acute lethal liver injury and peritonitis, although the pharmacologic mechanism, dynamics and toxicologic events as well as the clinical safety of CH remain to be elucidated and evaluated.

In conclusion, we have demonstrated that CH can attenuate and delay the inflammatory response in mice following LPS/D-GalN-induced acute lethal liver injury or peritonitis and can improve the survival of mice following LPS/D-GalN-induced acute lethal liver injury. This altered inflammatory response is associated with inhibitory effects of CH on *NF-κB* activity and serum levels of proinflammatory cytokines.

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