Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical Pharmacology 69 (2005) 233–240
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Protective effect of dextromethorphan against endotoxic shock in mice

Guorong Li[a,*], Yuxin Liu[a], Nian-ssheng Tzeng[b], Gang Cui[c], Michelle L. Block[a], Belinda Wilson[a], Liya Qin[a], Tongguang Wang[a], Bin Liu[a], Jie Liu[d], Jau-Shyong Hong[a]

[a]*Neuropharmacology Section, Laboratory of Pharmacology and Chemistry, NCI, National Institute of Environmental Health Sciences, National Institutes of Health Sciences, Mail Drop F1-01, P.O. Box 12233, Research Triangle Park, NC 27709, USA*
[b]*Departments of Psychiatry, Tri-Service General Hospital, National Defense Medical Center, Taipei, Taiwan*
[c]*Pathology and Lab Medicine, University of North Carolina at Chapel Hill, NC 27513, USA*
[d]*Inorganic Carcinogenesis Section, Laboratory of Comparative Carcinogenesis, NCI, National Institute of Environmental Health Sciences, National Institutes of Health Sciences, Research Triangle Park, NC 27709, USA*

Received 5 June 2004; accepted 5 October 2004

Abstract

Dextromethorphan (DM) is a dextrorotatory morphinan and an over-the-counter non-opioid cough suppressant. We have previously shown that DM protects against LPS-induced dopaminergic neurodegeneration through inhibition of microglia activation. Here, we investigated protective effects of DM against endotoxin shock induced by lipopolysaccharide/D-galactosamine (LPS/GalN) in mice and the mechanism underlying its protective effect. Mice were given multiple injections of DM (12.5 mg/kg, s.c.) 30 min before and 2, 4 h after an injection of LPS/GalN (20 μg/700 mg/kg). DM administration decreased LPS/GalN-induced mortality and hepatotoxicity, as evidenced by increased survival rate, decreased serum alanine aminotransferase activity and improved pathology. Furthermore, DM was also effective when it was given 30 min after LPS/GalN injection. The protection was likely associated with reduced serum and liver tumor necrosis factor alpha (TNF-α) levels. DM also attenuated production of superoxide and intracellular reactive oxygen species in Kupffer cells and neutrophils. Real-time RT-PCR analysis revealed that DM administration suppressed the expression of a variety of inflammation-related genes such as macrophage inflammatory protein-2, CXC chemokine, thrombospondin-1, intercellular adhesion molecular-1 and interleukin-6. DM also decreased the expression of genes related to cell-death pathways, such as the DNA damage protein genes *GADD45* and *GADD153*. In summary, DM is effective in protecting mice against LPS/GalN-induced hepatotoxicity, and the mechanism is likely through a faster TNF-α clearance, and decrease of superoxide production and inflammation and cell-death related components. This study not only extends neuroprotective effect of DM, but also suggests that DM may be a novel compound for the therapeutic intervention for sepsis.

Keywords: DM; LPS/GalN; Liver injury; Inflammation; ROS; Gene expression

1. Introduction

Dextromethorphan (DM) is one of the most widely used antitussives for the treatment of cough associated with acute upper respiratory tract infection. It is an over-the-count medicine; synthetic, non-narcotic and centrally acting cough suppressant. DM is the *d*-isomer of the codeine analog levorphanol, a dextrorotatory morphinan. We have recently demonstrated that naloxone, another analogue of morphinan, protects LPS-induced neurotoxicity in vitro and in vivo through the inhibition of release of proinflammatory factors and free radicals [1–5], and prevents endotoxin shock in lipopolysaccharide/galactosamine (LPS/GalN)-treated mice (unpublished data). DM is structurally similar to naloxone and has been shown to protect against LPS-induced dopamine neurodegeneration in mixed neuron-glia co-culture through the inhibition of microglia over-activation, and the subsequent reduction

Abbreviations: DM, dextromethorphan; LPS, lipopolysaccharide; GalN, D-galactosomine; ALT, alanine aminotransferas; TNF-α, tumor necrosis factor-alpha; ROS, reactive oxygen species; SOD, superoxide dismutase; *MIP-2*, macrophage inflammatory protein-2; TSP-1, thrombospondin1; *mKC*, a mouse CXC chemokine; *ICAM-1*, intercellular cell adhesion molecule-1; *IL-6*, interleukin-6; *GADD45*, growth arrest and DNA damage inducible protein 45; *GADD153*, growth arrest and DNA damage inducible protein 153

* Corresponding author. Tel.: +1 919 843 0183; fax: +1 919 843 0749.
E-mail address: guorongl@med.unc.edu (G. Li).

0006-2952/$ – see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2004.10.003

of pro-inflammatory cytokines (such as TNF-α) and free radicals, such as nitrite oxide (NO) and reactive oxygen species (ROS) [6]. Our in vitro data suggested that DM has even more potent anti-inflammatory effects than naloxone (unpublished data), which led us design more in vivo studies to verify its potent anti-inflammatory effects and to exploit the possible mechanism.

Endotoxemia occurs frequently in cases of liver failure [7,8] and is thought to play a role in the pathogenesis of liver disease [9]. GalN is an amino sugar selectively metabolized by the hepatocyte, which induces a depletion of the uridine triphosphate pool and thereby inhibits macromolecule (RNA, proteins and glycogen) synthesis in the liver [10]. When given together with a sublethal dose of LPS, GalN highly sensitizes animals to develop acute hepatoxicity and lethal shock [11–17]. Thus, LPS/GalN animal model has been widely used to study the effects of drugs/agents on endotoxin shock and the potential underlying mechanisms [11–15]. In this model, TNF-α is the major mediator leading to liver injury [11,14]. Significant increase in NADPH-oxidase activity and oxidative stress is another important toxicological mechanism [15].

In the present study, the protective effects of DM against LPS/GalN-induced liver injury were examined in mice. We demonstrated that DM remarkably protected animals from LPS/GalN-induced endotoxemia and liver damage. The mechanisms of the protection were further demonstrated by significant inhibition of DM from LPS-induced production of TNF-α, ROS, and relevant gene expressions associated with inflammation and hepatocellular death.

2. Material and methods

2.1. Chemicals

Lipopolysaccharide (LPS, from *Escherichia coli* 0111:B4) was purchased from Sigma (St. Louis, MO). Cell culture ingredients were obtained from Life Technologies (Grand Island, NY). D-(+/−) Galactosomine (GalN), dextromethorphan (DM) were purchased from Sigma. DM was dissolved in ddH_2O and freshly prepared. 2′7-Dichlorofluorescin diacetate (DCFH-DA) was obtained from Calbiochem (La Jalla, CA). WST-1 was purchased from Dojindo Laboratories (Gaithersburg, MD). TNF-α enzyme-linked immunosorbent assay (ELISA) kits were from R&D Systems (Minneapolis, MN). Other chemicals are of reagent grade.

2.2. Animals

Animal studies were performed in accordance with National Institutes of Health Guidelines, approved by the Institute's Animal Care and Use Committee, and NIH guidelines were followed. Male CD-1 mice (6-week-old) were purchased from Charles River Laboratories (Wilminton, MA). The animals were fed on a standard diet and tap water ad libitum for 2 weeks prior to experiments. Environmental conditions were standardized, including a room temperature of 21 °C and 12 h artificial lighting-cycle. Mice were fasted 12 h before use. Male, (8-week-old) B6.129S6-*Cybb*^{tm1Din} (PHOX−/−) and C57BL/6J (PHOX+/+) mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained in a strict pathogen free environment. The PHOX−/− mice lack the functional catalytic subunit gp91 of NADPH oxidase complex and are unable to generate extracellular superoxide in response to LPS or other immunological stimulus. PHOX+/+ and PHOX−/− mice were used for Kupffer cell isolation and culture.

2.3. Treatments

Endotoxin shock was induced by administering a single intraperitoneal dose of LPS/GalN (20 μg/700 mg/kg) in saline [14,16,17]. To examine whether DM has protective effects on endotoxin shock, DM (6.25, 12.5 and 25 mg/kg) was injected into mice subcutaneously 30 min before and 2, 4 h after the injection of LPS/GalN. Control mice received the same volume of saline. At different time points, animals were euthanized and serum and livers were collected for analysis. In another time-course study, animals were treated with DM 12.5 mg/kg at 30 min before, or at 30, 60 and 120 min after the injection of LPS/GalN to evaluate therapeutic effects of DM. Survival rate was evaluated within 12 h after endotoxin administration.

2.4. Sample collection and preparation

Blood was collected from the retro-orbital veins of the eyes under anesthesia, and the liver was then perfused with saline through the portal vein. Perfused livers were collected and frozen at −70 °C. Blood was stored at 4 °C overnight and then centrifuged at 1500 × *g* at 4 °C for 15 min. Serum was collected and stored at −70 °C prior to analysis.

2.5. Evaluation of hepatotoxicity

Serum alanine aminotransferase (ALT) activity was assayed as a marker of hepatocellular death using a commercially available kit (Infinite ALT, Sigma). A portion of the liver was fixed in 10% neutral formalin, processed by standard histological techniques, stained with hematoxylin and eosin, and examined for morphological evidence of liver injury.

2.6. TNF-α assay

Frozen liver samples were homogenized in 10 mg/ml cold lysis buffer (20 mM Tris, 0.25 M sucrose, 2 mM EDTA, 10 mM EGTA, 1% Triton X-100 and protein

cocktail inhibitor 1 tablet/10 ml), and then centrifuged at 35,000 × *g* for 40 min. Supernatant was then collected for protein assay using the BCA protein assay reagent kit (PIERCE, Milwaukee, WI). The levels of TNF-α in the serum, liver and cultured Kupffer cell supernatant were determined with TNF-α ELISA kits, according to the manufacturer's instructions.

2.7. Kupffer cell culture

Kupffer cells were isolated from CD-1, PHOX+/+ and PHOX−/− mice by collagenase digestion and differential centrifugation using Percoll (Pharmacia, Uppsala, Sweden) as described previously [18] with slight modifications. Briefly, following pentobarbital anesthesia, the liver was perfused with Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (HBSS) at 37 °C for 5 min at a flow rate of 13 ml/min, followed by perfusion with HBSS containing 0.05% collagenase IV (Sigma, St. Louis, MO) at 37 °C for 5 min. The liver was excised and cut into small pieces in collagenase buffer. The suspension was filtered through Nylon gauze mesh and parenchymal cells were removed by centrifugation at 50 × *g* for 3 min. The nonparenchymal cell fraction was pelleted at 450 × *g* for 10 min at 4 °C. Cells were centrifuged on a density cushion of 50% of Percoll at 1000 × *g* for 15 min and the Kupffer cell fraction was collected and washed. Viability of cells determined by trypan blue exclusion was >90%. Cells were seeded in 24-well culture plates and cultured in RPMI 1640 media supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin G and 100 μg/ml streptomycin sulfate) at 37 °C with 5% CO_2. Non-adherent cells were removed after 2 h by replacing media and cells were cultured for 24 h before experiments.

2.8. Measurement of extracellular superoxide and intracellular reactive oxygen species (iROS) production

Superoxide production in Kupffer cells was measured by the superoxide dismutase (SOD)-inhibitable reduction of tetrazolium salt, WST-1 [19]. Kupffer cells were plated in 96-well tissue-plates at 10^5 cells/well. Cells were cultured at 37 °C for 2 h in the media with 10% FBS and 2 h in the media with 2% FBS in the presence of 5–10 μM of DM. Cells were then washed twice with HBSS and incubated at 37 °C with 50 μl of HBSS, in the presence of DM or vehicle control. Thirty minutes later, 50 μl SOD (50 U/ml), 50 μl of WST-1 (1mM) and 50 μl of vehicle or LPS (10 ng/ml) in HBSS were added to each well. Absorbance at 550 nm was read immediately after LPS treatment with a spectramax plus microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). The amount of superoxide was calculated by subtracting the absorbance of the treatment including SOD from the absorbance of the amount of superoxide produced, and results were expressed as percentage of vehicle-treated control cultures.

Intracellular ROS was determined by DCFH oxidation, as described previously [20]. For each measurement, a fresh stock solution of CM-H2-DCFDA (5 mM) was prepared in methanol. CM-H2-DCFDA, diluted to a final concentration of 1 μM in HBSS, was added to cultures and incubated for 30 min at 37 °C. Cells were pre-treated with or without DM for 30 min, and LPS in HBSS were then added to cultures. After incubation for 1.5 h at 37 °C, fluorescence intensity was measured at 485 nm excitation and 530 nm emissions using a spectramax gemini XS fluorescence microplate reader (Molecular Devices, Sunnyvale, CA).

2.9. Real-time RT-PCR analysis

Total RNA was extracted from liver tissues by Tri reagent (Sigma) and purified with RNeasy columns (Qiagen, Valencia, CA). Expression of the selected genes was quantified using real-time RT-PCR analysis as previously described [21]. Briefly, total RNA was reverse transcribed with MuLV reverse transcriptase and oligo-dT primers. The forward and reverse primers for the selected genes were designed using primer express software (Applied Biosystems, Foster City, CA) and listed in Table 1. The SYBR green master mix kit was used for real-time PCR analysis. The relative differences in expression between

Table 1
Primer sequences used for quantitative real-time RT-PCR analysis of liver tissue

Gene	Accession number	Forward primer	Reverse primer
TNF-α	XM_110221	GACCCTCACACTCAGATCATCTTCT	CCTCCACTTGGTGGTTTGCT
MIP-2	NM_009140	CCTCAACGGAAGAACCAAAGAG	CTCAGACAGCGAGGCACATC
TSP1	M87276	GCCGGATGACAAGTTCCAA	GCCTCAAGGAAGCCAAGAAGA
mKC	NM_008176	TGGCTGGGATTCACCTCAAG	GTGGCTATGACTTCGGTTTGG
ICAM-1	NM_010493	GTCTCGGAAGGGAGCCAAGTA	CGACGCCGCTCAGAAGAA
IL-6	J03783	GCCCACCAAGAACGATAGTCA	GAAGGCAACTGGATGGAAGTCT
c-*myc*	X01023	CGCCGCTGGGAAACTTT	TCCTGGCTCGCAGATTGTAA
GADD45	L28177	CAGATCCATTTCACCCTCATCC	TCCAGTAGCAGCAGCTCAGC
GADD153	X67083	CTCCTGTCTGTCTCTCCGGAA	TACCCTCAGTCCCCTCCTCA
Beta-actin	M12481	GTATGACTCCACTCACGGCAAA	GGTCTCGCTCCTGGAAGATG

Abbreviations: *MIP-2*, macrophage inflammatory protein-2; *TSP-1*, thrombospondin1; *mKC*, a mouse CXC chemokine; *ICAM-1*, intercellular cell adhesion molecule-1; *IL-6*, interleukin-6; *GADD45*, growth arrest and DNA damage inducible protein 45;*GADD153*, growth arrest and DNA damage inducible protein 153.

groups were determined using cycle time (Ct) values and the differences between groups were expressed as relative increases, setting the control as 100%. Assuming that the Ct value is reflective of the initial starting copy and that there is 100% efficacy, a difference of one cycle is equivalent to a two-fold difference in starting copy.

2.10. Statistics

The data were expressed as the mean ± S.E.M. Statistical significance was assessed with an analysis of variance followed by Bonferroni's *t*-test using the stat view program (Abacus Concepts, Berkeley, CA). A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. DM protects against LPS/GalN-induced mortality

The LPS/GalN-induced murine endotoxin shock model [11–17] was used to assess the protective effect of DM. DM alone did not induce hepotoxicity and lethality, but its pretreatment (30 min prior to LPS) produced dose-related protection against endotoxin shock. Survival rate within 12 h after LPS/GalN treatment are presented in Table 2. Approximately 46% of animals in LPS/GalN alone group survived within 12 h of LPS/GalN challenge. Pretreatment with DM (25 and 12.5 mg/kg, s.c.) significantly increased the survival rate up to 90% (11/12 and 50/56 mice survived). Even at the lower dose (6.25 mg/kg, s.c.), DM pretreatment increased the survival rate to 67%. This clearly shows that DM is effective in protecting LPS/GalN-induced lethal shock in mice.

3.2. DM protects against LPS/GalN-induced hepatotoxicity

Hepatocyte hemorrhagic necrosis has been the most frequently reported symptoms associated with LPS/GalN-induced endotoxemia [17,22]. Liver histology was examined to evaluate the effect of DM treatment on LPS/GalN-induced hepatotoxicity. In LPS/GalN-treated mice, the liver sections showed apparent broad hemorrhagic necrosis and severe hepatocyte swelling and degeneration 12 h after LPS/GalN challenge (Fig. 1B, arrows). These pathological alterations were dramatically ameliorated in the liver of animals receiving DM pretreatments (Fig. 1C). In the LPS/GalN plus DM-treated animals, hepatic congestion and hepatocellular necrosis were rare.

Serum ALT, an indicator of acute hepatocellular death, was examined to determine the protective effect of DM. As shown in Fig. 2, LPS/GalN (20 μg/700 mg/kg, i.p.) treat-

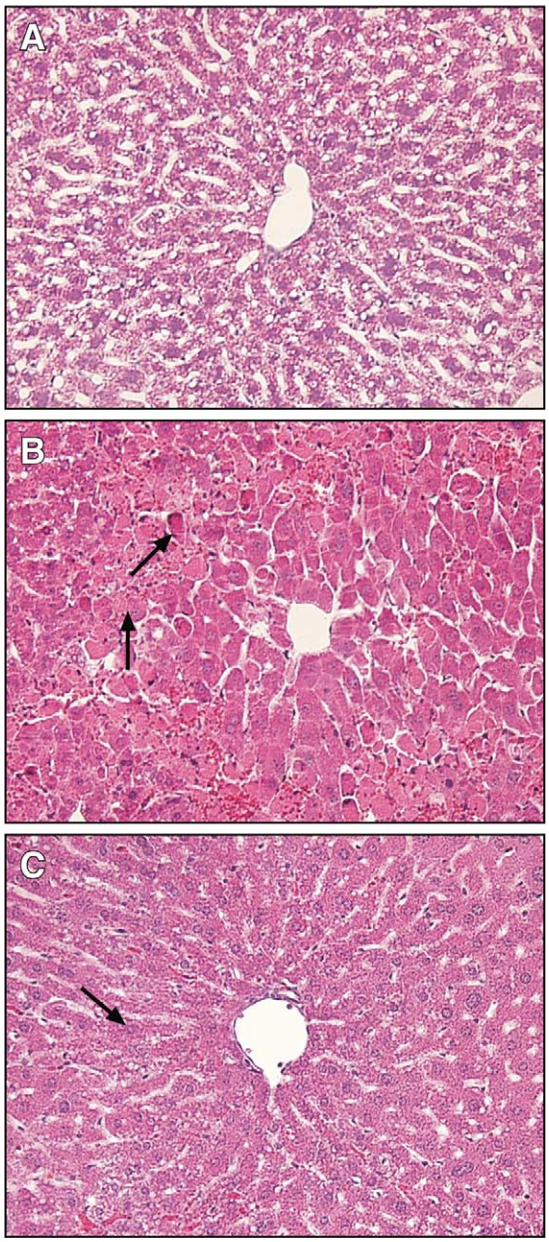

Fig. 1. Photomicrographs of the mouse liver. Sections were stained with hematoxylin and eosin and are shown at 100× magnification. Control (A), essentially normal morphology. LPS/GalN alone (B), foci of necrotic parenchymal cells, cell swelling and congestion. DM 12.5 mg/kg, 30 min pretreatment plus LPS/GalN (C), hepatic congestion and cell death are mild, while the cell swelling is the only notable lesion. The arrows indicate foci of necrotic cells.

Table 2
The effect of DM on sepsis mice survival

Dextromethorphan (mg/kg)	Animal number (*N*)	Survived animal	Rate of survival (%)
0	61	28	45.9
6.25	12	8	66.6*
12.5	56	50	89.3*
25	12	11	91.6*

Mice were given LPS/GalN (20 μg/700 mg/kg, i.p.) with or without the administration of DM (6.25–25 mg/kg, s.c.) 30 min before LPS/GalN. Animal survival rate was evaluated 12 h after LPS/GalN treatment. Data are from 12 to 61 mice. *$P < 0.05$ compared to control.

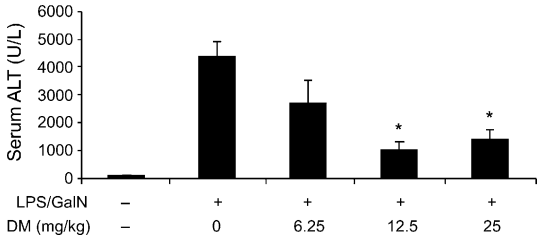

Fig. 2. Effect of DM treatment on LPS/GalN-induced liver injury in mice. Mice were given LPS/GalN (20 μg/700 mg/kg, i.p.) with or without the administration of DM (6.25–25 mg/kg, s.c.) 30 min before and 2 and 4 h after LPS/GalN. Liver injury was evaluated by serum alanine aminotransferase (ALT) activity. Data are mean ± S.E.M. ($N = 12$). $^{*}P < 0.05$ indicates significantly different from the LPS/GalN alone group.

ment produced 35-fold increase in serum ALT level compared to saline treated control animals, indicating sever liver injury. DM dramatically decreased serum ALT levels in a dose-dependent manner to ∼25-fold of LPS/GalN group at 12.5 and 25 mg/kg dosages. The time-dependent reduction of serum ALT levels is shown in Fig. 3. DM administration at different time points, including 30 min pre-treatment and 30, 60, 120 min post-treatments, decreased serum ALT levels. Compared with the LPS/GalN alone group, DM 30 min pre- and post-treatment have significant effects in reducing serum ALT levels.

3.3. Reduction of serum and liver TNF-α levels by DM

TNF-α, a cytokine implicated in sepsis, was studied in an effort to examine the mechanisms of the protective effects of DM in LPS/GalN-challenged mice. DM (12.5 mg/kg, s.c.) was administered to mice, followed by LPS/GalN challenge 30 min later. Serum and liver TNF-α level was assessed using ELISA at the indicated time points. As shown in Fig. 4, DM significantly decreased TNF-α levels in both serum (Fig. 4A) and liver (Fig. 4B) at 2 and 4 h after LPS/GalN challenge, but not at 1 h peak. The parallel reductions of both hepatic and serum TNF-α suggest that the site of action of DM for the reduction of serum TNF-α could be in the liver (see below).

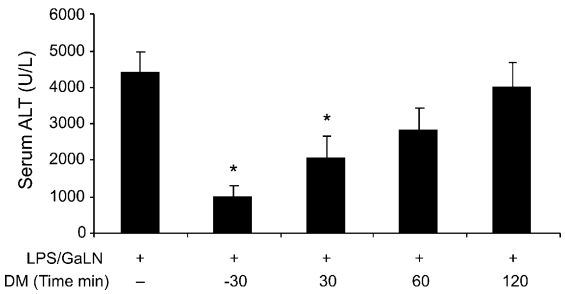

Fig. 3. Effect of DM treatment (time course) on LPS/GalN-induced liver injury in mice. Mice were given LPS/GalN (20 μg/700 mg/kg, i.p.) with or without the administration of DM (12.5 mg/kg, s.c.) 30 min before or 30, 60 and 120 min after LPS/GalN. Liver injury was evaluated by serum ALT activity. Data are mean ± S.E.M. ($N = 16$). $^{*}P < 0.05$ indicates significantly different from the LPS/GalN alone group.

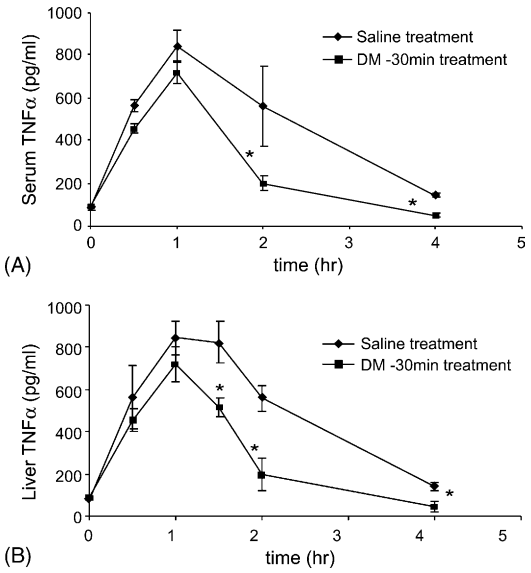

Fig. 4. Effects of DM on TNF-α production in the serum (A) and liver (B). Mice were given LPS/GalN (20 μg/700 mg/kg, i.p.) with or without the administration of DM (12.5 mg/kg, s.c.) 30 min before LPS/GalN. Serum and liver TNF-α protein levels were assessed by the TNF-α enzyme-linked immunosorbent assay (ELISA) kit. Data are mean ± S.E.M. ($N = 12$). $^{*}P < 0.05$ indicates significantly different from the LPS/GalN alone group.

This conclusion is consistent with the report indicating that liver is the major source for the increase in serum TNF-α in endotoxic shock animals [23].

3.4. DM suppressed superoxide and TNF-α production in primary Kupffer cell cultures by DM

The activation of Kupffer cells by LPS is a critical event in the endotoxemia or sepsis [24]. Endotoxin activates Kupffer cells to release inflammatory mediators such as free radicals and TNF-α. Therefore, Kupffer cells were isolated to study the possible mechanism of DM protection against liver injury in sepsis. We found that both extracellular superoxide and intracellular reactive oxygen species production were markedly increased in LPS-stimulated mouse Kupffer cells. The increase was dramatically diminished by DM at 5 and 10 μM concentrations, respectively (Fig. 5A and B). NADPH-oxidase is a critical enzyme induced in the phagocytic ROS signaling pathway. We found that the LPS-induced TNF-α production in PHOX−/− mice was only half of that in PHOX+/+ mice, and DM (5 and 10 μM) significantly decreased LPS-induced increase in TNF-α production only in PHOX+/+ Kupffer cell (Fig. 5C), suggesting that DM inhibits TNF-α through the PHOX-ROS pathway.

Neutrophil-derived peroxides can diffuse into hepatocytes and contribute to parenchymal cell death in vivo [25]. Since LPS could not significantly induce superoxide in isolated rat neutrophil, we used phorbol 12-myristate 13-acetate (PMA) as a stimulator to test effects of DM on superoxide production in neutrophils. We found that PMA alone increased superoxide production by 2.7-fold, and

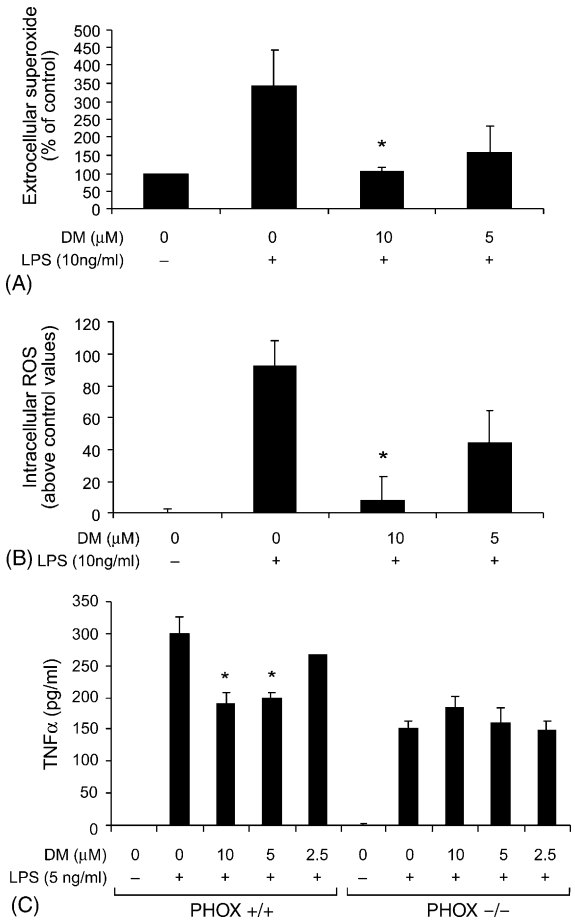

Fig. 5. Effects of DM on LPS-induced Kupffer cell activation: superoxide (A), iROS (B) and TNF-α (C). Kupffer cells were pre-treated with DM (2.5, 5 or 10 μM) or vehicle 30 min, and then stimulated with LPS 5–10 ng/ml or vehicle. Superoxide production was measured by a spectromax plus microplate spectorphotometer, and iROS was determined with a spectromax gemini XS fluorescence microplate reader as described in methods. TNF-α production (4 h after LPS stimulation) in PHOX+/+ and PHOX−/− was measured by ELISA kit. Data are mean ± S.E.M. of three–four individual experiments with three–six-well in same treatment. $^{*}P < 0.05$ indicates significantly different from LPS alone treated culture.

DM 10 μM significantly suppressed PMA-induction of superoxide to 1.7-fold (data not shown).

3.5. Real-time RT-PCR analysis of expression of genes of interest

To further examine the effects of DM's protection against LPS/GalN-induced liver injury, pertinent gene expression changes were examined with real-time RT-PCR. As shown in Table 3, 12 h after LPS/GalN, there were dramatic increases in the expression of genes encoding mouse macrophage inflammatory protein (*MIP*-2, 153-fold), thrombospondin-1 (*TSP1*, 94-fold), mouse chemokine (*mKC*, 14-fold), intracellular adhension molecule-1 (*ICAM-1*, 8-fold) and interleukin-6 (*IL-6*, 153-fold), strongly indicating that LPS/GalN induced dramatic inflammation in mice. DM significantly diminished the

Table 3
Real time RT-PCR analysis of liver tissue in control, LPS/GalN- and LPS/Gal + DM-treated mice

AA	LPS/GalN + vehicle LPS/CalN + DM (fold of control)		% Reduction by DM
Inflammatory markers			
TNF-α	13.0 ± 2.5	12.7 ± 2.3	2.3
MIP-2	153.3 ± 6.4	39.0 ± 2.1*	74.5
Thrombospondin-1	94.2 ± 2.5	56.0 ± 1.57*	40.6
mKC	13.7 ± 2.9	5.8 ± 1.6*	57.7
ICAM-1	8.45 ± 1.3	5.05 ± 2.7*	40.2
IL-6	153.4 ± 13.3	43.6 ± 6.3*	71.6
Cell-death markers			
c-*myc*	45.4 ± 3.6	18.4 ± 2.1*	59.5
GADD45	24.6 ± 12.4	5.3 ± 2.4*	78.5
GADD153	7.4 ± 0.3	2.7 ± 1.3*	63.5

Mice were given LPS/GalN (20 μg/700 mg/kg, i.p.), or LPS/GalN + DM (12.5 mg/kg, s.c., ×2). Liver samples were taken at 12 h after LPS/GalN administration, and total RNA was isolated for real-time RT-PCR analysis. In each individual sample, the expression level of each gene was first normalized with that of β-actin and then the relative differences between groups were expressed as relative increases setting controls as 1.0. Data represent means ± S.E. of $n = 4$–5 animals per group (compared to LPS/GalN alone). Gene abbreviations are listed in Table 1. $^{*}P < 0.05$.

LPS/GalN-induced increase in the expression of the *MIP-2, TSP1, mKC*, *ICAM-1* and *IL-6* genes, supporting the hypothesis that inhibition of LPS-induced inflammation is a mechanism of DM-mediated protection, and this finding is consistent with our in vitro findings [6]. However, DM did not reduce *TNF-α* mRNA expression.

LPS/GalN hepatotoxicity is shown to be associated with the increase in cell death-related genes [17,26]. LPS/GalN treatment greatly enhanced the expression of c-*myc* (45-fold), while this gene expression was diminished 60% with DM treatment. In addition, LPS/GalN increased the DNA damage proteins, such as *GADD45* and *GADD153*, by 25- and 7-fold, respectively, and *GADD45, GADD153* were significantly suppressed by DM to 5- and 3-fold respectively, corresponding to the liver toxicity measures showing reduced hepatic cell death by DM.

4. Discussion

This study demonstrated that DM is effective in protecting mice from LPS/GalN-induced lethal shock and acute hepatoxicity, as evidenced by significant decrease in mortality and the reduction of serum ALT levels (an index of acute hepatocellular death) in a dose- and time-dependent manner. LPS/GalN-induced mortality is predominantly associated with severe liver damage and congestion [11–17]. Thus, the reduction of mortality by DM in this study is probably due to DM's protection against LPS/GalN-induced liver injury. Indeed, liver histopathology clearly showed that LPS/GalN-induced hemorrhaged necrosis and hepatocyte degeneration are dramatically improved in DM-treated mice. This work is an important extension

of our in vitro findings showing that DM protected against inflammation-mediated neurodegeneration in neuron-glia cultures [6], and further supported important anti-inflammatory effects of DM. DM was effective even when given 30 min post LPS/GalN, suggesting a potential new therapeutic treatment for endotoxemia and acute liver damage with a novel mechanism of action.

Previous reports indicated that TNF-α was an important factor mediating LPS/GalN-induced liver damage [11,14,17,27]. TNF-α is a potent inflammatory cytokine produced by the monocyte-macrophage lineage, including Kupffer cells [24]. There is increasing evidence indicating that TNF-α may play a role in the initiation or progression of multiple organ failure in septic shock [24]. Inhibition of TNF-α synthesis or activity attenuates liver injury caused by LPS, indicating that TNF-α is a critical factor in the sepsis-related liver toxicity [11,14,17,26,27]. The current study showed that DM did not reduced *TNF-α* mRNA expression in liver, and had no effects on TNF-α protein production at 1 h peak. However, DM decreased TNF-α level at 2 and 4 h in both serum and liver, apparently by inducing a TNF-α faster clearance, which would partially contribute to liver protection and reduction of mortality. It has been reported that in this LPS/GalN mouse model, TNF-α is the central proximal mediator, which controls all subsequent events [28–30]. DM's protection may be related to the later events, since DM 30 min post-treatment was still effective.

Another important factor examined in this study is the reactive oxygen species (ROS). ROS are important cytotoxic and signaling mediators in the pathophysiology of inflammatory liver diseases [31] and play an important role in the onset of hepatic damage during endotoxemia or sepsis [32]. Neutrophils contribute to hepatocellular ROS production in the acute inflammatory reactions [25]. In this study, we determined the effect of DM on the production of extracellular superoxide and intracellular concentration of ROS in cultured Kupffer cells prepared from CD1 mouse liver and isolated neutrophils. Indeed, the results clearly showed that LPS could induce production of superoxide and iROS from Kupffer cells, and DM significantly reduced this production. DM also significantly decreased superoxide production in neutrophils. Our in vitro findings are consistent with reported literature indicating that sepsis is accompanied by hepatic oxidative stress, which occurs both intracellularly and extracellularly [31–33]. The overproduction of intracellular ROS could be the result of NADPH oxidase activation [33–35], suggesting that DM may decrease ROS at least in part through inhibition of NADPH oxidase.

We have recently demonstrated that DM is effective in reducing LPS-induced neurotoxicity through the inhibition of microglia NADPH oxidase [36] and the reduction of release of inflammatory factors [6]. In the central nervous system, LPS-induced neurotoxicity is thought partly due to superoxide free radicals, released extracellularly via the activation of NADPH oxidase from microglia [37]. To obtain further evidence that PHOX is a target for the reduction of ROS by DM, which in turn is linked with the reduction in the release of TNF-α, we isolated Kupffer cells from NADPH oxidase deficient (PHOX−/−) mice to examine the effects of DM on LPS-induced TNF-α production. The results clearly showed that DM inhibited TNF-α production in Kupffer cells in PHOX+/+, but not in PHOX−/− mice, similar to the observation in neuron-glia cultures [36]. Taken together, these results strongly support that the liver protective effects of DM seen in the in vivo study can be explained in part by the reduction of the production of ROS and the increase of TNF-α clearance.

To get more insight into the mechanism of DM protection, the expressions of genes related to inflammatory markers (*MIP-2*, *TSP-1*, *mKC*, *ICAM-1* and *IL-6*) in the liver were further studied. These markers reflect that LPS-induced liver injury is related to the activation of Kupffer cells and subsequent neutrophil infiltration [26]. In this study, LPS/GalN dramatically increased the expressions of these genes. As the increase in these inflammation markers has been reported to be associated with neutrophil-mediated inflammation and liver injury [26,38]. Here, we showed that DM significantly reduced these gene expressions, suggesting that DM may influence neutrophil recruitment to the liver, indicating another important protective mechanism of DM.

The expression level of *c-myc* gene, and cell-death markers *GADD45* and *GADD153* (an indicator of DNA damage) were dramatically increased by LPS/GalN [17], and were attenuated by DM. This observation is consistent with morphological study showing impressive protection of hepatic cells from LPS/GalN-induced toxicity. LPS induced hepatocellular death, including necrosis in the liver, was dramatically attenuated by DM, which is consistent with decreased serum enzyme activities with DM treatment, and suggests that the protection of DM could involve hepatocellular necrosis.

In summary, in the present study, we clearly demonstrated that DM, analogue of morphinan, has a potent effect against LPS/GalN-induced mortality and liver damage in mice. The protective effects of DM could be due to the inhibition of LPS-induced liver oxidative stress, reduction of TNF-α, prevention of neutophils infiltration, and the suppression of cell death genes. Given the impact of DM on multiple factors critical to the acute LPS inflammatory response, DM could be a novel compound for the development of an effective therapy in treatment of endotoxin shock, and endotoxin-induced liver injury and neurodegeneration.

Acknowledgments

The authors thank Drs. Larry Keefer and Jingbo Pi for critical review during preparation of this manuscript.

References

[1] Liu B, Du L, Hong JS. Naloxone protects rat dopaminergic neurons against inflammatory damage through inhibition of microglia activation and superoxide generation. J Pharmacol Exp Ther 2000;293(2): 607–17.

[2] Liu Y, Qin L, Wilson BC, An L, Hong JS, Liu B. Inhibition by naloxone stereoisomers of beta-amyloid peptide (1-42)-induced superoxide production in microglia and degeneration of cortical and mesencephalic neurons. J Pharmacol Exp Ther 2002;302(3):1212–9.

[3] Chang RC, Rota C, Glover RE, Mason RP, Hong JS. A novel effect of an opioid receptor antagonist, naloxone, on the production of reactive oxygen species by microglia: a study by electron paramagnetic resonance spectroscopy.. Brain Res 2000;854(1–2):224–9.

[4] Liu B, Du L, Kong LY, Hudson PM, Wilson BC, Chang RC, et al. Reduction by naloxone of lipopolysaccharide-induced neurotoxicity in mouse cortical neuron-glia co-cultures. Neuroscience 2000;97(4): 749–56.

[5] Liu B, Jiang JW, Wilson BC, Du L, Yang SN, Wang JY, et al. Systemic infusion of naloxone reduces degeneration of rat substantia nigral dopaminergic neurons induced by intranigral injection of lipopolysaccharide. J Pharmacol Exp Ther 2000;295(1):125–32.

[6] Liu Y, Qin L, Li G, Zhang W, An L, Liu B, et al. Dextromethorphan protects dopaminergic neurons against inflammation-mediated degeneration through inhibition of microglial activation. J Pharmacol Exp Ther 2003;305(1):212–8.

[7] Nolan JP. The role of endotoxin in liver injury. Gastroenterology 1975;69(6):1346–56.

[8] Nakao A, Taki S, Yasui M, Kimura Y, Nonami T, Harada A, et al. The fate of intravenously injected endotoxin in normal rats and in rats with liver failure. Hepatology 1994;19(5):1251–6.

[9] Nolan JP. Intestinal endotoxins as mediators of hepatic injury–an idea whose time has come again. Hepatology 1989;10(5):887–91.

[10] Decker K, Keppler D. Galactosamine hepatitis: key role of the nucleotide deficiency period in the pathogenesis of cell injury and cell death. Rev Physiol Biochem Pharmacol 1974;(71):77–106.

[11] Hishinuma I, Nagakawa J, Hirota K, Miyamoto K, Tsukidate K, Yamanaka T, et al. Involvement of tumor necrosis factor-alpha in development of hepatic injury in galactosamine-sensitized mice. Hepatology 1990;12(5):1187–91.

[12] Galanos C, Freudenberg MA, Reutter W. Galactosamine-induced sensitization to the lethal effects of endotoxin. Proc Natl Acad Sci USA 1979;76(11):5939–43.

[13] Chojkier M, Fierer J. D-galactosamine hepatotoxicity is associated with endotoxin sensitivity and mediated by lymphoreticular cells in mice. Gastroenterology 1985;88(1 Pt 1):115–21.

[14] Lehmann V, Freudenberg MA, Galanos C. Lethal toxicity of lipopolysaccharide and tumor necrosis factor in normal and D-galactosamine-treated mice. J Exp Med 1987;165(3):657–63.

[15] Ben-Shaul V, Sofer Y, Bergman M, Zurovsky Y, Grossman S. Lipopolysaccharide-induced oxidative stress in the liver: comparison between rat and rabbit. Shock 1999;12(4):288–93.

[16] Silverstein R, Turley BR, Christoffersen CA, Johnson DC, Morrison DC. Hydrazine sulfate protects D-galactosamine-sensitized mice against endotoxin and tumor necrosis factor/cachectin lethality: evidence of a role for the pituitary. J Exp Med 1991;173(2):357–65.

[17] Liu J, Saavedra JE, Lu T, Song JG, Clark J, Waalkes MP, et al. O(2)-Vinyl 1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate protection against D-galactosamine/endotoxin-induced hepatotoxicity in mice: genomic analysis using microarrays. J Pharmacol Exp Ther 2002;300(1):18–25.

[18] Valatas V, Xidakis C, Roumpaki H, Kolios G, Kouroumalis EA. Isolation of rat Kupffer cells: a combined methodology for highly purified primary cultures. Cell Biol Int 2003;27(1):67–73.

[19] Tan AS, Berridge MV. Superoxide produced by activated neutrophils efficiently reduces the tetrazolium salt, WST-1 to produce a soluble formazan: a simple colorimetric assay for measuring respiratory burst activation and for screening anti-inflammatory agents. J Immunol Methods 2000;238(1–2):59–68.

[20] Wang H, Joseph JA. Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. Free Radic Biol Med 1999;27(5–6):612–6.

[21] Walker NJ. Real-time and quantitative PCR: applications to mechanism-based toxicology. J Biochem Mol Toxicol 2001;15(3):121–7.

[22] Leist M, Gantner F, Bohlinger I, Tiegs G, Germann PG, Wendel A. Tumor necrosis factor-induced hepatocyte apoptosis precedes liver failure in experimental murine shock models. Am J Pathol 1995; 146(5):1220–34.

[23] Kumins NH, Hunt J, Gamelli RL, Filkins JP. Partial hepatectomy reduces the endotoxin-induced peak circulating level of tumor necrosis factor in rats. Shock 1996;5(5):385–8.

[24] Enomoto N, Takei Y, Hirose M, Kitamura T, Ikejima K, Sato N. Protective effect of thalidomide on endotoxin-induced liver injury. Alcohol Clin Exp Res 2003;27(Suppl. 8):2S–6S.

[25] Jaeschke H, Ho YS, Fisher MA, Lawson JA, Farhood A. Glutathione peroxidase-deficient mice are more susceptible to neutrophil-mediated hepatic parenchymal cell injury during endotoxemia: importance of an intracellular oxidant stress. Hepatology 1999;29(2):443–50.

[26] Jaeschke H, Gores GJ, Cederbaum AI, Hinson JA, Pessayre D, Lemasters JJ. Mechanisms of hepatotoxicity. Toxicol Sci 2002;65 (2):166–76.

[27] Hewett JA, Roth RA. Hepatic and extrahepatic pathobiology of bacterial lipopolysaccharides. Pharmacol Rev 1993;45(4):382–411.

[28] Leist M, Gantner F, Jilg S, Wendel A. Activation of the 55 kDa TNF receptor is necessary and sufficient for TNF-induced liver failure, hepatocyte apoptosis, and nitrite release. J Immunol 1995;154(3): 1307–16.

[29] Schlayer HJ, Laaff H, Peters T, Woort-Menker M, Estler HC, Karck U, et al. Involvement of tumor necrosis factor in endotoxin-triggered neutrophil adherence to sinusoidal endothelial cells of mouse liver and its modulation in acute phase. J Hepatol 1988;7(2):239–49.

[30] Essani NA, Fisher MA, Farhood A, Manning AM, Smith CW, Jaeschke H. Cytokine-induced upregulation of hepatic intercellular adhesion molecule-1 messenger RNA expression and its role in the pathophysiology of murine endotoxin shock and acute liver failure. Hepatology 1995;21(6):1632–9.

[31] Jaeschke H. Reactive oxygen and mechanisms of inflammatory liver injury. J Gastroenterol Hepatol 2000;15(7):718–24.

[32] Spolarics Z. Endotoxemia, pentose cycle, and the oxidant/antioxidant balance in the hepatic sinusoid. J Leukoc Biol 1998;63(5):534–41.

[33] Jaeschke H. Mechanisms of oxidant stress-induced acute tissue injury. Proc Soc Exp Biol Med 1995;209(2):104–11.

[34] Sies H. Oxidative stress: oxidants and antioxidants. Exp Physiol 1997;82(2):291–5.

[35] Kaplowitz N, Tsukamoto H. Oxidative stress and liver disease. Prog Liver Dis 1996;14:131–59.

[36] Zhang W, Wang T, Qin L, Gao HM, Wilson B, Ali SF, et al. Neuroprotective effect of dextromethorphan in the MPTP Parkinson's disease model: role of NADPH oxidase. FASEB J 2004;18(3):589–91.

[37] Qin L, Liu Y, Wang T, Wei SJ, Block ML, Wilson B, et al. NADPH oxidase mediates lipopolysaccharide-induced neurotoxicity and proinflammatory gene expression in activated microglia. J Biol Chem 2004;279(2):1415–21.

[38] Zhang XW, Wang Y, Liu Q, Thorlacius H. Redundant function of macrophage inflammatory protein-2 and KC in tumor necrosis factor-alpha-induced extravasation of neutrophils in vivo. Eur J Pharmacol 2001;427(3):277–83.