Naltrexone Protects Against Lipopolysaccharide/D-Galactosamine–Induced Hepatitis in Mice

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Received April 9, 2008; Accepted August 5, 2008

Abstract. Naltrexone, an opioid receptor antagonist, has been claimed to have anti-inflammatory and immunomodulatory effects both in vitro and in vivo. Thus, the aim of this study was to evaluate the effects of naltrexone on acute hepatitis induced by intraperitoneal (i.p.) administration of lipopolysaccharide (LPS, 20 μg/kg)/D-galactosamine (D-gal, 700 mg/kg) in conscious ICR mice. Results demonstrated that post-treatment with naltrexone (20 mg/kg, i.p.) significantly attenuated the deleterious liver function in mice treated with LPS/D-gal. It was also found that naltrexone significantly inhibited the elevation of plasma tumor necrosis factor-α (TNF-α) caused by LPS/D-gal. The overproduction of nitric oxide (NO) and superoxide anions induced by LPS/D-gal were also significantly reduced by naltrexone. Moreover, infiltration of neutrophils into the liver of mice 12 h after treatment with LPS/D-gal was also decreased by naltrexone. In conclusion, the beneficial effects of naltrexone on LPS/D-gal–induced hepatitis result from its inhibition of pro-inflammatory factors and antioxidative effects. Thus, naltrexone is of therapeutic potential for treating liver injury.

Keywords: lipopolysaccharide, D-galactosamine, naltrexone, tumor necrosis factor-α, nitric oxide

Introduction

Reactive oxygen species (ROS) are important cytotoxic and signaling mediators in the pathophysiology of inflammatory liver diseases (1). Most acute and chronic liver diseases have oxidative damage as a common feature of pathology. Phagocytic cells, including Kupffer cells, are present in large quantities in diseased liver tissue, release ROS in response to various diseases, infections, or other factors, and cause much of the inflammation and necrosis to the liver tissue. Neutrophils contribute to hepatocellular injury in a number of acute inflammatory reactions such as endotoxemia, sepsis, and alcoholic hepatitis (2 – 6). Liver cell necrosis depends on the transendothelial migration of neutrophils and the adherence to parenchymal cells (7 – 11). It was reported that in C3Heb/FeJ mice, lipopolysaccharide (LPS)/D-galactosamine (D-gal) induced a significant increase of hepatic and plasma levels of glutathione disulfide (GSSG), an indicator of oxidant stress, selectively during the neutrophil-mediated injury phase (12, 13). In glutathione peroxidase–deficient mice (Gpx1/2), LPS/D-gal or tumor necrosis factor-α (TNF-α)/D-gal caused more severe neutrophil-mediated liver injury compared with wild-type animals. These results suggest that neutrophils-derived ROS are responsible for an intracellular oxidative stress in hepatocytes after LPS/D-gal treatment. Because of the higher susceptibility of Gpx1/2 mice to a neutrophil-mediated injury, the authors concluded that peroxides generated by neutrophils diffused into hepatocytes and contributed to parenchymal cell death in vivo (12, 13).
Thus, strengthening defense mechanisms against ROS in target cells can attenuate excessive inflammatory injury without affecting host defense reactions and preventing the production and release of ROS by phagocytic and Kupffer cells may slow down the disease progression and help to reverse liver damage.

Pro-inflammatory cytokines, for example, TNF-α, can induce the formation of ROS in hepatocytes (14). In addition, various studies have shown that TNF-α, a major mediator of septic shock, induces tissue injury, loss of blood pressure, organ failure, and ultimately death (15). Increased level of hepatotoxic cytokines such as TNF-α are well documented in alcoholic liver disease and non-alcoholic steato-hepatitis and have been shown to play a mechanistic role in both of these disease processes (16). Severity of liver damage has been correlated with concentration of TNF-α in alcoholic patients (17).

Naltrexone is a relatively pure opioid receptor antagonist and a long-acting opioid antagonist without opioid agonist activity. It has been claimed to have anti-inflammatory and immunomodulatory effects both in vitro and in vivo (15, 18, 19). Our previous study indicates that pre-treatment with naltrexone has a protective effect against LPS-induced septic shock in rats (18). Naltrexone significantly reduced 1) the elevation of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), which are biochemical indicators of liver function; 2) the plasma level of TNF-α; and 3) the overproduction of superoxide anions in aortic rings, induced by LPS. In conclusion, pre-treatment with naltrexone significantly reduced circulatory failure and hepatic dysfunction in sepsis. These effects were associated with reduction of elevated TNF-α levels and superoxide anion formation.

Endotoxemia induced by LPS/d-gal is a known model of acute liver failure that involves a neutrophil-mediated parenchymal cell injury (1, 7, 11, 13). Therefore, LPS/d-gal–induced acute hepatitis in mice (ICR) was selected to further evaluate the post-treatment effect of naltrexone on hepatic injury.

Materials and Methods

In vivo experiments
Male ICR mice with body weight 30 – 35 g, whose stock originated from the Institute of Cancer Research of National Institutes of Health in the USA, were purchased from the National Animal Center (Taipei, Taiwan). This study was approved by the local Institutional Review Board according to the recommendations from Helsinki and the internationally accepted principles in the care and the use of experimental animals. In this study, ICR mice were randomly assigned to the following treatment groups: A) control group (n = 6): mice were treated with saline intraperitoneally (i.p.) at time = 0 and 30 min later; B) naltrexone group (n = 6): mice were treated with naltrexone hydrochloride (20 mg/kg, i.p.; Sigma Chemical Co., St. Louis, MO, USA) at time 0 and then with saline 30 min later; C) LPS/d-gal group (n = 10): mice were treated with d-gal (700 mg/kg, Sigma) plus Escherichia coli 3129 LPS (20 μg/kg, i.p.; Sigma) at time 0 and then with saline 30 min later; D) LPS/d-gal + naltrexone group (n = 11): mice were treated with naltrexone hydrochloride (20 mg/kg, i.p.) 30 min after LPS/d-gal administration. All animals were observed for 12 h and survived. Twelve hours after the LPS/d-gal treatment, the animals were sacrificed and organs were harvested. Histopathological examination of liver sections was done by light microscopy.

Prior to (i.e., at 0 h) treatment with vehicle (control group) or LPS/d-gal, 0.1 ml of blood was drawn from the cornea of the mice to measure the changes in TNF-α and nitrate (an indicator of NO). Before choosing a suitable dose of naltrexone to perform the study, a preliminary dose–response study with doses of 10, 20, 40, and 80 mg/kg was evaluated, and naltrexone at 20 mg/kg appeared to have a significant hepatic protective effect against injury induced by LPS/d-gal (data not shown). This effect was similar to those at 40 and 80 mg/kg. Hence, the dose of 20 mg/kg was chosen for the study. All biochemical parameters were recorded at time 0 and at 1.5 or 12 h in all animal groups; however, not all the animals were used for each particular measurement because of technical reasons.

Measurement of TNF-α in plasma levels
Blood samples (0.1 ml) for the measurement of TNF-α level in the plasma were obtained at 0, 1.5, and 12 h after the injection of saline or LPS/d-gal. These samples were collected from the left cornea of the mice at 0 and 1.5 h and from the heart of the mice at 12 h and centrifuged at 7200 \( \times \) g for 10 min to obtain the plasma to measure the levels of TNF-α and nitrate (as described below). The plasma samples (50 μl) were obtained, and TNF-α levels were measured by using commercial enzyme-linked immunosorbent assay (ELISA) kits, purchased from BioSource International Inc. (Camarillo, CA, USA).

Determination of plasma nitrate
Blood samples for the measurement of plasma nitrate level were obtained at 12 h after the injection of saline or LPS/d-gal. Each plasma sample (30 μl), which was kept in a −20°C freezer until use, was thawed and deproteinized by incubating it with 60 μl of 95% ethanol...
(4°C) for 30 min. The samples were subsequently centrifuged for an additional 5 min at 14,000 × g. It is noted that the nitrate concentration in plasma reported in the study is actually the total nitrite and nitrate concentration in plasma. In this method, nitrate is reduced to NO via nitrite. The amounts of nitrate in the plasma (2 μl) were measured by adding a reducing agent (0.8% VCl3 in 1 N HCl) to the purge vessel to convert nitrate to NO, which was stripped from the plasma by using a helium purge gas. The NO was then drawn into the Sievers Nitric Oxide analyzer (Sievers, Inc., Boulder, CO, USA). Nitrate concentrations were calculated by spectrophotometric kinetic assay (Sigma).

Determination of superoxide anions production in the liver

At 12 h after the initial treatment with saline, naltrexone, or LPS/D-gal, liver was harvested from all the mice, as described above. Hepatic superoxide anions content was determined by lucigenin-enhanced luminescence as described previously with some modifications (20, 21). They were incubated in warmed (37°C), oxygenated (95% O2, 5% CO2)-equilibrated modified Krebs-HEPES solution containing 20 mM HEPES (pH 7.4) for 30 min. The samples were then placed into a 96-well microplate containing 200 μl of the modified Krebs-HEPES solution with 50 μl of 1.25 mM lucigenin (in a final volume of 250 μl). The chemiluminescence of the sample was measured in a luminometer (MicroLumatplus LB 96V; Berthold TECHNOLOGIES GmbH & Co., KG, Bad Wildbad, Germany) as relative light units (RLU) emitted, integrated over a 15-min period at room temperature. The well containing the buffer and lucigenin was read first to determine background luminescence, then liver tissue was added, and luminescence was determined again. All liver tissues were dried in a 95°C oven for 24 h. Superoxide levels were reported as relative units of luminescence (RLU) after background luminescence subtraction per 15 min and were normalized to milligram dry tissue weight (i.e., RUL/15 min per mg). All manipulations were performed in the darkroom with minimal light.

Determination of plasma biochemical parameters

Plasma ALT and AST activity in mice were measured before and 12 h after LPS/D-gal treatment by means of a spectrophotometric kinetic assay (Sigma).

Histological analysis

Livers were obtained from each group 12 h after the injection of saline, naltrexone, or LPS/D-gal and these tissues were fixed in buffered formaldehyde (10% in phosphate-buffered saline) for histopathological examination as described previously (18, 21). The fixed liver tissue were dehydrated in graded ethanol and embedded in paraffin. Three-micron sections were stained with the hematoxylin and eosin reagent for light microscopy and examined in a blinded fashion. Histopathologic changes of necroinflammatory activity were scored by five grades of 0 to 4. Grade 0, no inflammation or necrosis; Grade 1, portal inflammation (1% – 25%) and/or lobular inflammation without necrosis; Grade 2, mild periportal inflammation (26% – 50%) and/or lobular focal hepatocellular necrosis; Grade 3, moderate periportal inflammation (51% – 75%) and/or lobular more extensive necrosis; Grade 4, severe periportal inflammation (>75%) and/or necrosis includes bridging necrosis.

Statistical analysis

Statistical evaluation was performed by statistical software (SPSS, Statistical Package for Social Science; SPSS Inc., Chicago, IL, USA). Data were analyzed using one-way analysis of variance (ANOVA) followed by Students t test. A P-value of less than 0.05 was considered to be statistically significant. Data are mean ± S.E.M. of n observations, where n represents the number of animals or the number of samples studied.

Results

Effect of naltrexone on plasma TNF-α level

The basal plasma level of TNF-α was not significantly different between any of the experimental groups studied. Administration of LPS/D-gal caused a significant increase in the plasma levels of TNF-α to 9774 ± 1239 pg/ml at 1.5 h (Fig. 1). The increase in TNF-α induced by LPS/D-gal is transient and usually peaks at 1 – 2 h and lasts for about 2 – 4 h. In the sham-treated group, there was no increase in plasma TNF-α level during the experimental period. However, the post-treatment of mice with naltrexone greatly reduced the elevation of TNF-α level in plasma (e.g., 4450 ± 754 pg/ml at 1.5 h, Fig. 1) induced by LPS/D-gal. In addition, naltrexone alone did not have any significant effects on the TNF-α level in plasma (Fig. 1).

Effect of naltrexone on plasma biochemical parameters

Plasma levels of ALT and AST are two important biochemical markers which are clinically used to monitor liver function of patients. Dramatic increase in plasma levels of ALT and AST at 12-h post-treatment of LPS/D-gal (Fig. 2: A and B) indicates that there was severe liver injury, characteristic of the animal model. The elevation was dramatically modulated by naltrexone to close to the basal level.
Effect of naltrexone on hepatic superoxide anions formation

Figure 3A showed the content of hepatic superoxide anions at 12 h after the LPS challenge. Hepatic superoxide anions production was significantly increased in LPS/D-gal–treated groups. However, post-treatment of naltrexone reduced the hepatic superoxide anions formation induced by LPS/D-gal.

Effect of naltrexone on plasma nitrate level

The basal plasma level of nitrate was not significantly different between any of the experimental groups studied. The administration of LPS/D-gal induced the elevation of plasma nitrate/nitrite level at 12 h (90.81 ± 12.47 μM, Fig. 3B). This increase of plasma nitrate/nitrite level was attenuated by the post-treatment of naltrexone (57.93 ± 6.84 μM, Fig. 3B).

Effect of naltrexone on LPS/D-gal–induced hepatotoxicity

The mice in the control group (Fig. 4A) showed a normal liver lobular architecture and hepatocytes. Histological examination of livers from LPS/D-gal–treated mice (Fig. 4C) revealed marked damage, including disruption of normal architecture, confluent necrosis, abundant infiltrated polymorphonuclear leukocytes, and hemorrhage. These pathological changes were ameliorated by the naltrexone treatment (Fig. 4D). In addition, naltrexone itself did not have any significant effects on the liver of mice (Fig. 4B).

Discussion

The present study demonstrated that naltrexone significantly prevented the deterioration of liver function and liver damage induced by LPS/D-gal. Furthermore, it also significantly suppressed the increases of plasma TNF-α and NO levels and reduced hepatic superoxide anion formation and neutrophil infiltration in the liver of mice induced by LPS/D-gal. Thus, naltrexone may be of potential to be used as a novel therapeutic agent for treating acute hepatitis.

Naltrexone was approved by the Food and Drug Administration (FDA) for the treatment of opiate dependence in 1984 and of alcoholism in 1994. Earlier clinical studies noted liver function test abnormalities...
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(such as elevated ALT and AST) in patients treated with naltrexone at a daily dosage of about 100 to 300 mg (22–24). Evidence of the hepatotoxic potential of naltrexone prompted FDA to issue a black box warning that naltrexone has capacity of causing hepatocellular injury when given at a dose six times of the recommended dose (50 mg/day), and the risk of hepatic injury has raised a great concern about the safety issue of treating patients with naltrexone clinically (25). However, there are published reports showing that naltrexone does not appear to be hepatotoxic at the daily dose of 50 to 200 mg, even when administered over a prolonged period of time (26–30). In our previous study, we reported the effects of hepatic enzymes on 74 alcohol addicts who received 25 mg of naltrexone daily in the first week and then 50 mg of naltrexone daily for the rest of the 12-week period (31). After the 12-week treatment, levels of the hepatic enzymes, AST and ALT, did not show any elevation and were within the normal range, indicating that naltrexone did not induce abnormalities in liver function tests or elevate the liver enzymes. Instead, statistically significant decreases in plasma levels of AST and ALT were shown throughout the study (31).

LPS/D-gal–induced liver injury in mice is a promising animal model for elucidating the mechanisms of clinical liver dysfunction and for evaluating the efficiency of hepatoprotectives (32). Based on our experiments and a previous report (33), LPS/D-gal at the dose of 20 μg/kg and 700 mg/kg, respectively, was employed in this mortality study. In LPS/D-gal–treated group, one mouse died within 24 h and the remaining mice survived for additional 48 h, while there was no death within 72 h in LPS/D-gal + naltrexone–treated group (data are not shown). Thus, there was no significant difference in survival rate between the untreated group (no naltrexone) and the naltrexone-treated group (data are not shown). However, naltrexone is effective in protecting mice from LPS/D-gal–induced acute hepatotoxicity, as evidenced by the significant reduction of plasma ALT/AST levels (an index of acute hepatocellular death) and decrease of liver injury.

Previous reports demonstrated that TNF-α was an important factor in mediating LPS/D-gal–induced liver damage (34–37). TNF-α is a potent pro-inflammatory cytokine produced by the monocyte-macrophage lineage, including Kupffer cells (38). There is increasing evidence indicating that TNF-α may play a role in the initiation or progression of multiple organ failure in septic shock (38). Taken together, that inhibition of TNF-α synthesis or activity leads to attenuation of LPS-induced liver injury, suggests that TNF-α plays a pivotal role in sepsis-related liver toxicity (34–37, 39). The current study showed that naltrexone significantly decreased TNF-α level at 1.5 h in plasma, which would partially contribute to liver protection. It has been reported that in this LPS/D-gal mouse model, TNF-α is the central proximal mediator, which triggers the lethal effects of septic shock syndrome (40–42). Although naltrexone blocked LPS-induced TNF-α production in vivo (Fig. 1), it did not significantly inhibit LPS-induced TNF-α production in macrophages (15). There are several possible mechanisms that could account for the ability of naltrexone to inhibit systemic TNF-α production in response to LPS, including the following: i) naltrexone may induce unknown intermediates that function to directly inhibit macrophage-derived TNF-α or ii)
Fig. 4. Effects of naltrexone post-treatment on LPS/\(\beta\)-gal–induced histological changes (A: control, B: naltrexone alone, C: LPS/\(\beta\)-gal, D: LPS/\(\beta\)-gal + Naltrexone) in livers from ICR mice. Mice were post-treated with naltrexone (20 mg/kg, i.p.) 30 min after LPS/\(\beta\)-gal administration. Liver sections were stained with hematoxylin and eosin and viewed by light microscopy (400×). E: An image at higher power field microscopically. The cell within the blue circle is an hepatocyte, and the cells with lobulated nucleus are neutrophils. F: Score of histopathological necroinflammatory activity of the livers. Data are mean ± S.E.M. (n = 6). *\(P<0.05\): LPS/\(\beta\)-gal vs control, †\(P<0.05\): LPS/\(\beta\)-gal + Naltrexone vs LPS/\(\beta\)-gal.
that naltrexone may induce the production of anti-inflammatory cytokines, such as IL-10, IL-4, and IL-13, which suppress LPS-induced TNF-α production (43).

In inflammatory responses, abundant ROS are produced, and several sources of oxygen radical species have been proposed as being the cause of tissue damage. Following transmigration and activation, infiltrating neutrophils produce abundant oxygen radicals via oxidative bursts. Other sources of oxygen radical species include activated macrophages and various extracellular molecular processes such as arachidonic acid metabolism and xanthine dehydrogenase oxidation (44). NO may combine with superoxide anion to form the more potent reactive oxygen metabolite peroxynitrite anion, which decomposes to form hydroxyl radical (45). Both peroxynitrite anion and hydroxyl radical are responsible for cellular lipid peroxidation, protein oxidation, and mitochondrial impairment function, which cause further damage to tissues and can induce cell death (46). In this study, post-treatment with naltrexone significantly reduced PMN infiltration in the liver, which may indirectly cause oxidative damage, suggesting that some of the beneficial effects of naltrexone depend on its anti-oxidant properties (as described previously in the report) that may participate in the recovery from lethal hepatitis in LPS/D-gal–treated mice.

Interestingly, we found that post-treatment with naltrexone (20 mg/kg, i.p.) significantly improved hepatic function after LPS/D-gal administration in mice. However, Greenelch et al. reported that pretreatment with naltrexone (10 mg/kg, i.p.) cannot reduce liver damage in mice with acute endotoxic shock even though naltrexone prevented LPS/D-gal–induced mortality (15). The following reasons may explain the failure of hepatic protection by naltrexone in Greenelch’s endotoxic mouse study: i) The dose of naltrexone (10 mg/kg) used for mice may be too low to protect liver from severe damage because the doses of naltrexone used in rats with endotoxic shock in some reports are at 10 mg/kg (18, 47) or higher (our unpublished data). ii) In Greenelch’s study, sepsis is induced by LPS/D-gal (1 and 25 mg/kg, respectively). These doses may lead to more severe hepatic damage than that in our study. iii) In the present study, we showed that administration of naltrexone at higher doses significantly reduced the injury, implying that naltrexone may exert its protective effects partially by blocking the opioid receptors.

Recently, Jaume and his colleagues reported that naltrexone reduces Fas-induced hepatitis in mice (48) and that opioid receptor antagonist improves the resistance of mice to Fas-induced hepatitis via a peripheral mechanism that does not involve a down-modulation of Fas mRNA in hepatocytes nor a decrease in pro-inflammatory activity of neutrophils. As mentioned in the previous discussions that liver damage has been associated with overproduction of ROS (1 – 6) or TNF-α (15 – 17, 34 – 42), these publications lend support to our current results and our earlier report indicated that naltrexone is capable of preventing and/or protecting against LPS or LPS/D-gal–induced liver damage via modulation of ROS or TNF-α (18). Although our findings argue differently from Jaume et al. in terms of how the compound protects against hepatitis, both results suggest that naltrexone is effective in preventing or treating acute hepatitis in animal models. The precise mechanism of actions responsible for the inhibitory effects of naltrexone on TNF-α release, NO synthesis, and superoxide anion generation remains to be further investigated.

In summary, the present study clearly demonstrated that naltrexone has a potent protective effect against LPS/D-gal–induced liver damage in mice. The protective effects of naltrexone could be due to the direct and/or indirect inhibition of pro-inflammatory factors and antioxidant properties. This finding suggests the potential therapeutic application of naltrexone for the treatment of liver damage.

Acknowledgments

This work was supported by a research grant from the National Science Council (NSC 95-2320-B-016-030) and by a research grant from Jenken Biosciences, Inc., Research Triangle Park, NC, USA. The authors acknowledge Dr. S.J. Wei for his assisting with statistic analyses of this work.

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