Delayed Administration of D-Ala2-D-Leu5-Enkephalin, a Delta-Opioid Receptor Agonist, Improves Survival in a Rat Model of Sepsis

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Sepsis is the major cause of death in intensive care units, despite enormous efforts in the development of antimicrobial therapies. Sepsis is mediated by early [e.g., tumor necrosis factor (TNF)-α and interleukin (IL)-1β] and late [e.g., high-mobility group box 1 protein (HMGB1)] proinflammatory cytokines. HMGB1, which is secreted into extracellular milieu by activated macrophages or passively released by destroyed macrophages, stimulates intensive inflammatory responses. D-Ala2-D-Leu5-enkephalin (DADLE), a synthetic δ-opioid receptor agonist, has been shown to protect rats from sepsis. Here we elucidated the mechanism for protective effect of DADLE against sepsis. Sepsis was established in Sprague-Dawley rats by means of cecal ligation and puncture (CLP). In this model, the serum levels of TNF-α and IL-1β were increased after 2-3 h, while those of HMGB1 were increased after 18 h. Administration of DADLE (5 mg/kg) concurrently with CLP improved survival, which was associated with the decreases in the serum levels of TNF-α, IL-1β and HMGB1. Importantly, DADLE administrated 4 h after CLP showed comparable protective effect as the concurrent administration, with decreased serum HMGB1 levels. Moreover, peritoneal macrophages isolated from rats were challenged with lipopolysaccharide (LPS). Concurrent or delayed DADLE administration at 10⁻⁶ M suppressed the LPS-induced cell death. DADLE also suppressed the release of HMGB1 from macrophages that was induced by LPS, TNF-α or interferon-γ. In conclusion, DADLE protects rats from sepsis probably by decreasing the serum level of HMGB1. We propose DADLE as a candidate for septic shock therapy, even if it is administered after the onset of sepsis.

Keywords: sepsis; high-mobility group box 1 protein; endotoxemia; proinflammatory cytokine; DADLE

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HMGB1 has been implicated as a late mediator of lethal endotoxemia and sepsis. Administration of anti-HMGB1 antibodies protects mice against LPS-induced acute tissue injury and lethal endotoxemia (Yang et al. 2004). Notably, anti-HMGB1 antibodies are capable of rescuing mice from lethal experimental sepsis, even when the first doses are given 24 h after the onset of sepsis (Fink 2007), indicating a wider window for HMGB1-targeted therapeutic strategies. Therefore, agents capable of attenuating HMGB1 release may have potential in the prevention and treatment of inflammatory diseases.

D-Ala2-D-Leu5-enkephalin (DADLE), a synthetic δ-opioid receptor agonist, has been shown to improve survival of septic rats (Feng et al. 2009). Here, we show that DADLE rescued animals from lethal endotoxemia, even when treatment began after the acute cytokine response. This protective role may be attributable to the inhibition of the late proinflammatory cytokine HMGB1 rather than the early proinflammatory cytokines TNF-α or IL-1β.

Materials and Methods

Materials

LPS from Escherichia coli serotype O111:B4 and DADLE were purchased from Sigma (Shanghai, China). Purified recombinant rat TNF-α and interferon (IFN)-γ were purchased from R&D Systems (Minneapolis, Minnesota, USA).

Rats and septic models

This study was approved by the Animal Care and Use Committee of Huzhou University Medical College. Adult male Sprague-Dawley (SD) rats (10-12 weeks old, purchased from SLAC Laboratory Animal Centre, Shanghai, China) were kept in the animal house in a temperature-controlled room with a 12-h light/dark cycle; free access to standard laboratory chow and water was allowed. Sepsis was established by the method of cecal ligation and puncture (CLP). Different doses of DADLE (0.5 mg/kg, 1 mg/kg, 5 mg/kg and 10 mg/kg) were administered by intra-peritoneal injection (i.p.) concurrently with CLP (DADLE group). Treatment with a single dose of DADLE (5 mg/kg, i.p.) concurrently with CLP conferred the greatest protection against sepsis and was used at 4 h after CLP (DADLE-post group). Animals (sepsis group) only received normal saline after CLP. Control animals underwent a sham operation.

Cell culture

Peritoneal macrophages were collected from male SD rats (10-12 weeks old) 3 days after i.p. injection of 4% thioglycollate, and were cultured in RPMI 1640 medium (Life Technologies, New York, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (GIBCO BRL, New York, US), 2 mM glutamine (GIBCO), and an antibiotic-antimycotic mix (GIBCO) in a humidified incubator with 5% CO₂ and 95% air. Isolated peritoneal macrophages were stimulated with LPS (100 µg/L), TNF-α (10 ng/ml), or IFN-γ (40 ng/ml) in the presence or absence of various concentrations of DADLE at 37°C for various durations. Then, the culture media were collected for determination of HMGB1 levels, and stimulated macrophages were harvested.

Western blot analysis of p38 mitogen-activated protein kinase (MAPK) in macrophages

Levels of activated, phosphorylated p38 MAPK were measured by Western blot analysis. Cytoplasmic extracts from harvested macrophages were obtained and protein concentrations were determined using the bicinchoninic acid assay with TCA precipitation using BSA as a reference standard (Pierce, IL, USA). Equal amounts of cytoplasmic protein (100 µg) were electrophoresed in a denaturing 10% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. Nonspecific binding sites were blocked with TBS (40 mM Tris, pH 7.6, 300 mM NaCl) containing 5% nonfat dry milk for 1 h at room temperature. Membranes were then incubated in a 1:1000 dilution of anti-p38 MAP kinase rabbit polyclonal antibody or anti-phospho-p38 MAP kinase rabbit monoclonal antibody (Cell Signaling Technology, Shanghai, China) in TBS with 0.1% Tween 20 (TBST). After three washes in TBST, membranes were incubated in a 1:2,000 dilution of horseradish peroxidase conjugated anti-rabbit IgG (Cell Signaling Technology, Shanghai, China). Immunoreactive proteins were detected by enhanced chemiluminescence.

Flow cytometry analysis and confocal imaging

Peritoneal macrophages from rats were stimulated with LPS
(100 μg/L), with DADLE (10⁻⁶ M) added concurrently or 4 h after LPS stimulation. Cells were harvested 18 h after LPS stimulation. The harvested macrophages were resuspended at a concentration of 1 × 10⁷ cells/ml. The Annexin V-FITC Apoptosis Detection Kit (R&D Systems) was used to measure apoptosis. Cells were stained with annexin V fluorescein isothiocyanate (annexin V-FITC) and propidium iodide (PI). Cell death was analyzed with a FACScan (Becton Dickinson, San Jose, CA, USA) and CELLQuest software (Becton Dickinson). Images were captured with a confocal laser-scanning microscope (Zeiss LSM510; Carl Zeiss, Germany).

Statistical analysis

Data were expressed as mean ± s.d. One-way ANOVA was used to compare between different groups. When the ANOVA was significant, post-hoc testing of differences between groups was performed using Tukey’s test. The Kaplan-Meier method was used to compare the differences in mortality rates. P < 0.05 was considered statistically significant.

Results

TREATMENT WITH DADLE PREVENTS SEPSIS LETHALITY AND DECREASES THE SERUM HMGB1

Rats underwent CLP that induced sepsis. Different doses of DADLE were administered concurrently with CLP. Treatment with a single dose of DADLE (5 mg/kg, i.p.) conferred significant protection from lethal endotoxemia (Fig. 1A). In parallel experiments, the early peaks of TNF-α and IL-1β in the serum appeared 2 and 3 h after CLP, respectively (Fig. 1B and C). HMGB1 levels peaked around 18 h after CLP (Fig. 1D). Treatment with DADLE (5 mg/kg, i.p.) decreased the levels of TNF-α (Fig. 1B), IL-1β (Fig. 1C), and HMGB1 (Fig. 1D) as compared to normal saline treatment.

We next assessed the therapeutic efficacy of DADLE when administered after the onset of sepsis. Treatment with DADLE was initiated 4 h after CLP, a time at which clinical signs of sepsis were already evident. DADLE was administered after the early peak of TNF-α and IL-1β in the serum, which occurred within 3 h after the onset of sepsis.

![Fig. 1. DADLE increases survival of septic rats and decreases serum levels of HMGB1, TNF-α, and IL-1β. (A) Treatment with a single dose of DADLE concurrently with CLP (5 mg/kg, i.p.) conferred the greatest protection against sepsis (P < 0.05, n = 20). Treatment with DADLE (5 mg/kg, i.p.) 4 h after CLP (post CLP) still conferred significant protection against sepsis (vs normal saline, P < 0.05, n = 20). There was no significant difference between concurrent and delayed administration of DADLE (5 mg/kg, i.p.) (P > 0.05, n = 20). The Kaplan-Meier method was used to compare the differences in mortality rates between groups. The early peaks of serum TNF-α (B) and IL-1β (C) appeared 2 to 3 h after CLP. Administration of DADLE concurrently with CLP significantly decreased the serum TNF-α or IL-1β level as compared with the saline treatment (*P < 0.05, n = 8, using Student’s t test). Administration of DADLE (5 mg/kg, i.p.) 4 h after CLP (DADLE-post) did not significantly influence the serum TNF-α or IL-1β level (P > 0.05, n = 8, using Student’s t test). (D) HMGB1 levels peaked around 18 h after CLP. Treatment with DADLE concurrently with CLP (5 mg/kg, i.p.) decreased HMGB1 levels as compared to normal saline treatment (*P < 0.05, n = 8, using Student’s t test). Treatment with DADLE (5 mg/kg, i.p.) 4 h after CLP decreased HMGB1 levels 18 h after CLP as compared to normal saline treatment (*P < 0.05, n = 8, using Student’s t test).]
Delayed treatment with DADLE also significantly protected rats from lethal systemic inflammation as compared to saline treatment (survival with DADLE treatment, 13/20; survival with normal saline, 5/20; \( P < 0.05 \)). There was no significant difference between the two DADLE treatment groups (13/20 vs 14/20, \( P > 0.05 \)) (Fig. 1A). Therefore, the significant protection achieved with DADLE suggests that it might target late-acting mediators of lethal systemic inflammation. HMGB1 is a late mediator of endotoxin lethality, and treating endotoxemic rats with DADLE 4 h after CLP significantly decreased the systemic level of HMGB1, which was measured 18 h after the onset of endotoxemia (Fig. 1D). This indicates that the protective role played by DADLE may be partly attributable to the inhibition of HMGB1 release during sepsis.

**DADLE inhibits the LPS-induced HMGB1 release from macrophages**

It is interesting to consider whether DADLE could inhibit LPS-induced HMGB1 release in vitro. Concurrent treatment of DADLE was effective in inhibiting the LPS-induced HMGB1 release from peritoneal macrophages in a dose-dependent manner, with a maximal effect at concentrations of \( 10^{-8} \) M (Fig. 2A), and significant inhibition was still achieved when DADLE was added 4 h after adding LPS (Fig. 2B). It is therefore feasible to attenuate late-act-
Protective Effect of DADLE against Sepsis

DADLE inhibits the TNF-α- or IFN-γ-induced HMGB1 release from macrophages

The regulation of HMGB1 release in sepsis is complex. Besides LPS, some proinflammatory factors such as TNF-α and IFN-γ can also stimulate HMGB1 release from macrophages/monocytes (Rendon-Mitchell et al. 2003; Yin et al. 2005). It was intriguing to consider whether DADLE could inhibit the release of HMGB1 induced by either TNF-α or IFN-γ. DADLE suppressed the TNF-α- and IFN-γ-induced HMGB1 release in a dose-dependent manner, with a maximal effect at a concentration of 10⁻⁶ M (Fig. 3).

DADLE inhibits the LPS-induced cell death of macrophages

To elucidate mechanisms that underlie DADLE-mediated protection, we determined whether DADLE inhibited cell death during LPS-mediated inflammatory responses. Both concurrent and delayed treatment of DADLE (10⁻⁶ M) suppressed the LPS-induced apoptosis and necrosis (Fig. 4).

DADLE modifies the signal transduction in macrophages after LPS stimulation

LPS treatment increased the activity of NF-κB and activation of p38 MAPK, as determined by increased NF-κB p65 subunit and phosphorylated p38 MAPK (p-p38 MAPK). Both concurrent and delayed treatment of DADLE (10⁻⁶ M) inhibited the p38 MAPK and NF-κB signal transduction pathways in macrophages after LPS stimulation (Fig. 5).

Discussion

HMGB1, a necessary and sufficient inflammatory mediator of lethal sepsis (Klune et al. 2008), has been identified as a therapy target because of its wide time window. In this study, we found that DADLE, a synthetic opioid peptide, can inhibit the HMGB1 release from macrophages through a δ-opioid receptor (DOR)-dependent pathway. Earlier studies have found that HMGB1 has immunomodulatory and signal transduction effects by activating δ-opioid receptors that are widely expressed on immune cells (Quock et al. 1999; Bidlack 2000; Husted et al. 2005), and those effects could be blocked by the δ-opioid-selective antagonist naltrindole. Our results indicate that this δ-opioid agonist suppressed HMGB1 release through a novel anti-
Because antibody-blocking strategies improved survival of lethal endotoxemia and sepsis (Yang et al. 2004; neutralizing antibodies or specific antagonists improved survival of sepsis, causing fever, derangement of the intestinal barrier function, acute lung injury and multiple organ failure (Bustin 2002; Bonaldi et al. 2003; Czura et al. 2004). Specifically inhibiting circulating HMGB1 using DADLE concurrently with stimulation. It seems that TNF-α and IL-1β are not critical mediators of severe sepsis, although they play central roles in the pathogenesis of septic shock (Czura et al. 2004). Most patients with sepsis do not have significantly increased TNF-α levels, and anti-TNF-α therapy has a limited clinical effect in critically ill patients (Fisher et al. 1994; Abraham et al. 1998, 2001).

Additionally, we showed that DADLE inhibited the secretion of HMGB1 from cultured macrophages. Administration of HMGB1 recapitulated the pathophysiology of severe sepsis, causing fever, derangement of the intestinal barrier function, acute lung injury and multiple organ failure (Bustin 2002; Bonaldi et al. 2003; Czura et al. 2004). Specifically inhibiting circulating HMGB1 using neutralizing antibodies or specific antagonists improved survival of lethal endotoxemia and sepsis (Yang et al. 2004; Klune et al. 2008). Because antibody-blocking strategies showed limited efficacy in clinical trials against sepsis, we searched for methods to inhibit HMGB1 release. However, the regulation of HMGB1 secretion is complex. Besides LPS, proinflammatory cytokines such as TNF-α and IFN-γ can also stimulate HMGB1 release from macrophages/monocytes (Rendon-Mitchell et al. 2003; Yin et al. 2005), and DADLE inhibited TNF-α- and IFN-γ-induced HMGB1 release from macrophages. Our results show that DADLE can significantly suppress the HMGB1 release that was induced by TNF-α or IFN-γ, which elucidates additional mechanisms that underlie the DADLE-mediated inhibition of HMGB1 secretion in endotoxemia and sepsis. Our results indicate that DADLE inhibits HMGB1 release from macrophages, decreases circulating HMGB1 levels, and improves survival in animal models of sepsis, including ‘established’ peritonitis.

Importantly, administering DADLE 4 h after stimulation, without affecting the early TNF-α and IL-1β peaks, resulted in comparable protective effect as administering DADLE concurrently with stimulation. It seems that TNF-α and IL-1β are not critical mediators of severe sepsis, although they play central roles in the pathogenesis of septic shock (Czura et al. 2004). Most patients with sepsis do not have significantly increased TNF-α levels, and anti-TNF-α therapy has a limited clinical effect in critically ill patients (Fisher et al. 1994; Abraham et al. 1998, 2001).

In conclusion, DADLE protects rats from sepsis probably by decreasing the serum HMGB1 level. We propose DADLE as a candidate for septic shock therapy, even if it is administered hours after the onset of sepsis.
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Conflict of Interest
We declare that we have no conflict of interest.

References


