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Dexmedetomidine inhibits the secretion of high mobility group box 1 from lipopolysaccharide-activated macrophages *In vitro*

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ABSTRACT

Background: High mobility group box 1 (HMGB1) is a critical proinflammatory factor that is closely related to mortality in sepsis patients. Dexmedetomidine has been proven to reduce the mortality rate from endotoxin shock and attenuate endotoxin-induced acute lung injury. These effects result from reduced secretion of many proinflammatory mediators, although it is not clear whether dexmedetomidine affects the secretion of HMGB1. In this study, we explored the effect of dexmedetomidine on the expression and secretion of HMGB1 from lipopolysaccharide (LPS)-activated macrophages.

Methods: We incubated RAW264.7 cells with LPS in the presence or absence of various concentrations of dexmedetomidine. We used an enzyme-linked immunosorbent assay to detect the secretion levels of HMGB1 in the culture supernatant. We employed real-time polymerase chain reaction to assess the expression of HMGB1 mRNA, and used a nuclear/cytoplasm extraction kit to extract the nuclear and cytoplasmic proteins. We employed Western blotting to observe changes in the translocation of HMGB1 from the nucleus to the cytoplasm. In addition, we used a nuclear factor (NF)-κB p50/p65 transcription factor assay kit to analyze NF-κB activity in the nuclear extract.

Results: Dexmedetomidine inhibited the translocation of HMGB1 from the nucleus to the cytoplasm and its extracellular secretion in LPS-activated macrophages while suppressing the expression of HMGB1 mRNA. Dexmedetomidine inhibited the translocation of NF-κB from the cytoplasm to the nucleus in LPS-activated macrophages in a dose-dependent manner. Moreover, these effects were significantly reversed by the α2-adrenergic receptor antagonist yohimbine.

Conclusions: Our study demonstrates that dexmedetomidine inhibits the translocation of HMGB1 from the nucleus to the cytoplasm and the expression of

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HMGB1 mRNA at clinically relevant dosages. The mechanism responsible for these effects may be through the NF-κB signaling pathway and the α2-adrenergic receptors.

1. Introduction

Sepsis can lead to upregulation of inflammatory molecules, including tumor necrosis factor-α (TNF-α), interleukin-1β(IL-1β), macrophage migration inhibitory factor, and high mobility group box 1 (HMGB1) [1–3]. Excessive generation of these inflammatory molecules contributes not only to tissue damage, but also to hemodynamic changes, multiple organ failure, and, ultimately, death [4–6]. Previous data demonstrated that efforts aimed at inhibiting the production of TNF-α, IL-1β, macrophage migration inhibitory factor, and HMGB1 were beneficial against sepsis [4–6]. High mobility group box 1, a ubiquitous and chromosomal protein, can be actively released from macrophage-like RAW264.7 cells and is a late mediator of endotoxin lethality [4]. Once released, HMGB1 can bind to cell-surface receptors (e.g., the receptor for advanced glycation end products, Toll-like receptor [TLR]-2, and TLR4) and mediate various inflammatory responses [7–9]. Specific inhibition of HMGB1 activity attenuated the development of experimental septic shock [10,11] and had good clinical effect in septic shock patients [12]. Therefore, HMGB1 may be an important therapeutic target in lethal systemic inflammatory diseases in which excessive amounts of HMGB1 are released [13].

Dexmedetomidine, a potent and highly selective α2-adrenergic receptor agonist, is widely used for sedation in intensive care units [14]. In addition, dexmedetomidine offers good perioperative hemodynamic stability and reduces intraoperative anesthetic requirements [15]. Moreover, dexmedetomidine has been shown to possess some anti-inflammatory capacity [16]. Dexmedetomidine reduces sepsis-related acute lung injury [17] and has a protective effect on the ischemia-reperfusion injury of heart, kidney, brain, testis, and intestine in animal models [18–22]. Recent data demonstrate that dexmedetomidine inhibits the expression of a variety of inflammatory molecules including nitric oxide, prostaglandin E_2, TNF-α, IL-1β,and IL-6 [23].These anti-inflammatory effects of dexmedetomidine are produced by inhibiting the nuclear factor (NF)-κB signaling pathway and reversed by α2-adrenergic receptor antagonists. However, whether dexmedetomidine influences HMGB1 secretion remains unclear. Based on the characteristics of dexmedetomidine, we hypothesized that dexmedetomidine could inhibit overexpression of HMGB1 during sepsis.

Therefore, we designed the current experiment to investigate the effects of different doses of dexmedetomidine on the expression and secretion of HMGB1 from LPS-activated macrophages, and to explore the potential mechanisms of these anti-inflammatory effects.

2. Materials and methods

2.1. Cell culture and stimulation

We cultured murine macrophage-like RAW264.7 cells (Shanghai Institute of Cell Biology, Chinese Academy of Sciences, Shanghai, China) in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal calf serum (Hyclone, Logan, UT), 100 U/mL penicillin, and 100 mg/mL streptomycin in an atmosphere of humidified 5% CO_2 at 37°C. At 80%–90% confluence, we washed the cells three times, then transferred them to six-well polystyrene culture plates at 1×10^6 cells/well in 2 mL medium per well. After overnight incubation, we removed the medium and replaced it with RPMI 1640 medium containing 0.25% fetal calf serum (for experiments designed to measure HMGB1 in conditioned media). We incubated RAW264.7 cells with LPS (500 ng/mL) in the absence or presence of graded concentrations of dexmedetomidine (0.01, 0.1, and1 μmol/L). Dexmedetomidine exerts clinical sedation when plasma concentrations reach the level of 0.01–0.1 μmol/L [24]. We then chose the dosages of dexmedetomidine chosen accordingly, to correspond to approximately 1 and 10 times the clinically relevant dosages, to facilitate further investigation [24]. To elucidate the role of the α2-adrenergic receptors, we allocated another five groups of macrophages to receive NS (normal saline), LPS, LPS plus yohimbine (100 μmol/L, an α2-adrenergic receptor antagonist; Sigma-Aldrich, St Louis, MO), LPS plus dexmedetomidine (1 μmol/L), and LPS plus dexmedetomidine (1 μmol/L) plus yohimbine (100 μmol/L), and denoted them as NS, L, L+Y, L+D, an L+D+Y, respectively. We collected cell-free supernatants to determine HMGB1 levels after 24 h stimulation. Meanwhile, we extracted total RNA to determine HMGB1 mRNA levels. We assayed the levels of HMGB1 in the cytoplasm and nucleus by Western blot analysis to detect the intracellular translocation of HMGB1. We used the nuclear proteins of RAW264.7 cells to measure NF-κB activity using the NF-κB p50/p65 transcription factor assay kit. For cell viability analysis, we incubated RAW264.7 cells with graded concentrations of dexmedetomidine (0.01, 0.1, and 1 μmol/L). Cells cultured in media alone acted as a negative control in all tests.

2.2. Enzyme-linked immunosorbent assay

We determined the secretion levels of HMGB1 in the culture medium using commercially available enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN) and performed them according to the manufacturer's instructions.

2.3. Cell Counting Kit–8 assay

We determined the viability of RAW264.7 cells using the Cell Counting Kit–8 assay kit (Beyotime, Wuhan, China) as previously reported [25]. We plated RAW264.7 cells at a density of 10^4 cells/well in 96-well plates in 100 μL RPMI 1640 medium. We added 20 μL Cell Counting Kit–8 (CCK-8) to RAW264.7 cells in each microwell and incubated it for 2 h at 37°C after the 24-h incubation period. We measured the absorbance of the colored solution using a microplate reader (Bio-Rad

Laboratories, Hercules, CA) at a test wavelength of 450 nm and a reference wavelength of 630 nm.

2.4. Extraction of cytoplasmic and nuclear proteins

At 24 h after treatment, we harvested RAW264.7 cells and washed them three times with cold phosphate-buffered saline (PBS). We extracted the cytoplasmic and nuclear protein fractions using NE-PER extraction reagents according to the manufacturer's protocol (Pierce Biotechnology, Rockford, IL). Cytoplasmic and nuclear protein extracts were used for Western blot analysis.

2.5. Western blot analysis

We assayed the levels of HMGB1 in the cytoplasm and nucleus by Western blot analysis. We quantified proteins using the Enhanced BCA Protein Assay Kit (Beyotime, Wuhan, China). Samples were denatured at 100°C for 5 min before adding dithiothreitol. We loaded equal amounts of protein in each well for electrophoresis in 12% sodium dodecyl sulfate–polyacrylamide gels, and then transferred them onto polyvinylidene fluoride microporous membranes (Millipore, Bedford, MA). Membranes containing the transferred proteins were blocked with PBS containing 0.1% Tween 20 (PBS-T) and 5% skim milk for 1 h at room temperature. After three washes with PBS-T, we then incubated membranes with rabbit anti-HMGB1 polyclonal antibody (1:300; Abcam, San Diego, CA), anti-β-actin antibody (1:1000; Santa Cruz Technology, Santa Cruz, CA), and anti-histone H3.1 antibody (1:1000; SAB, Pearland, TX) for 1 h at room temperature. After three washes with PBS-T, we incubated the membranes with horseradish peroxidase–linked secondary antibodies (1:1000; Golden Bridge, Beijing, China) for 1 h at room temperature. After three final washes in PBS-T and two washes in PBS, we impregnated the membranes with the ECL reagents (Amersham, Buckinghamshire, UK) and then exposed them digitally with the Image Reader LAS-4000 (FujiFilm, Tokyo, Japan) and quantified them for statistical analysis using Multi Gauge version 3.0 software (FujiFilm Life Science, Tokyo, Japan).

2.6. Real-time polymerase chain reaction analysis

We extracted total RNA from treated RAW264.7 cells by adding TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. We synthesized first-strand cDNA using 2 μg total cellular RNA as the template, 1 μL 10 mmol/L dNTP mix, 5 μL M-MLV 5× reaction buffer (with Mg^{2+}), 0.625 μL recombinant RNasin, and 1 μL M-MLV (Promega, Madison, WI), in a final volume of 25 μL. We performed quantitative real-time polymerase chain reaction (PCR) on a LightCycler 2.0 Real-Time PCR System (Roche Applied Science, Indianapolis, IN). The reaction contained 1 μL of the cDNA template, 2 μL of the forward primer, 2 μL of the reverse primer (HMGB1: CAC CGT GGG ACT ATT AGG AT; HMGB1 rev: GCT CAC ACT TTT GGG GAT AC; β-actin: CCT CTA TGC CAA CAC AGT; β-actin rev: AGC CAC CAA TCC ACA CAG), and 10 μL PCR Premix (BioTNT, Shanghai, China) in 20 μL. The PCR reaction involved initial denaturation (95°C, 5 min), followed by 40 cycles of denaturation (95°C, 5 s) and extension (60°C, 30 s), and then denaturation (95°C, 15 s), extension (72°C, 10 s), and a final denaturation (95°C, 15 s). We conducted the real-time PCR assays in triplicate for each sample to ensure experimental accuracy. We calculated the mean fold change in the expression of HMGB1 mRNA in the experimental group compared with the control group using the $2^{-\Delta\Delta Ct}$ method [26].

2.7. NF-κB binding assay

We determined the DNA binding activity of NF-κB (p50/p65) using the enzyme-linked immunosorbent assay–based nonradioactive NF-κB p50/p65 transcription factor assay kit (Chemicon, Temecula, CA) according to the manufacturer's instructions. We analyzed the absorbance at 450 nm using an automated plate reader (Bio-Rad Laboratories).

2.8. Statistical analysis

All data are presented as the mean ± standard deviation (SD) of results obtained from three replicates. We assessed differences between groups by one-way analysis of variance followed by the Student-Newman-Keuls test. A P value less than 0.05 was considered statistically significant. We analyzed all data using SPSS 13.0 statistical software (SPSS Inc, Chicago, IL).

3. Results

3.1. Effect of dexmedetomidine on HMGB1 levels secreted by cultured RAW264.7 cells after treatment with LPS

We collected the supernatant 24 h after LPS stimulation to determine the secretion of HMGB1 by enzyme-linked immunosorbent assay. We found that the HMGB1 levels in the culture supernatant increased after the administration of LPS

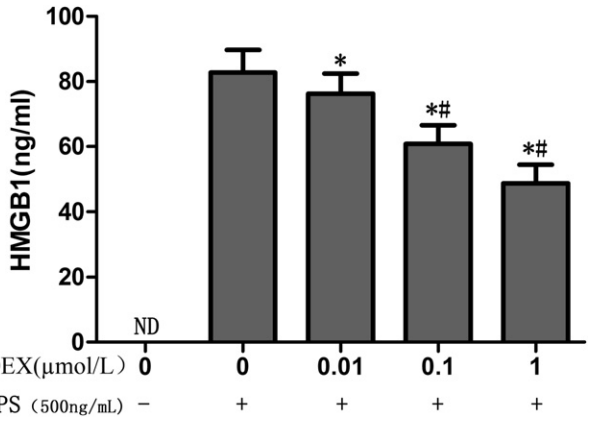

Fig. 1 – Effect of dexmedetomidine at different concentrations on HMGB1 release from RAW264.7 cells after 24-h stimulation with LPS. We stimulated RAW264.7 cells without or with LPS (500 ng/mL) in the absence or presence of dexmedetomidine (0.01, 0.1, and 1 μmol/L) for 24 h. We collected and assayed culture supernatants for HMGB1 with an enzyme-linked immunosorbent assay kit. Data are mean values ± SD ($n = 3$). $^{*}P < 0.05$ compared with the LPS-treated group and $^{\#}P < 0.01$ compared with the LPS-treated group. DEX = dexmedetomidine; ND = not detectable.

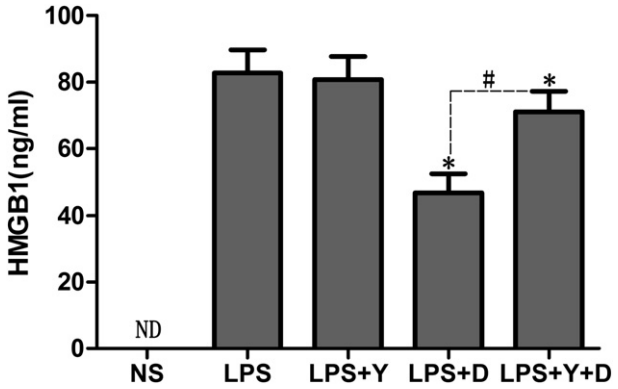

Fig. 2 – Effect of yohimbine (100 μmol/L) on HMGB1 release from RAW264.7 cells after 24-h stimulation with LPS. We stimulated RAW264.7 cells without or with LPS (500 ng/mL) in the absence or presence of dexmedetomidine (1 μmol/L) for 24 h. Yohimbine was added 5 min before LPS or LPS plus dexmedetomidine. We collected and assayed culture supernatants for HMGB1 with an enzyme-linked immunosorbent assay kit. Data are mean values ± SD ($n = 3$). $^{*}P < 0.05$ compared with the LPS-treated group and $^{\#}P < 0.05$ the LPS+D group *versus* the LPS+Y+D group. DEX = dexmedetomidine; Y = yohimbine; ND = not detectable.

(500 ng/mL), but the administration of dexmedetomidine significantly inhibited the secretion of HMGB1 in a dose-dependent manner (Fig. 1). The inhibitory effects of dexmedetomidine on HMGB1 release were significantly reversed by the α2-adrenergic receptor antagonist yohimbine (Fig. 2).

3.2. *Effect of dexmedetomidine on viability of RAW264.7 cells*

We measured RAW264.7 cell viability after treatment with graded dexmedetomidine using the CCK-8 assay. The results showed that increasing concentrations of dexmedetomidine did not have a significant effect on RAW264.7 cell viability (Fig. 3).

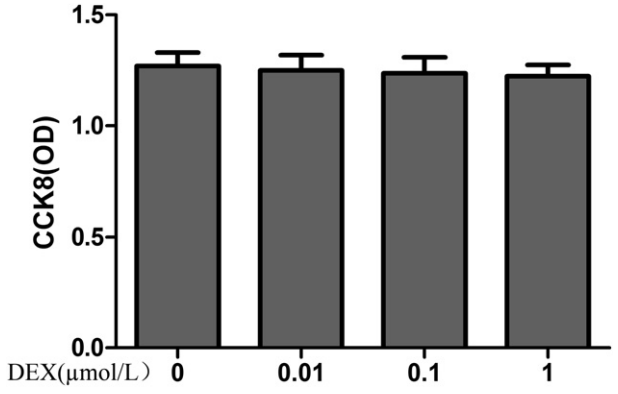

Fig. 3 – Effect of dexmedetomidine at different concentrations on RAW264.7 cell viability after incubation for 24 h. We incubated RAW264.7 cells with graded concentrations of dexmedetomidine (0.01, 0.1, and 1 μmol/L) or cultured them in RPMI 1640 medium alone for 24 h. We measured cell viability using the CCK-8 assay. Data are mean values ± SD ($n = 3$). $P > 0.05$ compared with the control group (0 μmol/L dexmedetomidine). DEX = dexmedetomidine.

3.3. *Effect of dexmedetomidine on translocation of HMGB1 from nucleus to cytoplasm*

The HMGB1 protein levels in the nucleus were significantly reduced after LPS stimulation, but addition of dexmedetomidine significantly reversed this effect. Likewise, the increased HMGB1 protein levels in the cytoplasm after LPS stimulation were reduced by treatment with dexmedetomidine. Thus, addition of dexmedetomidine caused reduced translocation of HMGB1 from the nucleus to the cytoplasm (Fig. 4).

3.4. *Effect of dexmedetomidine on HMGB1 mRNA expression*

To gain further insight into the mechanism responsible for the inhibition of dexmedetomidine on HMGB1 secretion, we next tested the effect of dexmedetomidine on HMGB1 transcription. We found that dexmedetomidine inhibited the LPS-induced increase in HMGB1 mRNA expression at the transcriptional level. Data indicated that the changes in HMGB1 levels in the cell culture supernatant are consistent with changes in intracellular levels of HMGB1 mRNA expression (Fig. 5).

3.5. *Effect of dexmedetomidine on LPS-induced increase in NF-κB activity*

The above results demonstrated that dexmedetomidine inhibited the expression of HMGB1 mRNA in LPS-activated RAW264.7 cells, so we next explored whether the upstream NF-κB signal transduction pathway and the α2-adrenergic receptor were also involved. Our results showed that NF-κB activity was increased in RAW264.7 cells stimulated with LPS (500 ng/mL); dexmedetomidine significantly reduced NF-κB activity in LPS-activated RAW264.7 cells in a dose-dependent manner (Fig. 6). The inhibitory effects of dexmedetomidine on the LPS-induced increase in NF-κB activity were significantly reversed by the α2-adrenergic receptor antagonist yohimbine (Fig. 7).

4. Discussion

Lipopolysaccharide-induced up-regulation of HMGB1 is mediated by TLR-4 [27]; TLR-4 activation subsequently induces the activation of NF-κB [27]. It is well established that NF-κB is a crucial factor that controls the release of HMGB1 [4]. Although the interaction between dexmedetomidine and TLR-4/NF-κB has not been studied, NF-κB activation has been shown to be influenced by the α2-adrenergic receptors, because previous data demonstrated that activation of the α2-adrenergic receptors induced NF-κB activation [28]. Judging from these data, we speculate that dexmedetomidine, a selective α2-adrenergic receptor agonist, may act through regulating the activation of TLR-4 and NF-κB to exhibit its effects on regulating the endotoxin-induced up-regulation of HMGB1 in LPS-activated macrophages.

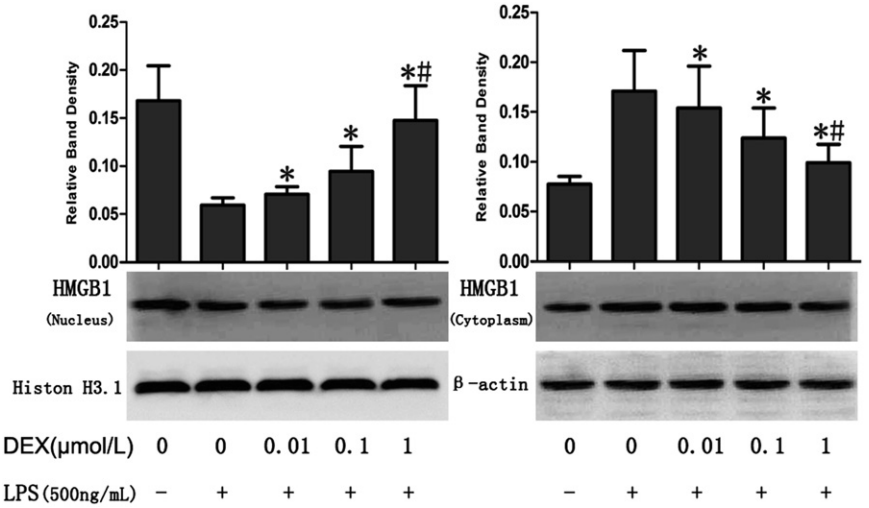

Fig. 4 – Effect of dexmedetomidine at different concentrations on the translocation of HMGB1 from the nucleus to the cytoplasm in RAW264.7 cells after 24-h stimulation with LPS. We extracted the nuclear and cytoplasmic proteins of RAW264.7 cells using a nuclear/cytoplasm extraction kit and assayed them by Western blot analysis. Data are mean values ± SD ($n = 3$). *$P < 0.05$ compared with the LPS-treated group and #$P < 0.01$ compared with the LPS-treated group. DEX = dexmedetomidine.

Our study clearly demonstrated that dexmedetomidine at clinically relevant dosages (i.e., 0.01 and 0.1 μmol/L) significantly inhibited HMGB1 secretion in LPS-activated murine macrophages [24]. The mechanism of inhibition of HMGB1 secretion involved inhibiting the translocation of HMGB1 from the nucleus to the cytoplasm, as well as inhibiting the LPS-induced up-regulation of HMGB1 mRNA expression at the transcriptional level. These effects are achieved by inhibiting the NF-κB signaling pathway. Data from this study also revealed that the inhibitory effects of dexmedetomidine on HMGB1 secretion could be significantly reversed by the α2-adrenergic receptor antagonist yohimbine. On the other hand, the finding that dexmedetomidine at 1 μmol/L (i.e., 10 times the clinically relevant dosage) significantly mitigated HMGB1 secretion in LPS-activated macrophages is clinically interesting. In recent studies, pediatric patients required

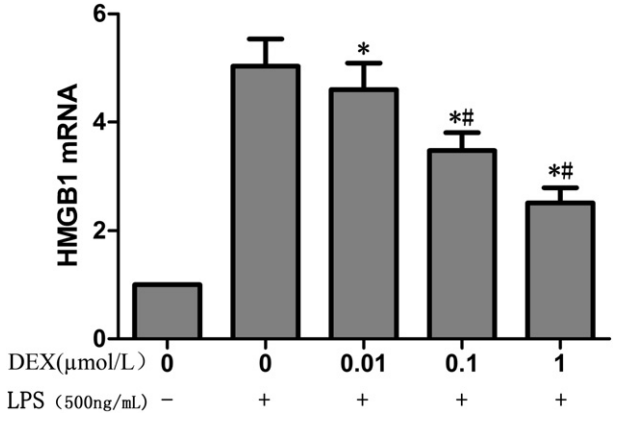

Fig. 5 – Effect of dexmedetomidine at different concentrations on HMGB1 mRNA expression in RAW264.7 cells after 24-h stimulation with LPS. We stimulated RAW264.7 cells with or without LPS (500 ng/mL) in the absence or presence of dexmedetomidine (0.01, 0.1, and1μmol/L) for 24 h. We extracted and assayed total RNA of RAW264.7 cells for HMGB1 mRNA by real-time polymerase chain reaction. Data are mean values ± SD ($n = 3$). *$P < 0.05$ compared with the LPS-treated group and #$P < 0.01$ compared with the LPS-treated group. DEX = dexmedetomidine.

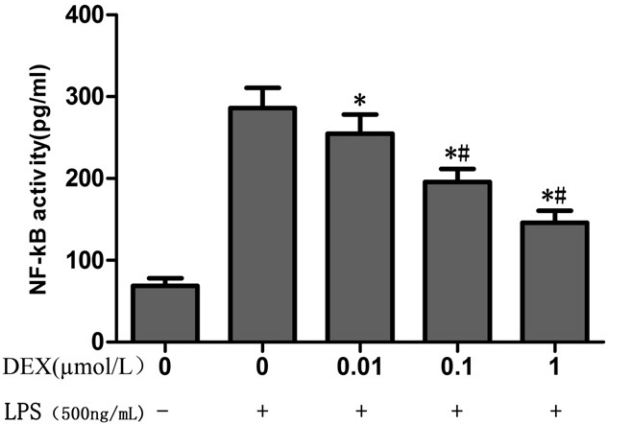

Fig. 6 – Dexmedetomidine reduces nuclear factor (NF)-κB activity in a dose-dependent manner. We stimulated RAW264.7 cells with or without LPS (500 ng/mL) in the absence or presence of dexmedetomidine (0.01, 0.1, and1μmol/L) for 24 h. We extracted the nuclear proteins and determined NF-κB activity using an NF-κB p50/p65 transcription factor assay kit. Data are mean values ± SD ($n = 3$). *$P < 0.05$ compared with the LPS-treated group and #$P < 0.01$ compared with the LPS-treated group. DEX = dexmedetomidine.

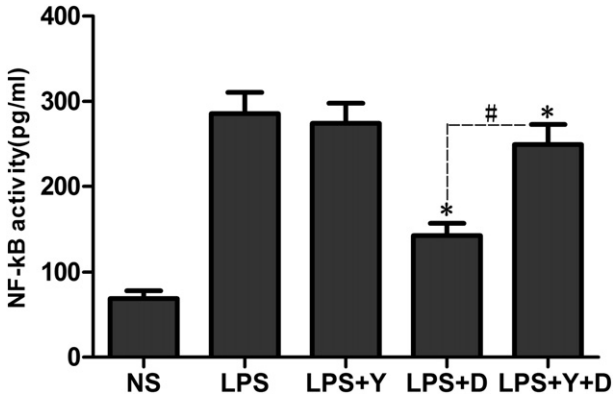

Fig. 7 – Yohimbine (100 μmol/L) reverses the LPS-induced increase in NF-κB activity. We stimulated RAW264.7 cells without or with LPS (500 ng/mL) in the absence or presence of dexmedetomidine (1 μmol/L) for 24 h. We added yohimbine 5 min before LPS or LPS plus dexmedetomidine. We extracted the nuclear proteins determined and NF-κB activity using an NF-κB p50/p65 transcription factor assay kit. Data are mean values ± SD ($n = 3$). $^{*}P < 0.05$ compared with the LPS-treated group and $^{\#}P < 0.05$ for the LPS+D group *versus* the LPS+Y+D group. DEX = dexmedetomidine; Y = yohimbine; ND = not detectable.

higher dosages of dexmedetomidine (up to approximately 5–10 times the clinical dosage) to achieve adequate conscious sedation [29,30]. Moreover, such dosages were well tolerated by those patients [29,30]. Therefore, dexmedetomidine administered at clinically relevant dosages (i.e., 0.01 and 0.1 μmol/L), even at higher dosages (up to approximately 5–10 times clinical dosages), may have the potential benefit of being used as an anti-inflammatory agent in septic patients.

Taniguchi et al [16] reported that dexmedetomidine reduced the mortality rate and had an inhibitory effect on inflammatory response during endotoxemia [16], and Lai et al [28] reported that dexmedetomidine mitigates endotoxin-induced up-regulation of inflammatory molecules in LPS-activated murine macrophages. Although they mentioned that dexmedetomidine significantly reduced the mortality rate in endotoxemia by slowing the secretion of several inflammatory molecules, whether dexmedetomidine can affect HMGB1 is unknown. Our results confirm that dexmedetomidine may reduce the synthesis and secretion of HMGB1. Because HMGB1 is a critical mediator involved in the development of hemorrhagic shock [31], neuropathic pain [32], ischemia-reperfusion injury [33], and even diabetes [34], dexmedetomidine may provide some protection against a number of clinical inflammatory disorders by inhibiting the synthesis and secretion of HMGB1. Thus, our current study is important for better understanding the anti-inflammatory effect of dexmedetomidine on sepsis and many other inflammatory disorders.

Nevertheless, the exact mechanism of the anti-inflammatory effect of dexmedetomidine has not been fully elucidated. Recent studies have shown that the inhibitory effects of dexmedetomidine on several inflammatory molecules involved the NF-κB signaling pathway [28]. Previous studies demonstrated that the NF-κB signaling pathway regulates the expression and secretion of HMGB1 [35,36]. Thus, our study supports the hypothesis that dexmedetomidine inhibits the NF-κB signaling pathway, which regulates the translocation of HMGB1 from the nucleus to the cytoplasm. Previous studies have also confirmed that the α2-adrenergic receptor is involved in regulating the secretion of inflammatory molecules by the NF-κB signaling pathway [28].Therefore, dexmedetomidine may act through α2-adrenergic receptor and other upstream pathways that regulate HMGB1. Therefore, our study supports the hypothesis that the inhibitory effects of dexmedetomidine on HMGB1 secretion could be significantly reversed by the α2-adrenergic receptor antagonist yohimbine.

There were several possible limitations to this study. α2-Adrenergic receptors have been shown to have three subtypes: α2A, α2B, and α2C [37]. Further study of the role of specific subtypes of α2-adrenergic receptors in mediating the effects of dexmedetomidine on regulating endotoxin-induced HMGB1 secretion is required. In addition, we also need to study whether the similar protective effects of dexmedetomidine on LPS-induced systemic inflammatory response and subsequent vital organs injuries in septic animals involve the inhibition of HMGB1.

Our study confirmed that dexmedetomidine at clinical dosages significantly inhibits both the translocation of HMGB1 from the nucleus to the cytoplasm and the LPS-induced increases in HMGB1 mRNA expression in LPS-activated macrophages. The inhibitory effects of dexmedetomidine were mediated, at least in part, by the NF-κB signaling pathway and the α2-adrenergic receptor. Our findings provide new insight for us to better understand the anti-inflammatory mechanisms of dexmedetomidine, and also help to explore new clinical treatments of inflammatory diseases in critical clinical settings.

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