Am J Transl Res 2017;9(12):5245-5258
www.ajtr.org /ISSN:1943-8141/AJTR0062390

Original Article
Dexmedetomidine mitigates CLP-stimulated acute lung injury via restraining the RAGE pathway

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Received July 27, 2017; Accepted November 17, 2017; Epub December 15, 2017; Published December 30, 2017

Abstract: Objective: RAGE pathway plays crucial effects in causing acute lung injury (ALI). Dexmedetomidine (DEX) is showed to mitigate sepsis-stimulated ALI. However, its mechanisms have not been verified. The study was to evaluate whether the RAGE pathway participated in the actions of DEX on sepsis-stimulated ALI in rats. Methods: Male rats were administrated with intravenously DEX 30 min after sepsis. At 24 h of sepsis, lung myeloperoxidase (MPO) and macrophages in the bronchoalveolar lavage fluid (BALF) were observed. The actions of DEX on pro-inflammatory molecules and related mechanisms were determined by immunological methods. Results: It was indicated that DEX markedly attenuated CLP-stimulated augment of lung inflammatory cells infiltration, along with significantly mitigated MPO activity. Besides, DEX obviously reduced lung wet/dry weight ratio and the levels of HMGB1 and RAGE in BALF and lung tissue. Moreover, DEX post-treatment apparently attenuated the histopathological lung injury compared with CLP model group. Furthermore, western blot analysis revealed that DEX efficiently restrained the activation of IκB-α, NF-κB p65, and MAPK. Conclusion: Our studies demonstrated that DEX attenuates the aggravation of sepsis-stimulated ALI via down regulation of RAGE pathway, which has a potential value in the clinical therapy.

Keywords: Dexmedetomidine, CLP, HMGB1, RAGE, ALI

Introduction

Acute lung injury (ALI) is characterized by a clinical disorders that derives from complicated reactions of the lungs to numerous direct and consequential injury [1]. ALI represents a strong inflammatory response to the interstitial edema, neutrophil infiltration, the destruction of the integrity of the epithelium, and lung parenchyma lesion [2]. The mechanism of ALI includes inflammation/anti-inflammation mal-adjustment, up-regulation of inflammatory mediators, and the increased production of adhesion molecules [3, 4]. Neutrophil activation is conducive to lung inflammation via producing reactive oxygen species, proteolytic enzymes, and pro-inflammatory molecules which contribute to the development of ALI. Inflammatory mediators, for example, HMGB1, IL-1β, and IL-6, exert a crucial effect in involving and enlarging ALI via triggering neutrophils infiltration and chemotaxis. There is no particular drug to relieve ALI in clinic so far. Therefore, exploring for the original treatment and searching the novel reagent to remedy ALI may stand for a latent target for treatment.

The receptor for advanced glycation end-products (RAGE) results in continuing downstream signaling pathway activation, mediates hyper-inflammatory responses, and contributes to high mortality during both endotoxin shock and severe sepsis [5-7]. Su et al. have reported that RAGE is a direct index of lung inflammation in experimental lung inflammation models [8]. RAGE regulates the expression of pro-inflammatory cytokines by promoting the activation of MAPK and NF-kB (NF-κB) [9]. These pro-inflammatory molecules are regarded as vital in the development of ALI. Consequently, it has been shown that control of RAGE activity may be serviceable as anti-inflammation drugs.

Dexmedetomidine (DEX) has been reported to guard creatures against harm via its immunoregulation and anti-inflammation roles [10-12].
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DEX can inhibit the pro-inflammatory molecules reactions in rats with sepsis [13], and likewise has underlying worth in dealing with several other disorders creature models, consisting of transient focal cerebral ischemia/reperfusion [14] and ALI [15]. Nevertheless, whether DEX attenuates RAGE expression and endow with its inhibition of ALI remain ill-defined. In the current research, we applied sepsis-stimulated ALI animal model to determine the role of DEX on the inflammation reaction, especially RAGE expression, and to further investigate its underlying pathological process.

Materials and methods

Animals

Six-week-old male RAGE deficient (RAGE⁻/-) rats (180-200 g) were obtained from the Shanghai Experimental Animal Center. All rats were arranged for a chamber (25±2°C, 50-75% humidity) and free choice feeding. The animal scheme was authorized by the Institutional Animal Ethics Committee of Zhejiang Provincial Hospital of TCM.

Experimental design and grouping

For the roles of DEX on CLP-stimulated ALI, WT rats were assigned to six groups: Sham, DEX, CLP, CLP+DEX (2.5), CLP+DEX (5), and CLP+DEX (10) groups (n=10). The details of various treatment of each group are summarized in Table 1. The rats were anesthetized by the intraperitoneal administration of sodium pentobarbital (50 mg/kg, Sigma-Aldrich, USA) before surgery. CLP animal model was performed to mimic polymicrobial sepsis as mentioned earlier [16]. Fluid resuscitation was performed to all animals by injecting 10 mL normal saline (NS) promptly after surgical procedures. DEX (2.5, 5 or 10 μg·kg⁻¹·h⁻¹) or NS were infused continuously through the femoral vein cannula 30 min
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after CLP. The dosages of these reagents were in accordance with Takumi Taniguchi’s former study and our preliminary experiments [17]. Lung samples and BALF were harvested at 24 h following sepsis for the following experiments.

For the roles of Anti-RAGE Ab on CLP-induced lung inflammatory mediators (such as, IL-1β, IL-6, TNF-α, and HMGB1) production in BALF, WT rats were allocated to one of the seven groups: Sham, Anti-RAGE Ab, DEX, CLP, CLP+Anti-RAGE Ab, CLP+DEX (10), and CLP+Anti-RAGE Ab+DEX (10) groups (n=10). The details of various treatment of each group are summarized in Table 1. WT rats were intraperitoneal administrated with Anti-RAGE Ab (10 mg/kg) 1 h prior to sepsis, and subsequently were administrated with DEX (10 μg·kg⁻¹·h⁻¹, i.v.) or a normal saline (NS) following sepsis; BALF was harvested at 24 h of sepsis.

For the roles of DEX on CLP-stimulated the activation of NF-κB and phosphorylation of MAPK, WT rats were assigned to five groups: Sham, CLP, CLP+DEX (2.5), CLP+DEX (5), and CLP+DEX (10) groups (n=10). The details of various treatment of each group are summarized in Table 1. Lung samples were harvested at 24 h of sepsis.

For the roles of DEX on sepsis-stimulated ALI, WT and RAGE⁻/⁻ rats were allocated to one of the six groups: Sham in WT rats, CLP in WT rats, CLP+DEX (10) in WT rats, Sham in RAGE⁻/⁻ rats, CLP in RAGE⁻/⁻ rats, and CLP+DEX (10) in RAGE⁻/⁻ rats groups (n=10). The details of various treatment of each group are summarized in Table 1. Lung samples and BALF were harvested at 24 h of sepsis.

Lung wet-to-dry ratio and MPO assay

The wet weight was recorded as the removed lung weight while the rats were euthanized. Dry weight was obtained from oven dried lung tissues (80°C for 48 h). Lung edema evaluation was performed to lung wet to dry ratio. Homogenates (5%) was obtained from homogenized lung samples (100 mg) by fluidizing in extraction buffer. MPO measurement was performed by the centrifuged homogenates (14,000 g for 30 min). MPO levels were determined by assay kits obtained from Beyotime Institute of Biotechnology (Shanghai, China) in accordance with the manuals.

Protein content assay and inflammatory cell counts of BALF

BCA test kit (Pierce, Rockford, Illinois) was used to determine the BALF total protein level in accordance with the manufacturer’s manuals. The cell pellets were collected from the centrifuged samples (800 g for 15 min at 4°C) and resuspended in PBS (1 mL). A hemocytometer was applied to measure the total cell counts. The Wright-Giemsa staining was used to differential cell counts in cytopsins.

Assessment of inflammatory mediators

Production of inflammatory mediators, including IL-1β, IL-6, TNF-α, and HMGB1, in lung samples and BALF, was determined via ELISA assay kits on the basis of the manufacturer’s instructions.

Histopathological examination of the lungs

Rats that were not performed to collect BALF were subjected to lung histopathological evaluation. The lung sections were obtained from the process of excising, paraformaldehyde (4%) fixing, imbedding in paraffin, and sliced. Pathogenic manifestations of lung samples were stained by H&E and evaluated under an optical microscope.

Western blot analysis

Liquid nitrogen was used for freezing and collecting lung samples for storage until homogenization at 24 h after CLP. Cell Lysis Buffer on ice was used to homogenize lung samples. The protein was quantified by BCA determination kit (Beyotime Institute of Biotechnology, Shanghai, China). A PVDF membrane (Millipore, MA, USA) was used to electro-transferee proteins (50 μg) separated by SDS-PAGE. Buffer (3% BSA in TBST) was used to block the membrane at room temperature for 1 h. Shortly afterwards, the primary antibody was used to incubate the membrane overnight at 4°C by diluting in blocking buffer. An appropriate HRP-conjugated secondary antibody was used to incubate the membrane by diluting in blocking buffer after washing three times in TBST. The enhanced chemiluminescence system (Santa Cruz) was used to visualize and capture the images.

Real-time PCR

Trizol (Invitrogen Corporation, Carlsbad, CA) was used to extract total RNA from lung in
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accordance with the manufacturer’s manuals. The LightCycler 2.0 System (Roche Applied Science, Indianapolis, IN) was used to accomplish Real-time PCR. Specific primers for inflammatory cytokine’s gene expression were used to amplify the complementary DNA, and β-actin gene expression was used to normalize the findings. The 2-ΔΔCt method was used to determine the relative mean fold change of inflammatory cytokine’s gene expression [18].

Statistical analysis

Data were expressed as means ± standard deviation (S.D.). Statistically significant differences between groups were measured by one-way analysis of variance (ANOVA) followed by post hoc Bonferroni test. P<0.05 was considered statistically significant.

Results

Role of DEX on CLP-stimulated vascular edema

CLP administration generated a marked enhancement in capillary leakage, as illustrated by the BALF total protein level and lung W/D ratio. The BALF total protein level and lung W/D ratio were investigated at 24 h after CLP. As shown in Figure 1A and 1C, CLP administration markedly enhanced BALF total protein level and lung W/D ratio. Nevertheless, post-treatment with DEX markedly reduced BALF total protein level and lung W/D ratio compared to CLP group. These findings showed that DEX could attenuate lung edema in CLP-stimulated ALI rats.

Role of DEX on MPO content and cell content in CLP-Stimulated ALI WT rats

24 h following CLP treatment, the number of total cells, macrophages, and neutrophils in the BALF were markedly enhanced relative to the sham group. As shown in Figure 1D-F, post-treatment with DEX markedly reduced the count of total cells, neutrophils, and macrophages compared to CLP-stimulated ALI rats. MPO content was evaluated to further explore the role of DEX on the infiltration of lung neutrophils in CLP-stimulated ALI rats, the activity of. As illustrated in Figure 1B, CLP administration markedly enhanced MPO activity compared to the sham group. In addition, this enhancement was obviously attenuated via post-treatment with DEX (Figure 1B). These findings demonstrated that DEX reduced lung hypernemic neutrophils infiltration in CLP-stimulated ALI rats.
Role of DEX on inflammatory molecule levels in lung tissue and BALF of CLP- Stimulated ALI WT rats

To determine the inflammatory molecules production, including IL-1β, IL-6, and TNF-α, in lung tissue and BALF, lung tissues and BALF were collected at 24 h following CLP treatment. As shown in Figure 2, CLP treatment markedly enhanced the production of IL-1β, IL-6, and TNF-α in lung tissues and BALF compared to the sham group. DEX markedly reduced IL-1β, IL-6, and TNF-α level compared to the CLP-stimulated ALI group (Figure 2).
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Role of DEX on CLP-stimulated lung histopathological changes in WT rats

To determine the role of DEX on CLP-stimulated ALI in rats, lung manifestations were evaluated under an optical microscope. As illustrated in Figure 3C, lung samples collected from the CLP group indicated markedly pathological lesion, such as thickened alveolar wall, pulmonary hemorrhage, and infiltration of inflammatory cell. In addition, these manifestations were ameliorated via post-treatment with DEX (Figure 3D-F).

Role of DEX on RAGE and HMGB1 levels in sepsis-stimulated ALI

ELISA indicated that HMGB1 releases in the CLP group were markedly higher than in the sham group. DEX administration has been
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shown to obviously attenuate HMGB1 production compared with the CLP group at 24 h (Figure 4A). Furthermore, ELISA indicated that BALF sRAGE release in the CLP group was obviously higher than in the sham group. DEX administration failed to markedly attenuate sRAGE expression compared with the CLP group at 24 h (Figure 4B). Western blot and Real-time PCR indicated that lung RAGE and HMGB1 levels in the CLP group were obviously higher than in the sham group (Figure 4C-F). DEX administration markedly downregulated RAGE and HMGB1 levels compared with the CLP group at 24 h (Figure 4C-F).

Role of anti-RAGE Ab on inflammatory molecule levels from sepsis-stimulated ALI

We demonstrated that sepsis-stimulated inflammatory molecule production was markedly attenuated by both DEX administration and Anti-RAGE Ab administration individually (Figure 5); the CLP+DEX (10) group has a stronger inhibitory effects on CLP-induced releases of inflammatory cytokines than the CLP+Anti-RAGE Ab group (P<0.05, Figure 5); furthermore, the CLP+Anti-RAGE Ab+DEX (10) group has a stronger inhibitory effects on CLP-induced release of inflammatory cytokines than the CLP+Anti-RAGE Ab group or the CLP+DEX (10) group (Figure 5). These findings suggested that RAGE is associated with DEX-involved amelioration of sepsis-stimulated inflammatory responses.

Role of DEX on the phosphorylation of NF-κB in CLP-stimulated ALI WT rats

The activation of NF-κB p65 and degradation of IκB-α were markedly enhanced after CLP administration (Figure 6). Post-treatment with DEX significantly attenuated the activation of NF-κB p65 and degradation of IκB-α (Figure 6). These findings demonstrated that DEX attenuated the phosphorylation of NF-κB in CLP-stimulated ALI rats.

Figure 5. Role of Anti-RAGE Ab on the levels of inflammatory molecules from WT rats with CLP-stimulated ALI. Rats were intraperitoneal administration with Anti-RAGE Ab (10 mg/kg) 1 h before CLP, and then the rats were administration with DEX (10 μg·kg⁻¹·h⁻¹, i.v.) or normal saline after CLP. BALF was harvested to measure the inflammatory molecules TNF-α (A), IL-1β (B), IL-6 (C), and HMGB1 (D). *P<0.05 CLP vs. CLP+DEX, **P<0.05 CLP+Anti-RAGE Ab/DEX vs. CLP+Anti-RAGE Ab+DEX, #P<0.05 CLP+Anti-RAGE Ab vs. CLP+DEX. DEX, dexmedetomidine.
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Role of DEX on activation of MAPK in sepsis-stimulated ALI

As shown in Figure 7, post-treatment with DEX markedly reduced MAPK activation, including ERK, JNK, and p38 compared to the CLP group. Our findings showed that DEX suppressed MAPK activation in CLP-stimulated ALI.

Role of DEX on the manifestations of sepsis-stimulated ALI

As shown in Figure 8, CLP administration markedly enhanced the lung MPO activity and W/D ratio in WT and RAGE-/- rats. Nevertheless, post-administration with DEX (10 μg·kg⁻¹·h⁻¹) significantly decreased lung MPO activity and W/D ratio compared to CLP group (Figure 8A and 8B). Amelioration role of DEX in RAGE-/- rats had larger count of total cells, macrophages, and neutrophils and the total protein concentration in the BALF at 24 hours after sepsis when relative to with those in WT rats (Figure 8C-F).

Role of DEX on inflammatory cytokines release in lung samples and BALF from sepsis-stimulated ALI

As shown in Figure 9, CLP administration markedly enhanced the production of inflammatory cytokines in lung samples and BALF from rats compared with the sham group. DEX (10 μg·kg⁻¹·h⁻¹) significantly decreased the count of total cells, macrophages, and neutrophils and the total protein levels in the BALF from WT and RAGE-/- rats. Post-administration with DEX (10 μg·kg⁻¹·h⁻¹) markedly decreased the count of total cells, macrophages, and neutrophils and the total protein levels in the BALF compared with CLP group in rats (Figure 8C-F). Amelioration roles of DEX in RAGE-/- rats had larger count of total cells, macrophages, and neutrophils and the total protein concentration in the BALF at 24 hours after sepsis when relative to with those in WT rats (Figure 8C-F).
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Role of DEX on CLP-stimulated lung injury in WT and RAGE⁻/⁻ rats

To investigate the effect of DEX on sepsis-stimulated ALI in WT and RAGE⁻/⁻ rats, lung histopathological injury was observed under an optical microscope. As illustrated in Figure 10, lung histopathological injury was significantly ameliorated in RAGE⁻/⁻ rats treated with DEX compared to those treated with CLP alone (Figure 10). These findings suggest that DEX has potential therapeutic benefits in reducing lung injury following sepsis in both WT and RAGE⁻/⁻ rats.

Figure 8. Roles of DEX on the pathological manifestations of CLP-stimulated ALI in WT and RAGE⁻/⁻ rats. Rats were administered continuously with DEX (10 μg·kg⁻¹·h⁻¹) or NS through the femoral vein cannula 30 min after CLP. The lung W/D ratio (A) and MPO activity (B) were evaluated in lung samples obtained at 24 h after CLP. BALF was harvested to evaluate the total protein levels (C) and the count of total cells (D), neutrophils (E), and macrophage (F).

*P<0.05 vs. CLP in WT or RAGE⁻/⁻ rats, #P<0.05 vs. CLP+DEX in WT rats. DEX, dexmedetomidine.
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samples collected from the CLP in WT and RAGE−/− rats exhibited markedly pathological lesion, such as thickened alveolar wall, pulmonary hemorrhage, and infiltration of inflammatory cell. Nevertheless, these manifestations were ameliorated via post-treatment with DEX (Figure 10). Furthermore, amelioration roles of DEX in RAGE−/− rats had severer manifestations in lung samples at 24 hours after sepsis when relative to those in WT rats (Figure 10).

Discussion

CLP triggered systemic inflammatory response represents an enhancement in systemic pro-inflammatory molecules and stimulates lung...
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Inflammatory responses that can result in ALI [19]. Since the manifestations of ALI in humans cannot be completely reproduced, CLP rat model was deemed as the best imitation model of the pathogenesis of CLP-stimulated ALI [20]. Widespread damage and leucocyte activation in ALI induces the increased permeability of the alveolar-capillary barrier [21]. Although some recent progress has been made, there is no useful therapy for ALI. Therefore, it is exceedingly significant to investigate a useful reagent and to explore the pathogenesis which can be applied to CLP-stimulated ALI. In the current research, we showed that DEX has a strong anti-inflammatory capacity which prevents the infiltration of leucocyte and protein leakage.

As one of the most abundant forms of human white blood cells, neutrophil is a key player of the lung inflammatory reaction [22]. Patients with the deterioration of pulmonary function showed that neutrophil is an important factor in lung inflammation [23]. Furthermore, MPO is considered to be a symbol of the presence and activity of neutrophil in the circulation of blood [24]. Our results indicated that the post-treatment of DEX inhibited lung MPO activity. This data indicated that DEX displays a protective effect against CLP-stimulated ALI by attenuating MPO activity.

DEX has been regarded as a highly selective α₂-adrenoceptor agonist, which is mostly applied to different clinical settings for sedative or analgesic requirements [25]. Clinical evidence suggested that DEX will suppress the inflammatory response mediated by TNF-α, IL-1β, IL-6, and so on, in septic patients [26]. In addition, MDA and MPO levels were obviously lower in the CLP+DEX group compared to those in the CLP group. DEX post-treatment provides protection roles and may be involved in its obvious biological activities in CLP-stimulated lung inflammation. Our results suggest that DEX administration may supply protection and may be related to pro-inflammatory mediators’ changes. Therefore, DEX may act as a remedial drug for multiple medical conditions relating to sepsis-associated lung inflammation.

Sepsis is involved in the production of HMGB1 and S100A12 [27]. Inhibition of RAGE by different ligands leads to the activation of NF-κB [27]. Besides, RAGE is considered to be an endothelial cell adhesion receptor and contributes to the recruitment of leukocytes [27]. Engagement of RAGE pathway inhibits the host defense in sepsis and S. pneumonia animal models [27]. HMGB1 and RAGE have been associated with the molecular mechanisms of ALI and adjusted with NF-κB and MAPK [28].

Figure 10. Roles of DEX on pathological manifestation in lung samples in WT and RAGE−/− rats. Rats were infused continuously with DEX (10 μg·kg⁻¹·h⁻¹) or NS through the femoral vein cannula 30 min after CLP. Lung samples (n=10) were subjected to histological measurements at 24 h after CLP. Representative pathological manifestation of lung samples collected from WT and RAGE−/− rats. (A) Sham group in WT rats, (D) Sham group in RAGE−/− rats, (B) CLP group in WT rats, (E) CLP group in RAGE−/− rats, (C) CLP+DEX (10) group in WT rats, (F) CLP+DEX (10) group in RAGE−/− rats (H&E staining, magnification 200×). DEX, dexmedetomidine.
These experimental researches provide evidence to further understand the effect of RAGE signaling in the inflammatory response during medically major infectious diseases, which ultimately promotes better remedies resisting in pathogenic bacteria. Therefore, we suggested that DEX attenuated CLP-stimulated ALI by inhibiting HMGB1 and RAGE activation.

According to previous studies, NF-κB is a main transcription factor and exerts a critical regulatory action in ALI to numerous stimuli [29-31]. NF-κB exists in an inactive form and binds to its inhibitor IκB in the cytosol. Activated NF-κB is associated with immune regulation, inflammatory response, and anti-apoptosis via phosphorylating IκB and shifting into the nucleus [32, 33]. Notably, previous researches indicated that the suppression of NF-κB pathway was recommended as a potential useful remedial tactics for ALI [34, 35]. Therefore, we explored whether DEX could regulate NF-κB signaling pathway. Our results confirmed that DEX inhibited the activation of NF-κB via reducing the phosphorylation of p65 and I-κBα. MAPK signaling pathway, including ERK, JNK, and p38 MAPK, is important for the administration of pro-inflammatory mediator genes and inflammatory responses in ALI [15, 28]. Thus, we investigated whether DEX exerts its anti-inflammatory effect through the MAPK signaling. Our data showed that DEX suppressed the activation of MAPK in CLP-stimulated ALI. The present study demonstrated that CLP resulted in the activation of NF-κB and MAPK, and DEX post-treatment attenuated these features. These findings showed that the control of CLP-stimulated ALI though DEX might be through the repress of NF-κB and MAPK activation.

Certain study limitations do exist. First, pulmonary gas exchange was not assessed even though a histologic change in the lung correlates with clinical lung injury. Secondly, the question of whether short term reduction in cytokine activation will translate to a sustained outcome of reduced inflammation once the DEX is metabolized in eventual clinical utility remains unanswered. Thirdly, our study only focuses on the activation of NF-κB and MAPK signal. Thirdly, the question of whether other pathways, e.g. PI3K/Akt and HO-1, participate in mediating the lung protective effects of DEX remains unanswered. Fourthly, this study did not use the antagonist of the α₂-adrenergic receptors. The roles of the α₂-adrenergic receptors on mediating the beneficial roles of DEX against sepsis-stimulated ALI remain to be determined.

In summary, our results indicated the alternatives of applying DEX as an impactful reagent with preventing CLP-stimulated ALI. The molecular mechanism of DEX is via downregulating HMGB1 and RAGE expression and reducing the activation of NF-κB and MAPK. Further researches have contributed to explore whether DEX is a potential agents to attenuate the occurrence of CLP-stimulated ALI.

Acknowledgements
This work was supported by the Zhejiang Province Traditional Chinese Medicine Research Fund (NO. 2012ZA048).

Disclosure of conflict of interest
None.

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