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Protective Effect of 20-Hydroxyecdysterone against Lipopolysaccharides-Induced Acute Lung Injury in Mice

Guoying Song[1], Xi-Chao Xia[1,2*], Ke Zhang[2], Yuhong Ma[3], Ruixue Yu[2], Bingjie Li[2], Mengbo Li[2], Xiaozhu Yu[2], Junfeng Zhang[2] and Shipeng Xue[3]

[1]Library of Pingdingshan University, Pingdingshan, 467000, Henan Province, China

*[*2]Basic Medical College, Pingdingshan University, Pingdingshan, 467000, Henan Province, China*

[3]Basic Medicine College, Nanyang Medical University, Nanyang, 473041, Henan Province, China.

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ABSTRACT

Aim: To investigate the protective effect of 20-hydroxyecdysterone (20E) against lipopolysaccharide-induced acute lung injury (ALI) in mice.

Materials and methods: Mice were respectively administrated a single intratracheal instillation with normal saline (normal group) and 4 mg/kg lipopolysaccharide (LPS) (LPS cohort). Next, animals of LPS cohort were subsequently divided into the model group, the control group, the low-dose group of 20E, middle-group of 20E and high-dose group of 20E. Histological changes of lung were examined by hematoxylin and eosin. Expression levels of tumor necrosis factor-α (TNF-α), interleukin-2 (IL-2), IL-6, IL-8, IL-4 and IL-10 were determined by real-time PCR and enzyme-linked immuno-sorbent assay, respectively.

Results: 20E treatment could result in a decrease of lung damage in the ALI mice. In the 20E treated groups, expression of TNF-α, IL-2, IL-6 and IL-8 was significantly inhibited compared with that model group, respectively. In addition, expression of IL-4 and IL-10 was induced in the 20E treated groups, respectively.

Conclusion: The results suggest that 20E play a protective-role in ALI of mice probably by inhibiting the pro-inflammory cytokine expression and enhancing the anti-inflammatory cytokine expression.

Keywords: 20-hydroxyecdysterone, Acute lung injury, Protective effects, Pro-inflammation cytokines, Anti-inflammatory cytokines

INTRODUCTION

Acute lung injury (ALI) is considered as a major health problem for elderly population and characterized by activation of the pulmonary endothelium, disruption of the endothelial and alveolar epithelial barriers, and increase of the microvascular permeability [1]. Lung, a crucial airway for pathogens into the body, is widely contacted with a large number of micro-organisms those can cause acute inflammation and ALI [2]. Evidences have demonstrated that that inflammation is closely associated with the occurrence of ALI. In the inflammation, over-production of inflammatory cytokines are involved into injury of lung tissues [3,4]. Lipopolysaccharides (LPS) presented in cell wall of gram-negative bacterium can cause ALI [5]. Lung injury induced LPS is close link with release of macrophage-derived pro-inflammatory cytokines as well as anti-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1, IL-2, IL-6, IL-8, IL-4, IL-10 and interferon-γ [6,7].

Ecdysterone was first isolated from insect and plays a key role in the molting, development and reproduction of animals. Afterward, it was confirmed that the active component of 20-hydroxyecdysterone (20E) is widespread

Corresponding author: Xi-Chao Xia, Associate Chief, Basic Medical College, Pingdingshan University, Pingdingshan 473061, China Henan, P. R. China; Tel: +86 0377 63526123, E-mail: xiaxichao8336@163.com

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across the plant kingdom and sustain higher concentration in them [8,9]. It has demonstrated that 20E have remarkable pharmacological properties in mammals, including lowering cholesterol levels and blood glucose, stimulating protein synthesis, promoting carbohydrate and lipid metabolism, and inducing stem cell differentiation [10]. Recently, it has been suggested that 20E has obvious antioxidant activity by radical scavenging tests *in vitro* and *in vivo* [11]. Meanwhile, our study indicates that 20E can function as an important player in counteracting memory deficits in rats with diabetes, possibly through enhancing the anti-oxidative ability in the brain [12]. But, little is known about the effect of 20E on the ALI. Thus, the current study was designed to shed light of the effect of 20E on the ALI.

MATERIALS AND METHODS

Reagents

The reagents used in the experiment and their sources were as follows: lipopolysaccharide (LPS) and 20E were obtained from Sigma-Aldrich Co (St. Louis, MO, USA), dexamethasone-21-acetate (Dex) and sodium pentobarbital were purchased from Aladdin (Shanghai, China). PCR primers were synthesized by Sangon Biological Engineering Technology Company (Shanghai China). Trizol reagent and RT-PCR kit including RTase M-MLV, dNTP mixture, Taq DNA polymerase, RNase inhibitor and SYBR Premix Ex TaqTM were obtained from Takara (Dalian, China). Enzyme-linked immuno-sorbent assay (ELISA) kit of mice was purchased from BOSTER (Wuhan, China). All other chemicals were of reagent grade.

Animals

The experimental study was performed following approval from the Pingdingshan University Animal Care and Ethics Committee. All experimental and surgical procedures were conducted by the Pingdingshan University Surgical Application and Research Center. Animal cultures were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male ICR mice (20-24 g) were obtained from Henan Laboratory Animals Center for Medical Science and Research. The animals were fed standard laboratory chow (Wuhan University Center for Animal experiment, Wuhan, China) with free access to water and housed individually at a controlled temperature of 19-25℃ with a 12:12 h light/dark cycle.

Animals were randomly divided in six groups (n=15) including normal group, model group, control group (Dex group), low-dose group of 20E, middle-dose group of 20E and high-dose group of 20E. Except normal group, mice in other groups were anaesthetized with an intraperitoneal injection of 40 mg/kg of sodium pentobarbital and treated with a single intratracheal instillation with 4 mg/kg LPS. Based on previous study [12], animals in the low-dose group of 20E, middle-dose group of 20E and high-dose group of 20E received 20E solution at a concentration of 0.1 mg/kg, 1 mg/kg and 10 mg/kg, respectively, and Dex group received Dex at 10 mg/kg. Times of animal treatment were 1, 12, 24 and 36 h before LPS instillation and 12, 24 and 36 h after LPS challenge. Animals from normal group and model group were treated with the same volume of normal saline at corresponding times. The mice were killed by cervical dislocation. Left and right part of lungs were separated, harvested, immediately frozen in liquid nitrogen, and then stored at -80℃ until used.

Histological analysis of lung

Left lungs were fixed in 10% formaldehyde and embedded in paraffin. Specimens were cut with 4 μm thick sections those were stained by routine hematoxylin and eosin (H&E) for histological analysis. Sections were analyzed under light microscope (Olympus BX50, Barcelona, Spain).

Real-time PCR assay for TNF-α, IL-2, IL-6, IL-8, IL-4 and IL-10 mRNA level in the lung

Total RNA was extracted from lung tissues using the Trizol reagent according to the manufacturer's protocol. Quality of RNA was monitored by 1.2% agarose gel electrophoresis. First-strand cDNA was synthesized using M-MLV reverse transcriptase.

To determine levels of TNF-α, IL-2, IL-6, IL-8, IL4 and IL10 transcript derived from lungs, real-time quantitative PCR was performed following the manufacture instruction of SYBR Premix Ex TaqTM. Primers used in this study were listed in **Supplemental Table 1**. PCR was performed using an ABI 7500 Real-Time Detection System (Applied Biosystems, USA) in accordance with manufacture. Based on standard curve constructed, the expression levels of TNF-α, IL-2, IL-6, IL-8, IL-4 and IL-10 were calculated by 2-△△CT.

Table 1. Primers used in the experiment.

Primer	Sequence (5′–3′)
TNF-α-F	ACTGGGCATCCTTTGTCGG
TNF- α-R	CTGTTGGTGAAGAGGTGCGG
IL-2-F	ATCGTGGGTGGCGTTGAG
IL-2-R	GTTCTGGCGGATGTTGTGG
IL-6-F	CTCAGGACGGTAAGTGTCGCTT
IL-6-R	TGGTAGAACTGGAAGGACGGCT
IL-8-F	GGGTTCCTTACTCCGCTTTCG
IL-8-R	GTCAGTGCCTTGGTTCAGGTCG
IL-4-F	TTGAAGAAAGCAGACGCCCC
IL-4-R	TGGCAGGTTGAGTGGCAGC
IL-10-F	ATCGCAACCCATCAAACCTCG
IL-10-R	ACAATCGTCGCAGACACGGT
β-actin-F	CATCCACGAGACCACCTACAAC
β-actin-R	GAAATACTGCCTCGCTCCCTC

ELISA assay for the TNF-α, IL-2, IL-6, IL-8, IL4 and IL10 protein levels in the lung

Right lungs were weighted, perfused with chilled normal saline, then cut into small pieces, placed in 0.2 M phosphate buffer (pH 7.4) and homogenized using homogenizer to obtain 20% homogenate. Resultant homogenate was centrifuged (5000x g for 15 min, 4℃) and the supernatant was sub packaged to sterile eppendorf tubes. Levels of TNF-α, IL-2, IL-6, IL-8, IL-4 and IL-10 in lung were measured by ELISA kits. All the procedures were performed in accordance with the manufacturer's instructions.

Statistical analysis

Original data were analyzed by Statistical Package for Social Sciences software (version 14.0) (SPSS, Inc., Chicago, IL). Results were expressed as mean ± SEM. Multiple sets of data comparison were carried out by one-way analysis of variance (ANOVA). Comparison of data between groups were treated by least significance difference (LSD) combined with Tamhane's way. Values with $P<0.05$ were considered as statistically significant.

RESULTS

Histopathologic analysis

Histopathological examination of normal animals showed normal cellular architecture with distinct alveolar cell **(Figure 1A)**. The lung sections of model group exhibited an obvious damage characterized by focal hemorrhage, distortion and alveolar thickening **(Figure 1B)**. Compared with that of model group, injury derived from LPS was ameliorated by Dex treatment and different-dose 20E treatment **(Figures 1C-1F)**. Furthermore, obvious protecting-role was observed in Dex and high-dose group of 20E **(Figures 1C and 1F)**.

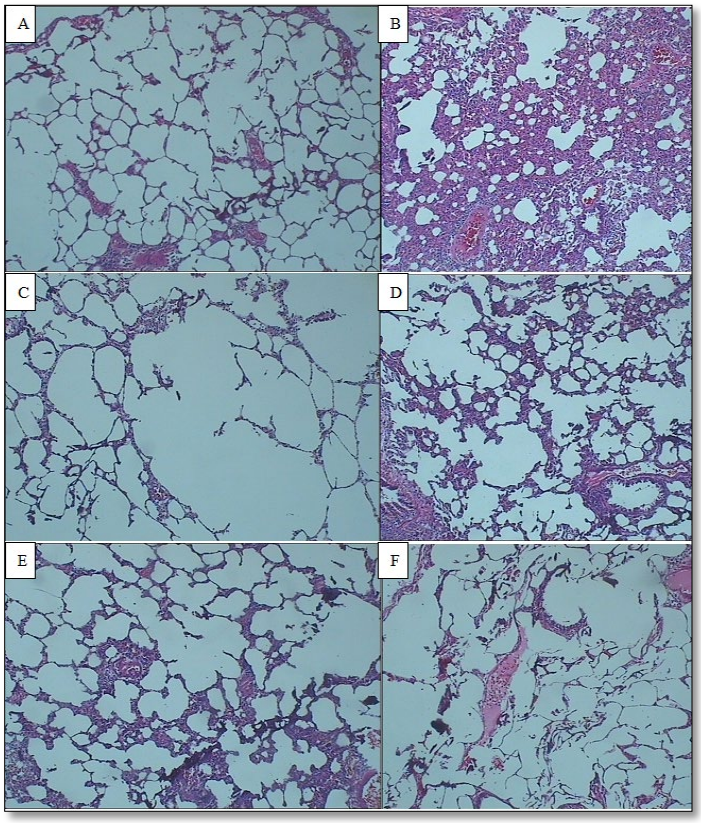

Figure 1. Effects of 20E on LPS-induced lung histopathological change (hematoxylin and eosin stain, magnification: 400). (A) Normal group, (B) LPS group, (C) Dex group, (D) Low-dose group of 20E, (E) Middle-dose group of 20E, (F) High-dose group of 20E.

Effects of 20E on the TNF-α, IL-2, IL-6, IL-8, IL-4 and IL-10 mRNA level in the lung

Compared with that of normal group and Dex group, the mRNA levels of TNF-α, IL-2, IL-6 and IL-8 were significantly increase in the model group **(Figure 2)**. Administration of different-dose of 20E could result in a dose-dependent decline of TNF-α, IL-2, IL-6 and IL-8 level. In the high-dose group of 20E, mRNA level of TNF-α, IL-2, IL-6 and IL-8 decreased 58.1% ($P<0.01$), 61.1% ($P<0.01$), 52.1% ($P<0.01$) and 47.7% ($P<0.01$) compared with that of model group **(Figure 2)**, respectively.

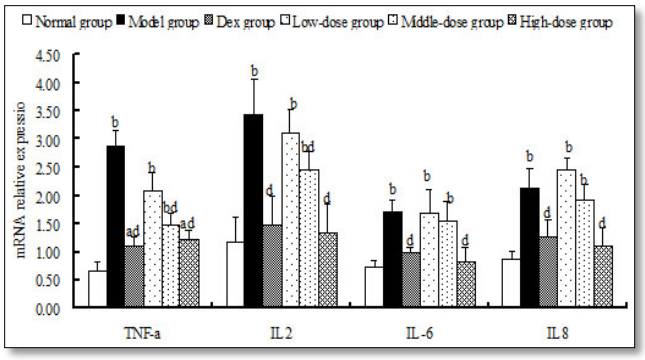

Figure 2. Effects of 20E on the TNF-α, IL-2, IL-6 and IL-8 mRNA level in the lung.
Note: n=5/each tissue. [a]P<0.05, [b]P<0.01 vs control group at normal group. [c]P<0.05, [d]P<0.01 vs control group at the model group

Compared with that of normal group and Dex group, mRNA levels of IL-4 and IL-10 were significantly decreased in the model group **(Figure 3)**. But, in high-dose group of 20E, mRNA level of IL-4 and IL-10 increased 80.6% (P<0.01) and 116.8% (P<0.01) in contrast with that of model group, respectively **(Figure 3)**.

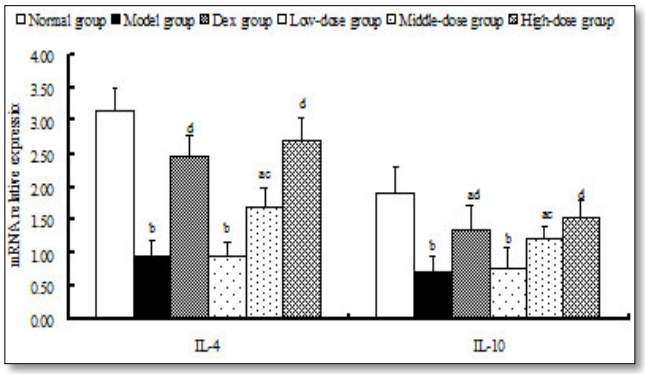

Figure 3. Effects of 20E on the IL-4 and IL-10 mRNA level in the lung.
Note: n=5/each tissue. [a]P<0.05, [b]P<0.01 vs control group at normal group. [c]P<0.05, [d]P<0.01 vs control group at the model group

Effects of 20E on the TNF-α, IL-2, IL-6, IL-8, IL-4 and IL-10 expression in the lung

Compared with that of normal group and Dex group, in a significant increase of TNF-α, IL-2, IL-6 and IL-8 level was observed in the model group (P<0.01) **(Table 2)**. Expression levels of TNF-α, IL-2, IL-6 and IL-8 showed a decline trend in different 20E treated groups compared with that of model group. In the high-dose group of 20E, level of TNF-α, IL-2, IL-6 and IL-8, respectively decreased 48.3% (P<0.01), 66.3% (P<0.01), 26.9% (P<0.05) and 45.7% (P<0.01) (Table 2), but the level of IL-4 and IL-10 increased 1.9 times (P<0.01) and 2.9 times (P<0.01) in contrasted with that of model group **(Table 2)**.

Table 2. Effects of 20E on the TNF-α, IL-2, IL-6, IL-8, IL-4 and IL-10 protein level in the lung.

Group	TNF-α	IL2	IL-6	IL8	IL-4	IL-10
Normal group	0.64 ± 0.17	1.17 ± 0.45	0.71 ± 0.13	0.88 ± 0.14	3.15 ± 0.35	1.89 ± 0.41
Model group	2.89 ± 0.27^{b}	3.43 ± 0.73^{b}	1.70 ± 0.21^{b}	2.13 ± 0.34^{b}	0.93 ± 0.27^{b}	0.70 ± 0.25^{b}
Dex group	1.11 ± 0.14^{ad}	1.48 ± 0.51^{d}	0.98 ± 0.11^{d}	1.26 ± 0.31^{d}	2.45 ± 0.32^{d}	1.33 ± 0.38^{ad}
Low-dose group	2.08 ± 0.33^{b}	3.12 ± 0.40^{b}	1.69 ± 0.40^{b}	2.43 ± 0.23^{b}	0.94 ± 0.22^{b}	0.76 ± 0.31^{b}
Middle-dose group	1.47 ± 0.21^{bd}	2.44 ± 0.34^{bd}	1.55 ± 0.33^{b}	1.91 ± 0.30^{b}	1.69 ± 0.31^{ac}	1.21 ± 0.18^{ac}
High-dose group	1.21 ± 0.16^{ad}	1.34 ± 0.51^{d}	0.81 ± 0.27^{d}	1.11 ± 0.29^{d}	2.71 ± 0.34^{d}	1.53 ± 0.27^{d}

Note: n=5/each tissue. [a]P<0.05, [b]P<0.01 vs control group at normal group. [c]P<0.05, [d]P<0.01 vs control group at the model group

DISCUSSION

In the current study, administration of 20E resulted in a decrease of the alveolar cell damage and decline of the TNF-α, IL-2, IL-6 and IL-8 expression in the ALI mice, which suggested a protective-role of 20E against LPS injury is closely associated with suppression of TNF-α, IL-2, IL-6 and IL-8 production. Inflammation cascade is initiated by the innate immune system, in which TNF-α, IL-2, IL-6 and IL-8 play a key role in the inflammatory response [13]. Generally, inflammatory response is taken as a direct pathogenic-signal in ALI [14]. It has been demonstrated that the over-expression of these pro-inflammatory mediators contribute to ALI or lung cancer [15]. Thus, down-regulation of TNF-α, IL-2, IL-6 and IL-8 expression derived from 20E is beneficial in reducing the lung damage of mice. Similar studies are also reported in ALI cases. Administration of dexamethasone-21-acetate can dramatically reduce the concentration of cytokines TNF-α, IL-2, IL-6 against the damage of LPS on lung [16]. In addition, 1α, 25-dihydroxyvitamin D3 and vitamin D3 analogue can attenuate the innate immunity response in a mouse model of ALI through suppression of IL-8 production [17,18]. Notably, wide applications of steroids are observed in medicine besides dexamethasone-21-acetate, 1α, 25-dihydroxyvitamin D3 and vitamin D3 analogue [19-21]. Thus, these studies should open a new road to explore of 20E functions.

Further research showed the expressions of IL-4 and IL-10 were obviously induced in the middle-dose group of 20E and high-dose group of 20E, suggesting multiple targets acted on by 20E are involved in the protection event against ALI. Inflammatory response of organs is coordinated by the interplay of anti-inflammatory cytokines and pro-inflammatory cytokines, where a low concentration of pro-inflammation cytokines and/or a high concentration of anti-inflammatory cytokines contribute to protection of tissues and cells against damage and sustain homeostasis of body [22,23]. Meanwhile, the IL-4 and IL-10, as anti-inflammatory cytokines, their expression levels that is induced by 20E t is helpful in inhibiting the expression of pro-inflammatory cytokines [24,25]. For example, IL-10 works as a key player to reduce the release of pro-inflammatory mediators during ALI, such as IL-6 and inducible nitric oxide synthase [24,26]. Therefore, we postulate that the relative level of anti-inflammatory cytokines and inflammatory cytokines play a crucial role in organs against injury [22,23].

In the current study, protective-role of 20E against ALI in the mice showed a dose-dependent effect suggesting that different targets acted on by 20E are likely involved in inflammation of ALI. In the insect and crustacean, 20E, as an important hormone that plays a key role in regulating development, growth, reproduction and innate immunity by acting on different targets [27,28]. Considering here, the study of TNF-α, IL-2, IL-6 and IL-8, IL-4 and IL-10 temporal and spatial expressions derived from 20E activation is an interesting work. Meanwhile, regulation of TNF-α, IL-2, IL-6 and IL-8, IL-4 and IL-10 derived from different-dose 20E treatment groups is complex event. Therefore, great efforts are required to elucidate the mechanisms of 20E in ALI.

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CONFLICT OF INTEREST

We declare that there is no conflict of interest.

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