

Immunologic Mechanism of Patchouli Alcohol Anti-H1N1 Influenza Virus May Through Regulation of the RLH Signal Pathway In Vitro

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Abstract Patchouli alcohol (PA) is a kind of methanol extracted from traditional Chinese medicine *Pogostemonis Herba*. Our research aimed to observe the anti-influenza virus role of PA in vitro. 16HBE (human respiratory epithelial cell) was infected by H1N1 (A/FM1/1/47) to set the cell model. Then the 16HBE was co-cultivated with three kinds of immune cells: dendritic cells, macrophages, and monocytes, PA (the concentration is 10 µg/mL) was added as a treatment intervention for 24 h. The immune cells and the supernate were collected for RT-PCR and ELISA detection related to RLH (RIG-1-like helicases) pathway. Results showed that the IL-4 and IFN-γ in supernate were increased after H1N1 infection, and the PA treatment suppressed the expression of cytokines and the mRNA of RLH pathway. PA anti-influenza virus may through regulate the RLH signal pathway.

Keywords Patchouli alcohol (PA) · Anti-influenza · H1N1 · Dendritic cells (DCs) · Macrophage · RLH

Introduction

Influenza virus can cause pandemic disease around the world, and seriously threaten human's health. According to statistics, over 10 % people on earth get the flu each year [1]. At present, human cannot control emergence and pandemic of the flu [2]. The current therapy of flu cannot acquire ideal effects. The current therapy is to use M2 protein inhibitor such as amantadine and neuraminidase inhibitor oseltamivir. But the viruses have three types and each type has several subtypes, and the antigenic drifts frequently, these affected the effects of treatment and vaccine [3].

Recent years, many scientists focus on Traditional Chinese Medicine to screen the anti-influenza virus drugs. Patchouli alcohol (PA) is a monomer extract from traditional Chinese medicine *Pogostemonis Herba*. PA has many pharmacological effects. Research showed that PA was a fragrant material used in decorative cosmetics and fine fragrances and so on [4]. PA can function as anti-inflammation drug, inhibiting the ear edema induced by xylene and suppressing the expression of inflammatory factor such as NF-κB, IL-1β in mice [5]. PA can also enhance the host immune responses against the influenza virus in mice [6].

RIG-1-like helicases (RLH) are involved in the induction of Type I interferon in response to viral infection [7]. It is a critical cytoplasmic double stranded RNA pattern receptor required for innate immune activation in response to viral infection. Activation of RIG-1 leads to type I interferon and inflammatory cytokine production [8]. RNA

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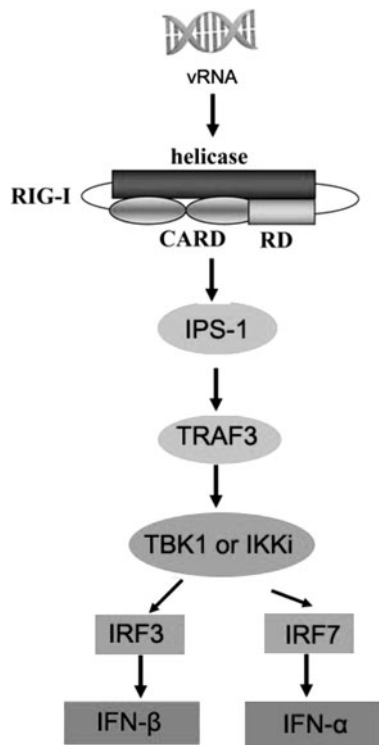


Fig. 1 Flowchart of RLH signal pathway

virus such as Influenza virus (IFV) generates dsRNA for replication in infected cell, RIG-I was implicated in the recognition of the viral dsRNA, it can also discriminates virus and host RNAs. RIG-I associate with IPS-1 (IFN promoter stimulator), and overexpression of IPS-1 induces the IFN production [9]. The production of IFN will play the roles of anti-influenza virus (Fig. 1).

In order to screen the effective anti-influenza monomer, our study was designed to observe the effect of PA anti-influenza role in vitro through the RLH pathway, hoping to reveal the mechanism, and promote the investigation of new anti-influenza drugs.

Materials and Methods

Cell Culture, Immune Cell Isolation, and Virus Inoculation

Cell line 16HBE was provided by Xiangfu Biotech Corporation in Shanghai, China.

The cells were cultured in RPMI-1640 containing 10 % heat-inactivated FBS (fetal bovine serum) at 37 °C and 5 % CO₂ incubator. Immune cells were isolated from umbilical cord blood (Guangzhou Overseas Hospital). Three types of immune cell were isolated from umbilical cord blood. Monocytes were directly isolated used

Lymphoprep. Mature DCs were stimulated by rhGM-CSF, rhIL-4 and rhTNF- α . Macrophages were stimulated by rhGM-GSF for 7 days. Virus of H1N1 influenza (A/FM1/1/47) was provided by Institute of Tropical Diseases, Southern Medical University of China.

Drug and its Original Ingredient

Patchouli alcohol and standard substance (molecular formula: C₁₅H₂₆O) were purchased from TCM Institute of Chinese Materia Medica, Nanjing. Patchouli alcohol was obtained from volatile and colorless solid, and the configuration was confirmed by X-ray (see Fig. 2). According to literature, the Patchouli alcohol dissolved in MeOH in which the concentration is 10 μ g/mL, and showed 99.8 % inhibition of influenza virus and low cytotoxicity, so our experiment used the concentration of 10 μ g/mL. According to literature, the Patchouli alcohol showed 99.8 % inhibition of influenza virus and low cytotoxicity when dissolved in MeOH in the concentration of 10 μ g/mL [10].

Cell Model Establishment and Experimental Group

We used the “Millicell culture insert apparatus” to co-culture the cells, it’s a special culture bottle, and the up and down of this apparatus was separated by a semipermeable membrane. 16HBEs were underneath and the immune cells were above, between them was a free-exchanged membrane. After co-culture 12 h, 100 \times TCID₅₀ of H1N1 (A/FM1/13) was added into the 16HBE to establish the infected cell model, after infected 2 h, 10 μ g/mL of PA was added, and then cultured for 24 h. The experiment was divided into nine groups: DC with infected and uninfected (16HBE + DC + Virus and 16HBE + DC), macrophage with infected and uninfected (16HBE + macrophage + Virus and 16HBE + macrophage), monocyte with infected and uninfected (16HBE + monocyte + Virus and 16HBE + monocyte), PA was added to the three infected groups as treatment intervention (16HBE + DC + Virus + PA, 16HBE + macrophage + Virus + PA, 16HBE + monocyte + Virus + PA).

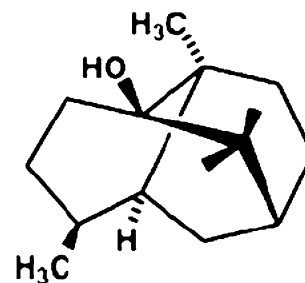


Fig. 2 Chemical structure of Patchouli alcohol (C₁₅H₂₆O)

RT-PCR Detection of the RLH Pathway

Three types of immunocytes were collected after co-cultured and treatment intervention for PCR detection. The RLH pathway has many genes, we chose part of them to detect. The total RNA of these immunocytes was extracted by Trizol reagent (Tiangen Biotech Co., LTD, Beijing), and the cDNA reverse transcription kit and Real Master Mix also purchased from the above company for RT-PCR detection. The primers for PCR detection were designed and synthesized by Generay Biotech CO., Shanghai. The amplify times were read by qPCR instrument (Bio-Rad, USA), and each gene expression was analyzed by $2^{-\Delta\Delta Ct}$ method. $\Delta Ct = Ct(\text{target gene}) - Ct(\text{reference gene})$, and $\Delta\Delta Ct = \Delta Ct(\text{treatment group}) - \Delta Ct(\text{control group})$. The data were described by (mean \pm SD), and analyzed by SPSS 13.0 for windows.

Elisa Assay of the IFN- γ and IL-4 in the Cell Line Supernatant

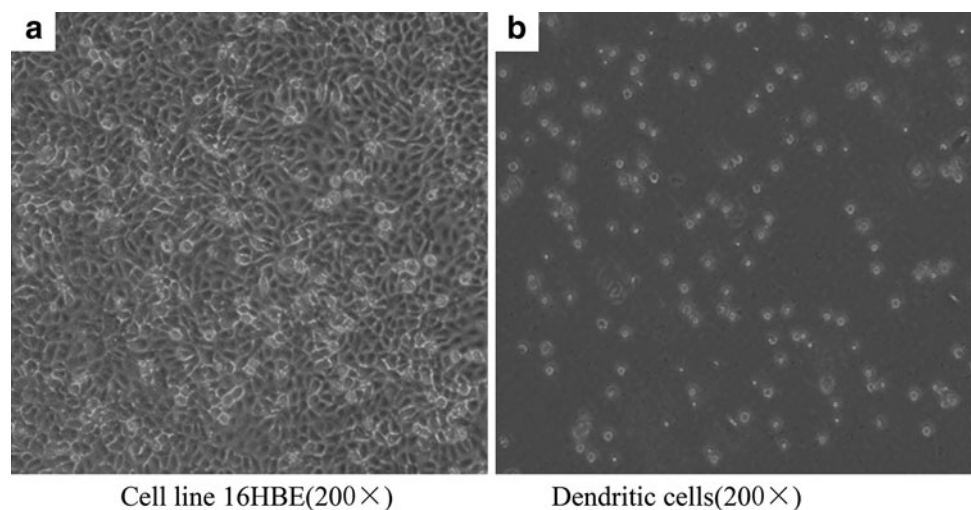
The supernatant was collected after co-cultured and treatment intervention by PA for 24 h, and the IFN- γ and IL-4 of the supernatant solution were assayed by ELISA (enzyme-linked immunosorbent assay). The ELISA kit was purchased from ExCell Biology Incorporated, Shanghai. The absorbance was assayed at 450 nm using M680 microplate reader (Bio-Rad, USA).

Result

Cell Culture of 16HBE and Immune Cells

16HBE is the cell line, it was cultured at 37 °C and 5 % CO₂ incubator, the cell culture medium is RPMI-1640

Fig. 3 **a** Human respiratory epithelial cell, resuscitated from cell lines, eighth generation cells. **b** Immune cell DCs extracted from human cord blood, induced by 50 ng/mL rhGM-CSF for 7 days. DCx immunophenotyping was determined by FCM (Flow Cytometry Method). During this process, we detected the expression of CD83, CD86, and HLA-DR. **a** Cell line 16HBE ($\times 200$). **b** Dendritic cells ($\times 200$)



containing 10 % heat-inactivated FBS (Fig. 3). DC and Macrophages must be stimulated by rhGM-GSF and rhIL-4 (Fig. 3).

RLH Pathway Detected by RT-PCR

We choose part gene of RLH pathway to detect, they are RIG-1, IPS-1, Irf-3, and Irf-7. In our experiment, after H1N1 infecting, the RLH pathway in immune cells was up-regulated, especially in DCs and macrophages. The mRNA expression of the above six genes were significantly enhanced. PA treatment groups showed that the expression of these genes were down-regulated (see Fig. 4).

IFN- γ and IL-4 in Cell Line Supernatant Assayed by ELISA

After infection procedure, the IFN- γ and IL-4 both increased significantly than the uninfected groups. Group in which 16HBE was co-cultured with immune cells showed anti-viral effects, they can reduce the level of these inflammatory factors, especially DC, and it has the strongest effect to reduce these factors. PA treatment can reduce the factors to anti-inflammation, groups which add PA showed that the IFN- γ and IL-4 were lower than other untreated infected groups. It means PA can reduce inflammatory response when influenza virus was invading (see Fig. 5).

Discussion

We should keep our memories of influenza virus because unanticipated outbreaks of influenza virus will repeatedly challenge human's health. Threat of IFV is real exists and the effective anti-virus drugs must be developed [11]. At

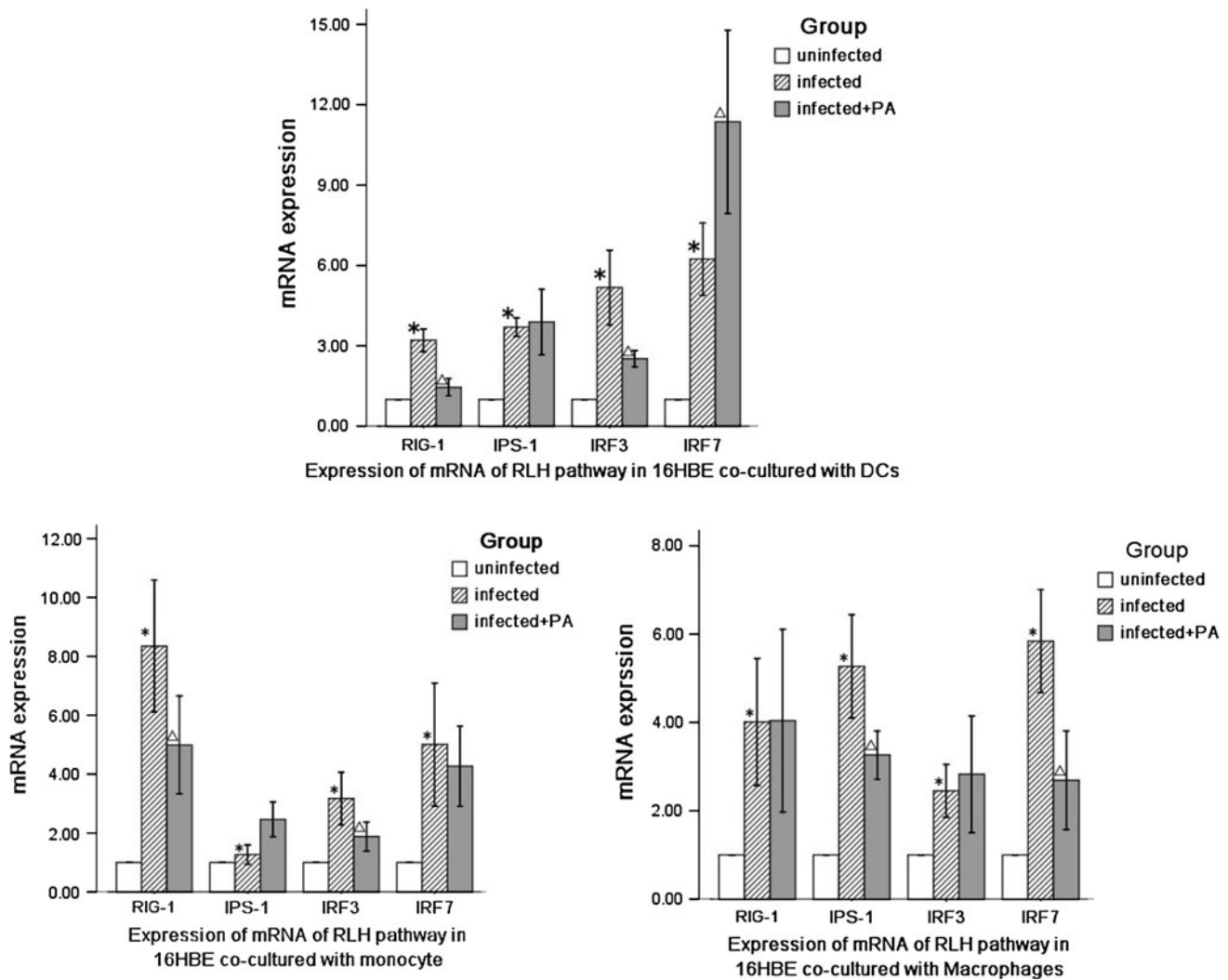


Fig. 4 Expression of mRNA in of RLH pathway in 16HBE co-cultured with immune cells detected by RT-PCR($2^{-\Delta\Delta}$). **a** RIG-1 expression of each groups, in DC and monocyte groups, PA can down-regulate the raised RIG-1 gene expression; **b** IPS-1 expression

of each groups, in monocyte group, virus up-regulate the IPS-1 expression; **c** IRF-3 expression of each groups, the changes were as **a**; **d** IRF-7 expression of each groups.(VS uninfected group, $*P < 0.05$; VS infected group, $\Delta P < 0.05$)

present, there is no specific medicine to anti-influenza, and the current drugs are seriously drug-resistant and have several side effects [12]. Facing the unknown outbreak of influenza, to develop more useful antibiotic becomes imperative.

Screen from the Traditional Chinese Medicine or Herbs to find an antibiotic is one of the research ideas to design the new drugs, such as Quercetin and Andrographolide. Research showed that the monomer Quercetin might be a potential substance to anti-viral and anti-inflammatory agents [13], and it has been demonstrated that it possessed a strong anti-influenza A/WS/33 virus by indirectly interacted virus particles [14]. The extract of Andrographolide also has a defended effect when influenza virus invading [15, 16].

Patchouli alcohol (PA, $C_{15}H_{26}O$) was a monomer extracted from Chinese Medicine Pogostemon cablin. Research showed that this monomer can directly anti-influenza virus in vitro [10]. Our experiment showed that, the inflammatory factors $IFN-\gamma$ and $IL-4$ in supernatant of co-culture cells system were elevated after infection by H1N1, and PA could reduce these factors in treatment groups. It suggested that PA was anti-inflammatory by down-regulating the inflammatory factors.

RLH (RIG-I-like helicases) pathway induces the antiviral innate immune response [17]. Research found that RIG-1 induce a type-1 IFN in vitro when virus invade, and RIG-1 has the function of recognition of viruses. Viral RNA in the viruses in the molecular signature was recognized by RIG-1 [18]. IPS-1 interacts with TRAF3 to activate TBK-1 [19],

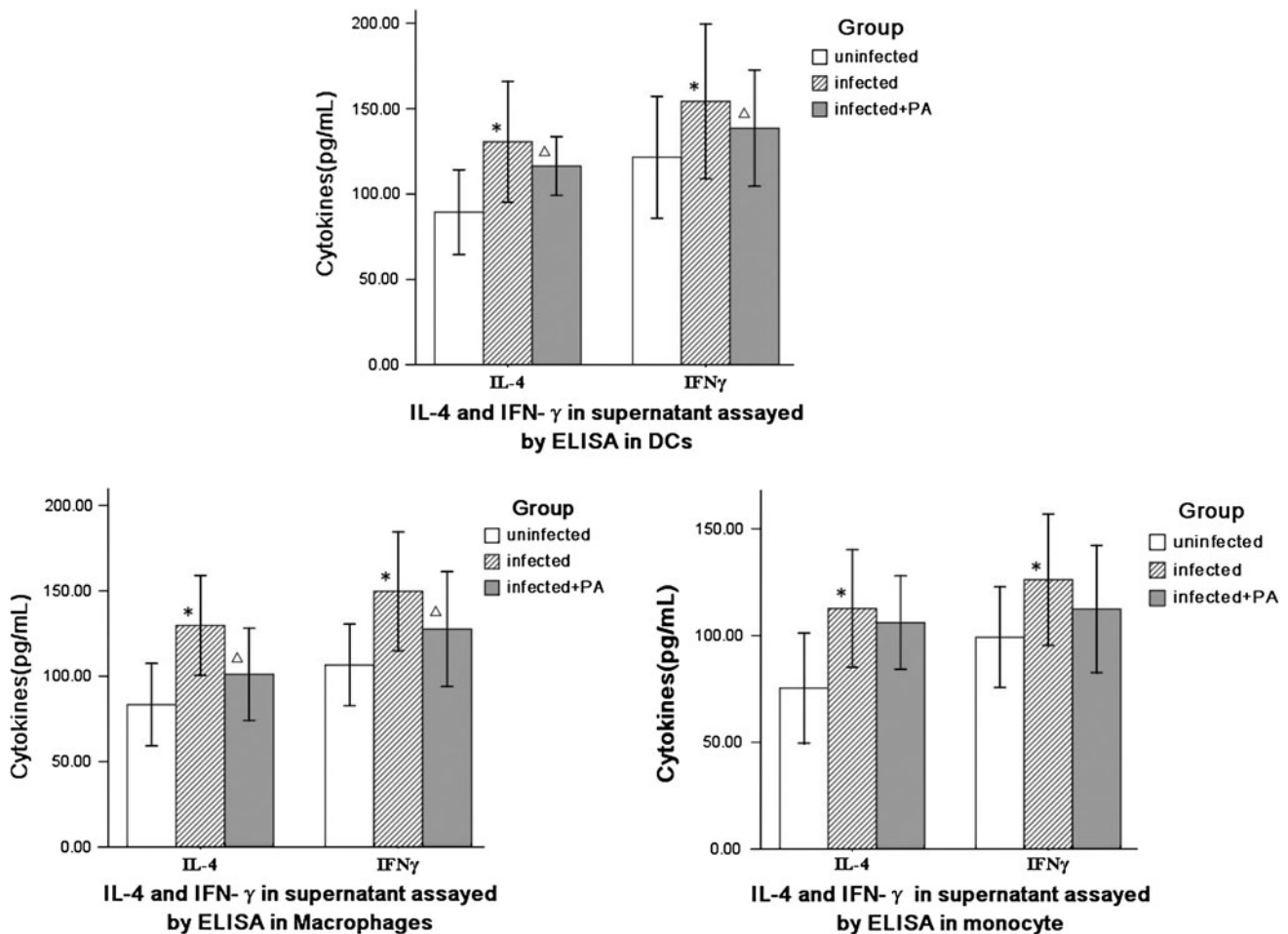


Fig. 5 IL-4 and IFN- γ in supernatant assayed by ELISA (VS uninfected group, * $P < 0.05$; VS infected group, $\Delta P < 0.05$)

and TBK1 phosphorylate IRF3 and IRF7, and then induce the IFN- α response [20]. IPS-1 also suggests a role for inducing the virus to apoptosis. Our experiment showed that RLH pathway was up-regulated when virus infected the mRNA expression of RIG-1, TBK-1, and IPS-1 was all up-regulated. It means that virus invasion promote the cell to produce more IFN- α to play anti-virus role. PA treatment down-regulate the RLH pathway, it means the PA can enhance the ability of innate immune recognition and response, and restrain the expression of IFN- α inflammatory factor to attenuate the inflammatory responses.

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