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Xijiao Dihuang Decoction combined with *Yinqiao* Powder reverses influenza virus-induced F-actin reorganization in PMVECs by inhibiting ERM phosphorylation

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Abstract *Objective:* It has been documented that ezrin/radixin/moesin (ERM) phosphorylation by the p38 mitogen-activated protein kinase (MAPK), Rho/ROCK, and protein kinase C (PKC) pathways leads to filamentous actin (F-actin) reorganization and microvascular endothelial cell hyperpermeability. In this study, we investigated the effects of *Xijiao Dihuang* Decoction combined with *Yinqiao* Powder (XDY) on influenza virus (IV)-induced F-actin restructuring and ERM phosphorylation regulated by the Rho/Rho kinase 1 (ROCK), p38 MAPK, and PKC signaling pathways in pulmonary microvascular endothelial cells (PMVECs).
Methods: Serum containing XDY (XDY-CS; 13.8 g/kg) was acquired using standard protocols for serum pharmacology. Primary PMVECs were obtained from male Wistar rats and cultured. After adsorption of IV A (multiplicity of infection, 0.01) for 1 h, medium with 20% XDY-CS was added to the PMVECs. The distributions of F-actin and phosphorylated ERM were determined by confocal microscopy, and F-actin expression was measured by flow cytometry. The expression levels of ROCK1, phosphorylated myosin phosphatase target-subunit (p-MYPT), phosphorylated MAPK kinase, phosphorylated p38 (p-p38), phosphorylated PKC (p-PKC), and phosphorylated ERM (p-ERM) were determined by western blotting.
Results: F-actin reorganization in IV-infected PMVECs was reversed by XDY-CS treatment, which was accompanied by reduced p-ERM production. The p-ERM protein accumulated at plasma membrane of PMVECs infected with IV, which was also inhibited by XDY-CS treatment.

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In addition, XDY-CS treatment drastically reduced the levels of p-p38, ROCK1, p-MYPT, and p-PKC induced by IV infection in PMVECs.
Conclusion: These results show that XDY-CS inhibited influenza-induced F-actin reorganization in PMVECs by down-regulating p-ERM expression via inhibition of the Rho/ROCK, p38 MAPK, and PKC pathways. In conclusion, XDY could reduce the damage to endothelial cytoskeleton induced by IV infection, thus protecting the barriers of PMVECs.

Introduction

Influenza virus (IV) is a prevalent virus that causes respiratory diseases worldwide. Seasonal viruses spreading among the human population cause annual epidemics that lead to approximately 500 000 deaths per year. One of the major health complications of IV is viral pneumonia, which can result in acute respiratory distress syndrome (ARDS).[1] In the acute phase of ARDS, symptoms are characterized by cyanosis, hypoxemia, pulmonary edema, and respiratory failure, which may ultimately lead to multiple organ failure and a low survival rate.[2]

Respiratory failure in the acute phase of ARDS can be attributed to damage to the epithelial-endothelial barriers of pulmonary alveoli, where gas exchange takes place.[3,4] Damage to these barriers leads to the flooding of proteinaceous edema fluid containing fibrin, erythrocytes, and inflammatory cells into the alveolar lumen. Although the pathogenesis of ARDS is only partially understood, it is thought that the key determinants of ARDS are endothelial barrier dysfunction and subsequent pulmonary microvascular endothelial leakage. Clinical analysis showed that mortality remains high among patients with severe influenza despite the use of anti-viral therapeutic strategies, which suggests that microvascular leakage may play a critical role in the pathogenesis of viral infections.[5]

Xijiao Dihuang Decoction combined with *Yinqiao* Powder (XDY) is a classic compound formula originally prescribed by Jutong Wu, an ancient Chinese physician from the Qing Dynasty. XDY can clear heat, remove toxins, cool blood, stop bleeding, and promote blood circulation.[6] Results from a clinical study indicated that modified *Yinqiao* Powder could ameliorate symptoms and shorten the duration of fever caused by H1N1 IV.[7] *Xijiao Dihuang* Decoction, currently referred to as *Qingre Dihuang* Decoction, has been evaluated as a treatment for blood-heat syndrome in modern studies. *Xijiao Dihuang* Decoction was beneficial in treating syndromes where toxins intrude into the blood, a phenomenon that occurs in severe acute respiratory syndrome (SARS).[8] For example, modified *Xijiao Dihuang* Decoction was used to treat fire-toxin syndrome caused by viral pneumonia by virtue of its abilities for clearing heat, cooling blood, nourishing yin, and dispelling phlegm.[9] In previous studies, we showed that XDY treatment clearly reduced the mortality rate, decreased pulmonary permeability, and alleviated pulmonary edema in mice with viral pneumonia.[10] The results from in vitro studies demonstrated that XDY could protect the endothelial barrier by reversing permeability increases in PMVECs caused by IV infection.[11] However, the mechanisms by which XDY mitigates hyper-permeability in IV-infected PMVECs requires further investigation.

The Ezrin/Radixin/Moesin (ERM) protein forms links between the cytomembrane and the cytoskeleton. The integrity of the cytoskeleton is mediated by ERM phosphorylation and is crucial for the maintenance of both endothelial morphology and permeability. It has been well documented that ERM can be phosphorylated via the p38 mitogen-activated protein kinase (MAPK), Rho/Rho kinase (ROCK), and protein kinase C (PKC) pathways, thus leading to rearrangement of filamentous actin (F-actin) and hyperpermeability of microvascular endothelial cells.[12–14]

Therefore, in this study, we focused on the effects of XDY on F-actin rearrangement in IV-infected PMVECs. Furthermore, we explored the underlying mechanisms by determining its effects on ERM phosphorylation, as well as its roles in activating the Rho/ROCK, p38 MAPK, and PKC pathways, which serve as upstream activators of the ERM protein.

Materials and methods

Reagents

Dulbecco's modified Eagle's medium, Medium 199 (M199), and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Endothelial cell growth supplement (ECGS) was purchased from Becton, Dickinson and Company (Bergen, NJ, USA). Fluorescein isothiocyanate labeled-phalloidin (phalloidin-FITC) was purchased from Sigma–Aldrich (St Louis, MO, USA). The reagent 4′,6-diamidino-2-phenylindole (DAPI) was purchased from Boster (Wuhan, Hubei, China). Bovine serum albumin (BSA) was obtained from Amresco (Solon, OH, USA). Paraformaldehyde and Triton-X-100 were purchased from Beijing Bioway Biotech Group (Beijing, China). Antibodies against phosphorylated ERM (p-ERM) and phosphorylated PKC (p-PKC) were obtained from Cell Signaling Technology (Danvers, MA, USA). Antibodies against phosphorylated MAPK kinase (p-MKK), phosphorylated p38 (p-p38), and ROCK1 were purchased from Abcam (Cambridge, England, UK). An antibody against phosphorylated myosin phosphatase target-subunit (p-MYPT) was purchased from Abnova (Taipei, Taiwan, China). Recombinant