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Effects of penehyclidine hydrochloride on pulmonary contusion from blunt chest trauma in rats

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ABSTRACT

Background and objective: Toll-like receptor 4 (TLR4) is widely recognised as a pattern recognition receptor (PRR) in the triggering of innate immunity. Lung inflammation and systemic innate immune responses are dependent on TLR4 activation undergoing pulmonary contusion. Therefore, the author investigated the effects of penehyclidine hydrochloride (PHC) on the expression of TLR4 and inflammatory responses of blunt chest trauma-induced pulmonary contusion.
Materials and methods: Male Sprague-Dawley (SD) rats were randomly assigned into three groups: normal control (NC) group, pulmonary contusion (PC) group and penehyclidine hydrochloride treatment (PHC) group. Pulmonary contusion was induced in anesthetised rats at fixed chest impact energy of 2.45 J. Lung injury was assessed by the histopathology changes, arterial blood gas and myeloperoxidase (MPO) activity of lung. The serum tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6) levels were measured using enzyme-linked immunosorbent assays (ELISA). The expression of TLR4 was determined by immunohistochemistry.
Results: Blunt chest trauma produced leucocytosis in the interstitial capillaries, hypoxemia, and increased MPO activity. The expressions of TNF-α, IL-6 and TLR4 in the lung were significantly enhanced during pulmonary contusion. PHC treatments effectively attenuated pulmonary inflammation responses, as shown by improved pulmonary oxygenation, histopathology damage, decreased the MPO activity, the expressions of TNF-α, IL-6, and TLR4 after lung injury.
Conclusion: It might be concluded that PHC exhibit anti-inflammatory and protective effects in traumatic lung injury via the inhibition of the TLR4 pathway.

Introduction

Blunt chest trauma resulting in pulmonary contusion (PC) is a common injury. These injuries contributed significantly to the morbidity and mortality of trauma patients.[1] It has been elucidated that PC from chest trauma is associated with a progressive inflammatory response mediated by local and systemic immunological alterations.[2] PC is an independent risk factor for acute lung injury (ALI), which characterised by an abrupt onset of hypoxemia with the presence of diffuse pulmonary infiltrate.[3] Lung inflammation and systemic inflammatory responses are dependent on toll-like receptor 4 (TLR4) activation during pulmonary contusion.[4] TLR4 activated intracellular signal transduction can ultimately activate overwhelming release of pro-inflammatory cytokines,[5] such as tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6), chemokines and additional pro-inflammatory mediators involved in lung injury.

Penehyclidine hydrochloride derived from hyoscyamine, is a new selective anti-cholinergic drugs which is the original owners of People's Republic of China owning intellectual property rights. Penehyclidine hydrochloride has strong anti-cholinergic effects in the central and peripheral nerves, owing to high selectively M_1, M_3 receptor and no significant effect on the M_2 receptor. Compared with other hyoscyamines, the notable advantage of penehyclidine hydrochloride is that it has few M_2 receptor-associated cardiovascular side effects.[6] Recently, some researches reported that penehyclidine hydrochloride might have the potential positive effects on sepsis/septic shock, acute lung/liver injury, ischemia/reperfusion injury.[7–11] However, at the present, it is still unknown whether penehyclidine hydrochloride has the same potential protective effect on pulmonary contusion from blunt chest trauma.

The present study was undertaken to explore the effects of penehyclidine hydrochloride on histopathology, lung wet-to-dry

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weight ratio (W/D), the arterial oxygenation, myeloperoxidase (MPO) activity, the serum TNF-α and IL-6 levels, the expression of TLR4 in lung tissues during pulmonary contusion from blunt chest trauma in rats.

Materials and methods

Animals

Male Sprague-Dawley (SD) rats weighing 250–300 g were maintained on sterile, standard laboratory chow and water ad libitum in individual ventilated cages under specific pathogen-free (SPF) conditions in the animal facility of the Experimental Research Centre of Huazhong University of Science and Technology. All animal experiments were approved by Huazhong University of Science and Technology Institutional Animal Care and Use Committee. Rats were allowed free access to food and water ad libitum.

Experimental protocols

Ninety-six animals were randomly assigned into three equal groups: normal control (NC) group, pulmonary contusion (PC) group and penehyclidine hydrochloride treatment (PHC) group. Except for the normal control group, all rats from PC and PHC group had a moderate pulmonary contusion. PHC group was intraperitoneally injected with PHC for 2.0 mg/kg[8] immediately after blunt chest trauma. The animals were allowed to recover from anaesthesia and then brought back to their cages with free access to food and water. All the animals were killed under anaesthesia by intra-peritoneal injection pentobarbital (45.0 mg/kg, i.p.) at 2, 8, 12 and 24 h. Blood samples and lung tissue specimen were harvested.

Pulmonary contusion model

Pulmonary contusion was induced using the model for isolated bilateral lung contusion described by Raghavendran et al.[12] Briefly, after induction of 2% isoflurane anaesthesia, a hollow cylindrical was dropped from a definite height; it was encased in a vertical stainless steel tube which was positioned onto a lexon platform. The precordial shield directed the impact force bilaterally to the lungs so as to prevent cardiac trauma (impact energy, 2.45 J). The impact energy created via this mechanism was calculated by using the equation $E = mgh$ (g: 9.8 m/s^2).

The histopathology of lung tissue (haematoxylin and eosin stain/HE)

At 8 h after blunt chest trauma, both lungs were removed. The right lung was fixed with 10% formaldehyde solution for 48 h, embedded in paraffin and cut into 4 mm pieces by microtome, and stained with haematoxylin and eosin stain (HE). All histopathology changes were detailed in each lung tissue, including intra-alveolar haemorrhage, disruption and capillary congestion and leucocyte infiltration.

Electron transmission microscopy

The fragments of the right lung were washed twice with phosphate-buffered saline (PBS) and fixed for 1 h in a solution containing 3.0% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2. After fixation, the samples were washed twice in the same buffer, and then post-fixed in a solution containing 1% osmium tetroxide, 2 mM $CaCl_2$ and 0.8% potassium ferricyanide in 0.1 M cacodylate buffer, pH 7.2, dehydrated in acetone and embedded in Epon resin. Polymerisation was done at 60 °C for 2 days. Ultra-thin sections were collected on 300-mesh copper grids, counter-stained with uranyl acetate and lead citrate and examined with a Hitachi H-600 transmission electron microscope (Hitachi, Tokyo, Japan).

Analysis of arterial blood gas (ABGs) and lactic acid

At 8 h after blunt chest trauma, whole blood was drawn via the right carotid artery (2.0 ml each). ABGs and blood lactic acid values were immediately determined using a portable blood gas analyser (i-STAT, Princeton NJ, USA).

Measurement of blood serum TNF-α and IL-6

After the arterial blood sample was drawn. The blood was immediately separated by centrifugation at 3000 rpm for 15 min at 4 °C. The serum was divided into aliquots and stored at −70 °C until assayed. The serum TNF-α and IL-6 (American R & D) concentration was measured using enzyme-linked immunosorbent assays (ELISA) kits.

Lung wet-to-dry weight ratio (W/D)

After lungs were drawn, and large airways were removed. The left lung was weighed and then dried in an oven at 60 °C for 72 h. Lung W/D weight ratio was assessed pulmonary oedema formation.

Myeloperoxidase (MPO) assay

1.0 g of snap-frozen lung tissue was homogenised in 0.05 M potassium phosphate buffer at pH 5.5 and centrifuged at 3000 rpm for 10 min at 4 °C. The pellet was redissolved in 10 ml of 0.05 M potassium phosphate buffer at pH 5.5 containing 0.5% hexadecyltrimethyl ammonium bromide. An aliquot of the supernatant was assayed by measuring the H_2O_2-dependent oxidation of tetramethyl benzidine in sodium phosphate buffer. Absorbance at 450 nm of visible light was measured and the MPO activity was calculated in units per gram of lung tissue (U/g).

Immunohistochemical staining for TLR4

Expressions of TLR4 were determined using an SABC kit (American, Sigma). After deparaffinisation, endogenous peroxidase was quenched with 0.3% H_2O_2 in 60% methanol for 30 min. The sections were permeabilised with 0.1% Triton X-100 in phosphate-buffered saline for 20 min. Nonspecific absorption was minimised by incubating the section in 2% normal goat serum in phosphate-buffered saline for 20 min. The sections were then incubated overnight with 1:500 dilution of primary rabbit anti- TLR4 polyclonal antibody, followed by biotin-conjugated secondary antibody at 1:1000 dilutions. Finally, the sections were incubated with avidin–biotin complex kit and detected by using a diaminobenzidine (DAB) reagent (Boster, China). The biotin-peroxide and diaminobenzidine were used as substrates to develop signals in brown-yellow colorisation. Five different visual fields under a light microscope (400×) of each respective section were chosen and the mean optical densities (OD) of TLR4 positive cells from each section were analysed by image cytometry with HIPAS-2000 image analysis software (Qianli Technical Imaging, Wuhan, China).

Statistical analysis

Data were presented as mean ± SD and were analysed using SPSS 15.0 software. The differences associated with main sources of variation were tested using one-way analysis of variance (ANOVA). When the F statistic was significant for ANOVA comparisons, the