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Penehyclidine prevents nuclear factor-κB activation in acute lung injury induced by lipopolysaccharide

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Abstract

Penehyclidine (PHCD) has been proposed to reduce lung and lethal toxicity. The present study was undertaken to investigate the mechanisms responsible for the protective effect of PHCD against acute lung injury (ALI) in rats. Tail-vein injection of lipopolysaccharide (LPS; 5 mg kg^{-1}) was used to induce ALI in rats. Secondary increases in total protein, lactate dehydrogenase activity in bronchoalveolar lavage fluid and myeloperoxidase in lung tissue were used to evaluate the effects of PHCD on ALI in rats. Activated DNA binding activity and expression of nuclear factor κB (NF-κB) in lung tissue were measured using electrophoretic mobility shift assays assay and immunohistological staining. Levels and mRNA expression of tumour necrosis factor α (TNF-α) and interleukin 1β (IL-1β) were measured by enzyme-linked immunosorbent assay and reverse transcriptase-polymerase chain reaction. Pretreatment with PHCD (0.03 mg kg^{-1}, 0.1 mg kg^{-1} and 0.3 mg kg^{-1} i.p.) significantly attenuated the LPS-induced changes in lung injury parameters and inhibited the activation and expression of NF-κB in lung tissue. Furthermore, PHCD also substantially reduced the LPS-induced TNF-α and IL-1β mRNA expression and production in lung tissue and suppressed neutrophil recruitment. The results suggest that PHCD attenuates LPS-induced acute lung responses through inhibition of NF-κB activation and LPS-induced TNF-α and IL-1β production and resulting neutrophil recruitment associated with acute lung inflammation and injury. PHCD may be a useful adjuvant to treatment strategies targeting clinical situations of acute inflammation.

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Introduction

The development of acute lung injury (ALI) and its more severe form, acute respiratory distress syndrome (ARDS), is associated with high morbidity and mortality in critically ill patients (Ware & Matthay 2000). Mortality is over the range of 30–70%, similar to that in intensive care (Bernard et al 1994; Vasilye et al 1995). The most common cause of ARDS is known to be sepsis (Repine 1992). The lipopolysaccharide (LPS) components of endotoxins are responsible for the enhanced inflammatory response of ALI and ARDS (Cannon et al 1990; Kolb et al 2001; Kabir et al 2002), which is characterized by the loss of epithelial integrity, widening of the alveolar–arterial O_2 gradient, and the development of interstitial pulmonary oedema.

The precise pathogenesis for sepsis-induced ARDS is not yet fully defined. However, massive accumulation of neutrophils in the lungs and increased pulmonary proinflammatory cytokine levels are major characteristics of ALI (Ferry et al 1997; Abraham et al 2000). Proinflammatory cytokines, including tumour necrosis factor α (TNF-α), interleukin 1β (IL-1β) and macrophage inflammatory peptide-2, can be produced by resident pulmonary cell populations, including alveolar macrophages and vascular endothelium (Kelley 1990; Thomassen et al 1996). However, neutrophils that accumulate in the lungs after endotoxaemia or haemorrhage also appear to be a significant intrapulmonary source of IL-1β and other immunoregulatory cytokines (Parsey et al 1998). Activation of the neutrophils within the lung causes microvascular injury, attributed to the release of neutrophil proteases and reactive oxygen species (Sibille & Reynolds 1990). This pulmonary response contributes to the pathogenesis of various acute inflammatory diseases.

Nuclear factor κB (NF-κB) is an essential transcription factor that regulates the gene expression of various cytokines, chemokines, growth factors, and cell adhesion molecules (Blackwell & Christman 1997). The most characterized NF-κB complex is a p50–p65 heterodimer, which, at rest, is associated with an inhibitor protein, IκB, and is retained in the

cytoplasm. The active NF-κB can then translocate to the nucleus where it binds to the NF-κB motif of a gene promoter and functions as a transcriptional regulator. Activation of NF-κB p50–p65 heterodimer has been identified in LPS-induced ALI (Liu et al 1999; Fan et al 2001). Recent evidence indicates that LPS-induced NF-κB activation in lung tissue is associated with lung neutrophilia, epithelial permeability and lipid peroxidation (Blackwell et al 1996; Liu et al 1999). In-vivo activation of NF-κB, but not other transcription factors, in alveolar macrophages from patients with ARDS has also been demonstrated (Schwartz et al 1996). Therefore, activation of NF-κB binding to various gene promoter regions appears to be a key molecular event in the initiation of LPS-induced pulmonary disease.

Modulating the inflammatory response to diminish the adverse consequences of ALI may be of therapeutic benefit, but our understanding of this is incomplete (Fulkerson et al 1996). In China, anisodamine, an alkaloid extracted from a Chinese herb, was clinically used to treat patients suffering from bacterial meningitis and toxic bacillary dysentery, with a significant reduction of mortality and improvement of microcirculation (Xiu et al 1982; Ruan et al 2001). The mechanism of action responsible for the effects of anisodamine is inhibition of LPS-induced TNF-α gene expression (Wang et al 1999) and activation of the NF-κB pathway (Ruan et al 2001). Penehyclindine (PHCD), a synthetic compound of completely new structure, is a cholinergic antagonist, which differs from anisodamine with a long duration and slight effect on M_2 receptors. PHCD has been widely used in preoperative medication (Yan et al 2006) and in the treatment of organic pesticide poisoning (Liang 2007). A recent study on ALI indicated that PHCD could improve microcirculation, reduce the permeability of capillaries, protect cells and decrease the release of lysosomal enzymes (Han et al 2005). Our previous study also demonstrated a protective effect against ALI in rats, even at a low dose of 0.03 mg kg^{-1} (Juan et al 2007). However, the mechanisms for the inhibitory effects of PHCD on ALI have not been fully elucidated. The purpose of the present study was to investigate if different doses of PHCD retained the inhibitory effects on NF-κB activation and expression and production of inflammatory agents, such as TNF-α and IL-1β.

Materials and Methods

Reagents

LPS (*Escherichia coli* serotype 055:B5) was obtained from Sigma Chemical Co (St Louis, MO, USA). Penehyclidine hydrochloride injection was kindly supplied by Chengdu Lisite Pharmaceutical Co., China. The clinical preparation of anisodamine (raceanisodamine hydrochloride; $C_{17}H_{24}NO_4$; 10 mg mL^{-1} in saline) was purchased from Hangzhou Drug Co. (Hangzhou, China).

Animal protocols and experimental design

The current study was approved by the Ethics Committee of Anhui Medical University (licence no. 2006DZ1008). A total of 42 male SD rats (250 ± 17 g) were provided by the Experimental Animal Center of Anhui Medical University. The animals were randomly divided into six groups (n = 7): the normal group received saline only via tail-vein injection; the model group received a tail-vein injection of LPS (5 mg kg^{-1}); the anisodamine group received 10 mg kg^{-1} anisodamine via intraperitional injection 0.5 h before injection of LPS; and the three PHCD groups (designated as P1, P2 and P3) received an intraperitoneal injection of 0.03 mg kg^{-1}, 0.1 mg kg^{-1} and 0.3 mg kg^{-1} PHCD, respectively, 0.5 h before injection of LPS. The rats were killed by exsanguination 4 h after LPS challenge.

Isolation of bronchoalveolar lavage (BAL) cells, lung tissue and cell counts

At 4 h after LPS treatment, the rats were killed and BAL was performed through a tracheal cannula with aliquots of 8 mL each of ice-cold Ca^{2+}/Mg^{2+}-free phosphate-buffered medium (145 mM NaCl, 5 mM KCl, 1.9 mM NaH_2PO_4, 9.35 mM Na_2HPO_4 and 5.5 mM dextrose; pH 7.4) for a total of 80 mL for each rat. The BAL fluid was centrifuged at 500 *g* for 5 min at 4°C and cell pellets were washed and resuspended in phosphate-buffered medium. Cell counts and differentials were determined using an electronic Coulter counter with a cell sizing analyser (Coulter Model ZBI with a channelizer 256; Coulter Electronics, Bedfordshire, UK) as described by Lane & Mehta (1990). The recovered cells were 98% viable, as determined by trypan blue dye exclusion. After lavage, lung tissue was removed, immediately frozen in liquid nitrogen and stored at −70°C.

Parameters for evaluating lung injury induced by LPS

At 4 h after LPS administration, rats were anaesthetized with urethane and killed by bleeding from the abdominal aorta. The right primary bronchus was ligated and BAL was then performed through a tracheal cannula with aliquots of 5 mL each of 4°C cold saline for a total of 15 mL for each rat. The BAL fluid was centrifuged at 1500 *g* for 5 min at 4°C. The supernatant was harvested and stored at −20°C and the cell pellets were smeared onto slides for cell classification and counting. After the cell smear was stained with Wright–Giemsa, neutrophils were measured by counting 200 cells under light microscopy.

To assess the capillary permeability of the bronchoalveolar capillary, we measured the total protein in BAL, using bovine serum albumin as the standard. The activity of lactate dehydrogenase (LDH), a cytosolic enzyme used as a marker for cytotoxicity, was measured at 490 nm using an LDH determination kit according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, China). LDH activity was expressed as units L^{-1}, using an LDH standard. The LDH activity and total protein were measured in the first aliquot of the acellular BAL fluid.

Lung tissue myeloperoxidase (MPO) content

Lungs were homogenized in a buffer containing 50 mM potassium phosphate and 0.5% hexadecyltrimethylammonium bromide and 5 mM EDTA, sonicated, and centrifuged. The MPO activity of the supernatant was measured at 420 nm

using an MPO determination kit according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, China). MPO activity was expressed as units $(g\,lung)^{-1}$.

Semiquantitative polymerase chain reaction (PCR)

Groups of seven rats, with results obtained from individual rats, were used for each experimental condition. RNA was isolated using the RNA easy kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. Primers and probes for IL-1β and TNF-α were designed using Primer Express software supplied by PerkinElmer (Foster City, CA, USA).

The IL-1β primer and probe sequence consisted of the following: forward primer 5′-GAGGCTGACAGACCCCAAAAGAT-3′; reverse primer 5′-GCACGAGGCATTTTTGTTGTTCA-3′. The TNF-α primer and probe consisted of the following: forward primer 5′-CTAGTACCGAGATGTGGAACTGGC-3′; reverse primer 5′-CTGGCTCAGCCACTCCAGC-3′; probe GAPDH (501bp), sense: 5′-GTGAAGGTCGGTGTCAACGGATTT-3′, antisense: 5′-CACAGTCTTCTGAGAGTGGCAGTGAT-3′. PCR reaction was performed with cDNA as a template, using the above primers, after an initial 5 min denaturation at 94°C, followed by 30 cycles at 94°C for 30 s, 51°C for 30 s, 72°C for 30 s, and 72°C for 10 min. To detect amplified cDNA, the PCR product was analysed by agarose gel electrophoresis. The number of PCR cycles was selected for each cytokine product so that the ethidium bromide-stained amplified DNA products were between barely detectable and below saturation levels; the gel was scanned with a gel documentation system. Cytokine densitometric results were normalized to the GAPDH products.

Determination of cytokines in lung tissue

The levels of TNF-α and IL-1β in lung tissue were measured by enzyme-linked immunosorbent assay kits (Gemzyme, Cambridge, MA, USA) according to manufacturer's instructions, and corrected by total protein in lung tissue using the Bradford biophotometer method (Bradford 1976).

Immunohistochemical staining for lung tissue location of p65

The left lungs were removed and post-fixed for 24 h in the fixative. The post-fixed lungs were cryoprotected in 25% sucrose in PBS and the lung sections (20-μm thick) were placed on a cryostat. Before staining, the coverslips were rinsed in PBS (pH 7.4) for 10 min, and then blocked with 2% goat serum for 15 min, briefly rinsed in PBS and stained with anti-p65 antibody (Santa Cruz) for 60 min in a humid chamber. The coverslips were then washed three times in PBS and biotinylated secondary antibody in 2% goat serum was added. The coverslips were incubated in peroxidase substrate for 5 min and then washed three times in PBS.

Nuclear extracts

Tissue nuclear proteins were extracted from whole lung by the method of Deryckere & Burgert (1996). A quantity of tissue (50–100 mg) was mechanically homogenized in liquid nitrogen to which 4 mL of buffer A (Nail 150 mM, HEPES 1 M, NP40, EDTA 0.2 M, phenylmethylsulfonylfluoride 0.1 M) was added. The homogenate was transferred to a 15-mL Falcon tube (Becton Dickinson Labware, Franklin Lakes, NJ, USA) and centrifuged at 850 *g* for 30 s to remove cellular debris. The supernatant was then transferred to a 50-mL Falcon conical and incubated on ice for 5 min before being centrifuged for 10 min at 3500 *g*. The supernatant was collected as cytoplasmic extract. The pellet was resuspended in 300 μL of buffer B (sterile water, glycerol, HEPES 1 M, NaCl 5 M, $MgCl_2$ 1 M, EDTA 0.2 M, PMSF 0.1 M, DTT 1 M, benzamidine 10 $mg\,mL^{-1}$, pepstatin 1 $mg\,mL^{-1}$, leupeptin 1 $mg\,mL^{-1}$, aprotinin 1 $mg\,mL^{-1}$) and incubated on ice for 30 min, Following a 2-min microcentrifugation at 14000 *g*, the supernatant was collected as the nuclear extract and frozen at −70°C. Protein concentrations in nuclear and cytoplasmic extracts were determined using the Bradford assay (Bradford 1976).

Electrophoretic mobility shift assays (EMSA)

Nuclear extracts were analysed for the presence of NF-κB by EMSA using sequence DNA polymerase: NF-κB, 5′-TTTTCGAGCTCGGGACTTTCCGAGC-3′ and 3′-GCTCGAGCCCTGAAAGGCTCGTTTT-5′. End-labelling was accomplished by treatment with T4 kinase in the presence of [γ-^{32}P]ATP. Extracts were then incubated for 50 min at room temperature with labelled NF-κB oligonucleotide in binding buffer in the presence or absence of unlabelled competitor oligonucleotide. Antibody supershift EMSA was performed by incubating nuclear extract proteins (10 μg) with 2 μg of a polyclonal rabbit antibody against p65. DNA–protein complexes were resolved in non-denaturing 5% acrylamide gels in 0.03% Tris boric acid plus EDTA acid buffer at 270 V. Gels were dried and exposed to X-OMAT KODAK film (Sigma) and developed after 6 h of exposure.

Statistical analysis

Data are expressed as means ± s.e.m. Tukey's test was used for comparison between the model group and others; one-way analysis of variance was used for comparison among PHCD-treated groups using the SPSS 10.0 statistical package (SPSS, Chicago, IL, USA). For quantitative analysis of p65 expression by histoimmunochemistry, each slice was observed under the light microscope (×200) and analysed by a computerized image analysis system (HPLAS-1000; Champion Imaging Group, Montreal, QC, Canada). Four microscopic fields per region per section were analysed. $P < 0.05$ was considered as a significant statistical difference.

Results

LPS-induced ALI

To evaluate the rat ALI model, some parameters related to the acute phase response were determined. It was found that BAL total protein content (Figure 1A) and LDH activity (Figure 1B) in the model rats were significantly increased by 4.7- and 6-fold, respectively, compared with saline control rats,

indicating that LPS induced ALI in the treated rats ($P<0.01$). To confirm this, the lung tissues were observed under the microscope. It was found that histological oedema, leukocyte infiltration and haemorrhage were all present in the hyperplastic alveolar septa (Figure 1).

Effect of PHCD pretreatment on LPS-induced neutrophil accumulation

Recruitment of neutrophils to the interstitial and alveolar space in response to LPS was documented by a myeloperoxidase (MPO) assay in lung tissue and neutrophil counts in BAL fluid. For quantification of interstitial neutrophil accumulation, lungs were homogenized 4 h after LPS application, and a MPO assay was performed. A significant increase of MPO was observed in the model group 4 h after LPS injection, compared with the normal group ($P<0.01$). PHCD at doses of 0.03, 0.1 and 0.3 mg kg^{-1} decreased the up-regulated activity of MPO ($P<0.05$) (Figure 2A). For quantification of alveolar neutrophil accumulation, cells were counted in the BAL fluid. As shown in Figure 2B, total numbers of neutrophil granulocytes in the model group were significantly increased compared with the normal group ($P<0.01$), indicating a significant increase in neutrophil influx into the alveolar spaces compared with the saline

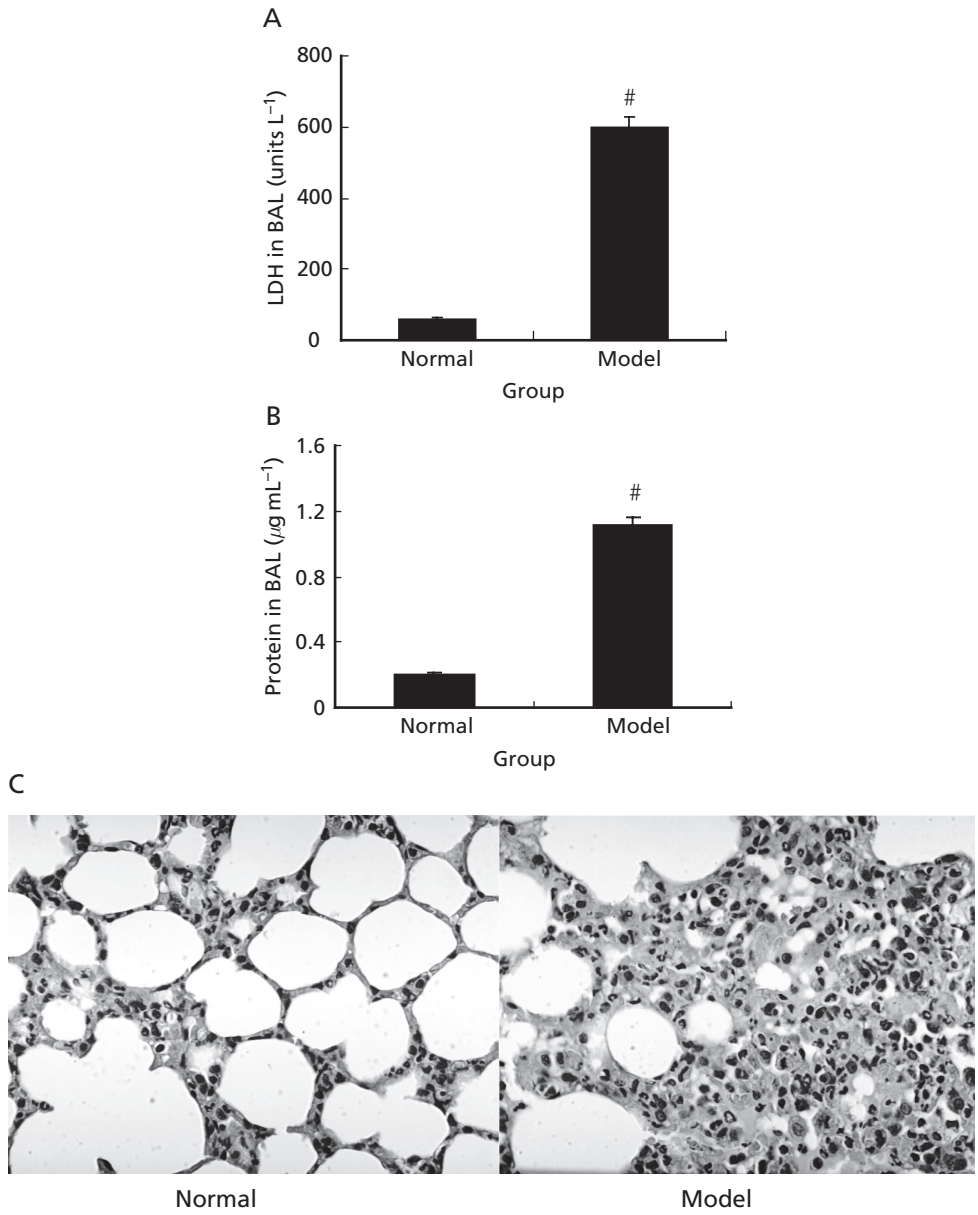

Figure 1 Levels of total protein (A) and activity of lactate dehydrogenase (LDH) (B) in bronchoalveolar lavage (BAL) fluid, and pathological characteristics of lung tissue observed under a light microscope (C). The groups represent rats treated as follows: saline (normal); LPS (5 mg kg^{-1}; model). Values represent means ± s.e.m. of results from two experiments. $^{\#}P<0.01$, significantly different compared with the saline control group.

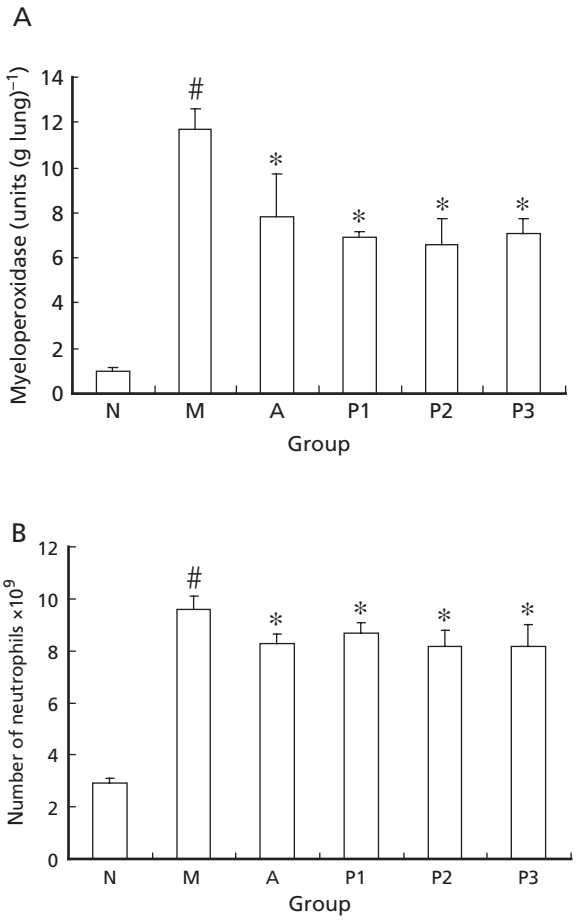

Figure 2 Effects of penehyclidine on lipopolysaccharide-induced neutrophil activation. A. Neutrophil accumulation in bronchoalveolar lavage fluid. B. Myeloperoxidase activity in the lung. The groups represent rats treated as follows: normal group (N); model group (M); acute lung injury rats treated with anisodamine at a dose of 10 mg kg^{-1} (A); rats treated with penehyclidine at doses of 0.03, 0.1 and 0.3 mg kg^{-1} (P1, P2, P3, respectively). Values are means ± s.e.m. from seven rats. $^{\#}P$ <0.01, compared with the normal group; $^{*}P$ < 0.05 compared with the model group.

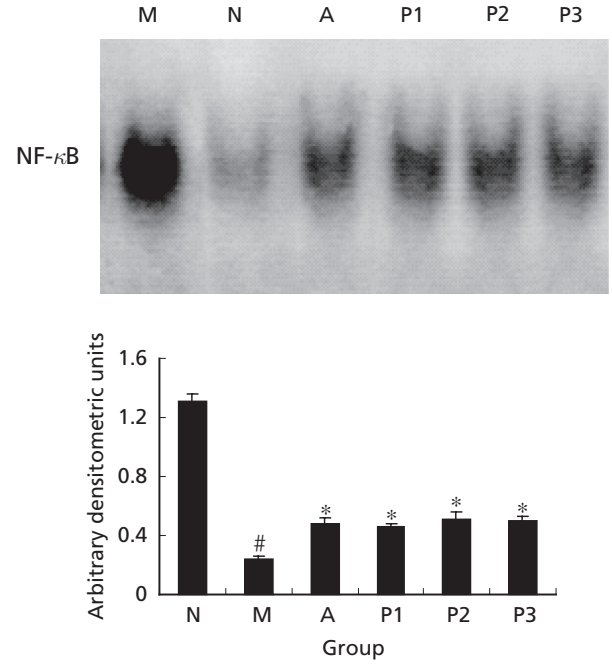

Figure 3 Effect of penehyclidine on NF-κB DNA binding activity in lung tissue. The groups represent rats treated as follows: model group (M); normal group (N); rats treated with anisodamine at a dose of 10 mg kg^{-1} (A); rats treated with penehyclidine at doses of 0.03, 0.1 and 0.3 mg kg^{-1} (P1, P2 and P3, respectively). Densitometry of NF-κB bands on electrophoretic mobility shift assays are expressed as arbitrary densitometric units. Values represent means ± s.e.m. of results from seven experiments. Supershift electrophoretic mobility shift assays using nuclear proteins in lung tissue. Lower panel: quantitative data of upper panel. Mean ± s.d., n = 7. $^{\#}P$ < 0.01, compared with normal group (N); $^{*}P$ < 0.05 compared with model group (M).

control animals. PHCD significantly suppressed the percentage of BAL neutrophils compared with the model group (P < 0.05).

Effects of PHCD on the expression of p65 and NF-κB activation in lung tissue

Figure 3 shows NF-κB activation in lung tissue. The DNA binding activity of NF-κB in lung tissue from saline control rats was slightly detectable. In LPS-treated animals, NF-κB activation was enhanced by 7-fold compared with the saline control animals. This enhancement was effectively depressed by a 0.5-h pretreatment with PHCD (0.03, 0.1, 0.3 mg kg^{-1}).

To confirm the translocation of the activity of NF-κB to the nucleus, immunohistological staining in lung tissue by p65 antibody was performed. Minimal nuclear staining for p65 in the cytoplasm of lung alveolar cells from the normal group was seen (Figure 4A) whereas lung alveolar cells from the model group showed intense nuclear staining (Figure 4B). This indicates that p65 was transferred to the nucleus where it activates gene transcription. The three doses of PHCD significantly reduced not only the total number of positive cells but also the number of positive nuclei stained by p65 antibody (Figure 4D–F). Quantitative analysis of p65 expression is also shown in the lower panel of Figure 4.

Effect of PHCD on expression of lung mRNA for TNF-α and IL-1β and cytokine levels in lung tissue

TNF-α and IL-1β were chosen in our experiments as representative inflammatory mediators. Figure 4C and 4D illustrates the representative mRNA expression of lung tissue for TNF-α and IL-1β, respectively. Expression of TNF-α and IL-1β mRNA was detectable in the samples of saline control animals, but was markedly increased by LPS treatment for 4 h (P < 0.01). PHCD decreased the levels of LPS-induced TNF-α and IL-1β mRNA expression in lung tissue (P < 0.05). Increases in the levels of TNF-α and IL-1β in lung tissue were noted in model rats after LPS injection. However, rats pretreated with the injection of PHCD presented a marked reduction in the amount of TNF-α and IL-1β (P < 0.05). Results are given in Table 1 and Figure 5.

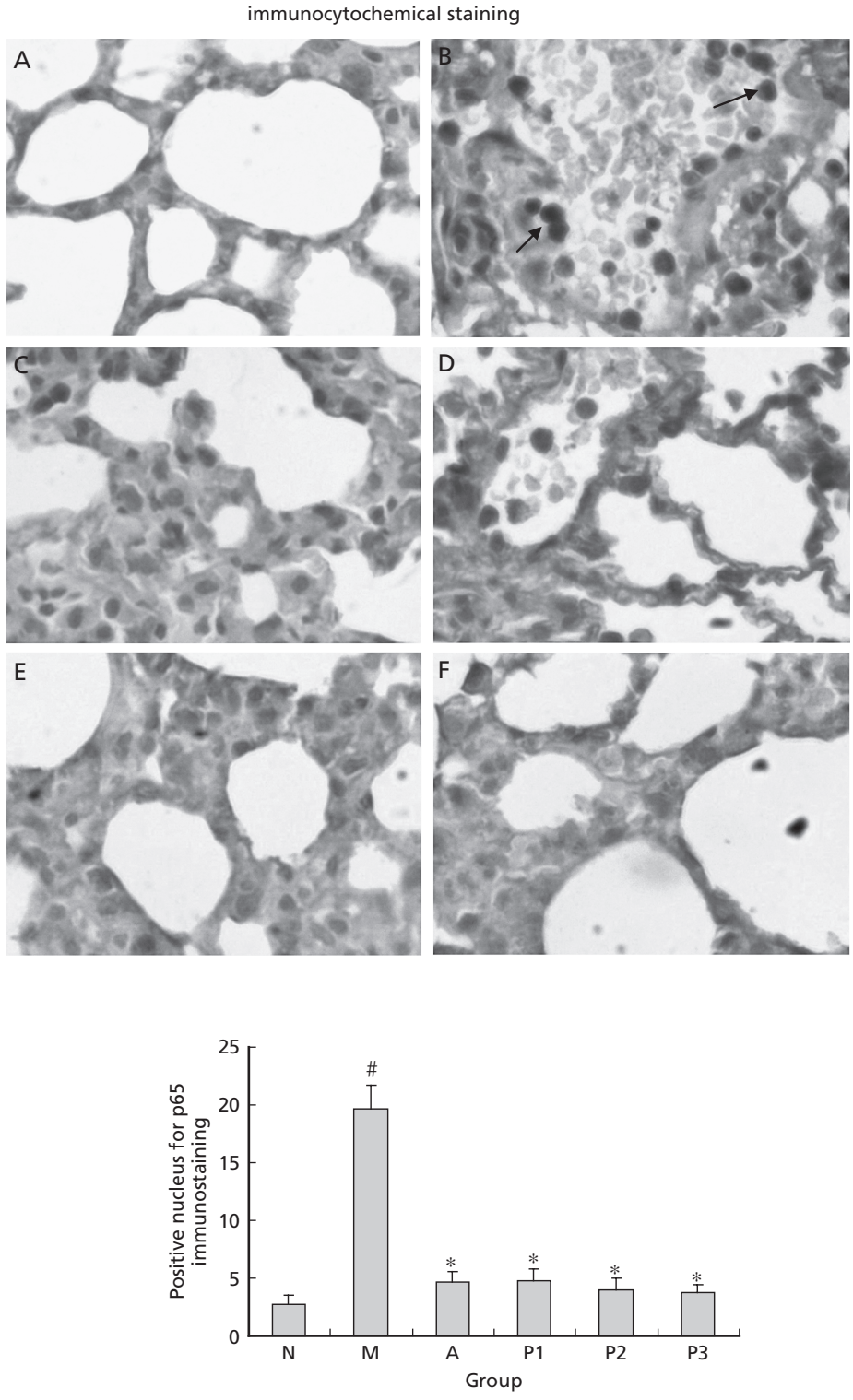

Figure 4 Effect of penehyclidine on NF-κB expression in lung tissue. Upper panel: immunocytochemical staining for intracellular location of p65. A. Normal group; B. model group (intense nuclear staining indicated by arrows); C. rats treated with anisodamine at a dose of 10 mg kg^{-1}; D, E, F. acute lung injury rats treated with penehyclidine at doses of 0.03, 0.1 and 0.3 mg kg^{-1}, respectively. Lower panel: quantitative data of upper panel.

Table 1 Effect of penehyclidine (PHCD) on tumour necrosis factor α (TNF-α) and interleukin 1β (IL-1β) mRNA expression and production in the lungs of rats with lipopolysaccharide-induced acute lung injury

Group	TNF-α (pg mL^{-1})	TNF-α mRNA	IL-1β (pg mL^{-1})	IL-1β mRNA
Normal	45.74 ± 1.34	0.12 ± 0.03	332.26 ± 42.77	0.15 ± 0.21
Model	155.50 ± 10.65††	0.69 ± 0.19††	2636.82 ± 211.19††	0.55 ± 0.16††
Anisodamine 10 mg kg^{-1}	103.12 ± 4.95*	0.37 ± 0.18*	1181.85 ± 85.84*	0.33 ± 0.12*
PHCD 0.03 mg kg^{-1}	101.46 ± 3.94*	0.30 ± 0.21*	1150.18 ± 66.65*	0.31 ± 0.17*
PHCD 0.1 mg kg^{-1}	100.96 ± 4.72*	0.27 ± 0.15*	929.30 ± 43.97*	0.33 ± 0.15*
PHCD 0.3 mg kg^{-1}	91.29 ± 3.29*	0.24 ± 0.14*	802.67 ± 59.29*	0.29 ± 0.11*

Data are mean ± s.d., n = 7 per group. Levels of expression were quantified by densitometry. Results were normalized to GADPH expression and presented relative to the level attained for lipopolysaccharide-induced TNF-α and IL-1β mRNA. Anisodamine was the positive control. $^{\dagger\dagger}P < 0.01$, significantly different compared with the normal group; $^{*}P < 0.05$, significantly different compared with the model group.

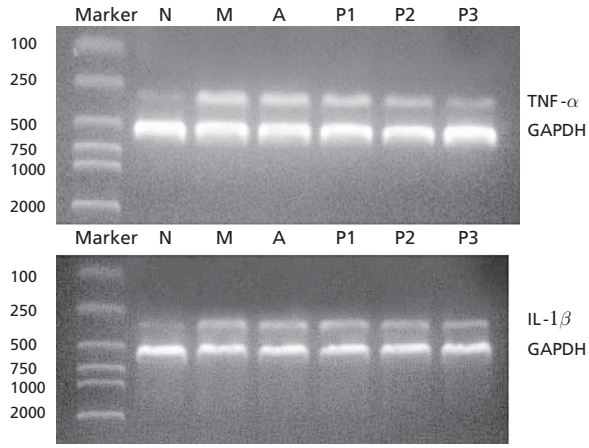

Figure 5 Effect of penehyclindine hydrochloride on mRNA expression of tumour necrosis factor α (TNF-α) and interleukin 1β (IL-1β) in lung tissue. The groups represent rats treated as follows: saline (normal group, N); lipopolysaccharide (model group, M); anisodamine (10 mg kg^{-1}) (A); rats treated with penehyclindine at doses of 0.03, 0.1 and 0.3 mg kg^{-1} (P1, P2, P3, respectively).

Discussion

In the present study, we determined the in-vivo relationship between NF-κB activation and LPS-induced ALI, the inhibition of NF-κB activation and ALI by PHCD, and the effects of inhibiting NF-κB activation on LPS-induced proinflammatory gene products, such as TNF-α and IL-1β, as well as neutrophil influx into the lungs.

ALI is characterized by an excessive inflammatory response to both pulmonary and extrapulmonary stimuli that ultimately leads to a disruption of alveolar–capillary integrity, with severe consequences for pulmonary gas exchange. In previous reports, we demonstrated that PHCD markedly reduced lung water content, lung wet-to-dry weight ratio, lung permeability index and the BAL fluid protein concentration, and significantly reduced the MDA level in lung and serum and increased SOD level in lung and serum in LPS-induced ALI in rats (Juan et al 2007). All that indicated that PHCD attenuates LPS-induced acute lung responses by inhibiting the massive accumulation of neutrophils in the lungs and the production of reactive oxygen species.

Emigrated neutrophils are a hallmark of acute inflammation and contribute to lung injury (Martin 2002). Therefore, the inflammatory response involved in ALI can be evaluated by the influx of polymorphonuclear leukocytes into the lungs. MPO in neutrophil primary granules catalyses the release of chloramine, halides and hypochlorous acid (Weiss et al 1982). This enzyme is stable enough to be detectable in-vivo and MPO levels have been found to be elevated in BAL fluid from patients with acute lung injury (McGuire et al 1982). In addition, BAL fluid from patients with ALI but not from normal control subjects was cytotoxic for normal lung parenchymal cells when incubated in the presence of H_2O_2 and halides, suggesting that free MPO in the air spaces is an active peroxidase. Our results show that treated animals had significantly higher levels of lung MPO 4 h after LPS administration compared with controls. Neutrophil extravasation in BAL fluid was also significantly increased at 4 h after LPS injection. Pretreatment with PHCD could inhibit the recruitment of polymorphonuclear leukocytes into the lung in the LPS-induced rat ALI model, even at a low dose (0.03 mg kg^{-1}).

The activation of NF-κB has been associated with lung injury in LPS-treated rats (Lim et al 2004; Tian et al 2004) and patients with ARDS (Schwartz et al 1996). After LPS stimulation, NF-κB is activated and translocated from the cytoplasm to the nucleus where it regulates several pro-inflammatory genes responsible for the synthesis of various cytokines and adhesion molecules. Kang et al (2003) reported that NF-κB activation in lung tissue peaked by 4 h after LPS treatment. Because of the key role played by this transcriptional factor, we investigated its activation in lung tissue 4 h after acute endotoxaemia by EMSA. In addition, the use of immunostaining techniques allowed us to better define cell populations in the lung parenchyma in which NF-κB was mostly expressed. Results from our laboratory indicate that NF-κB activation in the lung tissue was markedly attenuated by PHCD pretreatment (EMSA). We also observed an increased expression of NF-κB not only in cytoplasm but also in the nucleus of lung cells of animals receiving LPS. In contrast, PHCD-treated animals showed decreased expression of NF-κB in lung tissues.

The excessive expression of NF-κB-associated genes may be responsible for inflammatory injury to the lungs. TNF-α

and IL-1β are necessary and sufficient for injury in laboratory animal models of inflammation (Abraham 2000; Fan et al 2001). Blackwell et al (1996) and Liu et al (1999) have reported that LPS-induced NF-κB activation was temporally correlated with the expression of TNF-α and IL-1β mRNA in lung tissue. Since TNF-α and IL-1β can stimulate neutrophils to release inflammatory mediators (Abraham et al 2000), inhibition of these cytokines may contribute to the inhibition of LPS-induced pulmonary vascular injury in which activated neutrophils are involved. The present experiments indicate an increase in LPS-induced TNF-α and IL-1β mRNA expression and production in lung tissue. Its correlation with NF-κB activation and lung injury, which presented as a change in lung histopathology and an increase in protein and activity of LDH in BAL, is consistent with recent in-vivo studies (Juan et al 2007). Our results showed that pretreatment with PHCD not only significantly suppressed LPS-induced TNF-α and IL-1β mRNA expression, but also decreased the levels of TNF-α and IL-1β in rat lung tissue. The results of the present study indicate that the inhibition of LPS-induced NF-κB activation by PHCD may explain the inhibition of downstream LPS-induced NF-κB-dependent responses, and demonstrate that PHCD caused effective reduction of LPS-induced TNF-α and IL-1β expression and production in lung, consistent with suppression of neutrophil influx into the lung. These results support the concept of a correlation between inhibition of NF-κB and suppression of the inflammatory response, and suggest that NF-κB is an important intracellular target for the early detection and prevention of lung injury.

Conclusion

The results of the present study suggest that PHCD reduces NF-κB activation, expression of NF-κB in lung tissue, mRNA expression and production of TNF-α and IL-1β, and neutrophilic lung inflammation in response to LPS. We have shown that pretreatment with PHCD is efficacious in blocking the development of neutrophilic lung inflammation in response to LPS. We propose that pretreatment with PHCD may provide protective effects against LPS-induced ALI by attenuating the inflammatory cascade, and thus PHCD may be a useful adjuvant to treatment strategies targeting clinical situations of acute inflammation.

References

Abraham, E. (2000) NF-κB activation. *Crit. Care Med*. **28** (Suppl.): N100–N104

Abraham, E., Carmody, A., Shenkar, R., Arcaroli, J. (2000) Neutrophils as early immunologic effectors in hemorrhage- or endotoxemia-induced acute lung injury. *Am. J. Physiol. Lung Cell Mol. Physiol*. **279**: L1137–L1145

Bernard, G. R., Artigas, A., Brigham, K. L., Carlet, J., Falke, K., Hudson, L., Lamy, M., Legall, J. R., Morris, A., Spragg, R. (1994) The American-European Consensus Conference on ARDS. Definitions, mechanisms, relevant outcomes, and clinical trial coordination. *Am. J. Respir. Crit. Care. Med*. **149**: 818–824

Blackwell, T. S., Christman, J. W. (1997) The role of nuclear factor-kappa B in cytokine gene regulation. *Am. J. Respir. Cell. Mol. Biol*. **17**: 3–9

Blackwell, T. S., Blackwell, T. R., Holden, E. P., Christman, B. W., Christman, J. W. (1996) In vivo antioxidant treatment suppresses nuclear factor-kappa B activation and neutrophilic lung inflammation. *J. Immunol*. **157**: 1630–1637

Bradford, M. M. (1976) A rapid and sensitive for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*. **72**: 248–254

Cannon, J. G., Tompkins, R. G., Gelfand, J. A., Michie, H. R., Stanford, G. G., van der Meer, J. W., Endres, S., Lonnemann, G., Corsetti, J., Chernow, B. (1990) Circulating interleukin-1 and tumor necrosis factor in septic shock and experimental endotoxin fever. *J. Infect. Dis*. **161**: 79–84

Deryckere, F., Burgert, H. G. (1996) Tumor necrosis factor alpha induces the adenovirus early 3 promoter by activation of NF-kappaB. *J. Biol. Chem*. **271**: 30 249–30 255

Fan, J., Ye, R. D., Malik, A. B. (2001) Transcriptional mechanisms of acute lung injury. *Am. J. Physiol. Lung Cell Mol. Physiol*. **281**: L1037–L1050

Ferry, G., Lonchampt, M., Penel, L., Nanteuil, G., Canet, E., Tucker, G. C. (1997) Activation of MMP-9 by neutrophil elastase in an in vivo model of acute lung injury. *FEBS Lett*. **402**: 111–115

Fulkerson, W. J., Maclntyre, N., Stamler, J. (1996) Pathogenesis and treatment of adult respiratory distress syndrome. *Arch Intern. Med*. **15**: 29–38

Han, J. Y., Cao, F. S., Wang, Y. T., Mao, Z. C. (2005) The clinical practice of Chang tuo ning. *Chin. J. Emerg. Med*. **14**: 173–174

Kabir, K., Gelinas, J. P., Chen, M., Chen, D., Zhang, D., Luo, X., Yang, J. H., Carter, D., Rabinovici, R. (2002) Characterization of a murine model of endotoxin-induced acute lung injury. *Shock* **17**: 300–303

Kang, J. L., Lee, H. W., Lee, H. S., Pack, I. S., Castranova, V., Koh, Y. (2003) Time course for inhibition of lipopolysaccharide-induced lung injury by genistein: relationship to alteration in nuclear factor-κB activity and inflammatory agents. *Crit. Care Med*. **31**: 517–524

Kelley, J. (1990) Cytokines of the lung. *Am. Rev. Respir. Dis*. **141**: 765–788

Kolb, M., Margetts, P. J., Anthony, D. C., Pitossi, F., Gauldie, J. (2001) Transient expression of IL-1 beta induces acute lung injury and chronic repair leading to pulmonary fibrosis. *J. Clin. Invest*. **107**: 1529–1536

Juan, L., Jun, L., Jian-hui, P., Cai, F., Kun-zhou, C. (2007) Effects of penehyclidine pretreatment on endotoxin-induced acute lung injury in rats. *Chin. J. Anesthesiol*. **27**: 70–74

Lane, F. C., Mehta, J. R. (1990) In vitro human tumor sensitivity assay using cell counting and sizing. *Am. Biotechnol. Lab*. **8**: 12–16

Liang, J. (2007) Clinical observation of penehyclidine in the treatment of organic pesticides poisoning. *China Modern Doctor* **4**: 56–57

Lim, C. M., Kim, E. K., Koh, Y., Kim, W. S., Kim, D. S., Kim, W. D. (2004) Hypothermia inhibits cytokine release of alveolar macrophage and activation of nuclear factor κB in endotoxemic lung. *Intensive Care Med*. **30**: 1638–1644

Liu, S. F., Ye, X., Malik, A. B. (1999) Inhibition of NF-kappa B activation by pyrrolidine dithiocarbamate prevents in vivo expression of proinflammatory genes. *Circulation* **100**: 1330–1337

Martin, T. R. (2002) Neutrophils and lung injury: getting it right. *J. Clin. Invest*. **110**: 1603–1605

McGuire, W. W., Spragg, R. C., Cohen, A. B., Cochrane, C. G. (1982) Studies on the pathogenesis of the adult respiratory distress syndrome. *J. Clin. Invest*. **69**: 543–553

Parsey, M. V., Tuder, R. M., Abraham, E. (1998) Neutrophils are major contributors to intraparenchymal lung IL-1β expression after hemorrhage and endotoxemia. *J. Immunol*. **160**: 1007–1013

Repine, J. E. (1992) Scientific perspectives on adult respiratory distress syndrome. *Lancet* **339**: 466–469

Ruan, Q. R., Zhang, W. J., Hufnagl, P., Kaun, C., Binder, B. R., Wojta, J. (2001) Anisodamine counteracts lipopolysaccharide-induced tissue factor and plasminogen activator inhibitor-1 expression in human endothelial cells: contribution of the NF-kappa B pathway. *J. Vasc. Res*. **38**: 13–19

Schwartz, M. D., Moore, E. E., Moore, F. A., Shenkar, R., Moine, P., Haenel, J. B., Abraham, E. (1996) Nuclear factor-kappa B is activated in alveolar macrophages from patients with acute respiratory distress syndrome. *Crit. Care. Med*. **24**: 1285–1292

Sibille, Y., Reynolds, H. Y. (1990) Macrophages and polymorphonuclear neutrophils in lung defense and injury. *Am. Rev. Respir. Dis*. **141**: 471–501

Thomassen, M. J., Divis, L. T., Fisher, C. J. (1996) Regulation of human alveolar macrophage inflammatory cytokine production by interleukin-10. *Clin. Immunol. Immunopathol*. **80**: 321–324

Tian, J., Lin, X., Guan, R., Xu, J. G. (2004) The effects of hydroxyethyl starch on lung capillary permeability in endotoxic rats and possible mechanisms. *Anesth. Analg*. **98**: 768–774

Vasilye, S., Schaap, R. N. Mortensen, J. D. (1995) Hospital survival rates of patients with acute respiratory failure in modern respiratory intensive care units. An international, multicenter, prospective survey. *Chest* **107**: 1083–1088

Wang, L. Z., Liu, Y. Q., Cui, Y. H., Zhu, F. H., Wang, B. S., Lun, N. (1999) Effects of dexamethasone, cyproheptadine, anisodamine, and dinoprostoneon TNF alpha production in endotoxic shock. *Zhong guo Yao Li Xue Bao* **20**: 171–174

Ware, L. B., Matthay, M. A. (2000) The acute respiratory distress syndrome. *N. Engl. J. Med*. **342**: 1334–1349

Weiss, S. J., Klein, R., Slivka, A., Wei, H. (1982) Chlorination of taurine: evidence for hypochlorous acid generation. *J. Clin. Invest*. **70**: 262–270

Xiu, R. J., Hammerschmidt, D. E., Coppo, P. A., Jacob, H. S. (1982) Anisodamine inhibits thromboxane synthesis, granulocyte aggregation, and platelet aggregation. A possible mechanism for its efficacy in bacteremic shock. *J. Am. Med. Assoc*. **247**: 1458–1460

Yan, M., Guo, X. Y., Ye, T. H., Xiao, H., Wu, X. M., Jia, N. G., Wu, A. S. (2006) Clinical study on penehyclidine hydrochloride as premedication of general anesthesia. *J. Clin. Anesth*. **11**: 179–181