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Experimental

Inhibition of inflammatory responses by ambroxol, a mucolytic agent, in a murine model of acute lung injury induced by lipopolysaccharide

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Abstract

Objective The aim of this study is to investigate whether ambroxol inhibits inflammatory responses in a murine model of lipopolysaccharide-induced acute lung injury (ALI).

Methods Mice (n=295) were first intratracheally instilled with lipopolysaccharide (LPS) to induce ALI and then received an intraperitoneal (ip) injection of either normal saline (NS), ambroxol (30 or 90 mg/kg per day) or dexamethasone (2.5 or 5 mg/kg per day) for 7 days. Metabolism (n=10, each), lung morphology (n=5, each) and wet-to-dry lung weight ratio (n=10, each) were studied. The levels of tumor necrosis factor (TNF-α), interleukin-6 (IL-6) and transforming growth factor (TGF-β1) and the protein concentration (n=5 or 7, each) in bronchoalveolar lavage (BAL) were measured.

Results Mice with LPS-induced ALI that were treated with ambroxol at a dosage of 90 mg/kg per day significantly gained weight compared to the control and dexamethasone-treated groups. Ambroxol and dexamethasone significantly reduced the lung hemorrhage, edema, exudation, neutrophil infiltration and total lung injury histology score at 24 and 48 h. In addition, ambroxol and dexamethasone significantly attenuated the lung wet-to-dry weight ratio at 24 and 48 h (p<0.05). Compared to the control group, TNF-α, IL-6 and TGF-β1 levels in the BAL in both ambroxol- and dexamethasone-treated groups were significantly reduced at 24 and 48 h. The

protein in BAL, an index of vascular permeability, was also significantly decreased in the ambroxol- and dexamethasone-treated groups ($p<0.05$).

Conclusion Ambroxol inhibited proinflammatory cytokines, reduced lung inflammation and accelerated recovery from LPS-induced ALI.

Keywords Lipopolysaccharide - Acute lung injury - Ambroxol - Dexamethasone - Inflammation - Cytokines - Animal experimentation

Introduction

Acute lung injury (ALI) can result from either a primary pulmonary process or a systemic insult. The murine model of lipopolysaccharide (LPS)-induced ALI is characterized by increased capillary permeability, interstitial and alveolar edema and an influx of circulating inflammatory cells [1, 2]. Proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and transformation growth factor-β1 (TGF-β1) were also dramatically increased before alveolar flooding after the induction of lung injury [3, 4]. TGF-β1 may play a critical role in the development of pulmonary fibrosis after ALI [4].

Ambroxol, a mucolytic agent, has been used for the treatment of chronic bronchitis and neonatal respiratory distress syndrome [5, 6, 7]. It exhibits antioxidant [8, 9] and anti-inflammatory properties with reduction of the release of inflammatory cytokines from bronchoalveolar macrophages, monocytes and granulocytes [10, 11]. Glucocorticoids are the most frequently used anti-inflammatory drugs in the treatment of acute respiratory distress syndrome (ARDS) [12]. However, previous studies with glucocorticoids in animal models of lung inflammation/injury have shown variable results [13].

In view of these findings, we reasoned that ambroxol, like dexamethasone, might be used to treat ALI, through its anti-inflammatory effects, to reduce pulmonary edema. As a first step to assess the therapeutic role of ambroxol in ALI, we investigated the dosage and its side effects in a murine model of LPS-induced ALI, compared to saline and dexamethasone. More directly to assess the role of ambroxol, we concentrated on one dosage, determined from the finished experiment, to study its influence on lung histology, proinflammatory cytokines in BAL and lung permeability.

Methods and materials

Animals

Pathogen-free Swiss strain mice (n=295) weighing 18–21 g were used in this experiment. Mice were purchased from the Animal Center of Fudan University (Shanghai, China). They were

housed in air-filtered, temperature-controlled units (24±2°C), kept in a 12:12-h night-day rhythm and had food and water *ad libitum*. The protocol was approved by the Committee of Animal Care of Fudan University.

Drug and reagents

Ambroxol (2-amino-3,5-dibromo-N-(trans-4-hydroxy-cyclohexyl) benzylamine, (Boehringer Ingelheim, Germany), dexamethasone (Shanghai Pharmaceutical, Shanghai, China), lipopolysaccharide (LPS) from *Escherichia coli 055:B5* (Sigma, St. Louis, MO) and ovalbumin (Sigma, St. Louis, MO) were used as well as a TNF-α, IL-6 and TGF-β1 ELISA kit (Biosource International, Camarillo, CA).

Murine model of lipopolysaccharide-induced acute lung injury

The mice were anesthetized with inhalation of ether and fixed on a board at an angle of 50° in a supine position. After sterilization, a mid-line incision was performed in the neck to isolate the trachea. LPS, 1 mg, was diluted in 1 ml sterile saline and intratracheally instilled at a dosage of 5 mg/kg with a 29 gauge-needle syringe (Terumo, Japan) as previously described [*14*, *15*, *16*]. After intratracheal instillation, all mice were put in a vertical position to ensure that the fluid was evenly distributed in both lungs.

Experimental protocol

To determine the appropriate dosage of ambroxol to treat LPS-induced ALI, the mice were divided into five groups (control, ambroxol 30 mg/kg and 90 mg/kg, dexamethasone 2.5 mg/kg and 5 mg/kg). In the control group, mice were given 0.9% saline intraperitoneally (0.3 ml twice a day at 8:00 a.m. and 8:00 p.m.). In the ambroxol-treated group, mice were ip injected with ambroxol at a dosage of either 15 or 45 mg/kg twice a day at 8:00 a.m. and 8:00 p.m. In the dexamethasone group, the mice were ip administered dexamethasone at a dosage of either 2.5 or 5 mg/kg one time at 8:00 a.m. and the other time with NS 0.3 ml at 8:00 p.m. Food consumption, water intake and weight were carefully monitored at 24, 48, 72, 96, 120 and 168 h after treatment.

In the preliminary study, we found that the weight of mice with LPS-induced ALI increased significantly if they were treated with an ip injection of ambroxol at a dosage of 45 mg/kg twice a day. Therefore, we focused on these dosages of ambroxol (45 mg/kg twice a day and dexamethasone (5 mg/kg one time at 8:00 a.m. and the other time with NS at 8:00 p.m.) in the following experiments. To study the histological changes of the lung, the saline-, ambroxol- and dexamethasone-treated mice were exsanguinated at 24 h (n=5, each) and 48 h (n=5, each) after intratracheal instillation of LPS, five normal mice were taken as controls. To measure the lung wet-to-dry weight ratio, the saline-, ambroxol- or dexamethasone-treated groups were killed at 0 h (n=10, each), 24 h (n=10, each) and 48 h (n=10, each). To measure cytokines in the BAL, mice in different groups were killed at 0 h (n=5, each), 24 h (n=6, each), 48 h (n=5, each), 72 h (n=7, each), 120 h (n=5, each) and 168 h (n=7, each) via aortic transection and then BAL was performed. To investigate lung permeability, the protein concentration in BAL was measured at

0, 24, 48 and 72 h.

Metabolism

Food and water was accurately weighed before it was accessible to the mice. At 24, 48, 72, 96, 120 and 168 h after treatment, the remaining food and water was collected and weighed. The food consumption and water intake was determined by the difference between the total amount of food or water that was given to the mice and the total remaining food or water in each group (n=10) at each time point. Weight was also measured at 24, 48, 72, 96, 120 and 168 h after treatment.

Lung histology

After euthanasia by over-inhalation of ether, the chests of the mice were opened by a median sternotomy and the whole lungs were excised. Following inflation with 10 cmH_2O pressure, the right middle lobe of each mouse was removed and fixed in 10% buffered formalin for 24 h. Lung tissue was embedded in paraffin and 4 μm sections were cut. Hematoxylin and eosin stained sections were prepared by standard techniques. The degree of microscopic injury was scored based on the following variables: hemorrhage, edema, exudation, necrosis, congestion, neutrophil infiltration and atelectasis. The severity of injury was judged according to the following criteria [17]: no injury =0; injury to 25% of the field =1; injury to 50% of the field =2; injury to 75% of the field =3 and diffuse injury =4. A pathologist, who was blinded to the experimental protocol, provided a score for each variable based on the severity of injury [18, 19].

Lung wet-to-dry weight ratio

After euthanasia, the whole lungs were excised. The wet weight of lung was measured on an electronic scale and the lung was then desiccated in an oven at 55°C for 72 h to determine the dry weight [20].

Bronchoalveolar lavage

The mice were intubated with a 24-gauge cannula after isolation of the trachea by surgery. The lungs were flushed with 0.9% saline in 0.2-ml increments. The fluid recovery rate was 87±2%. Fluid was filtrated by a 0.4 μm Millipore filter to remove debris and then centrifuged at 2500 rpm for 15 min. Supernatant was collected and frozen at –70°C for later measurements.

Measurements of cytokines and protein concentration in bronchoalveolar lavage

Cytokines (TNF-α, IL-6, TGF-β1) in BAL were measured by ELISA under the same experimental conditions. The total TGF-β1 was measured after pre-activation with acidification to pH 2–3 for 1 h and then corrected to pH 6.5–7.5 prior to the ELISA assay. The protein

concentration in cell-free BAL was measured by the Bradford method with ovalbumin as a standard [21]. The ratio between BAL urea and serum urea was used to calculate the dilution of the original protein concentration by BAL saline.

Statistical analysis

Statistics were conducted by SPSS software (SPSS, Chicago, IL) and the results are presented as means ± SD. One-way analysis of variance (ANOVA) procedures and post hoc analysis (significance level set at $p<0.05$) were used to test for differences in the levels of cytokines and the protein concentration in BAL, and lung wet-to-dry weight ratio. The Mann-Whitney test was used to testify the significance of the histological scores.

Results

Mortality and metabolism

There was no mortality in any group. Generally, mice fur was shinier in the ambroxol-treated group than in the control group and the dexamethasone-treated group. No side effects, such as diarrhea or vomiting, were found in the ambroxol-treated group.

In the control group, the mice lost their appetites, compared to the experimental groups, and could not quickly overcome the symptoms induced by LPS instillation. Mice that were administered with dexamethasone 2.5 and 5 mg/kg per day exhibited diabetes-like symptoms—more food consumption and water intake compared to the NS and ambroxol-treated groups—but did not gain weight. Ambroxol-treated mice (30 or 90 mg/kg per day) drank and ate more than the control group, but not as much as the dexamethasone-treated group, and significantly gained weight after 72 h ($p<0.05$ vs the control and $p<0.01$ vs dexamethasone) (Fig. 1A–C). After 7 days, the net increase of weight from the baseline was: control 20.6±0.5 g, ambroxol 30 mg/kg per day 21.1±0.3 g, ambroxol 90 mg/kg per day 28.1±0.5 g, dexamethasone 2.5 mg/kg per day 4.9±0.1 g and dexamethasone 5 mg/kg per day 3.3±0.5 g.

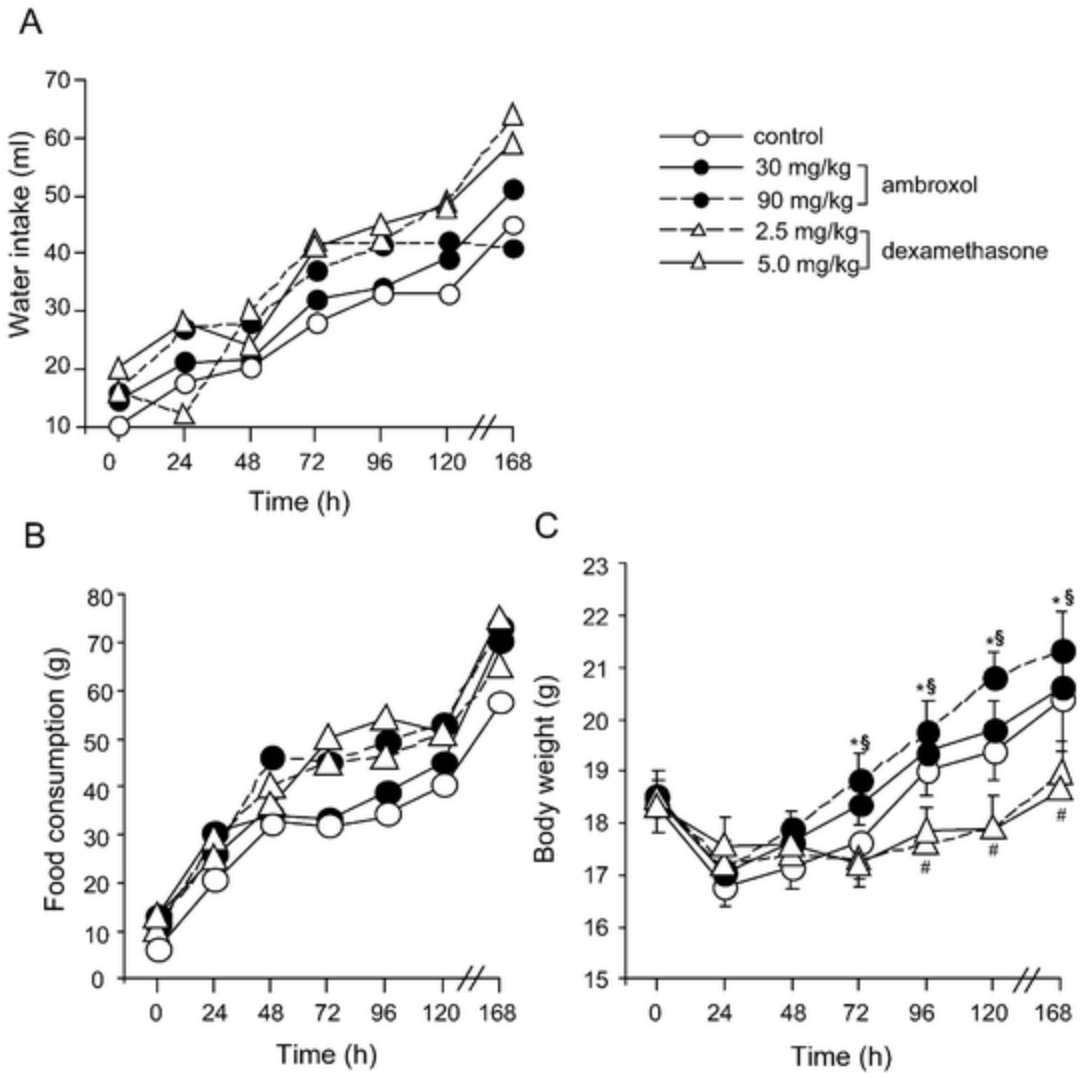

Fig. 1. The influence of ambroxol on metabolism in the mice with LPS-induced ALI. (**A**) Water intake; (**B**) Food consumption (**C**) The changes of body weight. *p<0.05, for ambroxol 90 mg/kg vs control at 72, 96 120 and 168 h; $^{\S}p$<0.01, for the dexamethasone-treated group vs ambroxol 90 mg/kg group at 72, 96 120 and 168 h; $^{\#}p$<0.01, for dexamethasone 2.5 and 5 mg/kg vs control at 96, 120 and 168 h. Values are presented as means ± SD, n=10 in each group

Lung histology

Ambroxol and dexamethasone significantly reduced the lung hemorrhage, edema, exudation, neutrophil and infiltration at 24 h (p<0.05) (Fig. 2A) and at 48 h (p<0.01) (Fig. 2B). This difference is shown in the representative histological sections (a-d, Fig. 2A,B).

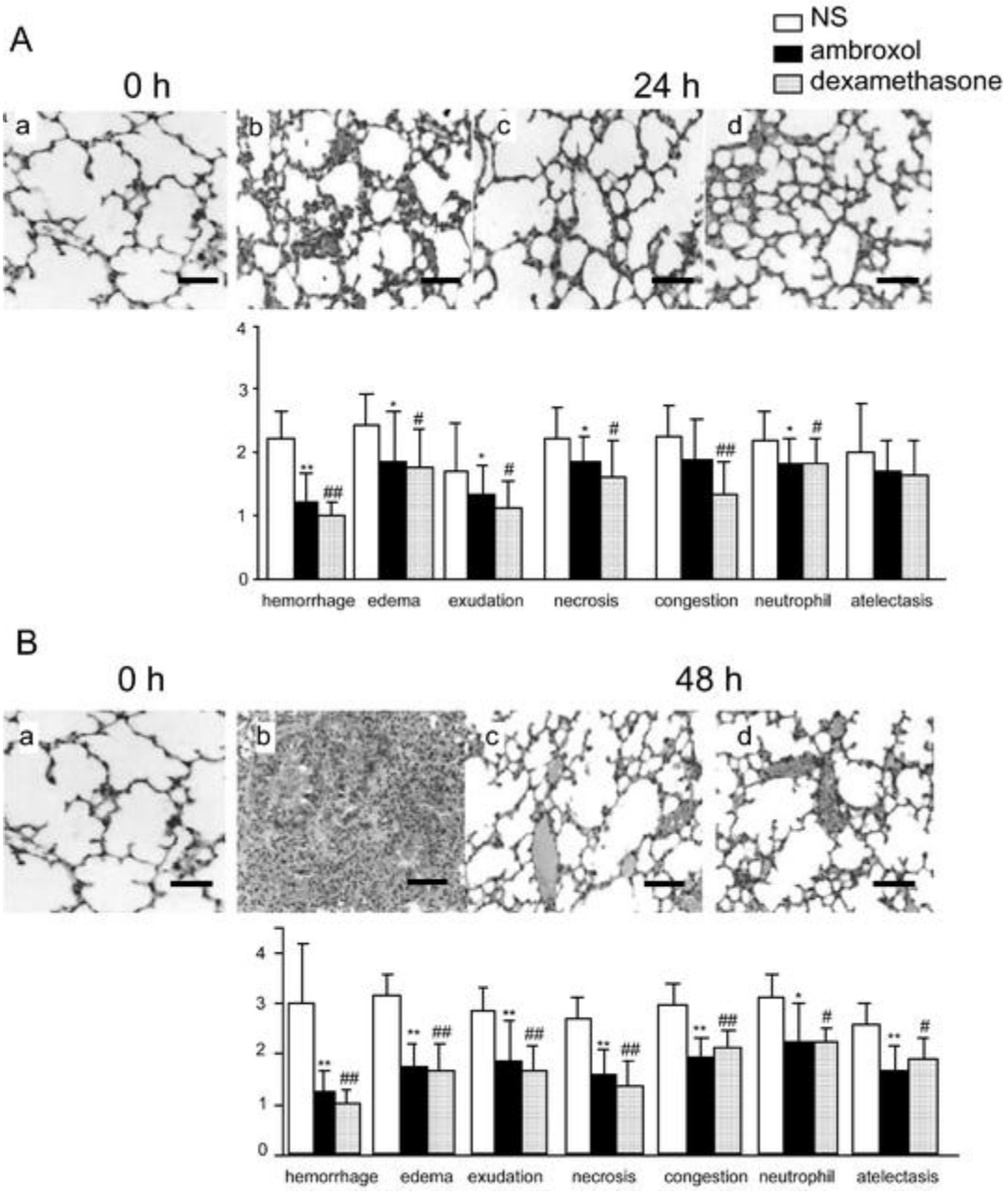

Fig. 2. (**A**) Changes of histology and lung injury scores of lung at 24 h after LPS instillation in the NS-, ambroxol- and dexamethasone-treated groups. *a* representative of histological change at 0 h in the NS-treated group; *b-d* represent histological changes at 24 h in the NS-, ambroxol (90 mg/kg)- and dexamethasone (5 mg/kg)-treated groups, respectively. **p*<0.05, ***p*<0.01 at 24 h in the ambroxol-treated group vs in the NS-treated group; $^{\#}p$<0.05, $^{\#\#}p$<0.01 at 24 h in the dexamethasone-treated group vs in the NS-treated group. (**B**) Changes of histology and lung injury scores of lung at 48 h after LPS instillation in the NS-, ambroxol- and dexamethasone-treated groups. *a* representative of histological change at 0 h in the NS-treated group. *b-d* represent histological change at 48 h in the NS-, ambroxol- and dexamethasone-treated groups. **p*<0.05, ***p*<0.01 at 48 h in the ambroxol-treated group vs in the NS-treated group. $^{\#}p$<0.05, $^{\#\#}p$<0.01 at 48 h in the dexamethasone-treated group vs in the NS-treated group (Magnification ×100, *bar* =50 μm)

Wet-to-dry lung weight ratio

Lung wet-to-dry weight ratio was significantly increased from 4.53±0.23 at 0 h to 6.01±0.64 at 24 h (p<0.05) and 6.48±0.91 at 48 h (p<0.01) after LPS instillation in the NS-treated group. However, lung wet-to-dry weight ratio was significantly decreased at 24 h: 5.44±0.42 (p<0.05) and 48 h: 5.39±0.45 (p<0.05) in the ambroxol-treated group, and at 24 h: 5.33±0.22 (p<0.05) and 48 h: 5.32±0.39 (p<0.05) in the dexamethasone-treated group, compared to that in the NS-treated group at the same time point (Fig. 3).

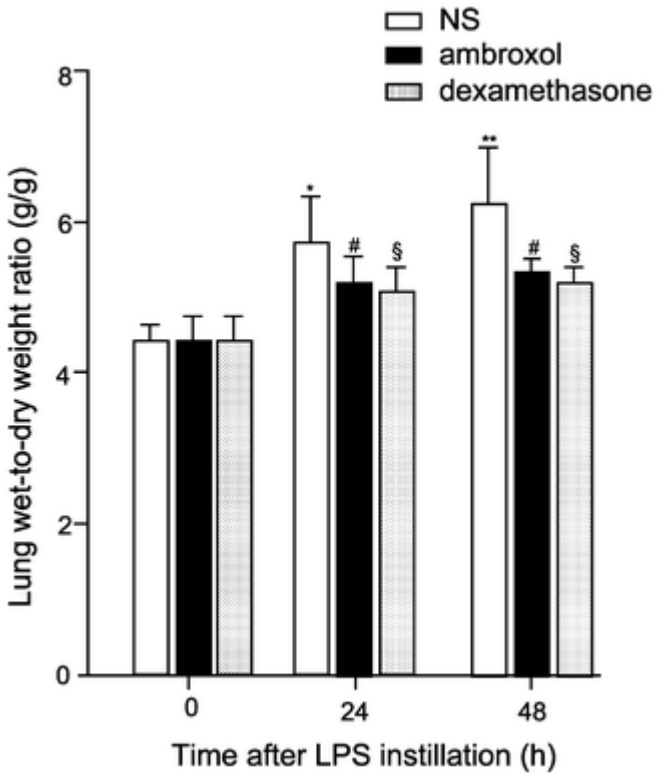

Fig. 3 . Lung wet-to-dry weight ratios were significantly decreased at 24 and 48 h after LPS instillation. $^{*}p$<0.05 24 h vs 0 h in the NS-treated group; $^{**}p$<0.01 24 h vs 0 h in the NS-treated group; $^{\#}p$<0.05 the ambroxol-treated group vs the NS-treated group at 24 h and 48 h; $^{\S}p$<0.05 the dexamethasone-treated group vs the NS-treated group at 24 h and 48 h. Values are presented as means ± SD

The levels of cytokines in bronchoalveolar lavage

Tumor necrosis factor-α

The level of TNF-α in BAL was significantly decreased from 943±46 pg/ml in the NS-treated group to 606±39 pg/ml in the ambroxol-treated group (p<0.05), and to 453±20 pg/ml in the dexamethasone-treated group (p<0.01) at 24 h after LPS instillation. At 48 h, the levels of TNF-

α in BAL were decreased in the dexamethasone-treated groups compared to the NS-treated groups ($p<0.05$). After 72 h, TNF-α levels in BAL fell to the same level in the three groups (Fig. 4A).

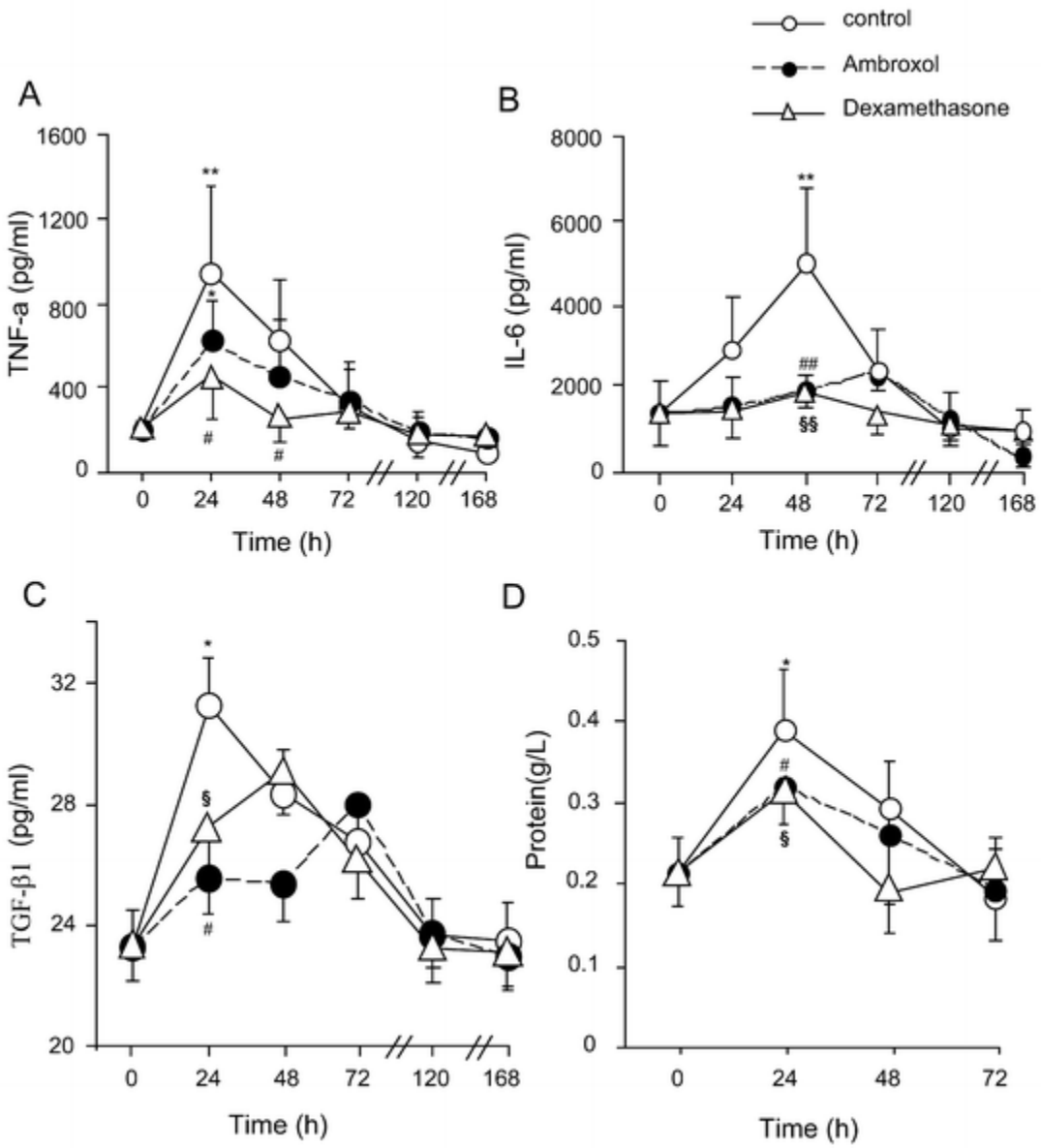

Fig. 4. (**A**) Ambroxol reduced the level of TNF-α in BAL at 24 h after intratracheal instillation of LPS. $^{**}p<0.01$ 24 h vs 0 h in the NS-treated group; $^{*}p<0.05$ the ambroxol-treated group vs the NS-treated group at 24 h; $^{\#}p<0.01$ the dexamethasone-treated group vs the NS-treated group at 24 h and 48 h. (**B**) Ambroxol inhibited the level of IL-6 in BAL at 48 h after intratracheal instillation of LPS. $^{**}p<0.01$ 48 h vs 0 h in the NS-treated group; $^{\#\#}p<0.01$ the ambroxol-treated group vs the NS-treated group at 48 h; $^{\S\S}p<0.01$ the dexamethasone-treated group vs the NS-treated group at 48 h. (**C**) Ambroxol delayed the occurrence of the peak value and reduced the level of TGF-β1 in BAL in LPS induced ALI. $^{*}p<0.05$ 24 h vs 0 h in the NS-treated group; $^{\#}p<0.05$ the ambroxol-treated group vs the NS-treated group at 24 h; $^{\S}p<0.05$ the dexamethasone-treated group vs the NS-treated group at 24 h. (**D**) Ambroxol decreased the protein concentration in BAL at 24 h after instillation of LPS. $^{*}p<0.05$ 24 h vs 0 h in the NS-treated group;

#$p<0.05$ the ambroxol-treated group vs the NS-treated group at 24 h; §$p<0.05$ the dexamethasone-treated group vs the NS-treated group at 24 h. Values are presented as means ± SD

Interleukin-6

The level of IL-6 in BAL was significantly decreased from 5002±3072 pg/ml in the NS-treated group to 1960±307 pg/ml in the ambroxol-treated group ($p<0.01$) and 1891±203 pg/ml in the dexamethasone-treated group ($p<0.01$) at 48 h after instillation of LPS. At 72 h, there were no differences in the levels of IL-6 in BAL in the three groups (Fig. 4B)

Transforming growth factor

The level of TGF-β1 in BAL was significantly increased from a baseline level of 23±2 pg/ml to 31±2 pg/ml at 24 h after LPS instillation in the NS-treated group ($p<0.01$). At 24 h, the levels of TGF-β1 in BAL in the ambroxol- or dexamethasone-treated groups were significantly decreased ($p<0.05$). At 48 and 72 h there were no differences in the levels of TGF-β1 in BAL among the three groups (Fig. 4C).

Protein concentration in bronchoalveolar lavage

The protein concentration in BAL in the NS-treated group significantly increased from the baseline to 0.39±0.04 g/l at 24 h ($p<0.05$). At 24 h after LPS instillation, the level of protein in BAL in the ambroxol-treated group (0.32±0.02 g/l) and in the dexamethasone-treated group (0.31±0.04 g/l) was significantly decreased compared to that in the NS-treated group ($p<0.05$; Fig. 4D).

Discussion

The potential efficacy of ambroxol to treat ALI is based in part on the hypothesis that it will suppress the proinflammatory cytokines of ALI, resulting in less lung edema. To test this hypothesis, we used a well-established murine model of ALI induced by LPS to study the effects of ambroxol on metabolism, lung histology, lung fluid balance, cytokines in BAL and lung permeability.

An earlier study showed that when male BALB/c mice were orally administered ambroxol (3–30 mg/kg) 1 h before intratracheal injection of HOCl or LPS, the ratios of lung wet weight to body weight and lung dry weight to body weight were not markedly suppressed [22]. However, polymorphonuclear neutrophil leukocytes (PMNs) in the lung vessels and alveolar septa of rats with LPS-induced ALI were significantly reduced when they were injected ip with ambroxol 70 mg/kg for 3 days [23]. Therefore, we attempted to increase the dosage of ambroxol to

90 mg/kg to treat mice with LPS-induced ALI. The growth of normal mice injected with ambroxol at a dose of 90 mg/kg per day was not affected (data not shown).

This study showed that there was no mortality in the five groups. Mice treated with ambroxol at a dosage 90 mg/kg per day gained more weight than the NS- and dexamethasone-treated groups. Mice treated with 30 mg/kg of ambroxol did not gain weight compared to the control group. This indicates that a significant increase in weight in the ambroxol-treated group was possibly related to decreased lung inflammation and, as such, relieved the systemic symptoms and, furthermore, accelerated recovery from lung injury. However, mice in the dexamethasone (5 mg/kg)-treated group did not gain weight even if they consumed more food and water, which suggested dexamethasone could not improve the outcome of mice with LPS-induced ALI. Recently, a report showed that ambroxol 10 mg/kg per day significantly improved the survival rate of mice infected with a lethal dose of influenza-A virus, but its effect at a dose of 30 mg/kg per day was less significant [24]. Whether the variance of the result is due to different strains of mice or animal models deserves further study.

Neutrophils play a major role in ALI [25]. Our results showed that intratracheal instillation of LPS in mice triggered an influx of neutrophils into airway spaces from 24 to 48 h [26]. In addition, the lung injury score of edema, exudation, atelectasis and hemorrhage was significantly increased. Ambroxol, like dexamethasone, could significantly reduce the lung hemorrhage, edema, exudation and neutrophil infiltration score at 24 and 48 h. At 24 and 48 h, the lung wet-to-dry weight ratio was also significantly decreased in the ambroxol- and dexamethasone-treated groups. Ambroxol directly decreases recruitment of neutrophils into the lung tissue and scavenges oxygen free radicals released from neutrophils [8, 17, 27].

In the present study there were significantly increased levels of TNF-α (at 24 h), IL-6 (at 48 h) and TGF-β1 (at 24 h) in BAL in the NS-treated group compared to baseline. This suggests that TNF-α, IL-6 and TGF-β1 play an important role in LPS-induced ALI. This finding is also consistent with other investigations [1, 3]. Ambroxol significantly decreased the levels of TNF-α (at 24 h), IL-6 (at 48 h) and TGF-β1 (at 24 h) in BAL. Other studies have also demonstrated that ambroxol is not only able to inhibit acute mediator release from mast cells and leukocytes, but also reduces immunomodulatory cytokine generation from basophils [11].

A significant increase in the level of TGF-β1 in BAL at 24 h after LPS instillation suggests that TGF-β1 might participate in the early inflammatory responses [3, 4]. Ambroxol reduced the level of TGF-β1 in BAL at 24 h and this level returned to the baseline at 72 h in the control group, which might be beneficial for the resolution of lung inflammation.

There was a significant increase in protein concentration in BAL, an index of microvascular permeability, at 24 h after LPS instillation. A previous study has also demonstrated that albumin concentration in the BAL at 24 h after intratracheal instillation of 30 μg of LPS significantly increased, compared with that after instillation of either a lower dose of LPS or 0.9% NS [28]. In our ambroxol-treated group, the protein concentration in BAL was significantly reduced. These

findings indicated ambroxol effectively decreased the lung vascular permeability and promoted the resolution of lung edema.

In this study ambroxol and dexamethasone are effective anti-inflammatory agents in the early phase of ALI. To exert anti-inflammatory effects, 18 mg of ambroxol is equivalent to 1 mg of dexamethasone. However, the mice which received dexamethasone developed a severe diabetes-like syndrome in the late phase. This may be related to side effects of dexamethasone on the metabolism of glucose, lipid, protein, salt and water of the mice. The growth of the dexamethasone-treated mice was stagnant in the late stage.

Intratracheal LPS-induced ALI is a model of self-limited lung inflammation, but it reproduces many features of sepsis-induced acute lung injury [28]. In the control group, the levels of TNF-α, IL-6 and TGF-β1 in BAL returned to baseline after 72 h of LPS instillation. Also, mice did well in eating and drinking, and gained weight. This indicates that the significant increase of weight in the ambroxol 90 mg/kg per day group may possibly be related to effective suppression of inflammatory responses in the first 2 or 3 days.

As to the limitations of our LPS-induced ALI model, we need to test the effects of ambroxol in the other models, such as acid aspiration. Our unpublished results show that intratracheal instillation of hydrochloride acid (pH 1.2) induces ALI 3 h later. Ten mice intratracheally instilled with acid and treated with ambroxol at a dose of 90 mg/kg per day survived and gained weight 7 days after treatment. However, at 48 or 72 h after acid instillation, 2 of 10 saline-treated mice died of severe lung injury confirmed by postmortem anatomy. The surviving mice did not grow as fast as the ambroxol-treated group.

High doses of ambroxol might have negative effects on host defenses [24]. However, ambroxol is well tolerated in pregnant women in doses of 1 g, which is administrated to accelerate fetal lung maturity [23]. Therefore, more work will be necessary in clinics to find an optimal therapeutic dosage of ambroxol with minimal side effects.

In addition, administration of ambroxol (70 mg/kg ip) once a day for 3 days protected lung and heart lipids from LPS-induced oxidative stress in mice [29]. Ambroxol can sufficiently enhance the antioxidant defense in lung tissue and can act as a lung lipid antioxidant [30]. This effect may have contributed to less lung injury and better recovery in the ambroxol-treated group.

In summary, ambroxol reduces the levels of proinflammatory cytokines in BAL, promotes resolution of inflammation of the lung in the early stage of ALI and accelerates recovery from LPS-induced lung injury.

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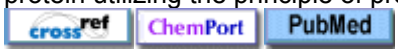

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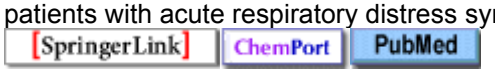

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