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Thalidomide Reduces Lipopolysaccharide/Zymosan-Induced Acute Lung Injury in Rats

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Key Words
Anti-inflammation · Nitric oxide · Sepsis · Vascular permeability · Zymosan

Abstract
Pharmacological therapies targeting fulminant lung inflammation in acute lung injury (ALI) need to be improved. We evaluated the effect of thalidomide, a chemical modulating both acute and chronic inflammation, on ALI induced by intravenous administration of lipopolysaccharide (LPS) and zymosan in male Sprague-Dawley rats. Injection of LPS and zymosan induced significant lung inflammation, as evidenced by increased neutrophil sequestration in lung tissue as well as enhanced nitric oxide metabolite (NO_x^-) production in the serum and bronchoalveolar lavage (BAL) fluid. Lactate dehydrogenase (LDH) activity and protein concentration in BAL fluid were significantly increased after administration of LPS and zymosan. Pulmonary microvascular permeability was determined using the Evans blue retention method, which showed a significant increase in microvascular permeability after LPS and zymosan administration, indicating the development of ALI. Animals that received thalidomide (100 mg/kg) 2 h prior to LPS injection had significantly reduced pulmonary NO_x^- production, pulmonary microvascular permeability, and LDH activity and protein concentration in BAL fluid. We therefore conclude that thalidomide ameliorates lung inflammation and reduces ALI induced by combined LPS and zymosan administration in rats.

Severe sepsis is the most common risk factor associated with the development of acute lung injury (ALI), with a reported incidence in 6 to 42% of lung injury cases [36]. The characteristic features of sepsis-induced lung injury include activation and subsequent sequestration of polymorphonuclear leukocytes in the pulmonary microvasculature with resultant endothelial cell injury [1, 36], and disruption of pulmonary capillary integrity leading to non-cardiogenic pulmonary edema [21]. The support provided by both respiratory therapy and pharmacological intervention in the management of ALI are, to a large degree, nonspecific. Strategies of ventilatory support have been extensively investigated, aiming to improve PaO_2 and to avoid complications [4, 27]. Adjunctive pharmacological therapies targeting systemic inflammation and hypoxemia of ALI have also been widely studied [19, 31].

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Despite a variety of treatment strategies, the mortality from sepsis-related ALI has not changed, indicating that the management of fulminant lung inflammation needs to be improved.

Extensive studies have focused on various pharmacologic approaches to modulating the inflammatory response that propagates ALI induced by severe sepsis. Antiadhesion molecule antibodies specifically block the interactions between leukocytes and endothelial cells and were demonstrated to reduce tissue inflammation and organ injury in neutrophil-dependent lung injury [3]. Antioxidants such as N-acetylcysteine and vitamin C prevent tissue damage in a variety of animal models of sepsis-induced lung injury [11]. Corticosteroids inhibit proliferation and activation of inflammatory cells and have been shown to reduce lung injury and organ failure when administered in doses reflecting levels of this hormone during stress [25]. Drugs that enhance the intracellular level of cyclic AMP such as pentoxifylline and lisofylline have been shown to inhibit chemotaxis and activation of neutrophils in animal models with sepsis and ALI [12, 14]. However, clinically, none of the above-mentioned treatments provides a survival advantage to patients with ALI [37]. An effective pharmacologic intervention remains to be established.

Thalidomide (α-N-phthalimidoglutarimide), a synthetic sedative derived from glutamic acid, has been administered to patients with chronic inflammatory diseases such as erythema nodosum leprosum [28] and rheumatoid arthritis [2]. It was recently reported that thalidomide might be used to control acute inflammation because of its anti-inflammatory, immunomodulatory, and anti-angiogenic properties [22]. Thalidomide inhibits neutrophil chemotaxis into the site of inflammation [9], suppresses the generation of reactive oxygen substances from activated neutrophils [29], and modulates the neutrophil-endothelial cell interaction via an adhesion cascade [28]. Furthermore, thalidomide may inhibit production of TNF-α by enhancing mRNA degradation [30]. These findings indicate that thalidomide is a potential anti-inflammatory drug. In the present study, we investigated the protective effect of thalidomide on ALI induced by lipopolysaccharide (LPS).

Methods

Animal Preparation

The experiment was conducted in accordance with the *Guiding Principles in the Care and Use of Animals* approved by the Institutional Animal Care and Use Committee of the National Defense Medical Center, Taiwan, ROC. Male Sprague-Dawley rats, weighing 250–350 g, received thalidomide pretreatment 2 h before the experiment. Thalidomide (100 mg/kg) was suspended in 200 μl Tween 80/H_2O and was given by gavage. Rats were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg). The trachea was cannulated to facilitate respiration. The femoral artery and vein were catheterized with PE50 tubing in order to monitor systemic blood pressure and to administer drugs.

Experimental Protocols

Rats were randomly divided into three groups as follows: (1) the control group (n = 18), which received normal saline injection intravenously, (2) the LPS/zymosan group (n = 18), which received intravenous injection of LPS (*Escherichia coli* 055:B5, 10 mg/kg) and zymosan (*Saccharomyces cerevisiae*, 10 mg/kg), and (3) the thalidomide group (n = 18), which received thalidomide pretreatment followed by injection of LPS and zymosan.

Arterial blood pressure was continuously monitored for 4 h after injection of LPS and zymosan. In one third of the rats in each group, arterial blood was sampled periodically during the experiment for analysis of blood gas and nitrite/nitrate (NO_x^-). At the end of each experiment, the rat was killed by pentobarbital overdose and the thorax was opened via midline thoracotomy. The right lung was clamped and excised for myeloperoxidase (MPO) measurement and wet/dry (W/D) lung weight ratio determination. Bronchoalveolar lavage (BAL) was completed on the left lung for measurement of nitrite/nitrate (NO_x^-), protein concentration, and lactate dehydrogenase (LDH) activity. An increase in the W/D ratio of the lung weight represents fluid accumulation in the lung or acute pulmonary edema. Protein concentration and LDH activity in the alveolar space indicate protein influx and alveolar cell damage, respectively. The W/D ratio of the lung weight, as well as the protein concentration and LDH activity in BAL fluid, have been widely used as indices of ALI [16, 23, 32, 39]. To determine pulmonary vascular permeability, another 6 rats from each group received Evans blue dye injection via a venous catheter 1 h before the left lung was harvested to measure dye concentration. The histopathology of the lungs was analyzed in another 6 rats from each group.

Bronchoalveolar Lavage

The rats were sacrificed and BAL was performed in the left lung using 5 ml phosphate-balanced saline in 2.5-ml aliquots. The recovered BAL fluid was centrifuged at 250 *g* for 10 min. Activity of LDH was measured by a method described previously [24]. In brief, part of the supernatant was incubated with 0.24 m*M* NADH in a Tris/NaCl pH 7.2 buffer at room temperature for 5 min. The reaction was then initiated by the addition of 9.8 m*M* pyruvate and assessed using a spectrophotometer at 340 nm for 2 min. The protein concentration of the supernatant was determined using BCA protein assay reagents (Pierce, Rockford, Ill., USA) and utilizing bovine serum albumin as a standard.

Nitrate/Nitrite (NO_x^-) Determination

Nitrate/nitrite (NO_x^-) concentrations in the serum and BAL fluid were measured using a chemiluminescence technique [15]. Briefly, blood samples for nitrite/nitrate (NO_x^-) were centrifuged cold at 10,000 *g* for 5 min, within 15 min of collection. Serum samples were stored at −70 °C and analyzed within 1 month. For analysis, serum and BAL fluid were deproteinized with an equal volume of ice-cold absolute ethanol. The NO_x^- in the supernatants were reduced with

vanadium to NO, which in turn reacted with O_3 to produce $^{\cdot}NO_2$. The energy emitted from $^{\cdot}NO$ can be detected using chemiluminescence.

W/D Ratio Determination

The rats were killed with an overdose of pentobarbital and a midline thoracotomy was performed. The right lung was excised after clamping the right pulmonary trunk and immediately weighed (wet weight) before being dried for 48 h at 60 °C, and then weighed again (dry weight).

Measurement of Pulmonary Vascular Permeability

Increased pulmonary microvascular permeability is an early marker of lung vascular injury as well as an important determinant in the development of noncardiogenic pulmonary edema. Evans blue dye has a high affinity for plasma protein, and is often used as a marker for determining vascular wall permeability [23]. In brief, Evans blue dye (2 mg/kg) was injected via the right femoral vein 1 h before sacrifice. At the end of the experiment, a midline thoracotomy was performed and the right lung was clamped at the hilar level. A catheter was placed into the pulmonary artery via the right ventricle, through which the left pulmonary circulation was flushed with 50 ml normal saline. Another large bore catheter was inserted into the left ventricle for drainage. The left lung was excised and weighed and placed in 5 ml dimethyl formamide for 24 h. The concentration of Evans blue in the extract was then assayed using a spectrophotometer at 620 nm. The results were expressed as micrograms of dye per gram of lung tissue.

MPO Activity

Pulmonary neutrophil infiltration was quantified by measuring MPO activity using a spectrophotometric method. Briefly, the right lung tissues were harvested and wet weights were recorded before the tissues were frozen in liquid nitrogen. Upon thawing, the lung tissues were suspended in 50 m*M* potassium phosphate buffer (pH 6.0), and homogenized. The homogenates were then centrifuged at 15,000 *g* for 10 min at 4 °C, and the resultant tissue pellets were rehomogenized with 0.5% hexadecyltrimethylammonium bromide. The specimen was freeze-thawed and sonicated thrice. The homogenates were again centrifuged at 15,000 *g* for 10 min at 4 °C, and the supernatants were collected for measurement of MPO activity. Supernatant (25 µl) was mixed with 975 µl of 50 m*M* phosphate buffer (pH 6.0) containing 0.167 mg/ml of *o*-dianisidine dihydrochloride and 0.0005% hydrogen peroxide. One unit of peroxidase activity is equal to the amount of enzyme decomposing 1 µmol of hydrogen peroxide per minute at 25 °C. MPO activity was assayed by measuring the change in absorbance at 460 nm (expressed as the change in absorbance/min/g of wet lung tissue).

Histopathology

After midline thoracotomy, the lungs were exposed and fully inflated with 10% paraformaldehyde in 0.1 *M* PBS at an airway pressure of 20 cm H_2O. The right lower lobe was then removed after ligation and fixed further by immersion in the same fixative for 24 h. The lung tissues were embedded in paraffin wax and cut into 4- to 6-µm-thick sections using a microtome. After these procedures, the sections were stained with hematoxylin and eosin for assessment of interstitial edema and the degree of neutrophil infiltration. Neutrophil sequestration was determined by counting the number of neutrophils in a high power field (×400) using light microscopy.

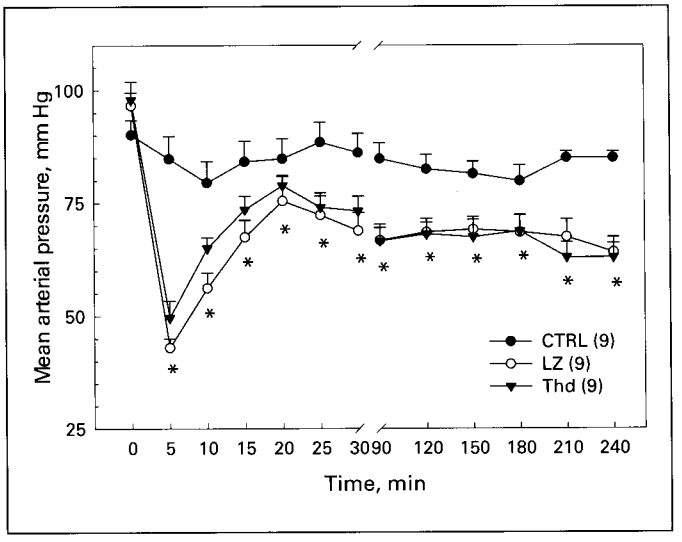

Fig. 1. Effect of thalidomide pretreatment (Thd) on mean arterial pressure in rats after LPS and zymosan injection (LZ). * $p < 0.05$ compared with the control group (CTRL).

Statistical Analysis

The data were expressed as means ± SEM. The changes of serum NO_x^- were evaluated using ANOVA with repeated measures. The differences of other parameters among groups were evaluated by using one-way ANOVA. When the variables were found to be statistically different, a multiple comparison test (Fisher's PLSD) was performed as the post-hoc examination. A value of $p < 0.05$ was considered to be statistically significant.

Results

Effects of Thalidomide on Blood Pressure Changes

Intravenous administration of LPS and zymosan caused a biphasic hypotensive response (fig. 1). The initial profound hypotension recovered within 20 min and was followed by a slow decline in blood pressure. Pretreatment with thalidomide did not alter the hypotensive response to LPS and zymosan injection.

Effects of Thalidomide on Protein Concentration and LDH Activity in BAL Fluid

Intravenous administration of LPS and zymosan caused a fivefold elevation of LDH activity and a twofold increase in the protein concentration in the BAL fluid compared with those in the control animals (fig. 2). Rats pretreated with thalidomide 2 h prior to LPS and zymosan administration had significantly reduced protein levels and LDH activity in the BAL fluid.

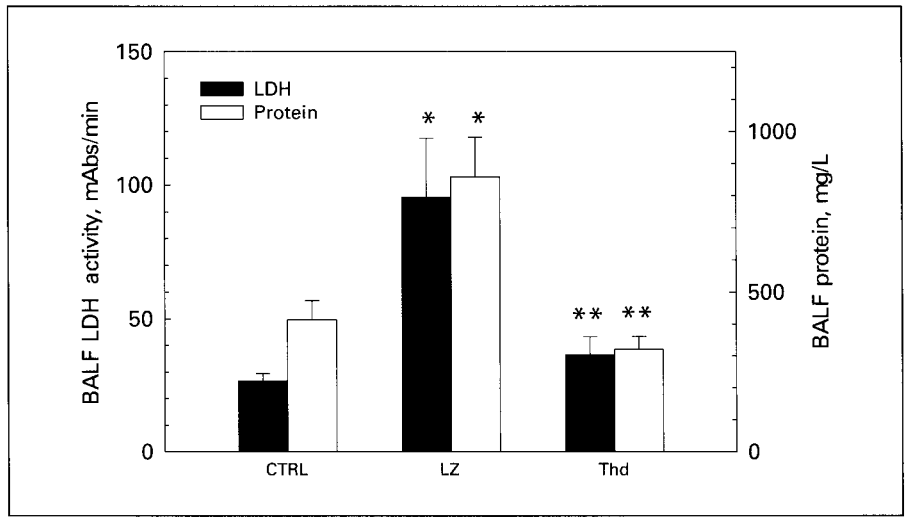

Fig. 2. Effect of thalidomide pretreatment (Thd) on the changes in protein concentration and LDH activity in the BAL fluid induced by venous injection of LPS and zymosan (LZ). * $p < 0.05$ compared with the control group (CTRL); ** $p < 0.05$ compared with the LZ group.

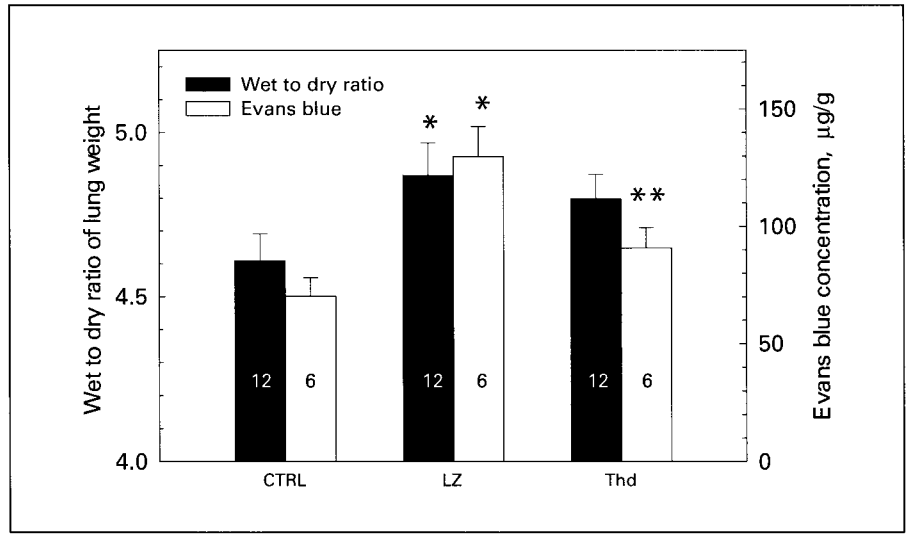

Fig. 3. Changes in W/D lung weight ratio and pulmonary microvascular permeability in rats after LPS and zymosan injection. The numbers in the columns represent group sizes. * $p < 0.05$ compared with the control group (CTRL); ** $p < 0.05$ compared with the LPS-zymosan group (LZ). Thd = Group receiving thalidomide pretreatment before LPS-zymosan injection.

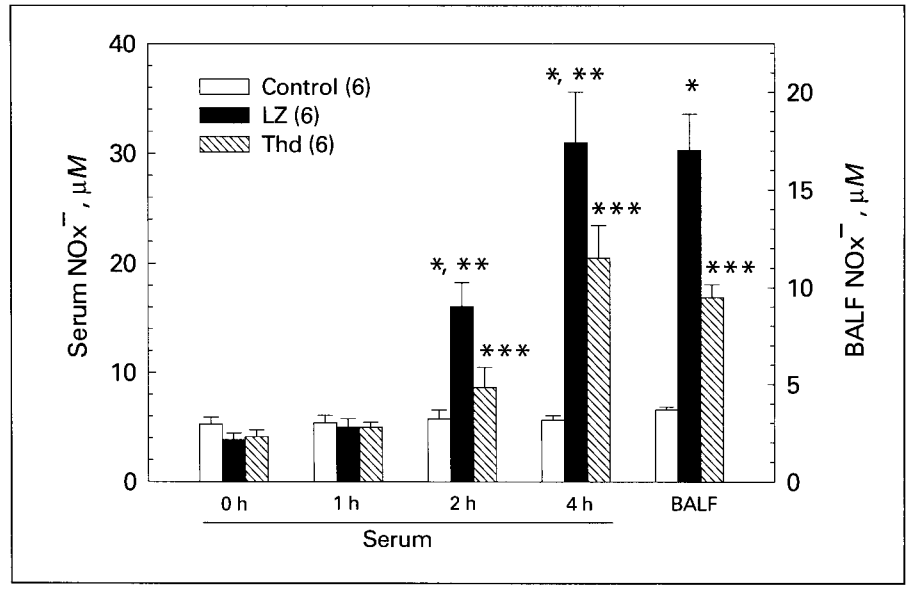

Fig. 4. Serum and BAL fluid concentration of NO_x^- in rats after LPS and zymosan injection (LZ). * $p < 0.05$ compared with the control group; ** $p < 0.05$ compared with serum level at 0 hour; *** $p < 0.05$ compared with the LZ group. Thd = Group receiving thalidomide pretreatment before LPS-zymosan injection.

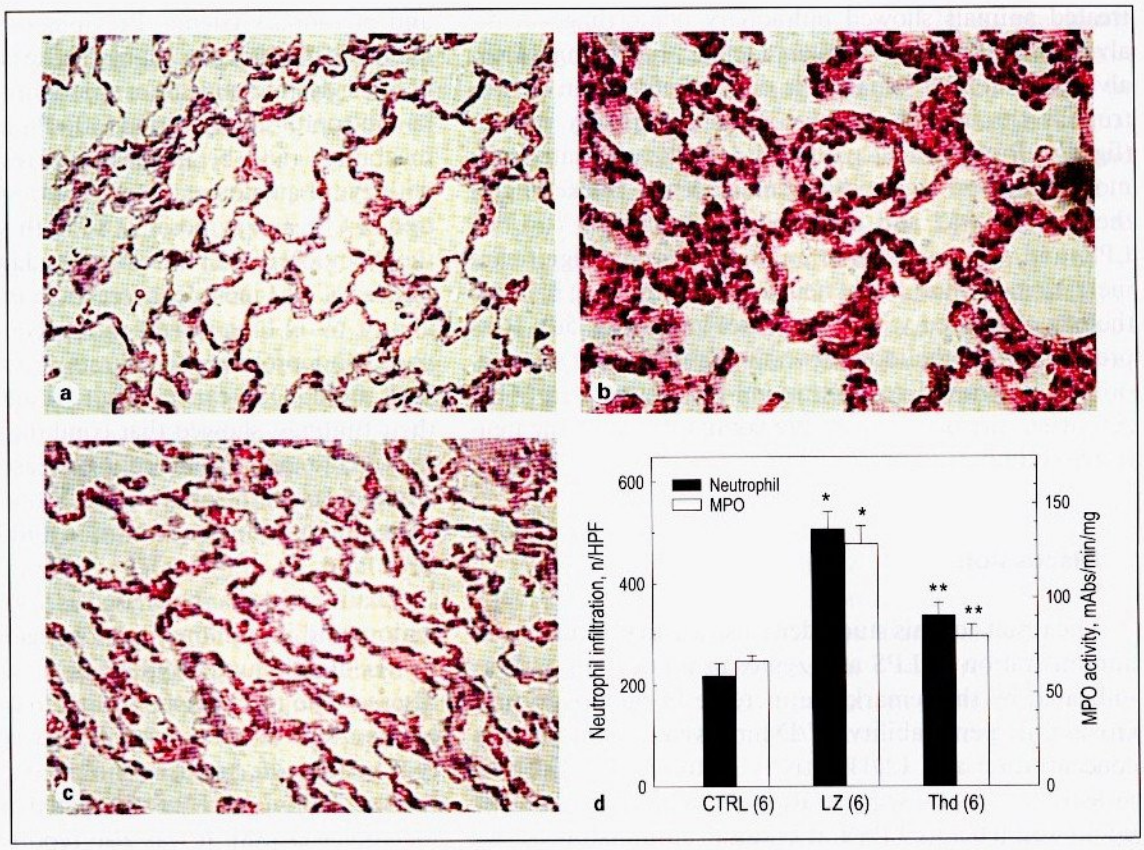

Fig. 5. Histological appearance of lung tissue from rats with LPS- and zymosan-induced lung injury. **a** Control group (× 400). **b** Lung tissue from an animal treated with intravenous injection of LPS and zymosan (× 400). **c** Lung tissue from an animal pretreated with thalidomide 2 h prior to intravenous administration of LPS and zymosan (× 400). **d** Neutrophil count per high-power field (HPF) and MPO activity in the lung tissue. * $p < 0.05$ compared with the control group (CTRL); ** $p < 0.05$ compared with the LZ group. Thd = Group receiving thalidomide pretreatment before LPS-zymosan injection.

Effects of Thalidomide on Pulmonary Microvascular Permeability

The effects of treatment with LPS and zymosan on the change of pulmonary microvascular permeability were assayed using the W/D lung weight ratio and Evans blue dye in lung tissue. Rats treated with LPS and zymosan showed an 85% increased pulmonary microvascular permeability compared with the control group and had a significantly greater W/D ratio of lung weight (fig. 3). Rats pretreated with thalidomide 2 h prior to LPS and zymosan administration had a significantly attenuated increase in pulmonary microvascular permeability, but the reduction in the W/D ratio was not statistically significant.

Effects of Thalidomide on BAL Fluid and Blood NO_x^- Production

LPS- and zymosan-treated animals showed a time-dependent increase in serum NO_x^- production. Parallel to the changes in the serum levels, the concentrations of NO_x^- in BAL fluid determined at the end of the experiment were significantly greater in these animals than in the control group (fig. 4). Rats pretreated with thalidomide 2 h prior to LPS and zymosan administration showed a significant attenuation of NO_x^- production in BAL fluid compared to the LZ group.

Effects of Thalidomide on Pulmonary Neutrophil Sequestration

Histologically, lungs taken from the control group were of normal architecture (fig. 5a). LPS- and zymosan-

treated animals showed pulmonary hemorrhage in the alveolar spaces, perivascular edema, thickening of the alveolar wall, and remarkable diffuse infiltration of neutrophils throughout the alveolar and capillary regions (fig. 5b). Pretreatment with thalidomide attenuated pulmonary hemorrhage, perivascular edema, thickening of the alveolar wall and neutrophil sequestration (fig. 5c). LPS- and zymosan-treated animals showed a significant increase in the number of neutrophils in the lung histopathology compared with the control group (fig. 5d). Rats pretreated with thalidomide showed a significant attenuation in neutrophil sequestration. The changes in MPO activity in the lung tissue were compatible with the neutrophil number.

Discussion

The results of this study demonstrate that intravenous administration of LPS and zymosan induces an ALI, as indicated by the remarkable increase in pulmonary microvascular permeability, W/D lung weight ratio, protein concentration and LDH activity in the BAL fluid, and massive neutrophil sequestration. Pretreatment with thalidomide 2 h before LPS and zymosan administration significantly attenuates the increases in pulmonary microvascular permeability, protein concentration and LDH activity in the BAL fluid, and pulmonary neutrophil sequestration. These findings suggest that thalidomide has a protective effect on ALI caused by intravenous administration of LPS and zymosan.

Clinically, ALI usually occurs early in severe sepsis, with a reported incidence in 6–42% of cases [36]. Several clinical conditions have been associated with the development of ALI, with severe sepsis being the most common risk factor. A characteristic feature of sepsis-induced lung injury is the activation and subsequent sequestration of polymorphonuclear leukocytes in the pulmonary microvasculature, with resultant endothelial cell injury [1]. LPS, a cell membrane component of gram-negative bacteria, is a potent microbial product inducing a severe host inflammatory response and a series of tissue injuries, including injury to the lungs. LPS may produce its toxic effects on the lungs by direct injury to the endothelium and indirect activation of pro-inflammatory cytokines as well as other cytotoxic agents [35]. To establish a septic lung model, LPS has been administered intravenously [6] or intraperitoneally [7] to evoke a septic response in rats. However, these insults primarily induced cardiovascular collapse or only mild ALI without significant hypoxemia and pulmonary edema. Previous data showed that LPS alone did not induce a change in the vascular permeability of mice, despite causing an approximately sixfold increase in neutrophil sequestration [40]. Pulmonary vascular permeability could be increased in response to treatment with zymosan alone, as well as in response to a combination of LPS and zymosan [18]. In this study, we modified the rat model of ALI developed by Jadwiga et al. [18]. The indices of ALI include increases in microvascular permeability, W/D lung weight ratio, sequestration of neutrophils, and protein concentration and LDH activity in BAL fluid. Our experimental results, compatible with their findings, showed that combined LPS and zymosan treatment caused significant increases in pulmonary microvascular permeability, the sequestration of neutrophils, and protein concentration and LDH activity in the BAL fluid.

Thalidomide has been used as an effective anti-inflammatory and immunomodulatory agent in rheumatoid arthritis, discoid lupus erythematosus and graft-versus-host disease. The mechanism of thalidomide is still not clear, but its effects have been ascribed to its anti TNF-α property. Thalidomide has been shown to suppress the production of TNF-α by LPS-stimulated monocytes [33] and macrophages [38]. It was also reported that thalidomide downregulates monocyte-specific surface markers, as well as the surface expression of integrin adhesion molecules of lymphocytes, monocytes, and granulocytes [26]. Recent data suggested that expression of adhesion molecules is differentially regulated by thalidomide, with decreased density of L-selectin on polymorphonuclear leukocytes and enhancement of ICAM-1 expression on TNF-activated HUVEC [10]. Thalidomide may exert its inhibitory action on TNF-α by enhancing mRNA degradation [30] or by suppressing transcription factor NF-κB activity [20]. Although these anti-inflammatory effects may account for the benefits of thalidomide in LPS-induced ALI, their involvement was not examined in the present study and therefore remains to be further investigated.

In the present study, the lung injury response to combined LPS and zymosan was inhibited by pretreatment with oral administration of thalidomide. However, pretreatment obviously lacks clinical relevance because sepsis-induced lung injury is unpredictable. This limitation would not be solved until water-soluble thalidomide becomes available. The drug is available in a crystal/powder form and is virtually insoluble in aqueous, alcohol and oil media, and thus only attains adequate serum levels after oral administration. The serum levels are detectable by high-pressure liquid chromatography within 1 h of oral

administration and peak at 2–4 h [8, 34]. Therefore, we administered thalidomide 2 h prior to LPS injection. In our animal model, the rats were sacrificed 4 h after zymosan administration, so the serum thalidomide level should have peaked within this experimental period.

Neutrophils sequestered in the lung play a fundamental role in the pathogenesis of ALI. An important relationship between the sequestration of neutrophils and increased pulmonary microvascular permeability is supported by demonstration that the magnitude of the increase in lung vascular permeability resulting from endotoxemia is diminished by neutrophil depletion [13]. Pretreatment of rats with thalidomide significantly decreased pulmonary microvascular permeability, although the W/D lung weight ratio was not significantly reduced when compared with the combined LPS and zymosan group. This finding indicates that thalidomide can inhibit neutrophil recruitment and activation, concurring with a previous report [5]. On the other hand, the lack of attenuation of the W/D lung weight ratio may be explained by two observations. First, the lung injury in this rat model was at an earlier stage than that found in acute pulmonary edema or in the acute respiratory distress syndrome. Second, the mechanism of fluid reabsorption may be slower than that of endothelium repair.

Whether NO plays a protective or deleterious role in lung function remains debatable. Evidence has shown that inhibition of NO synthesis by L-NAME enhanced LPS-induced ALI [41]. Other observations suggested that inhaled NO inhibits neutrophil migration and oxidative activity, and then significantly attenuates sepsis-induced ALI. It was also reported that NO inhibits LPS-induced TNF synthesis in vitro and in vivo [17]. As previously mentioned, excessive NO production plays an important role in the induction of lung injury either by itself or by the formation of peroxynitrite. Recent data suggested that infusion of an NOS inhibitor attenuates lung injury and microvascular leakage induced by endotoxemia [21]. Kristof et al. [21] demonstrated that LPS injection in wild-type mice elicits a significant degree of ALI that is associated with extensive nitrotyrosine formation and induction of iNOS protein. Our present results showed that injection of LPS and zymosan increased NO production in the blood and bronchoalveolar space, which was abolished by thalidomide administration. These findings suggest that inhibition of NO formation may play a role in counteracting the detrimental effects of lung injury caused by combined LPS and zymosan treatment.

In summary, our study demonstrates that combined LPS and zymosan administration causes remarkable lung injury and pulmonary microvascular dysfunction. Pretreatment with thalidomide significantly reduces neutrophil sequestration, NO_x^- production, pulmonary microvascular permeability, and protein concentration and LDH activity in the BAL fluid. We conclude that the combination of LPS and zymosan produces a specific, neutrophil-mediated ALI model, and that this lung injury can be alleviated by thalidomide administration.

Acknowledgments

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