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PROTECTIVE EFFECTS OF EARLY TREATMENT WITH LIPOIC ACID IN LPS- INDUCED LUNG INJURY IN RATS

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A lipopolysaccharide (LPS) stimulates the synthesis and releases several metabolites from phagocytes which can lead to an endotoxic shock characterized by multiple organ injury with the earliest to occur in the lungs. Among LPS-induced metabolites, reactive oxygen species are considered to play a crucial pathogenetic role in the lung damage. In this study, the effect of early administration of an antioxidant, α-lipoic acid (LA), on pulmonary lipid peroxidation, lung hydrogen peroxide (H_20_2) concentration, and lung sulfhydryl group content was evaluated in rats with endotoxic shock induced by administration of LPS (*Escherichia coli* 026:B6, 30 mg/kg, *i.v.*). In addition, lung edema was assessed with wet-to-dry lung weight (W/D) ratio. Animals were treated intravenously with normal saline or LA 60 mg/kg or 100 mg/kg 30 min after LPS injection. After a 5 h observation, animals were killed and the lungs were isolated for measurements. Injection of LPS alone resulted in the development of shock and oxidative stress, the latter indicated by a significant increase in the lung thiobarbituric acid reacting substances (TBARS) and H_20_2 concentrations, and a decrease in the lung sulfhydryl group content. The increase in the W/D ratio after the LPS challenge indicated the development of lung edema in response to LPS. Administration of LA after the LPS challenge resulted in an increase in the sulfhydryl group content and a decrease in TBARS and H_20_2 concentration in the lungs as compared with the LPS group. An insignificant decrease in the W/D ratio was observed in rats treated with either dose of LA. These results indicate that the LPS-induced oxidative lung injury in endotoxic rats can be attenuated by early treatment with LA. Administration of LA could be a useful adjunct to conventional approach in the management of septic shock.

Key words: *endotoxin, antioxidants, oxidative stress*

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INTRODUCTION

Lipopolysaccharide (endotoxin) from gram-negative bacteria when introduced into bloodstream of the organism causes a number of pathophysiological effects including septic shock. The septic shock is invariably associated with the acute lung injury, the magnitude of which influences mortality rates. Endotoxin-induced acute lung injury has been associated with exaggerated production of reactive oxygen species (ROS) by polymorphonuclear leucocytes (PMNs) sequestrated within pulmonary vasculature in response to endotoxemia (1, 2). Also pulmonary macrophages secrete extra amounts of ROS upon LPS stimulation (3). Reactive oxygen species include oxygen-derived free radicals, such as superoxide radical anion and hydroxyl radical and non-radical oxidants, e.g. H_20_2. When present at high concentrations, ROS cause cellular damage by lipid peroxidation as well as oxidation of proteins and DNA (4, 5).

Lipoic acid is a dithiol derivative of octanoic acid serving as a prosthetic group of various cellular enzymes (6). Recent studies have proved that α-lipoate acts as a potent antioxidant by inhibiting the process of lipid peroxidation and by revitalizing other antioxidants in the brain, heart, liver, and skeletal muscles of rats (7, 8). Animal studies have shown that LA protects against diabetic neuropathy (9), lipopolysaccharide-induced liver injury (10), vancomycin-induced nephrotoxicity (11), all of them result from oxidative damage.

However, studies concerning the protective effect of lipoic acid on rat lung in the early phase of endotoxic shock are sparse (12, 13). In contrary to our previous and present experiments other studies used LA as pretreatment in LPS-induced lung injury (12). The results of this previous experiment were promising so that we decided to extend the experiment with the use of a higher LA dose after LPS challenge. Therefore, the aim of the present study was to assess the effect of two doses of LA on the intensity of lung oxidative injury as indicated by lipid peroxidation, H_20_2 concentration and the content of sulhydryl groups in the pulmonary tissue, a short time after *i.v.* administration of LPS in rats. Additionally, the influence of early treatment with lipoic acid on lung edema, a clinical manifestation of endotoxin-induced lung damage, was also addressed.

MATERIALS AND METHODS

Chemicals

Lipopolysaccharide (*Escherichia coli* LPS 026:B6; lyophilized powder chromatographically purified by gel filtration; protein content < 1%), α-lipoic acid, thiobarbituric acid (TBA), butylated hydroxytoluene, sodium acetate trihydrate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were obtained from POCh (Gliwice, Poland) and were of analytical grade. Shortly before use, LPS was dissolved in sterile pyrogen-free normal saline. α-Lipoic acid was mixed with sterile normal saline in a dark bottle, and NaOH was added until the solid had been dissolved. The solution pH was then brought to 7.4 with HCl.

TBA solution was prepared by dissolving 0.67 g TBA in 100 ml deionized water and then diluted 1:1 with glacial acetic acid. Sterile, deionized water (resistance > 18 MΩ cm, HPLC Water Purification System USF ELGA, England) was used throughout the study.

Animals

Experiments were performed on 63 male Wistar rats weighing 260 - 300 g, aged 3 - 4 months. The animals were housed 6 per cage under standard laboratory conditions in a 12/12 h light-dark cycle (light on at 7.00 a.m.) at 20 ± 2°C ambient temperature and air humidity of 55 ± 5%. All animals received a standard laboratory diet and water *ad libitum*. All animals were given a one-week acclimation period before the onset of experiment. The experimental procedures followed the guidelines for the care and use of laboratory animals, and were approved by the Medical University of Lodz Ethics Committee.

Experimental protocol

Animals were randomly divided into six groups as follows:
I group (control group, n = 10) received twice 0.2 ml of saline, half an hour;
II group (LPS group, n = 12 with 2 dead animals during the experiment) received 0.2 ml of saline, and half an hour later endotoxic shock was induced by injection of *Escherichia coli* lipopolysaccharide at a dose of 30 mg/kg;
III and IV group (LA groups, n = 10 each) were given 0.2 ml of saline, and half an hour later rats were injected with a single dose of DL-α-lipoic acid, 60 mg/kg and 100 mg/kg, respectively;
V and VI group (LPS/LA groups, n = 10 each) received a single dose of LPS (30 mg/kg) and after half an hour a single dose of LA (60 mg/kg or 100 mg/kg, respectively).

All agents were injected intravenously into the tail vein between 8.00 a. m. and 9.00 a. m. The rats' body temperature was measured in the rectum with a thermistor thermometer (type PU 391/1, Czechoslovakia) before the first injection and then 1, 2, 3 and 5 h later.

Preparation of isolated lungs and lung edema assessment

At 5 h after the last injection animals were sacrificed. The lungs were removed from animals immediately after decapitation and rinsed with ice-cold saline to remove excess of blood. All subsequent steps were carried out at 0 - 4°C. Following rinsing, approximately 200 mg fragments of the organs were cut off for oxidative injury indices measurements. The remaining parts of lungs were weighed (wet weight) and placed in a drying oven at 80°C for 8 h, and again weighed (dry weight). Finally, the lung wet-to-dry weight ratios were determined as an indicator of lung edema.

Determination of lipid peroxidation

The lipid peroxidation products content in lungs was assayed as TBARS, previously described by Kasielski and Nowak (14). Briefly, 50 mg of lung tissue fragments were homogenized with 2 ml of 1.15% potassium chloride. Then, 4 ml of 0.25% hydrochloric acid containing 0.375% thiobarbituric acid (TBA), 15% trichloroacetic acid (TCA), 0.015% butylated hydroxytoluene (BHT) were added. The samples were boiled for 30 min at 100°C in tightly closed tubes. After cooling to 10°C, 2.5 ml of butanol was added to each tube and samples were centrifuged for 10 min (3800 r.p.m., 20°C). TBA-reactive substances in the butanol layer were measured spectrofluorometrically using Perkin Elmer Luminescence Spectrometer LS-50 (Norwalk, CT, U.S.A.). Excitation was at 515 nm and emission was measured at 546 nm. Sample TBARS concentrations were calculated by the use of the regression equation as follows: Y= 0.39 (X-Xo) -

1.32, where Y= TBARS concentration (μM); X, Xo= fluorescence intensity of the samples and control, respectively (arbitrary units; AU). The regression equation was prepared from triplicate assays of six increasing concentrations of tetramethoxypropane (range 0.01 - 50 μM) as a standard for TBARS. A mixture of 2 ml of 1.15% potassium chloride, 4 ml of 0.25 N hydrochloric acid was used as a control. Finally, the results were calculated for 50 mg of pulmonary tissue.

Determination of lung H_20_2 concentration

The intensity of oxidative burst of lung resident phagocytes from LPS-challenged animals was assessed by measuring hydrogen peroxide concentration. Lung tissue fragments were washed in ice-cold saline and stored at -80°C for no longer than 2 weeks. Generation of H_20_2 was determined according to the Ruch *et al*. method (15). Briefly, 50 mg of the lung tissue fragments were homogenized with 2 ml of 1.15% potassium chloride. Then, 10 μl aliquot of tissue homogenate was mixed with 90 μl of PBS (pH 7.0) and 100 μl of horseradish peroxidase (1 U/ml) containing 400 μmol homovanilic acid (HRP+ HVA assay) or with 90 μl of PBS and 100 μl of 1 U/ml horseradish peroxidase only (HRP assay). Both homogenates were incubated for 60 min at 37°C. Subsequently, 300 μl of PBS and 125 μl of 0.1 M glicyne-NaOH buffer (pH 12.0) with 25 mM EDTA were added to each homogenate sample. Excitation was at 312 nm and emission was measured at 420 nm (Perkin Elmer Luminescence Spectrometer, Beaconsfield UK).

Readings were converted into H_20_2 concentration using the regression equation: Y= 0.012X - 0.0007, where Y=H_20_2 concentration in homogenate (μM); X=intensity of light emission at 420 nm for HRP+HVA assay reduced by HRP assay emission (arbitrary units; AU). The regression equation was prepared from three series of calibration experiments with 10 increasing H_20_2 concentrations (range10-1000 μM).

The lower limit of H202 detection was 0.1 nM, with intraassay variability not exceeding 2%.

Measurement of total sulfhydryl groups in lung homogenates

The total sulfhydryl group content in lungs was determined according to the 2.2-dithio-bis-nitrobenzoic acid (DTNB) assay as described by Ellman (16). Briefly, 50 mg of the organ sample was thawed and homogenized with 6% trichloroacetic acid (TCA) in 1:20 volume proportion at 0°C. Then homogenate was centrifuged for 20 min (10000 g, 4°C). The volume of 0.5 ml of the supernatant was mixed with 0.5 ml of 0.3 M Na_2HPO_4 and 0.5 ml of 0.04% Ellman reagent (DTNB) freshly dissolved in 10% sodium citrate water solution. All reagents added were previously cooled to 0°C. The absorbance of the obtained solution was measured at 412 nm using spectrophotometer Pharmacia LKB-Ultrospect III. Readings were converted into sulfhydryl group content (μM) by the use of the regression equation Y = -0.2998 + 241.954X, where Y = free sulfhydryl group content (μM), X= intensity of light emission at 420 (arbitrary units, AU).

The regression equation was prepared from three repeats of standard experiments with increasing concentrations of glutathione (range 2 - 200 μM).

The control assay was carried out with the solution: 0.5 ml of 6% TCA; 0.5 ml of 0.3 M Na_2HPO_4- sodium phosphate; 0.5 ml of 0.04% DTNB.

Statistical analysis

Data are presented as mean ± SE, if not stated otherwise, from 10 survived animals in each group. The statistical analysis was done by ANOVA followed by the Duncan's multiple range test as a post-hoc. A p value less than 0.05 was considered significant.

RESULTS

Lung edema

As expected, the lungs of rats challenged with LPS had significantly higher wet-to-dry (W/D) ratio as compared to the control group and the LA groups (5.42 ± 0.2 *vs* 4.52 ± 0.3 and 4.48 ± 0.3, mean ± SE, p< 0.001, respectively). Treatment with LA after LPS challenge, irrespective of dose, insignificantly decreased W/D ratio comparing to the LPS group [5.26 ± 0.1, p= 0. 07, (60 mg/kg) and 5.27 ± 0.1, p= 0. 08, (100 mg/kg), mean ± SE].

Body temperature and general behavior

In the control group, there was no change in body temperature throughout the experiment (33.2 ± 0.1°C). Similarly, infusion of LA alone had no effect on body temperature. The injection of LPS resulted in a body temperature decline by about 2°C compared to saline and LA groups (p<0.001). In the LPS/LA group a decrease in body temperature occurred 2 h after LPS injection, than a gradual increase in body temperature was observed (*Fig.1*).

Animals challenged with LPS alone displayed reduced activity and adopted a hunched posture. The changes in animal behavior were already visible within 1 h after LPS injection. In contrast, behavior of animals from the LPS/LA group was similar to that from the control group.

In the LPS group, approximatelly 17% mortality rate was observed.

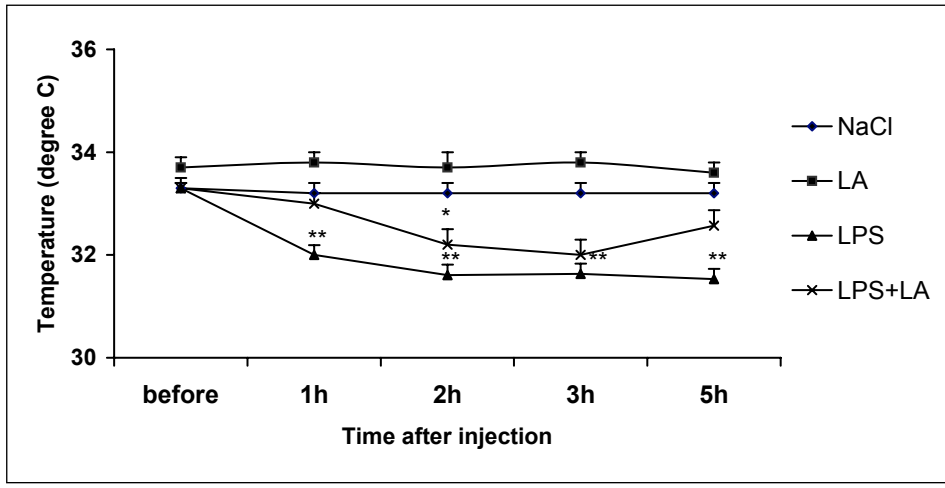

Fig. 1. The effect of lipoic acid on the LPS-induced change in body temperature in rats. LPS (30 mg/kg) or 0.2 ml saline was administered into the tail vein and 0.5 h later the animals were injected with lipoic acid (60 or 100 mg/kg, *i.v.*). Body temperature was measured in the rectum before and 1, 2, 3 and 5 h after the last injection. Data represent the mean ± SE (n=10, * p< 0.05, ** p<0.001 *vs* baseline temperature). Data for LA 100 mg/kg are similar to that for LA 60 mg/kg and are not shown.

Table 1. The influence of administration of lipoic acid (LA) on oxidative stress parameters in lung homogenates of rats with lipopolysaccharide-induced shock. Two doses of LA were used, 60 and 100 mg/kg b.w.; data for a dose of 100 mg/kg did not differ from that for 60 mg/kg and they are not shown here. Mean ± SE. * $p < 0.001$ *vs* control, ** $p < 0.001$ *vs* LPS, # $p< 0.01$ *vs* control, † $p < 0.05$ *vs* LPS, †† $p < 0.05$ vs control, ‡ $p< 0.02$ *vs* control

Parameter (μM)	Saline	LA	LPS	LPS+LA60
TBARS	1.40 ± 0.046	2.02 ± 0.54	6.1 ± 0.15*	3.65 ± 0.2**
H_20_2	0.075 ± 0.005	0.004 ± 0.004#	0.189 ± 0.004*	0.014 ± 0.005**
Sulfhydryl groups	2.36 ± 0.14	3.01 ± 0.2	0.87 ± 0.08*	1.26 ± 0.1†

LPS-lipopolysaccharide; LA60- lipoic acid, 60 mg/kg body weight; TBARS-thiobarbituric acid reacting substances; all administrations were done intravenously between 8.00 and 9.00 a.m.

Changes in lung oxidative injury indices

Animals challenged with LPS produced in the lung homogenates about 4.5-fold increase in lipid peroxidation levels, as measured by the formation of TBARS, compared to the control group (1.40 ± 0.05 *vs* 6.1 ± 0.15μM, $p<0.001$, n=10) (*Table 1*). The administration of LA (60 mg/kg body weight) after LPS challenge significantly reduced the extent of lipid peroxidation in the lung tissues when compared to the LPS group (3.65 ± 0.2 μM, $p<0.001$, n=10). A similar decrease was obtained with LA at the dose of 100 mg/kg body weight (3.59 ± 0.19 μM, $p<0.001$, n=10) (*Table 1*).

Mean lung H_20_2 concentration in animals challenged with LPS was approximately 3-fold elevated when compared to the control group (0.189 ± 0.004 *vs* 0.075 ± 0.005 μM, $p<0.001$, n=10). This increase in hydrogen peroxide generation in lungs in response to LPS was significantly attenuated by LA (0.014 ± 0.005, $p<0.001$, n=10) (*Table 1*).

Infusion of LPS resulted in a significant decrease in the sulfhydryl group content when compared to the control group values (0.87 ± 0.08 *vs* 2.36 ± 0.14, $p< 0.001$, n=10). Treatment of LPS-challenged animals with either dose of LA restored sulfhydryl group content in the lung homogenates [1.26 ± 0.1 μM, (60 mg/kg) and 1.22 ± 0.08 μM, (100 mg/kg), $p< 0.05$, n=10] (*Table 1*).

DISCUSSION

Systemic administration of LPS generally leads to acute lung injury which is manifested with hypoxemia and lung edema as a result of leukocytes pulmonary infiltrates, increased microvascular permeability and endothelial barrier disruption. The interaction of LPS with pulmonary resident phagocytes as well as infiltrating phagocytes lead to their activation and release of many proinflammatory mediators including ROS (17, 18). The uncontrolled production

of proinflammatory cytokines and ROS exceeds the antioxidant defense capacity of cells and extracellular fluids and provokes oxidative damage in all organs, as happens in endotoxic shock. The involvement of ROS in tissue damage has been reported in animal models of endotoxic shock and in humans with septic shock (18, 19). Cowley *et al*. (20) described decreased total antioxidant potential in patients with sepsis and organ dysfunction associated with poor survival. Nielsen *et al*. (21) indicated that free oxygen radicals induce lipid peroxidation in cells, and malondialdehyde formed during oxidative degeneration is regarded as an indicator of lipid peroxidation. Moreover, peroxidation of membrane lipids accompanying sepsis has been shown as an early contributor to septic organ injury (22).

Concordant with previous observations (1, 2), we reported in this study that intravenous infusion of LPS causes oxidative lung injury as shown by the increased lipid peroxidation and decreased sulfhydryl groups concentration in lung homogenates in rats. In our experiment, LPS-induced lung injury was clinically manifested by lung edema development as evidenced with a significant increase in wet-to-dry ratio of the lungs from septic rats compared to the ratio in the control animals. The increase in lipid peroxidation in our study is a result of increased production of ROS. This is indicated by increased levels of H_20_2 in the lungs of LPS-challenged rats. Hydrogen peroxide is an important component in the cascade of events during which ROS are produced. The main sources of H_20_2 produced in the respiratory tract are neurotrophils, monocytes (macrophages), type II pneumocytes and other epithelial cells.

The present findings show that LA at a dose of 60 mg/kg is highly effective in reducing free radicals and lipid peroxides in lungs of septic rats. Administration of LA, 0.5 h after *i.v.* LPS injection, caused significant decreases both in TBARS and H_20_2 concentrations in lung homogenates. It was also demonstrated in the present study that the early administration of LA after LPS challenge significantly increased the sulfhydryl group lung content. The LA-related attenuation of lung oxidative injury induced by LPS was accompanied by the trend toward decreased lung edema. Moreover, LA administration also resulted in improved survival rates and less advanced hypothermic reaction of septic rats compared to untreated animals. However, more than 1.5 fold increase in the dose of LA administered did not result in either further reduction of oxidative lung damage or clinical course of sepsis.

All these effects of LA in LPS-induced sepsis can be explained on the basis of its antioxidant properties. It was also shown that LA (1,2 dithiolane-3-pentanoic acid ($C_8H_{14}O_2S_2$), is both water and lipid soluble, and protects several macromolecules against ROS (23, 24). The LA exerts its effects mainly through its reduced form, dihydrolipoic acid (DHLA), a conversion to which is catalyzed by lipoamide dehydrogenase. This endogenous thiol antioxidant quenches reactive oxygen species (singlet oxygen, H_20_2, hydroxyl radicals), regenerates reduced glutathione (GSH) and chelates metals such as iron, copper, mercury and cadmium, which are known to mediate free-radical production in biological

systems. Both LA and DHLA also protect the integrity of cell membranes by interacting with other antioxidants, namely glutathione and vitamins E and C. It has been also shown that lipid peroxyl radicals can be directly scavenged by lipoic acid during the course of lipid peroxidation (25). Han *et al*. (26) indicated that LA has the ability to correct deficient thiol status of the cell by increasing the de novo synthesis of GSH, which is regarded to be a main endogenous antioxidant.

In conclusion, the results of the present study indicate that lipoic acid is effective in antagonizing the LPS-inducing lung injuries. The attenuation of oxidative lung stress is accompanied by moderately decreased lung edema and reduced mortality. Contrary to our study, the others used pretreatment with LA to attenuate LPS-related lung inflammation. Obviously, effectiveness of LA in developed endotoxic lung damage evidenced by our experiments seems to be more encouraging for its potential use in the treatment of endotoxemia.

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