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Anti-oxidative and anti-inflammatory effects of lipoic acid in rat liver*

Działanie antyoksydacyjne i przeciwzapalne kwasu liponowego w wątrobie szczura

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Summary

Introduction:

Lipopolysaccharide (LPS) is a key inflammatory component of Gram-negative bacteria, which after entering the systemic circulation contributes to the development of septic hepatic failure.

Aim:

The aim of this study was to evaluate the effects of alpha lipoic acid (LA) on oxidative stress parameters and inflammation in endotoxemic rat liver.

Material/Methods:

Male Wistar rats were divided into 4 groups, each group consisting of 8 animals. Group I received saline and served as a control, Group II received a single dose of LA (60 mg/kg i.v.), Group III received lipopolysaccharide (LPS) (15 mg/kg i.v.), and Group IV received LPS (15 mg/kg i.v.) and 30 min later received LA (60 mg/kg i.v.). Five hours after LPS or LA administration, the animals were sacrificed and the liver was isolated for measurements of levels of thiobarbituric acid reactive substances (TBARS), hydrogen peroxide (H₂O₂), total sulfhydryl groups (-SH), total glutathione (tGSH) and reduced glutathione (GSH).

Results:

Injection of LPS caused a significant increase in liver TBARS and H₂O₂ levels and a significant decrease in levels of -SH groups, tGSH and GSH. LPS-treated rats also showed an increase in TNF- α and IL-6 levels and edema in the liver. The administration of LA to endotoxemic rats significantly reduced TBARS, H₂O₂, TNF- α , and IL-6 levels and reduced edema in the liver when compared to the LPS group. This antioxidant also resulted in an increase in -SH groups and tGSH and GSH levels and ameliorated the glutathione redox status when compared to the LPS group.

Conclusions:

The results indicated that LA administered 30 min following LPS infusion may effectively prevent oxidative stress and inflammation in the liver. Thus LA is a potent antioxidant that can be useful in rebuilding LPS-induced damaged liver tissue.

Keywords:

lipoic acid • endotoxemic shock • liver damage

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INTRODUCTION

Lipopolysaccharide (LPS) is a highly conserved wall component of Gram-negative bacteria, which after entering the systemic circulation contributes to the development of septic hepatic failure. Sepsis and endotoxemia cause acute whole body inflammation and release of inflammatory cytokines and reactive oxygen species (ROS), which in turn damage vascular endothelium and lead to impairment of tissue respiration. The cascade of all these mentioned processes contributes to multiple organ dysfunctions. Despite medical advances, high plasma levels of LPS are still associated with a high mortality rate. In the liver, the LPS binds to LPS binding proteins, which facilitates its translocation to CD14 receptors localized on the surface of resident liver macrophages (Kupffer cells). Next, the signal of LPS through CD14 receptors is mediated by Toll-like receptor-4 and leads to abundant production of toxic mediators including proinflammatory cytokines such as interleukins (IL-1, IL-6), tumor necrosis factor (TNF- α) and ROS [31]. A massive increase in ROS and other radical species can lead to oxidative stress, cell injury and apoptosis.

The defense mechanism against ROS, mediators of liver damage, includes enzymatic antioxidants, such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GSHPx), glutathione reductase (GR), and glutathione S-transferase (GST), and non-enzymatic compounds, such as glutathione (GSH), thioredoxin (Trx), vitamins A, E, C and LA [6].

LA is a disulphide compound which serves as a coenzyme for the mitochondrial respiratory enzymes pyruvate dehydrogenase and α -ketoglutarate dehydrogenase. LA in many tissues is rapidly converted to its redox couple, dihydrolipoic acid (DHLA). LA and DHLA antioxidant properties are reflected by their direct ability to quench free radicals and indirect ability to recirculate cellular antioxidants [2]. Furthermore, LA and DHLA can chelate redox active transition metals and prevent the production of ROS in Fenton and Haber-Weiss reactions [19].

In addition, LA is both water and fat soluble, which makes it highly effective in reducing free radicals such as lipid peroxides in cellular membranes [12,21]. LA also reduces apoptosis in liver cells by its antioxidative potential [16]. LA and LA derivatives exhibit anti-

-inflammatory effects [18]. Many studies have reported the effectiveness of LA treatment in the prevention of pathologic conditions mediated by oxidative stress [5,28]. It was demonstrated recently that LA has the ability to prevent hepatic steatosis and fatty liver diseases such as nonalcoholic fatty liver disease (NAFLD) in rats fed a long-term high-fat diet [25,27]. Because of its antioxidative abilities, LA could be considered as a therapeutic factor for the treatment of sepsis. Taking the above into consideration, we wanted to evaluate the effect of LA on inflammation and some crucial indicators of oxidative stress induced by LPS in rat livers.

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 (BHT), sodium acetate trihydrate, triethanolamine hydrochloride (TEA), 5-sulfosalicylic acid hydrate (5-SSA), 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB), β -NADPH (β -nicotinamide adenine dinucleotide phosphate), glutathione reductase (GR), and 2-vinylpyridine 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. TBA solution was prepared by dissolving 0.67 g of TBA in 100 ml of deionized water and then diluted 1:1 with glacial acetic acid. Sterile, deionized water (resistance > 18 M Ω cm, HPLC Water Purification System USF ELGA, England) was used throughout the study.

Animals

The experiments were performed on male Wistar rats weighing 260-280 g, aged 2-3 months. The animals were acquired from the Medical University of Lodz animal quarters and were housed in individual cages under standard laboratory conditions: 12/12 h light-dark cycle (light on at 7.00 a.m.) at 20 \pm 2°C ambient temperature and 55 \pm 5% air humidity. All rats received a standard lab-

oratory diet and water *ad libitum*, and they were maintained for 1 week in the laboratory for adaptation. 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

The animals were randomly divided into four groups: Group I (n=8) received two doses of 0.2 ml saline, 0.5 h apart, and served as a control. Group II (n=8) received one dose of 0.2 ml saline and 0.5 h later received a single dose of LA (60 mg/kg b.w.). Group III (n=8) received one dose of 0.2 ml saline and 0.5 h later received LPS (15 mg/kg b.w.). Group IV (n=8) received LPS (15 mg/kg) and 0.5 h later received LA (60 mg/kg b.w.). All compounds were injected into the tail vein between 8.00 a.m. and 9.00 a.m. After the administration of α -LA or LPS, each group of animals was observed for a period of 5 h.

Tissue preparation

The animals were euthanized with an overdose of i.p. pentobarbital (100 mg/kg) after 5 h of observations. The liver was surgically removed and cleaned of extraneous tissue. It was rinsed with cold isotonic saline, dried by blotting between two pieces of filter paper and weighed. The liver weight to body weight (LW/BW) ratios were then calculated and used as an index of liver edema. Then the livers were stored at -80°C for the purpose of measuring oxidative parameters and pro-inflammatory cytokines.

DETERMINATION OF OXIDATIVE STRESS MARKERS

Estimation of tissue lipid peroxidation contents

The content of lipid peroxidation products in liver homogenates was assayed as thiobarbituric acid reactive substances (TBARS), previously described by Yagi et al. [29]. TBARS were measured spectrofluorometrically using a Perkin Elmer Luminescence Spectrometer LS-50 (Norwalk, CT, USA). Excitation was set at 515 nm and emission was measured at 546 nm. Readings were converted into the μM range using the calibration curve obtained for tetramethoxypropane (0.01–50 μM).

Estimation of tissue hydroxyl peroxide content

The H_2O_2 concentration in homogenates was measured using HRP/HVA systems. In brief, 50 mg of the frozen liver tissue fragments was homogenized with 2 ml of 1.15% KCl. Then, a 10 μl aliquot of the resulting homogenate was divided between two Eppendorf tubes. In one tube, a mixture of phosphate buffered saline (PBS) (pH 7.0) and horseradish peroxidase (HRP) (1 U/ml) containing 400 μmol homovanillic acid (HVA) was added to assay HRP + HVA, while PBS and 1 U/ml HRP were added to the other tube to assay HRP. Both tubes were simultaneously incubated for 60 min at 37°C . Subsequently, PBS and 0.1 M gly-

cine-NaOH buffer (pH 12.0) with 25 mM EDTA were added to each Eppendorf tube to stop the enzymatic reaction. Excitation was set at 312 nm and emission was measured at 420 nm (Perkin Elmer Luminescence Spectrometer, Beaconsfield, UK). Readings were converted into H_2O_2 concentration using the regression equation prepared from three series of calibration experiments with 10 increasing H_2O_2 concentrations (range 10–1000 μM).

Measurement of total sulfhydryl groups in liver tissue

Protein thiol group measurement was performed using the 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB or Ellman reagent) method [10]. The absorbance of the obtained solution was measured at 412 nm using a Pharmacia LKB-Ultrospect III spectrophotometer. The total concentration of thiol groups was calculated from the regression equation prepared from three repeats of increasing concentration of glutathione (2–200 μM). Concentrations of SH groups were expressed as μM .

GSH and GSSG assays

The total GSH content of the supernatant was measured in a 1 ml cuvette containing 0.7 ml of 0.2 mM NADPH, 0.1 ml of 0.6 mM DTNB, 0.15 ml of H_2O and 50 μl of a sample. The cuvette with the mixture was incubated for 5 min at 37°C and then supplemented with 0.6 U of GR. The reaction kinetics were traced spectrophotometrically at 412 nm for 5 min by monitoring the increase in absorbance. Glutathione disulfide (GSSG) concentration was determined in supernatant aliquots using the same protocol after optimization of pH to 6–7 with 1 M TEA and derivatization of endogenous GSH with 2-vinylpyridine (v:v). The reduced GSH level in the supernatant was calculated as the difference between total GSH and GSSG. The increments in absorbance at 412 nm were converted to GSH and GSSG concentrations using a standard curve (3.2–500 μM of GSH for total GSH and 0.975–60 μM of GSSG for GSSG). The results were expressed in μM .

TNF- α and IL-6 assay

Tissue TNF- α and interleukin-6 (IL-6) levels were measured by commercial rat ELISA kits (R&D Systems, USA) according to the manufacturer's instructions. A standard curve was prepared from standard dilutions of cytokines in duplicate. The TNF- α and IL-6 concentrations were determined from the standard curves and expressed in pg/ml. Experiments were repeated twice. The results were read using a TEK Instruments EL340 BIO-spectrophotometer (Winooski VT, USA).

Statistical analysis

The data are presented as mean \pm SEM from 8 animals in each group. The statistical significance was evaluated by ANOVA followed by Duncan's multiple range test as post-hoc. P values of less than 0.05 were considered significant.

RESULTS

Changes in the ratio of LW (liver weight)/BW (body weight)

The LW/BW ratio markedly increased following LPS infusion when compared to the control and LA group ($P < 0.001$). LA administration 30 min after LPS infusion produced suppression of liver edema when compared to the LPS group ($P < 0.001$) (Table 1).

Effect on lipid peroxidation level

LPS infusion caused a marked increase in lipid peroxidation when compared to control and LA groups ($P < 0.001$). Treatment with LA after LPS infusion resulted in a significant attenuation in TBARS level ($P < 0.001$) when compared to the LPS group (Table 1).

Effect of LA on LPS-induced H_2O_2 level

In order to investigate endogenous generation of ROS we measured H_2O_2 in liver homogenates. As shown in table 1, LPS caused a marked increase in H_2O_2 level in liver ($P < 0.01$) when compared to the control ($P < 0.01$) or LA group ($P < 0.001$). LA effectively inhibited LPS-induced ROS generation in liver homogenates, compared with the LPS group ($P < 0.01$). This effect indicates mitigation of oxidative damage to the liver.

Assessment of sulfhydryl groups

As shown in Table 1, administration of LPS to rats significantly reduced the level of -SH groups in liver compared with the control ($P < 0.05$) or LA group ($P < 0.001$). Administration of LA 30 min after LPS infusion resulted in a significant increase in the level of -SH groups when compared to the LPS group ($P < 0.05$).

Assessment of glutathione

Fig. 1 shows the levels of tGSH, GSH and GSSG in the liver of the control and experimental groups of rats. Liver GSH and tGSH levels were significantly decreased following LPS infusion when compared to the control group ($P < 0.001$). The decreases of liver GSH and tGSH content following LPS infusion were significantly reversed in those with LA administration ($P < 0.001$) (Fig. 1). Administration of LA 30 min after LPS infusion resulted in a marked increase in the GSH/GSSG ratio, when compared to the LPS group ($P < 0.001$), which indicates a displacement of the redox state of the cell toward a more reduced level (table 1).

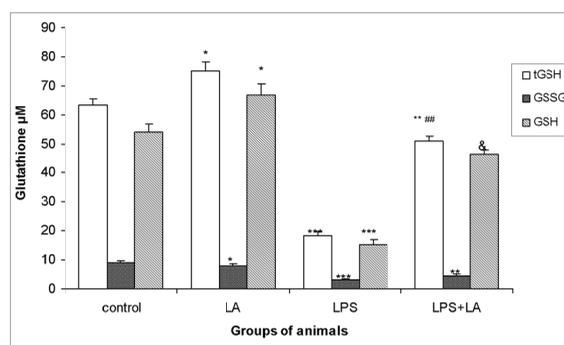


Fig. 1. Effect of lipoic acid (LA) (60 mg/kg, i.v.), lipopolysaccharide (LPS) (15 mg/kg i.v.) and their combination on total, oxidized and reduced glutathione in liver homogenates. tGSH-total glutathione, GSSG-oxidized glutathione, GSH-reduced glutathione. Values are expressed as means \pm SEM ($n = 8$ rats). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs control; & - $P < 0.001$ vs LPS

Assessment of TNF- α and IL-6

As shown in Table 2, LPS challenge caused a marked rise in the levels of TNF- α and IL-6 compared to the control

Table 1. Effect of lipoic acid (LA) (60 mg/kg, i.v.), lipopolysaccharide (LPS) (15 mg/kg i.v.) and their combination on oxidative stress parameters in liver homogenates. Values are expressed as means \pm SEM ($n = 8$ rats)

Parameter	Groups	Control (0.9% NaCl)	LA 60 mg/kg	LPS 15 mg/kg	LPS (15 mg/kg) + LA (60 mg/kg)
TBARS (μ M)		7.71 \pm 0.45	3.11 \pm 0.63***	17.97 \pm 2.31 ***	7.23 \pm 0.59&
H_2O_2 (μ M)		1.18 \pm 0.13	0.68 \pm 0.09**	2.09 \pm 0.23**	1.14 \pm 0.08##
Free -SH groups (μ M)		12.18 \pm 1.11	20.31 \pm 1.71*	6.04 \pm 0.59*	10.97 \pm 0.77#
LW/BW ratio (x100)		3.37 \pm 0.15	2.82 \pm 0.14	4.94 \pm 0.21***a	3.29 \pm 0.13&
GSH/GSSG ratio		6.04 \pm 0.638	9.23 \pm 0.91\$	3.71 \pm 0.42	8.65 \pm 0.78##

TBARS- thiobarbituric acid-reactive substances; H_2O_2 - hydrogen peroxide; free -SH groups- free sulfhydryl groups; GSH/GSSG ratio - reduced glutathione to glutathione disulfide ratio; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs control; # - $P < 0.05$, ## - $P < 0.01$, \$ - $P < 0.02$, & - $P < 0.001$ vs LPS, a - $P < 0.001$ vs LA

group ($P < 0.001$). Liver TNF- α and IL-6 levels were significantly reversed in rats treated with LA ($P < 0.001$).

Table 2. Effect of lipoic acid (LA) (60 mg/kg, i.v.), lipopolysaccharide (LPS) (15 mg/kg i.v.) and their combination on tumor necrosis factor (TNF- α) and interleukin (IL-6) levels in liver homogenates. Values are expressed as means \pm SEM ($n = 8$ rats)

Groups	TNF- α (pg/ml)	IL-6 (pg/ml)
0.9% NaCl	36.9 \pm 6.32	28.48 \pm 2.69
LA	45.57 \pm 4.55	34.23 \pm 2.92
LPS	226.06 \pm 17.85***a	194.19 \pm 11.15 ***a
LPS+LA	99.39 \pm 7.93 &	82.42 \pm 6.93 &

*** - $P < 0.001$ vs 0.9% NaCl; & - $p < 0.001$ vs LPS; a - $P < 0.001$ vs LA

DISCUSSION

Numerous studies have demonstrated that LPS treatment causes steatosis, inflammatory reactions and hepatocyte damage in the liver of experimental animals [8]. Circulating LPS binds to Toll-like receptor-4 (TLR-4) on hepatic phagocytes and macrophages, leading to their stimulation. Activated macrophages subsequently release ROS, reactive nitric species (RNS) as well as proinflammatory cytokines, such as TNF- α and IL-6 [31]. Additionally, LPS induces the migration of activated polymorphonuclear leukocytes (PMNs) into the liver, which constitutes another source of free radicals [32]. Recently, it was shown that LPS treatment results in an increase in necrotic hepatocytes and expression of antiapoptotic Bcl-2 and proapoptotic Bax proteins. ROS and necrotic changes play an important role in LPS-induced liver injury [8]. The increase in ROS generation in our study was reflected in an increase in TBARS and H₂O₂ levels and a decrease in the concentration of free -SH groups and tGSH and GSH. This effect was also indicated by the increase in liver weight/body weight ratio. The cell membranes possess a high content of polyunsaturated fatty acids (PUFAs), which are particularly sensitive to the effect of ROS. By oxidizing membrane PUFAs and intracellular macromolecules, ROS causes cellular dysfunction and tissue damage. In this study, the assay of TBARS reflected the degree of lipid peroxidation injury in hepatocytes.

H₂O₂ is formed by superoxide dismutase and converted to hydroxyl radicals in the presence of transient metal ions (the Fenton reaction). The hydroxyl radical is considered to be a highly reactive and potentially dangerous compound that can damage peripheral organs. Increased production of ROS, mainly superoxide anion (O₂⁻) and H₂O₂, was observed in experimental sepsis and endotoxemia in animals and patients [6,26].

In this study the increases in hepatic TBARS and H₂O₂ contents following intravenous LPS infusion were signif-

icantly reversed in animals with LA administration. In accordance with the present results, it was shown that LA reduces the increased ROS generation and protein oxidation in the liver [24]. This effect may result from the potent antioxidant capacity of LA. Previous studies have shown that α -lipoic acid and its reduced form dihydrolipoic acid (DHLA) have an amphiphilic property that easily allows them to cross the cell membranes and help to activate other antioxidants such as vitamin C, vitamin E, coenzyme Q10, and ubiquinone [13].

In our study, a decrease in the content of total free -SH groups in LPS-treated rats may be attributed to the increase in ROS concentration in the liver homogenates. It was indicated that ROS oxidize thiol groups, which in turn can influence the structure and function of numerous proteins. LPS is also a potent stimulator of nitric oxide (NO) [23], which can react with O₂⁻, leading to the formation of peroxynitrite (ONOO⁻), which in turns oxidizes sulfhydryl groups and generates hydroxyl radicals.

The tripeptide GSH is an important endogenous antioxidant responsible for free radical scavenging in all cell types. The decrease in the content of GSH after LPS administration results in a parallel shift in the redox state of the thiol system to become more oxidized. The decrease in GSH level and GSH/GSSG ratio in the liver of endotoxic rats constitutes evidence of severe oxidative stress and a breakdown of the redox balance in cells during endotoxemia [15].

In this study we proved that LA-treated rats demonstrated enhanced levels of -SH groups, tGSH and GSH when compared with the control. This may imply that LA prevents the oxidation of free or protein-bound thiols. The present results indicate that LA improves a deficient thiol status by increasing the levels of hepatic GSH and total thiols [1]. The maintenance of the thiol groups protects against oxidative stress and therefore influences the function of some thiol-containing proteins [4].

In the present study, the administration of LA alone or followed by LPS contributed to an increase in the level of tGSH and GSH, thus improving the hepatic redox status. Both LA and its derivate DHLA may act as extra- and intracellular redox couples and potent free radical scavengers [2]. It is well documented that LA is a highly reactive free radical scavenger able to increase GSH concentration in tissues [2, 20, 16]. In our study, the administration of LA to LPS-induced oxidative stressed rats resulted in an increased GSH/GSSG ratio in the liver. It was observed that maintaining higher GSH and lower GSSG levels enhances the antioxidant properties of tissues and protects cell membranes from free radicals [7].

One of the symptoms of oxidative stress and inflammation induced by LPS is edema of tissues. LPS stimulates monocytes and tissue macrophages to secrete cytokines, such as TNF- α , IL-6 or IL-1 β , that destabilize endothelial cells and damage the vascular barrier, which causes leakage of plasma into the tissue [22,17]. Furthermore,

during oxidative stress the secretion of vascular endothelial growth factor (VEGF) increases. This pro-inflammatory cytokine increases the vascular permeability and contributes to liver injury [3]. In the present study, LPS infusion caused an increase in the ratio of liver weight to body weight (LW/BW), which indicates an increase in vascular permeability and liver edema. LA infusion reduced the ratio of liver LW/BW, which confirms the protective effect of this antioxidant in the liver tissue. LA stabilizes endothelial cells and reduces passage of fluid into the tissue, thereby reducing damage of tissues [9]. Moreover, treatment of LA inhibits VEGF expression by blocking the superoxide anion, causing a decrease in vascular leakage and tissue edema reduction. Taken together, edema formation is the result of synergism between various inflammatory mediators that increase vascular permeability and/or the mediators that increase blood flow. This is the first report on the inhibitory effect of LA on LPS-induced liver edema.

In our study, the significant increase of TNF- α and IL-6 levels in the liver of LPS-infused rats was reflective of

the hepatic inflammatory response. This action was significantly reversed in those treated with LA. TNF- α has direct cytotoxic effects; it activates nuclear transcription factor kappa B of hepatocytes and Kupffer cells, leading to increased expression of adhesion molecules, which further impel the inflammatory injury of hepatocytes [30]. The inflammatory process can activate macrophages and monocytes and contribute to release of several inflammatory cytokines. In this study, treatment with LA after LPS-induced oxidative stress markedly reduced the levels of TNF- α and IL-6, suggesting that the hepatoprotective effect of LA may also be related to inhibition of proinflammatory cytokines [11,14] and induction of endogenous antioxidants [3,24].

CONCLUSION

These results suggest that LA is a potential anti-oxidative and anti-inflammatory agent and may be a candidate compound for protective and therapeutic effects on hepatocyte injury.

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