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PHARMACOLOGY

ANTI-INFLAMMATORY ACTIVITY OF LIPOIC ACID IN MICE PERITONITIS MODEL

MAŁGORZATA ZYGMUNT[1*], MAGDALENA DUDEK[2], ANNA BILSKA-WILKOSZ[3], MAREK BEDNARSKI[1], SZCZEPAN MOGILSKI[2], JOANNA KNUTELSKA[1] and JACEK SAPA[1]

[1]Department of Pharmacological Screening, Chair of Pharmacodynamics, [2]Chair of Pharmacodynamics, Jagiellonian University Medical College, Medyczna 9 St., 30-688 Kraków, Poland
[3]Chair of Medical Biochemistry, Jagiellonian University, Medical College, Kopernika 7, 31-034 Kraków, Poland

Abstract: This work aimed to investigate the effect of lipoic acid (LA) on sulfane sulfur (S*) level, infiltration of neutrophils and vascular permeability in a model of zymosan-induced peritonitis. The study showed that lipoic acid increased the sulfane sulfur level. Also, it decreased count of neutrophils and inhibition of intensity of early vascular permeability compared to the control group. These studies indicated that LA exhibits anti-inflammatory activity. LA serves as a sulfane sulfur acceptor and releases sulfane sulfur in the form of hydrogen sulfide (H_2S), which is probably responsible for its anti-inflammatory activity.

Keywords: lipoic acid, antiinflammatory and antioxidative effects, sulfane sulfur, peritonitis

Lipoic acid (LA) is a natural compound, chemically it is (*R*)-5-(1,2-dithiolan-3-yl)pentanoic acid (1-3). Lipoic acid is predominantly a lipophilic molecule having an amphipathic character due to its carboxylic acid group attached to the ring structure. In humans, LA is synthetized by the liver and other tissues with high metabolic activity: heart, kidney. High concentrations of this acid are found in animal tissues with extensive metabolic activity such as heart, liver and kidney. LA is widely distributed in both cellular membranes and cytosol. LA is easily absorbed from the gastrointestinal tract, is able to cross the blood-brain barrier, and does not exhibit any serious side effects (4).

LA contains an asymmetric carbon atom, which results in two possible optical isomers (R and S). Only the R-isomer is endogenously synthesized. LA contains two thiol groups, which may be oxidized or reduced. As with the thiol antioxidant - glutathione, LA is part of a redox pair, being the oxidized partner of the reduced form - dihydrolipoic acid (DHLA), which also possesses biological activity. Both LA and DHLA are easily soluble (5).

The chemical reactivity of LA is mainly due to its dithiolane ring. Therapeutic action of LA is based on unique antioxidant properties of LA/DHLA system, which possesses one of the lowest standard biological redox potential (6-10). Thus, DHLA is able to reduce not only reactive oxygen species (ROS) but also oxidized forms of other antioxidants. For this reason, it is called an antioxidant of antioxidants (5, 11). The reaction of LA with hydroxyl radical, for example, yields lipoic acid cation radical, which is transformed into LA by other antioxidants that can be regenerated by DHLA. Exogenous LA can be reduced to DHLA by several enzymes, including mitochondrial lipoamide dehydrogenase, which utilizes NADH, and cytoplasmic NADPH-dependent reductases: glutathione reductase and thioredoxin reductase (5).

Endogenously synthesized LA is covalently bound to specific proteins, which function as cofactors for mitochondrial dehydrogenase enzyme complexes (12, 13). LA has long been known as a coenzyme of multienzymatic complexes catalyzing the decarboxylation of α-ketoacids, but the present investigations are focused on its antioxidative properties and its reduced form DHLA (13). Studies have shown that both the oxidized and reduced forms of lipoic acid are antioxidants. LA scavenges hydroxyl

* Corresponding author: e-mail:gogol67@interia.pl

radicals, hypochlorous acid and singlet oxygen. It may also exert antioxidant effects in biological systems through transitional metal chelation, resulting in reduced ROS production. Both LA and DHLA are also responsible for the regeneration of active forms of other cellular antioxidants, including vitamins C and E (7). Dihydrolipoic acid has been shown to have antioxidant but also pro-oxidant properties in systems in which hydroxyl radical was generated. Lipoic acid was initially included in the vitamin B complex. However, at present, LA is not considered to be a vitamin (14).

It is probable that LA/DHLA system plays a significant role in the processes with sulfane sulfur, since it is known from *in vitro* studies that DHLA serves as a sulfane sulfur acceptor in rhodanese-catalyzed reactions. Sulfane sulfur containing dihydrolipoic acid hydropersulfide, formed in this reaction, releases sulfane sulfur in the form of hydrogen sulfide (H_2S) (15). Sulfane sulfur is a labile reactive sulfur atom in the 0 or –1 oxidation state, covalently bound to another sulfur atom (15). Rhodanese catalyzes sulfane sulfur transfer to different acceptors (15). Sulfane sulfur transfer to thiol groups of receptor and enzymatic proteins leads to the formation of hydropersulfides and/or trisulfides that often changes activity of these proteins. The role of sulfane sulfur in the formation of iron-sulfur center of iron-sulfur proteins has also been suggested.

Other properties of lipoic acid, apart from the antioxidant function, comprise: modulation of mitogen-activated protein kinase activity, reduction of production of inflammatory mediators, lowering of endothelin expression, effect on secondary messengers of the nuclear factor β and peroxisome proliferator-activated receptors (PPAR) activation cascade, and implication in the regulation of carbohydrate and lipid metabolism (8, 16).

LA has some neuroprotective properties (8, 17, 18). The precise mechanism of its neuroprotective effects remains unclear. It is known that it can inhibit microglial activation and caspase-related apoptotic pathways (18).

Various studies have shown, that LA exerts powerful anti-inflammatory and anti-oxidant effects *in vitro*. Treatment of cultured monocytes with LA inhibits lipopolysaccharide-mediated induction of pro-inflammatory factors. LA, by virtue of its antioxidant effect, has been shown to be beneficial in many metabolic and vascular diseases (16). Consistent with these effects, LA has been shown to be protective in human diseases associated with abnormal oxidative stress and energy metabolism (13). A number of experimental as well as clinical studies point to the usefulness of LA as a therapeutic agent for such diverse conditions like atherosclerosis, insulin resistance, neuropathy, neurodegenerative diseases and ischemia-reperfusion injury. LA significantly improves diabetic neurovascular and metabolic abnormalities, glycemic control and improves glucose utilization by cells due to stimulation of insulin-dependent Akt/PKB signaling pathway (4).

LA is a medication recommended in many countries in diabetic neuropathy. Also, it may play a role in cardiovascular protection and as an antiinflammatory agent (19). It also improves allergic inflammation (19). Studies have revealed that LA effectively suppresses allergic inflammation in a murine model of asthma by reducing the level of reactive oxygen species. LA represents a potential agent on the vascular endothelium. LA has also been considered as a therapeutic agent candidate for the treatment or prevention of pathologies associated with an imbalance of oxidoreductive status, such as neurodegeneration, ischemia-reperfusion, hepatic disorders, and asthma (19). Studies currently in progress support its use in the treatment of other diseases (autoimmune diseases, cancer, AIDS).

LA can easily cross blood–brain barrier (18). Thus, its use has been proposed in the treatment of neural disorders. LA has been shown to have a variety of properties which can interfere with the pathogenesis or progression of Alzheimer disease (17, 20).

All these features make lipoic acid a very promising drug. Therefore, we first posed a question whether LA has anti-inflammatory activity in animals and what is the mechanism of action of anti-inflammatory effect. To answer this question, we administered LA to mice and then we assayed S* and effects of LA on inflammatory parameters (infiltration of neutrophils and vascular permeability) during zymosan peritonitis. As a reference drug for anti-inflammatory activity we have used indometacin – a nonselective COX inhibitor that is effective in many murine models of inflammation (21).

EXPERIMENTAL

Animals

The experiments were carried out on male albino Swiss mice (body weight 20-26 g). The animals were housed in constant temperature facilities exposed to 12 : 12 h light-dark cycle and maintained on a standard pellet diet, tap water was given *ad libitum*. Control and experimental groups consisted of

six to eight animals each. The investigated compounds were administered intraperitoneally (*i.p.*) as a suspension in 1% Tween 80.

Methods

Infiltration of neutrophils in a model of zymosan-induced peritonitis

In a model of zymosan-induced peritonitis, zymosan A was freshly prepared (2 mg/mL) in sterile 0.9% saline (22). Thirty minutes after the *s.c.* injection of the LA - zymosan A (0.5 mL) was injected *i.p.* LA was suspended in 1% Tween 80 and pitched in ultrasonic cleaner. A dose of 50 mg/kg b.w. was chosen for test, because it is frequently used dose of LA in different studies. Animals were killed by decapitation after 4 h. The peritoneal cavity was lavaged with 1.5 mL of saline and after 30 s of gentle manual massage the exudate was retrieved. Cells were counted with the automatic cell counter (Countess, Invitrogen) following staining with Turk's solution. The control group was given *s.c.* 1% Tween 80 in a volume of 0.25 mL (23). The zymosan group, 30 min prior to zymosan, was given *s.c.* 1% Tween 80 in a volume of 0.25 mL.

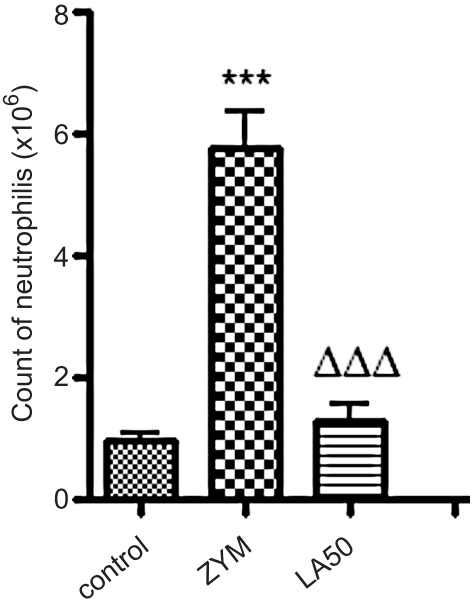

Figure 1. Infiltration of neutrophils changes during zymosan-induced peritonitis in mice. The mean ± SD of count of neutrophils, n = 6–8. Student *t*-test, *differences significant for control group *vs.* zymosan: *** $p < 0.001$, Δ - differences significant *vs.* zymosan, groups: LA50 – LA 50 mg/kg b.w.; ZYM – zymosan, control – Tween 80

Vascular permeability in a model of zymosan-induced peritonitis

LA suspended in 1% Tween 80 was injected *s.c.* in doses 10, 30, 50, 100 mg/kg b.w. Then, after 30 min, Evans blue suspended in saline (10 mg/mL) was injected *i.v.* into the caudal vein (0.2 mL/mouse) immediately followed by *i.p.* injection of zymosan A. Thirty minutes later, the animals were killed and their peritoneal cavities were lavaged with 1.5 mL of saline as described above. The lavage fluid was centrifuges and the absorbance in the supernatant was measured at 620 nm (23). The intensity of early vascular permeability was measured at 30 min of zymosan-induced peritonitis. The control group, 30 min prior to zymosan, was given *s.c.* 1% Tween 80 in a volume of 0.25 mL (23). Indomethacin injected *s.c.* in a dose of 10 mg/kg b.w. was used as a reference compound.

Determination of sulfane sulfur level in the peritoneal exudates

The level of S* in homogenates was determined by cold cyanolysis. To 100 µL of peritoneal

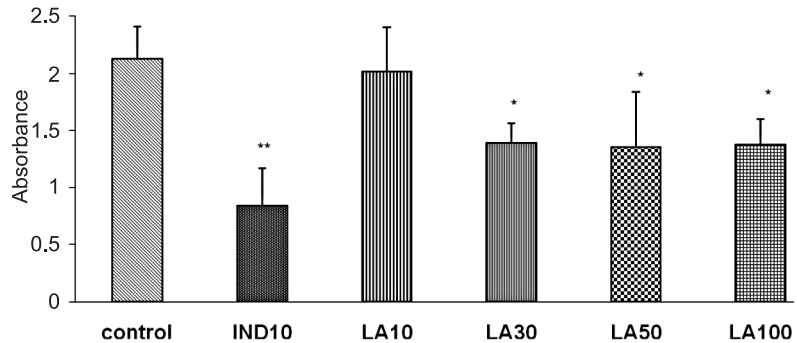

Figure 2. Vascular permeability changes during zymosan-induced peritonitis in mice. The mean ± SD of absorbance, n = 6–8. Students *t*-test, differences significant *vs.* control group - zymosan-induced peritonitis: * $p < 0.05$; ** $p < 0.01$. Groups: IND10 – indomethacin 10 mg/kg b.w.; LA10 – LA 10 mg/kg b.w.; LA 30 – LA 30 mg/kg b.w.; LA 50 – LA 50 mg/kg b.w.; LA 100 – LA 100 mg/kg b.w.

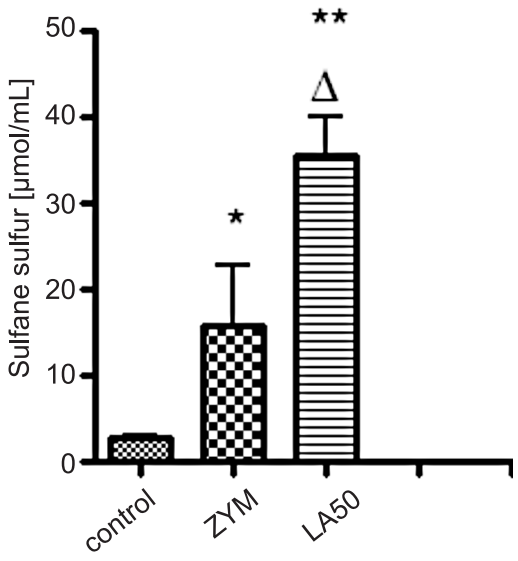

Figure 3. Sulfane sulfur level in the peritoneal exudates. The mean ± SD of sulfane sulfur level, n = 6–8. Student *t*-test, differences significant *vs*. control group :* $p < 0.05$, ** $p < 0.01$. Groups: LA50 – LA 50 mg/kg b.w.; ZYM – zymosan, Δ - differences significant *vs*. zymosan, control – Tween 80

exudates, 80 µL of 1 M amonia, 720 µL of distilled water and 100 µL of 0.5 M KCN were added. The samples were incubated at room temperature for 45 min. Then, 20 µL of 38% formaldehyde and 200 µL of Goldstein's reagent [$Fe(NO)_3$ + HNO_3 + H_2O] were added. After centrifugation at 12,000 × g for 10 min, the absorbance at 460 nm was determined. A standard curve was prepared with 1 mM KSCN (6). The control group was given *s.c.* 1% Tween 80 in a volume of 0.25 mL. The zymosan group, 30 min prior to zymosan, was given *s.c.* 1% Tween 80 in a volume of 0.25 mL.

Statistical analysis

The statistical significance was calculated using Student's *t*-test. Differences were considered statistically significant at $p \leq 0.05$.

RESULTS

Effects of lipoic acid on infiltration of neutrophils during zymosan peritonitis

The intensity of early infiltration of neutrophils measured at 30 min of zymosan peritonitis was significantly inhibited in LA-group compared to the control group (Fig. 1). LA decreased by 78% count of neutrophils in a model of zymosan-induced peritonitis.

Effects of lipoic acid on vascular permeability during zymosan peritonitis

The study showed that the intensity of early vascular permeability was significantly inhibited in groups received lipoic acid (LA100, LA50, LA30 groups) by 34.64-36.26% compared to the control group (Fig. 2). LA given at a dose of 10 mg/kg b.w. had no significant influence on vascular permeability. As a reference compound indomethacin was used at a dose of 10 mg/kg b.w. (IND10). The effect of reducing the vascular permeability of LA was not greater than that of indomethacin.

Effects of lipoic acid on sulfane sulfur level in the peritoneal exudates

LA administered at a dose 50 mg/kg b.w., increased of sulfane sulfur level in the peritoneal exudates by 11.5 times compared to the control group (Fig. 3). Zymosan increased the sulfane sulfur level in the peritoneal exudates by 4.5 times compared to the control group. However, after the administration of LA, sulfane sulfur increase was 2.5 times greater in comparison to zymosan.

DISCUSSION AND CONCLUSION

LA/DHLA system is one of the most important redox systems in human body. Exogenous LA can be reduced to DHLA by several enzymes so that LA and DHLA are in dynamic equilibrium, and can replace each other. In this paper, we tested only LA because it is now the drug with well-established therapeutic use. The aim of the current study was to evaluate of anti-inflammatory activity of LA. For this purpose we used zymosan-induced peritonitis in mice. Murine zymosan peritonitis was described as a suitable model of acute inflammation, characterized by vascular changes and mediator production leading to leukocyte accumulation at the inflammatory focus (22, 23). Two major events are critical for development of zymosan peritonitis, namely: the early increase in vascular permeability (< 1 h) and the infiltration of neutrophils into the peritoneum that follows after some hours (23). The mechanisms operating during the above stages have been investigated and these studies revealed that early vascular permeability depends mostly on cysteinyl-leukotrienes released by resident peritoneal macrophages and, to lesser extent, on mast cell histamine and prostaglandins (PGE_2, prostacyclin) of multiple cellular origin (23).

The studies focused on two major events leading to the onset of inflammation – the early increase

in vascular permeability and neutrophil infiltration into peritoneum. The study showed that LA has demonstrated anti-inflammatory activity in both tests. In the study of cellular infiltration, it significantly limited the migration of leukocytes to the site of inflammation, which was the peritoneum. The statistical analysis showed that LA significantly reduced the vascular permeability.

To explain the mechanism for anti-inflammatory activity of lipoic acid, sulfane sulfur level in the peritoneal exudates was determined and it was found that LA increased this level.

A number of both *in vivo* and *in vitro* studies provide evidence that the gaseous transmitter (H_2S) plays an important role as a modulator of inflammatory processes in various tissues, by acting on multiple targets. Recent data also suggest that H_2S may contribute to inflammatory processes. Several recent reports provide evidence suggesting a role for H_2S in inflammation. H_2S can scavenge peroxynitrite and can interfere with the ability of neutrophils, through hypochlorous acid, to kill microbes and other cells. It can also induce neutrophil apoptosis, thereby contributing to resolution of inflammatory reactions (24).

The precise sites and mechanisms of action of H_2S as an inflammatory mediator are not well established, although the existing data indicate diverse targets. It directly stimulates capsaicin-sensitive primary afferent neurons in the rat urinary bladder *via* an unknown molecular interaction. Some anti-inflammatory and anti-nociceptive effects of H_2S seem to be mediated *via* activation of ATPsensitive K^+ channels (KATP), as these effects were effectively prevented by glibenclamide, a KATP channel blocker. Other anti-inflammatory effects of H_2S occur *via* up-regulation of heme oxygenase-1 and CO production, leading to inhibition of the nuclear factor κB (NF-κB) pathway and down-regulation of inducible NO synthase (iNOS) expression and NO production by inflammatory stimuli (24-26).

In summary, the results of the present study have demonstrated anti-inflammatory activity of lipoic acid in mice peritonitis model. LA has decreased the count of neutrophils in a model of zymosan-induced peritonitis and significantly inhibited early vascular permeability compared to control group. LA serves as a sulfane sulfur acceptor and releases sulfane sulfur in the form of hydrogen sulfide (H_2S), which is probably responsible for its anti-inflammatory activity. Hydrogen sulfide inhibits the release of proinflammatory cytokines (IL-1β, IL-6, TNF-α), NO• oraz PGE_2. These effects depend on the dose and rate of release of hydrogen sulfide at various stages of infection. The most efficient way affects low concentrations of H_2S released slowly for a long time. It is possible that in this way hydrogen sulfide is released, which would explain the anti-inflammatory action of lipoic acid. In the future research, the levels of cytokines and eicosanoids as a pro-inflammatory mediators should be evaluated.

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