Liver-mediated effects of linoleyl-hydroxamic acid on lymph node cells and neutrophils

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Abstract

The response of cultivated lymph node cells and blood neutrophils on soluble messengers produced by intact or toxically injured CBA mouse liver explants after in vitro *0.06 or 0.6 μM linoleyl-hydroxamic acid (LHA, lipoxygenase inhibitor) application was studied. The results showed that the action of LHA on intact liver resulted in stimulation of the lymph node cell proliferation and differentiation as well as of the neutrophil oxygen radical production and phagocytosis by liver supernatants. The tetrachlormetan-injured liver supernatants induced strong activation of both lymph node cell and neutrophil reactions. When LHA was added, injured liver more markedly activated lymph node cells, and selectively regulated neutrophil function: high oxygen radical production in neutrophils was reduced, but phagocytosis was kept up. These findings extend the support for a participation of lipoxygenase products in toxic hepatitis development through liver-mediated up-regulation of neutrophil reactions and limitation of lymph node cell activation. LHA as lipoxygenase blocker may be preferable for toxic hepatitis treatment as regards to favorable pattern of liver-mediated neutrophil reactions.*

Key words: *liver, tetrachlormetan, linoleyl-hydroxamic acid, lymph node cells, neutrophils.*

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Introduction

Leukotrienes are known as important inflammatory mediators in the liver injury [1, 2]. Lipoxygenase inhibitors including novel hydroxamate derivatives were shown to have hepatoprotective effects on tetrachlormetan (CCl4)-, endotoxin- or galactosamine/endotoxin-induced liver injury [2-7]. However, the precise mechanisms by which these blockers influence liver inflammation are unknown.

Linoleyl-hydroxamic acid (LHA, octa-9, 12-dienehydroxamic acid [z,z]-) is suicide 5-lipoxygenase blocker with strong inhibitory activities on 12- and 15-lipoxygenases [8-12], which was successfully tested for experimental treatment of hypoxic disorders, anaphylactic heart dysfunction, atherosclerosis and arthritis [13-15]. Besides, LHA is shown to inhibit lipid peroxydation, and enzymatic oxidation of linoleic acid known as hepatoprotective substance [11, 12, 14]. Because of its properties LHA can be of interest in hepatic disorders treatment.

Our aim was to investigate the functional response of lymph node cells and blood neutrophils on modulatory factors secreted in culture by intact or CCl4-injured mouse liver explants after treatment by LHA.

Materials and Methods

Materials

The lipoxygenase blocker linoleyl-hydroxamic acid (LHA) was obtained from Prof. I. Butovich (Institute of Bioorganic Chemistry and Petrolchemistry, N.A.S. of Ukraine, Kyiv). The culture media components and equipment, and neutrophil function assay reagents were purchased from Sigma, USA.

Animals and intoxication

Male CBA mice weighing 20-22 g (n=56) were used in all experiments. CCl_4 (50% vol/vol solution in corn oil) was administered to mice (5 mg/kg, s.c.) three times with two-day intervals. The control animals were not treated.

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24 hours after last administration all mice were anaesthetized with ether, exsanguinated by decapitation, and their livers were removed under sterile conditions.

Liver explants cultivation and treatment

Liver fragments (1 mm^3) prepared in Hanks buffered salt solution with 2% bovine serum albumin, 0.1 g/l benzylpenicillin and 0.1 g/l streptomycin were placed into teflon diffusion chambers covered with Millipore membrane filter, 0.23 μm pore size [16]. The chambers were incubated at 37°C in humidified atmosphere with 5% CO_2 in polystyrene 6-well plates, 9 liver explants per well in 1 ml culture medium, which contained Dulbecco's modified Eagle's medium and RPMI-1640 medium (1:1), 10% heat-inactivated fetal bovine serum, 10 mmol/l Hepes, 0.1 g/l benzylpenicillin, 0.1 g/l streptomycin and 0.02 g/l gentamycin.

After 2 hours, the explants were cultivated for 2 hours in culture medium additionally containing 0.06 μM or 0.6 μM LHA. The control chambers were incubated in the culture medium without LHA. After such a treatment, the chambers were cultivated 24 hours in the culture medium, and then the supernatants were collected and kept at -20°C. Six wells per condition were harvested in four similar experiments. The explants were fixed in 10% neutral formalin solution, stained with hematoxylin-eosin and prepared for histological examination.

Evaluation of lymph node cell proliferation

Cells were obtained from lymph nodes of intact mice. The cell viability was greater than 90% as assessed by Tripan Blue exclusion. Lymph node cell suspension in culture medium was placed in diffusion chambers (5×10^6 cells/30 mcl per chamber) for the cultivation as described above for the liver explants, six wells per condition in four similar experiments. The supernatants from liver explants were used as the culture medium (1 ml per well). The cell suspensions were removed after 24 hours, and the samples were prepared for microscopic examination. After the drying in air, the samples were fixed with May-Grunvald fixative and stained by Romanovsky-Giemsa method. The lymph node cells of different stage of maturity were determined by light microscopy ×900. The results were expressed as a cytogramma in percent.

Measurement of oxygen radical production in neutrophils

The isolated neutrophils were obtained by centrifugation of peripheral blood of mice in Ficoll-Verografin density gradient [17]. The oxygen radical production in neutrophils was studied using micromodification of nitroblue tetrazolium (NBT) test [18]. Briefly, neutrophil suspension in medium 199 with Hanks' salts (1×10^5 cells/10 μl) was mixed with 5 μl of 0.2% NBT solution in medium 199 prepared ex tempore, and 20 μl of each kind of liver explant supernatants, or non-modified culture medium with or without a standard stimulator (0.1 μM fMLP), as positive and negative control respectively. Each assay was performed three times in four analogous experiments. After the 30 min incubation at 37°C, the samples were prepared for microscopic examination. After the drying in air, the samples were fixed in methanol, stained with 1% safranine solution, and examined by light microscopy ×1000. The number of activated neutrophils was expressed as percent of formasan-positive cells/100 neutrophils. The index of neutrophil activation reflecting the intensity of oxidative metabolism in neutrophils was calculated per 100 cells by Astaldi and Verga's formula: (A×0 + B×1 + C×2 + D×3)/100, in which A – number of formasan-negative cells; B, C, and D – number of cells, in which formasan deposits come to 1/3, 2/3 or 1 nuclear area, respectively.

Phagocytosis assays

The phagocytic activity was studied using the polystyrol latex ingestion method. Briefly, 10 μl of neutrophil suspension was mixed with 5 μl of latex suspension (0.8 μm) and 20 μl of supernatant or control solution such as those used in NBT-test. The samples were incubated and prepared as described for NBT-test. After fixing, the samples were stained by Romanovsky-Giemsa method, and examined by light microscopy ×1000. Phagocytic activity was expressed as percent of latex-containing cells/100 neutrophils. Intensity of phagocytosis was calculated as number of ingested particles per latex containing cell.

Data analysis

Values are given as mean ± SEM, 100 neutrophils per group. Considering that the data had normal distribution, the statistic analysis was done by Student's *t*-test.

Results

Morphofunctional state of liver explants

The histological examination showed that the action of 0.6 μM LHA on intact liver explants resulted in weakly expressed dystrophy of hepatocytes, edema of vessel walls, and extension of around-sinusoidal spaces. The influence of 0.06 μM LHA did not significantly change the morphological state of explants. Both doses of LHA caused the opposite effect on injured liver explants. The toxic action of CCl_4 was reduced. The small- and big-drop fatting of hepatocytes observed in injured liver explants turned into small-center fatting after application of 0.6 μM LHA. The influence of low doses of LHA minimized necrobiotic changes of parenchyma, which were observed in 40% of samples only. The appearance of basophilic colored hepatocytes evidenced the metabolic process intensification, and the young cells formation (data not shown).

Proliferative activity of lymph node cells

The LHA application to intact liver resulted in activation of the lymphocyte proliferation and differentiation (Table 1). The quantity of stromal lymph node cells was decreased after the treatment with the blocker. The LHA treatment caused dose-dependent inhibition of reticulocyte proliferation. Supernatants from injured liver activated lymphocyte proliferation, the quantity of mature form of the cells was significantly increased ($p<0.01$ vs. influence of intact liver supernatants, Tables 1, 2). The influence of LHA on the injured liver, similarly to its effect on intact liver, intensified these changes.

Oxygen radical production in neutrophils

The oxidative metabolism assays demonstrated (Fig. 1) that intact liver supernatants did not influence the neutrophil activation. The action of 0.06 μM LHA on liver resulted in elevation of number of activated neutrophils ($p<0.01$, Fig. 1A) and intensity of oxidative reactions ($p<0.001$, Fig. 1B). After 0.6 μM LHA application, the number of activated neutrophils was significantly less than after low dose treatment ($p<0.01$).

The injured liver supernatant significantly increased neutrophil activation ($p<0.001$ compared to intact liver action). The influence of LHA on liver completely depressed this activation in dose-dependent manner.

Phagocytic activity of neutrophils

As indicated in Figure 2, the intact liver supernatants caused the stimulation of the neutrophil phagocytic activity ($p<0.01$ compared to negative control, Fig. 2A) without increase of the number of ingested particles/cell (Fig. 2B). The action of LHA on the liver resulted in followed rise of phagocytic activity of neutrophils ($p<0.05$) but intensity of phagocytosis was increased only after influence of high dose of LHA ($p<0.001$).

The higher phagocytic activity of neutrophils was caused by the supernatants of the injured liver ($p<0.001$ in comparison to intact liver), and was more increased after treatment of liver with LHA, in dose-dependent manner ($p<0.05$ in comparison to injured liver without blocker). However, the intensity of phagocytosis was reduced after LHA action on liver ($p<0.01$), but in the less extent than the intensity of oxidative metabolism in neutrophils.

Discussion

Our results confirmed that LHA demonstrated anti-inflammatory effects during the toxic hepatitis. These data are in agreement with known results due to favorable effects of other lipoxygenase blockers under hepatitis [2-6].

However, our data evidenced that 0.6 μM LHA caused the injury of intact liver tissue. This phenomenon appears to be connected with physiological role of leukotrienes in

Table 1. Lymph node cell proliferation and differentiation caused by the supernatants from intact liver explants treated with LHA. Data are percentage of cells, means ± SEM

Group	Lymphocytes			P	L	B	M	R	DC
	large	middle	small						
Intact liver explants	39.4±0.17	28.13±0.16	11.47±0.15	1.11±0.03	0.52±0.03	0.60±0.04	4.23±0.05	13.3±0.06	0.5±0.04
+ LHA, 0.6 μM	13.2±0.06*	36.43±0.17*	39.04±0.07*	0.32±0.02*	0	0.35±0.03*	2.04±0.04*	4.17±0.05*	4.1±0.1*
+ LHA, 0.06 μM	6.64±0.06*	30.13±0.11*	50.18±0.19*	0.44±0.03*	0	0.56±0.01	3.36±0.05*	8.16±0.09*	4.3±0.1*

** p<0.05 vs. intact liver explants; R – reticulocytes; M – macrophages; B – basophils; P – prolymphocytes; L – lymphoblastes; DC – divisible cells.*

Table 2. Lymph node cell proliferation and differentiation caused by the supernatants from CCl_4-injured liver explants treated with LHA. Data are percentage of cells, means ± SEM

Group	Lymphocytes			P	L	B	M	R	DC
	large	middle	small						
Intact liver explants	14.1±0.03	37.42±0.02	31.17±0.04	1.14±0.04	0.72±0.03	0.91±0.04	5.42±0.07	9.15±0.05	2.6±0.1
+ LHA, 0.6 μM	6.12±0.02*	34.23±0.02*	44.81±0.09*	0.54±0.03*	0.36±0.03*	1.11±0.07*	3.71±0.03*	8.12±0.08*	3.9±0.1*
+ LHA, 0.06 μM	4.22±0.03*	21.46±0.03*	58.18±0.14*	0.21±0.02*	0	1.74±0.05*	5.12±0.11*	8.81±0.05*	4.1±0.1*

** p<0.05 vs. injured liver explants; R – reticulocytes; M – macrophages; B – basophils; P – prolymphocytes; L – lymphoblastes; DC – divisible cells.*

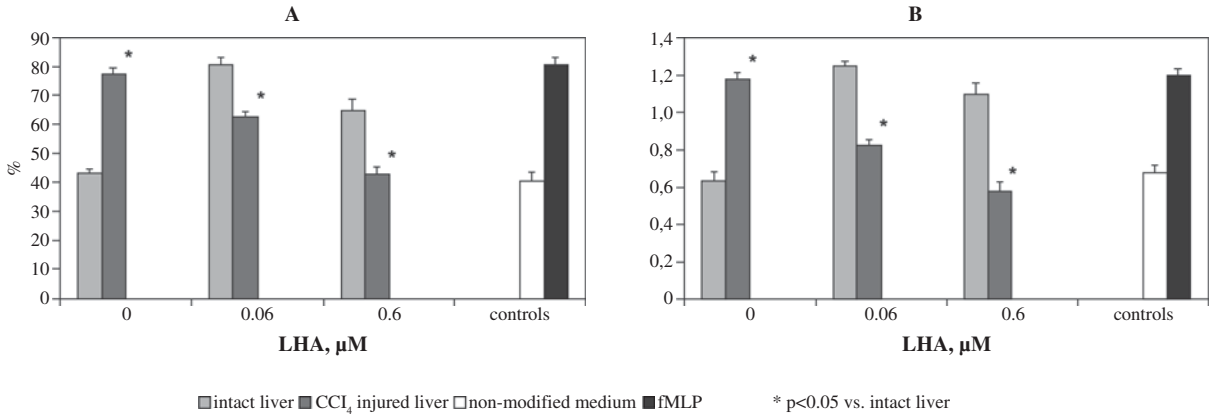

Fig. 1. NBT reduction in neutrophils influenced by the cultural supernatants from intact or CCl4-injured mouse liver explants after lipoxygenase blocker LHA application. **A:** percent of neutrophils containing formasan deposits; **B:** index of intensity of NBT reduction in neutrophils. Controls: NBT reduction in neutrophils in non-modified cultural medium with or without 0.1 µM fMLP as standard stimulator

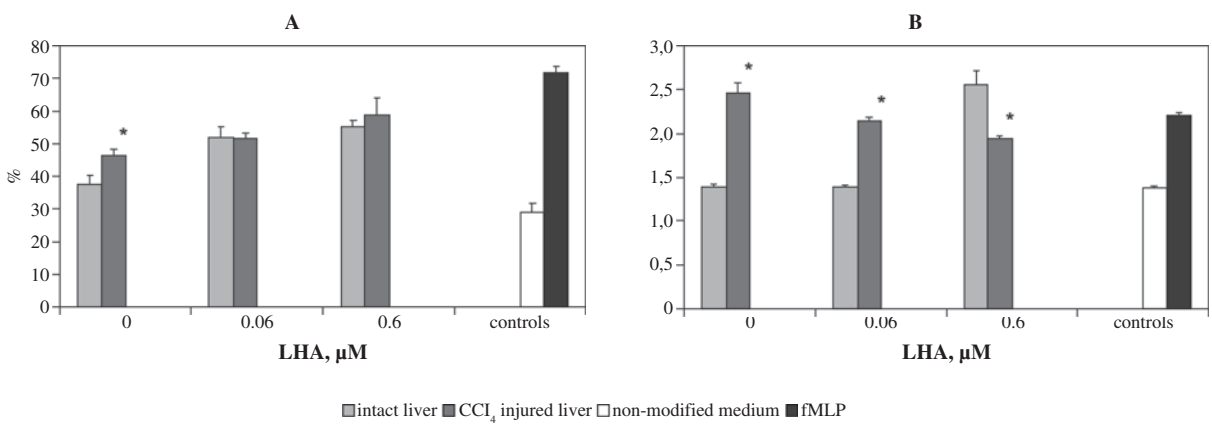

Fig. 2. Latex phagocytosis in neutrophils influenced by the cultural supernatants from intact or CCl4-injured mouse liver explants after lipoxygenase blocker LHA application. **A:** percent of neutrophils containing latex particles; **B:** intensity of phagocytosis (number of latex particles per latex-containing cell). Controls: phagocytosis in neutrophils in non-modified cultural medium with or without 0.1 µM fMLP as standard stimulator

the liver. Indeed, Tang et al. [19] shown that lipoxygenase metabolites play a physiological role in regulating cell survival and apoptosis. Makogon et al. [20] reported that LHA increased the oxygen consumption and viability of cultured hepatocytes but not of them in co-culture with sinusoidal liver cells. *In vivo* administration of LHA had not significant influence on body and tissue oxygen consumption [14]. Taken together, these and our data suggest that the presence of sinusoidal liver cells can play a key role in maintenance of regulatory functions of lipoxygenase metabolites in liver.

When the liver was toxically injured, LHA reduced the toxic morphological changes. These data showed that generation of large amounts of lipoxygenase products is involved in pathogenesis of toxic hepatitis, and blockade of lipoxygenase has direct protective effect.

It was known that cysteinyl leukotrienes, derived from 5-lipoxygenase, mediate T cell migration and homing from spleen and peripheral blood to lymph nodes by activation of the LTC4 transporter Abcc1 (Mrp1) [21]. However, *in vitro* studies showed that LTB4 and LTB5 may directly suppress lymphocyte proliferation [22]. Besides, the liver macrophages modulate immune responses via suppression of T cell activation by paracrine actions of IL-10, prostanoids, and TNF-alpha [23]. Our results extend these data for suppressive effect (direct or indirect) of liver-derived leukotrienes on lymph node cells.

Furthermore, we evidenced anti-inflammatory influence of liver on neutrophils after lipoxygenase blockade in injured liver, despite the fact that LHA-treated intact liver stimulated neutrophil activation.

The last effect appears to be at variance with the facts that leukotrienes are potent pro-inflammatory agents and that lipoxygenase blockers including hydroxamate derivatives have inhibitory effects on neutrophil activation [7, 24, 25]. First of all, LHA may elicit liver damage and leukocyte activation, at least in part, through inhibition of 15-lipoxygenase, which produces anti-inflammatory lipoxins [26]. However, in case of toxic hepatitis we did not observe such reactions (except phagocytosis activation), despite the fact that acute inflammation can switch lipoxygenase pathways toward generation of lipoxins [26].

We speculate that the limitation of liver leukotriene production by LHA may induce direct or cytokine network mediated activation of host defense and immune cells [22-25] to eliminate of liver-influencing agents. Besides immunostimulatory action, the last effect was confirmed in our experiments with administration LHA to both intact and injured liver as selective activation of neutrophil phagocytosis.

The effects of activation of both phagocytic and non-phagocytic reaction of neutrophils by intact liver cells are important for possible administration lipoxygenase blockers as LHA in extrahepatic disorders [13, 15]. LHA application during toxic hepatitis, as distinct from intact liver, limited non-phagocytic reaction of neutrophils, thereby preventing the second alterations of liver cells by oxygen radicals and granular enzymes from activated leukocytes [27]. It was formed positive pattern of neutrophil activation, namely: reduction of toxin-induced non-phagocytic reaction with maintenance of active phagocytosis.

Taken together, our data demonstrate that LHA administration in toxic hepatitis may attenuate liver injury, positively change inflammatory cell activity, and maintain immune system activation.

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