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Effect of L-carnitine on liver cell membranes in ethanol-intoxicated rats

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ABSTRACT

Ethanol intoxication is characterized by changes in cell metabolism which alter the structure and function of cell membrane components, including phospholipids and integral membrane proteins. The interaction of food nutrients with ethanol may modulate alcohol toxicity. One such compound is L-carnitine (L-3-hydroxy-4-N,N,N-trimethylaminobutyrate), which is also an antioxidant. Here we investigate L-carnitine as an antioxidant and assess its effect on the composition and electrical charge of liver cell membranes in ethanol-intoxicated rats. Qualitative and quantitative phospholipid composition and the presence of integral membrane proteins were determined by high performance liquid chromatography (HPLC). Electrophoresis was used to determine the surface charge density of the rat liver cell membranes. Ethanol increased phospholipid levels and altered the level of integral proteins as determined by decreased phenylalanine (Phe), cysteine (Cys) and lysine (Lys). Ethanol significantly enhanced changes in the surface charge density of the liver cell membranes. L-Carnitine administration to ethanol-intoxicated rats significantly protects phospholipids and proteins against oxidative modifications. Therefore, the beneficial effect of L-carnitine may be connected to its ability to scavenge free radicals.

1. Introduction

The cell membrane an integral part of a live cell isolates the cell from the environment and determines specific cell properties and its capacity for movement. The most important properties of a biological membrane are its electric charge and the potential difference between the membrane and surrounding solution [1,2]. Electrical properties of the membrane are determined by acid–base pairs and complex formation equilibria and the solution components. This equilibrium is established by membrane components, including phospholipids and proteins.

The oxidative stress induced by chronic ethanol consumption has been implicated in changes in structure and functions of liver cell components, including membrane phospholipids and proteins. Ethanol is rapidly absorbed from the gastrointestinal tract, and about 90% of it is metabolized in the liver. There, ethanol is oxidized into acetaldehyde and then into acetate: these processes are accompanied by free radical generation [3,4]. Ethanol reduces membrane hydration [5], thereby affecting the protein–lipid structure of cell membranes. Acetaldehyde and reactive oxygen species (ROS) can react with amino acids, peptides and proteins, modifying their composition and function [6,7]. ROS can also react with lipids causing peroxidation [8]. Free radical peroxidation, especially of unsaturated lipids, disrupts the important structural and protective functions associated with biomembranes. Certain *in vivo* pathological events result from this oxidation [9]. Free radical peroxidation also disrupts lipid membrane asymmetry, permeability and integrity.

L-Carnitine (CA; β-hydroxy-γ-trimethyl ammonium butyrate), a constituent of plasma and tissues, is synthesized in liver from lysine and methionine [10]. Enzymes for carnitine biosynthesis are found in the liver and in many other tissues, but not in skeletal and heart muscles. These latter tissues, therefore, are entirely dependent on carnitine uptake from the blood [11]. Carnitine is an absolute requirement for the transport of activated long chain acyl units into the mitochondria [12,13]. However, L-carnitine also protects against ROS by acting as an antioxidant scavenging hydroxyl radicals and inhibiting hydroxyl radical formation by the Fenton reaction system [14].

The interaction of ethanol metabolism with nutrients from food may modulate alcohol toxicity. For example, L-carnitine retards alcohol clearance and the development of hepatic steatosis in rats. In the current study, we examined whether L-carnitine consumption affects the phospholipid and integral membrane protein

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content, as well as the electrical properties, of liver cell membranes from ethanol-intoxicated rats.

2. Materials and methods

All experiments were approved by the Local Ethic Committee in Białystok (Poland) referring to Polish Act Protecting Animals of 1997.

2.1. Animals (treatment)

Two-month-old male Wistar rats were used for the experiment. They were housed in groups with free access to a granular standard diet and water and maintained under a normal light-dark cycle. Rats from each group were divided for four groups (by six animals in each).

2.2. Control group

Rats were treated intragastrically with 1.8 mL of physiological saline every day for 4 weeks ($n = 6$).

2.3. L-Carnitine group

Rats received L-carnitine solution (1.5 g L-carnitine dissolved in 1 L of water) ad libitum instead of water for 1 week. Next, the rats were treated intragastrically with 1.8 mL of physiological saline and received L-carnitine solution ad libitum instead of water every day for 4 weeks ($n = 6$).

2.4. Ethanol group

Rats were treated intragastrically with 1.8 mL of ethanol in doses from 2.0 to 6.0 g/kg body weight (b.w.) every day for 4 weeks. The dose of ethanol was gradually increased by 0.5 g/kg b.w. every 3 days ($n = 6$).

2.5. Ethanol and L-carnitine group

Rats received L-carnitine (1.5 g/1 L) solution ad libitum instead of water for 1 week. Next, it was treated intragastrically with 1.8 mL of ethanol in doses from 2.0 to 6.0 g/kg b.w. and received L-carnitine solution ad libitum instead of water every day for 4 weeks.

Animals from control and L-carnitine groups received an isocaloric amount of dextrose in physiologic saline.

2.6. Isolation of liver cell membranes

Livers (approximately 1.5 g) were homogenized in a solution containing 1 mM-$NaHCO_3$ (pH 7.6) and 0.5 mM $CaCl_2$ in a loose-fitting Dounce homogenizer. The addition of 0.5 mM $CaCl_2$ increased the cell membrane sedimentation, as determined by measurement of 5′-nucleotidase activity [15]. Membrane fragments were separated from nuclei and mitochondria by rate-zonal centrifugation of the 'low-speed' pellet as described previously [16]. The sediment was homogenized in sucrose (1.22 g/cm^3 density) and in the next step was covered with sucrose (1.16 g/cm^3 density). The cell membranes were separated by centrifugation at 2000 × g for 25–35 min. Membrane purity was determined by spectrophotometric determination of 5′-nucleotidase (EC 3.1.3.5) activity as described previously [15].

2.7. Isolation and analysis of phospholipids by HPLC method

The Folch method was used to extract phospholipids [17]. The cell membrane was homogenized in chloroform–methanol mixture of (2:1 volume ratio). The solution was then filtered with degreased paper filters, and the precipitate was washed with an extracting solution (8:4:3 chloroform:methanol:aqueous calcium chloride solution 0.05 M calcium chloride). The suspension was centrifuged at 500 × g for 2 min, the organic and the aqueous phases were separated, and the aqueous phase was shaken again with chloroform, methanol and water mixture of (3:48:47 volume ratio) and the phases were separated. The organic phases were combined and evaporated to dryness. The extract was dissolved in 200 μl of hexane:isopropanol mixture (3:2) [18]. Addition of 0.03% tert-butylhydroxytoluene (BHT) and flushing with nitrogen at each step in the procedure were used to prevent oxidation during lipid extraction.

HPLC analysis was performed on the extracted phospholipids to assess the quantities phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylethanolamine (PE) and phosphatidylcholine (PC). The isolated phospholipids were separated by group analysis in a silica gel column using normal phase (NP)-HPLC; acetonitryle–methanol–phosphoric acid (85%) mixture (130:5:1 volume ratio) by isocratic elution at 1 mL/s flow rate and 214 nm wave length [19].

2.8. Extraction of membrane proteins

The liver cell membranes were homogenized in 5 mM NaOH. PMSF (phenyl–methyl–sulfonyl fluoride) was added to a final concentration of 1 μM to inhibit proteolysis. The suspension was centrifuged for 45 min at 1000 × g [20].

The residual cell membranes were solubilized in 30 mL buffer containing 20 mM Tris/HCl (pH 7.4) and 1% Triton X-100 at 4 °C. The suspension was centrifuged at 1000 × g for 10 min. The supernatant was incubated at 32 °C for 2 h [21] and was then dialysed against distilled water and evaporated until dry.

2.9. Trypsin hydrolysis of proteins

The protein extract was weighed and dissolved in phosphate buffer (pH 7.4) to yield a final protein concentration of approximately 0.10 mg/mL. A stock solution of trypsin (0.05 mg/mL in H_2O) was added, at an enzyme:substrate ratio of 1:25. The reaction mixtures were incubated at 37 °C for 1 h. Hydrolysis was stopped by the addition of PMSF to a final concentration of 1 μM) [22] and the hydrolysate was then evaporated until dry and dissolved in 200 μl H_2O.

2.10. Separation of the peptide mixture of integral membrane proteins by HPLC

Following hydrolysis, the peptides were separated by HPLC on a LichroCART RP-18 column 100A (5 μm, 250 mm × 4.0 mm) equilibrated with solvent A (0.1% trifluoroacetic acid (TFA) in H_2O) and eluted with a linear gradient to 20% solvent B (0.1% TFA in acetonitryle) during the first 8 min, to 70% solvent B during the next 20 min and to 100% solvent B during the final 4 min at 220 nm: the flow rate was 1 mL/min [23]. The Merck HPLC system was equipped with a pump, an ultra violet (UV) detector, an analog interface module D-6000 A and System Manager Software. A typical separation of the peptide mixture containing liver integral membrane proteins is provided in Fig. 1.

2.11. Peptide assignment

The amino acid compositions of isolated peptides (6.2 min; 8.2 min; 9.1 min; 11.7 min; 12.6 min; 14 min; 17.1 min; 27.4 min; 28.9 min; 30.7 min) were determined by HPLC after acid hydrolysis under vacuum in the presence of 6 N HCl for 24 h at 110 °C.