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Effect of Vitamin E Derivative (U83836E) on Membranes of Rat Liver Cells after Methanol Intoxication

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Abstract

Methanol oxidation in vivo to formaldehyde and next to formate, which takes place mainly in the liver, is accompanied by free radicals generation. The goal of this paper is to desribe the effect of vitamin E derivative (U83836E) on rat liver cell membranes during methanol intoxication (3.0 g/kg body weight). This study has been concerned with measurement of lipid peroxidation and surface charge density of liver cells and activity of lysosomal enzyme - cathepsin B - in cytosol of liver cells and the blood serum. An ultrastructural study of liver cells has also been conducted. It has been proven that methanol administration causes an increase in lipid peroxidation products (by 33%) as well as in surface charge density (by 64%) of the liver cells. This might have resulted from the membrane liver cell damage visible in the electron microscope and in leak of cathepsin B into cytosol (increase in activity by 28%) as well as the leak of cathepsin B from cytosol into the blood (increase in serum activity by 27%). Administration of U83836E to rats intoxicated with methanol partially prevented the above changes. Obtained results suggest that U83836E acts as an effective antioxidant in methanol intoxication.

Keywords: methanol, vitamin E derivative (U83836E), lipid peroxidation, surface charge density, liver cell

Introduction

Methanol is oxidized at about 80% in the liver, so acute and chronic methanol intoxication is characterized by severe structural alterations of the liver cells [1]. The main metabolites are formaldehyde and formate and methanol metabolism occurs with a concomitant increase in NADH level. Methanol metabolites are highly toxic. Formaldehyde reacts both with low and high molecular weight compounds, especially with proteins [2]. Formic acid causes, among other things, metabolic acidosis and a decrease in ATP synthesis [1]. Metabolic acidosis an the increase in NADH concentration can lead to intesive production of hydroxyl radicals and superoxide anions [3]. These compounds are especially harmful to membrane lipids and proteins. Lipid peroxidation processes induce site-specific oxidative changes in the lipid bilayer phospholipid organization of hepatocytes membranes and lead to their disfunctions [4]. It is visible in the increase of the leak of cytosolic enzymes (ALT and AST) into the blood after methanol intoxication [5].

The cell membrane has a net negative electric charge which is determined by the greater part of negative charge carriers such as phosphatidylserine, sialic acid of glycophorine and free carboxyl groups of polypeptide chains [6]. Changes in the value of cell membrane charge may manifest cell condition, e.g. the development of tu-

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mors is accompanied by an increase in the membrane potential, whereas the necrosis of a cell is accompanied by a decrease in the surface charge [7]. The surface charge can also be influenced by active exogenic compounds like methanol or its metabolites, free radicals and others.

The generation of reactive methanol metabolites and the decrease observed in the liver antioxidant capacity during methanol intoxication [5] requires proper antioxidant therapy. The most important chain-breaking antioxidant in the lipid phase is vitamin E. An analog of the vitamin - trolox - acts more intensively. Owing to its structure it possesses lipophylic and, to a higher degree, hydrophilic properties and can be transported in the aqueous phase of blood and interstitial fluid and yet be readily taken up by membranes [8]. A new antioxidant, U-83836E, which contains the reactive trolox ring, can inhibit lipid peroxidation in vivo, has recently been synthesized.

The aim of this study has been to investigate, by means of biochemical, electrochemical and ultrastructural methods, the effect of U-83836E on the disturbances in the structure of lysosomal and liver cell membrane during methanol intoxication.

Materials and Methods

Male Wistar rats (approximately 230 g b.w.) 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. All procedures were in accordance with the guide for care and use of laboratory animals and the protocol was approved by the local Animal Care Committee. The minimal lethal dose of methanol in the rat is 9.5 g/kg b.w. [9] (doses from 1 to 6 g methanol/kg b.w. had been previously used in rats for experimental acute methanol intoxication [5]). The animals were divided into 4 main groups:

A. Control group. The rats were treated intragastrically with 1.0 ml of saline (n=42).

B. U83836E group. The rats were treated intraperitoneally with U83836E at doses of 10 mg/kg b.w. (n=42). After 12 hours the rats were given a second dose of U83836E (10 mg/kg b.w.).

C. Methanol group. The rats received intragastrically 3.0 g methanol/kg b.w. as a 50% solution in isotonic sa line through a plastic tube with a syringe (n=42).

D. Methanol + U83836E group. The rats were treated intraperitoneally with U83836E in doses of 10 mg/kg b.w. and subsequently with 3.0 g methanol/kg b.w. as above (n=42). 12 hours after intoxication the rats were given the second dose of U83836E - 10 mg/kg b.w.

After 6, 12, 24 h and 2, 5, and 7 days the intoxicated rats were sacrificed with ether anaesthesia (six animals in each group). The livers were quickly removed and placed in iced 0.15 M NaCl solution.

Biochemical Analysis

The livers for biochemical measurements were perfused with 0.15 M NaCl solution to remove blood cells, next blotted on filter paper, quickly weighed and homogenized in 9 ml ice-cold 0.25 M sucrose and 0.15 M NaCl with addition of 6 µl 250 mM butylohydroxytoluene in ethanol to stop peroxide formation during the assay. The homogenization procedure was performed as quickly as possible under fully standardized conditions. The homogenates in sucrose solution were centrifugated at 100,000 x g (4°C) for 60 min to settle the organelles to obtain the cytosol. The activity of cathepsin B in the cytosol and blood serum was determined with benzoyl-DL-arginine-p-nitroanilide (BAPA) as a substrate at pH 6.0 [10]. 10% homogenates in saline were centrifuged at 10,000 x g for 15 min at 4°C [11], and the supernatant was kept on ice until assayed. The extent of lipid peroxidation was assayed by measuring malondialdehyde (MDA) by direct HPLC method with spectrophotometric detection [12]. The concentration of malondialdehyde was expressed in nmoles MDA per g of the liver.

Electrochemical Analysis

The surface charge density of the hepatocyte membrane was measured by the electrophoresis method [13]. These studies were performed according to a method described by Mine and Krysinski [14]. Liver cells were suspended in phosphate buffered saline (PBS) solution in a measuring container and the velocity of hepatocyte membrane movement was measured by the change in electric field intensity from 0.4 to 0.2 V/m. Electrophoretic mobility (u) was calculated from the formula

$$u=v/E$$

where: v- speed of the cell movement, E- electric field intensity.

The electrophoretic mobility depends on the charge density, shape and size of the object. For a spherical cell of radius r, mobility is related to the surface charge density by the equation

$$\delta=3\eta u/2r,$$

where: η| - viscosity of solution, u - electrophoretic mobility, r - cell radius [15].

Ultrastructural Analysis

One-cubic-millimetre sections of the livers were collected for ultrastructural examinations, five from each animal. They were immediately fixed in 3.6% glutaraldehyde, then in 2% osmium tetroxide to be finally embedded in Epon 812 after dehydration in alcoholic series and propylene oxide. Ultrathin sections were contrasted with lead citrate and uranyl acetate and evaluated under transmission electron microscope OPTON 900 PC.

Statistical Analysis

The data obtained in this study have been expressed as mean ± SD. These data were analysed by standard statistical analyses, two-way ANOVA with Tukey's test for multiple comparisons to determine significance between different groups. The values for $p < 0.05$ were considered significant.

Results

Table 1 shows changes observed in the amount of MDA, the main lipid peroxidation product, up to 7 days after intoxication. Hepatic MDA concentration was significantly increased from 6 h to 5 days after intoxication with the highest increase by about 33% after 2 days. The treatment of intoxicated rats with U83836E significantly prevented changes in the MDA quantity caused by methanol. The increase in this parameter was by about 15% after U83836E ingestion, in comparison to control group.

Figure 1 shows the changes in surface charge density of liver cells from control rats and those which received methanol and methanol with U83836E. The surface charge density increased after methanol administration, reaching the highest value after 24 h (64% in comparison to the control). Recovery to the control value was observed 5 days after intoxication. The increase in surface charge density was smaller in rats receiving both methanol and U83836E, by about 28% in comparison to the control group.

Methanol intoxication caused, in comparison to control, statistically significant increase in the activity of cathepsin B in cytosol from 6 h to 5 days after intoxication with the highest increase after 24 h (by about 28%) (Fig. 2). Administration of U83836E alone did not bring any effect. When given with methanol, however, U83836E partially prevented the methanol-induced increase in cathepsin B activity which was enhanced only by 1% after trolox derivative administration.

Numerous changes pointing to a damage of membranes have been determined in microscopic-electronic examination of hepatocytes of methanol-intoxicated rats. These changes were connected with cell membrane damage (irregular decomposition or total disappearance of cell membrane microvilluses, and occasionally their segmental damage with leakage of the cells' components into the sinusoids lumen have been observed on the hepatocytes surface directed to the Disse space) (Fig. 3). Methanol also caused increases in numerous lysosomes which were characterized by different shapes and occasional obliteration of enclosing membranes (Fig. 4). The highest degree of subcellular structural damage was observed after 24 h and 2 days following methanol intoxication. Administration of U83836E to methanol-intoxicated rats also resulted in a smaller degree of liver cell membrane damage in comparison to the injury caused by methanol. It was particularly visible on the vascular surface of hepatocytes (more regular quantity

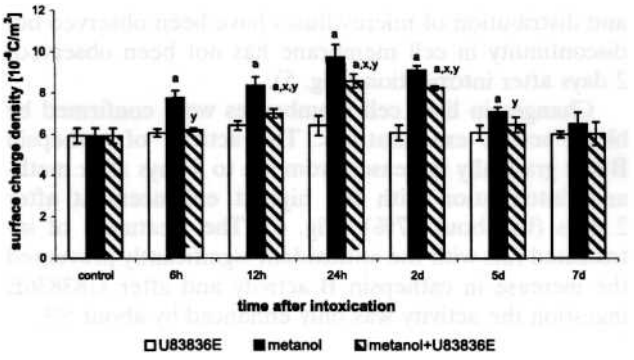

Fig. 1. Effect of U83836E on the surface charge density of the liver cells of rats intoxicated with methanol (3.0 g/kg b.w. given intragastrically); U83836E was given intraperitoneally twice: before methanol administration - 10 mg/kg b.w. and 12 hours after methanol administration - 5 mg/kg b.w.; data points represent the means ± SD; n = 6. Statistically significant differences for p < 0.05: a - in comparison with control group x - in comparison with U83836E group y - in comparison with methanol group.

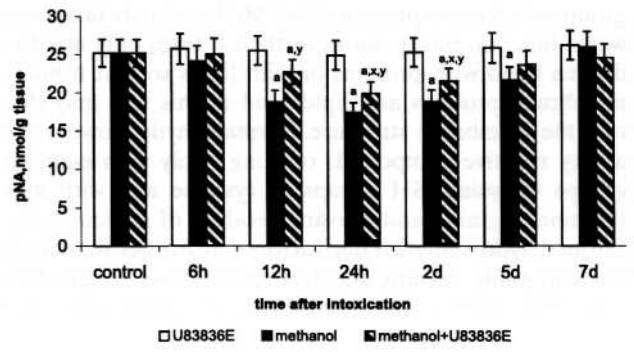

Fig. 2. Effect of U83836E on the catepsin B activity in the cytosol of rats intoxicated with methanol (3.0 g/kg b.w. given intragastrically); U83836E was given intraperitoneally twice: before methanol administration - 10 mg/kg b.w. and 12 hours after methanol administration - 5 mg/kg b.w.; data points represent the means ± SD; n = 6.
Statistically significant differences for p < 0.05:
a - in comparison with control group
x - in comparison with U83836E group
y - in comparison with methanol group.

Table 1. Effect U83836E on the malondialdehyde concentration (nmol/g) in the liver of rats intoxicated with methanol (3.0 g/kg b.w. given intragastrically); U83836E was given intraperitoneal^ twice: before methanol administration - 10 mg/kg b.w. and 12 hours after methanol administration - 5 mg/kg b.w.; data points represent the means ± SD; n = 6.

Group of animals	Control	Time after intoxication					
		6 h	12 h	24 h	2 days	5 days	7 days
Methanol	62.3 ± 3.8	66.7 ± 4.1[a]	1.4 ± 4.5[a]	79.8 ± 4.7[a]	82.6 ± 4.9[a]	73.4 ± 4.2[a]	63.9 ± 4.0
U83836E	62.3 ± 3.8	61.9 ± 4.0	59.9 ± 3.7	60.3 ± 3.8	60.9 ± 3.7	61.4 ± 4.0	62.2 ± 3.9
Methanol + U83836E	62.3 ± 3.8	63.1 ± 3.9	64.8 ± 4.0[y]	68.2 ± 4.0[a,x,y]	71.8 ± 4.2[a,x,y]	66.3 ± 4.0[y]	62.9 ± 3.8

Statistically significant differences for p < 0.05: [a] - in comparison with control group, [x] - in comparison with U83836E group, [y] - in comparison with methanol group.

and distribution of microvilluses have been observed but discontinuity in cell membrane has not been observed) 2 days after intoxication (Fig. 5).

Changes in liver cell membranes were confirmed by blood serum examinations. The activity of cathepsin B was gradually increased from 6 h to 5 days after methanol intoxication with the highest enhancement after 2 days (by about 27%) (Fig. 6). The treatment of intoxicated rats with the antioxidant significantly prevented the increase in cathepsin B activity and after U83836E ingestion the activity was only enhanced by about 5%.

Discussion

Methanol intoxication in rats is associated with its rapid metabolism in the liver. These processes are accompanied by free radicals formation [1]. As a consequence the changes in liver cell membrane structure and function have been observed in the present study. Methanol as well as its oxidation products may influence the structure and physicochemical properties of liver cell membranes in various ways.

Methanol may change the protein-lipid structure of the membrane which is supported by water molecules forming two hydrogen bonds with glicolipids and amino groups of receptor proteins [16]. Methanol may take over water function but it can nevertheless form only one hydrogen bond with proteins or with lipids so that it binds membrane proteins and lipids and in this way may disturb the membrane structure. Formaldehyde, however, is a very reactive compound, reacting easily with e-amino groups of lysine, SH groups of cysteine and with methionine, arginine and tyrosine residues of proteins [17]. Formed hydroxymethyl derivatives may further react with nucleopophilic groups and form methylene bridges [17, 18]. This results in changes in the structural and physicochemical properties as well as in the function of the proteins [18].

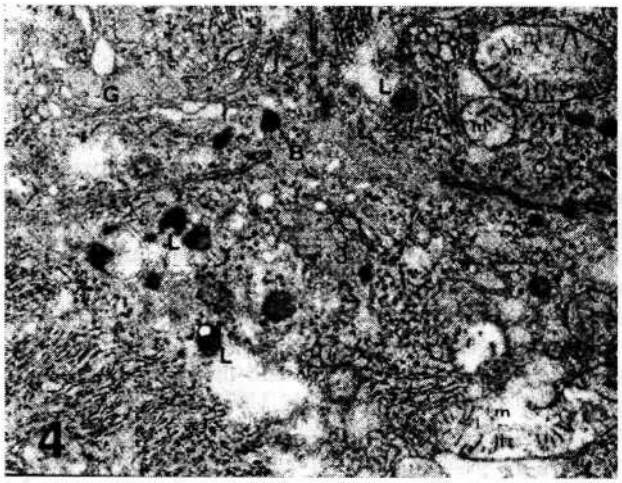

Fig. 4. Part of hepatocytes from liver of rats treated with methanol after 24 h of intoxication. Numerous lysosomes (L) collected near bile canaliculuc (B), mitochondrium (m) damaged, fragments of the Golgii complex (G). TEM, x7000.

Free radicals generation and the following decrease in antioxidant potential of the liver observed in methanol intoxication indicates the possibility of lipid peroxidation [19]. The reactions with lipids result in the formation of small molecular aldehydes measured as malondialdehyde, which has been observed in this work. The time of maximum concentration of lipid peroxidation product corresponds with the time of maximum surface charge density. Both reactive oxygen species and small molecular aldehydes can react with membrane proteins and modify their structure [20]. As a result, surface mem-

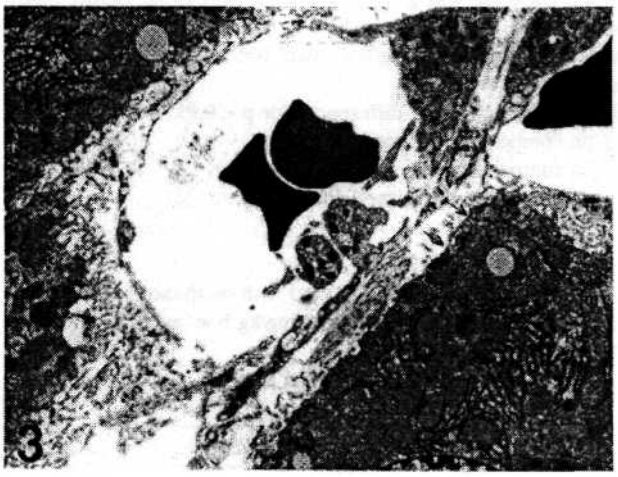

Fig. 3. Microscopic-electronic picture of the vascular pole and the vascular sinus. Smoothing of cell membrane (arrows) mitochondria (m). Hepatocytes of the rat liver after 48 h following methanol intoxication. TEM, x3000.

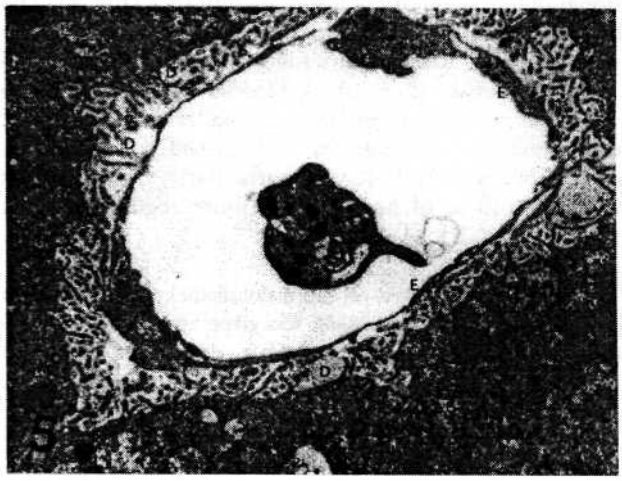

Fig. 5. Microscopic-electronic picture of the vascular pole and the vascular sinus and fragments of neighbouring parenchymatous cells of the rat liver after 2 days following methanol and U83836E administration. The Disse space (D) sinusoidal endothelium (E), mitochondria (m). TEM, x4400.

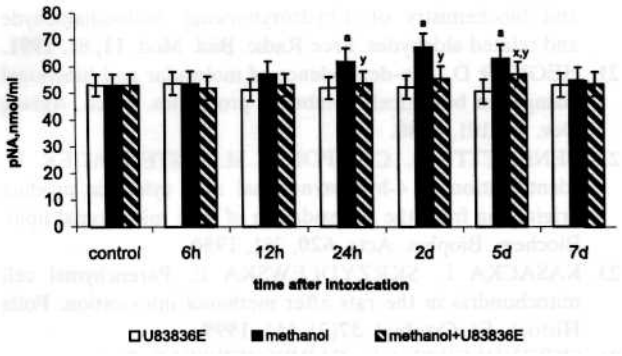

Fig. 6. Effect of U83836E on the catepsin B activity in serum of rats intoxicated with methanol (3.0 g/kg b.w. given intragastrically); U83836E was given intraperitoneally twice: before methanol administration - 10 mg/kg b.w. and 12 hours after methanol administration - 5 mg/kg b.w.; data points represent the means ± SD; n = 6.
Statistically significant differences for $p < 0.05$:
a - in comparison with control group
x - in comparison with U83836E group
y - in comparison with methanol group.

brane hepacyte positive charges of proteins decrease and negative charges occur. Changes in charge could also be caused by impairment in distribution of phosphatidiloserine, which is the component of the skeleton, and which could cause an increase in negative charge density [4].

Changes in lipid and protein structures caused by free radicals and formaldehyde may be also a reason for disturbances in lipid-protein interaction necessary for membrane functions [21]. First of all membrane fluidity is changed and an increase in their permeability is observed [22]. Like plasmolemma, lysosomes are subject to the potential membrane-disrupting effects of free radicals causing increased lysosomal fragility and release of lysosomal hydrolases into the cystosol and, next, into the blood. The ATP synthesis decrease also participates in membrane destabilization during methanol intoxication [23]. Damage of lysosomal membrane structures and leak of lysosomal enzyme (cathepsin B) into cytosol observed in methanol intoxication may lead to uncontrolled extralysosomal proteolysis in the liver cells and to the onset of liver tissue destruction. In the observed progression of effects, blabbing and enhanced autophagocytosis were the earliest signs of cell damage. Increase, observed in ultrastructure evaluation, in the amount of rough endoplasmic repiculum containing autophagosomes, mitochondria fragments and glycogen granules testifies to this. The cell protection mechanism against further destruction is cytoplasmic vacuolization.

It has been reported that antioxidant U-83836E given simultaneously with methanol to rats brought partial restoration of antioxidant enzyme activity, as well as low molecular antioxidants level [24]. In the present study, it has been observed that U83836E also partially prevents biological membrane damage resulting from methanol intoxication. U83836E owes its properties to its reactive trolox ring, belonging to the benzophyran family. It is possible that U-83836E, owing to its lipophilic properties, like α-tocopherol, is intercalated into biological membranes enhancing their stability. Antioxidants that are only water soluble do not act as efficient antioxidants, when reaction is initiated in the lipid phase of bilayers [25]. It could be suggested that vitamin E -derivative diffuses into the bilayer phase sufficiently to meet the polar peroxyl radicals [25]. The aromatized polar region of the trolox molecule has been implicated in the quenching of reactive oxygen species generated during methanol intoxication. In consequence it diminishes peroxidative processes within liver cell membranes and subsequently reduces the generation and accumulation of lipid peroxidation products.

In conclusion, it may be assumed that U83836E decreases the changes in liver cell membrane structures and functions observed after methanol intoxication.

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