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Original Research

Effects of Green Tea on Physico-Chemical Properties of Liver Cell Membranes of Rats Intoxicated with Ethanol

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Abstract

Changes in the composition and physicochemical properties of liver cell membranes appearing as the result of ethanol intoxication and the aging process are mainly due to reactive oxygen species (ROS). The destructive action of free radicals can be neutralized by administering antioxidants, especially natural ones like green tea. For this reason, the purpose of this study was to estimate the efficacy of green tea's influence on the physicochemical and biochemical properties of the rat liver as affected by the aging process and/or chronic ethanol intoxication. Several methods were used to evaluate this effect. Qualitative and quantitative composition of phospholipids in the membrane was determined by High Performance Liquid Chromatography (HPLC). The electrophoresis technique was used to determine the surface charge density of the rat liver cell membrane. The process of aging causes a decrease in the total amount of phospholipids, increases lipid peroxidation products and surface charge density of liver cell membrane. The results also demonstrate that ethanol administration provoked increase phospholipid composition, lipid peroxidation products and surface charge density in liver cell membrane. The ingestion of green tea with ethanol partially prevented these ethanol-induced and/or aging changes. Moreover, long-term drinking of green tea partially prevents changes in structure and function of membrane phospholipids caused by chronic ethanol intoxication.

Keywords: ethanol, green tea, phospholipids, lipid peroxidation products, surface charge density, aging, liver

Introduction

The oxidative stress induced by chronic ethanol consumption, particularly in aging, has been implicated in changes in structure and functions of liver cell components including membrane phospholipids. Food components may cause increases as well as decreases in cellular antioxidative ability. One such food component that influences ROS generation and antioxidant status is alcohol. Ethanol is rapidly absorbed from the gastrointestinal tract and about 90% of it is metabolized in the liver [1]. There ethanol is oxidized into acetaldehyde and next into acetate, and these processes are accompanied by free radicals generation [2]. Ethanol and active products of its metabolism influence the cell components, including biological membranes. An important electric property of a biological membrane is its surface charge and its electrokinetic potential, i.e. the potential drop between the membrane and its environment. Electrical properties of the membrane are the result of acid-

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base and complex formation equilibria between cell membrane components and the environment components [3, 4]. Most membrane components are involved in such equilibria, in particular phospholipids and proteins. The cell membrane charge is an indication of cell "form," e.g. tumour formation is accompanied by increase in membrane surface charge whereas cell necrosis provokes charge decrease [4]. Surface charge density can also be affected by metabolic transformation of xenobiotics in which reactive products are formed. Such is the case with ethanol metabolism.

Ethanol reduces membrane hydration [5], thereby affecting the protein-lipid structure of cell membranes. Acetaldehyde and ROS can react with aminoacids, peptides and proteins modifying their composition and function [6, 7]. ROS can also react with lipids, causing their peroxidation [8]. Free radical peroxidation – especially of unsaturated lipids in biomembranes – disrupts the various important structural and protective functions associated with biomembranes, and various *in vivo* pathological events are implicated as a result of this oxidation [9]. Free radical peroxidation also disorders lipid membrane asymmetry, permeability and integrality. The aging process is accompanied by increasing amount of reactive oxygen species and by changed antioxidative abilities of cells, resulting in perturbed redox homeostasis [10, 11].

The deleterious consequences of membrane peroxidation have stimulated studies on the efficacies and mechanisms of actions of biologically relevant antioxidants such as phenolic compounds. One of their most important natural sources is green tea [12].

The aim of this work has been to estimate the biochemical and electric properties of aged rats liver cells after chronic ethanol intoxication. In addition, the efficacy of green tea in terms of its protective action was investigated.

Materials and Methods

Green Tea

Green tea – Camellia sinensis (Linnaeus) O. Kuntze (standard research blends – lyophilized extract) was provided by TJ Lipton (Englewood Cliffs, NJ). Green tea extract contained epigalloctechin gallate (97mg/g dried extract), epigallocatechin (82mg/g dried extract), epicatechin (90mg/l), epicatechin gallate (15mg/g dried extrac) and coffeic acid (10mg/g dried extract), determined by HPLC [13].

Animals

Two- (200-220g b.w.), 12- (520-550g b.w.) and 24-month-old (750-780g b.w.) male Wistar rats were used for all experiments. Rats were fed the liquid diet for 5 weeks before death. Dietary intake was comparable in all groups, with all rats demonstrating consistent weight gain throughout the 5-week feeding period. Ethanol-fed rats had slightly decreased rates of weight gain (about 10% less weight gain compared with control-fed rats), consistent with the well-studied effects of isocaloric ethanol feeding on intermediate metabolism. All experiments were approved by the Local Ethics Committee in Białystok, Poland, referring to the Polish Act for Protecting Animals of 1997. Rats were housed in individual cages and pair-fed with either nutritionally control-adequate liquid Lieber DeCarli diet containing 47% of total energy as carbohydrate, protein 18%, lipid 35% or the identical diet with ethanol substituted isocalorically for carbohydrate (36% of total energy) [14]. Liquid diet (control and ethanol) containing 7g green tea extract/l diet was also prepared.

The animals from each age group were divided into the following groups:

The control group was fed for 5 weeks on a control Lieber DeCarli liquid diet (n=6).

The green tea group was fed for 5 weeks on a control liquid Lieber DeCarli diet containing green tea (7g/l) (n=6).

The ethanol group was fed for one week on a control liquid Lieber DeCarli diet and for the next 4 weeks on ethanol Lieber DeCarli liquid diet (n=6).

Ethanol and green tea group were fed for one week on a control Lieber DeCarli liquid diet containing green tea (7g/l) and next for 4 weeks with ethanol Lieber DeCarli liquid diet also containing green tea (7g/l) (n=6).

After 5 weeks of the experiment all rats were sacrified under ether anaesthesia (six animals in each group). Animals were put to sleep 10 minutes before the beginning of the experiment and this anaesthesia was sufficient until the end of experiments, since we did not observe any pain reflexes elicited by paw-pinch.

Isolation and Analysis of Phospholipids by HPLC Method

The tissues (about 1.5g) were homogenized in 1mM-$NaHCO_3$ (pH=7.6)- 0.5 M $CaCl_2$ in a loose – fitting Dounce homogenizer. Membrane fragments were separated from nuclei and mitochondria by rate-zonal centrifugation of the 'low-speed' pellet as described by Evans (1970) [15]. The sediment was washed and partially separated in two following centrifugationing at 1000xg. The sediment was homogenated in saccharose of 1.22 density and in the next step was covered with saccharose of 1.16 density. The cell membranes were separated by centrifuge at 2000xg for 25-35 minutes.

Then a method of Folch was applied to extraction of phospholipids [16]. The cell membrane was homogenized in a chloroform-methanol mixture of 2:1 volume ratio using 20 cm^3 per g of tissue. The solution was then filtered out with degreased paper filters, the precipitate was washed with the extracting solution containing 0.05 M calcium chloride; the volume ratio of chloroform, methanol and aqueous calcium chloride solution was 8:4:3. The suspensions were centrifuged at 500xg for 2 minutes, the

organic and the aqueous phases were separated, 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 were dissolved in 200 µl of chloroform-methanol mixture (3:2) [16].

HPLC analysis was then carried out. The Merck HPLC system was equipped with a pump (Model L-6200A), an ultra violet (UV) detector (Model L-4500), analog interface module D-6000A, and System Manager software. Normal phase high performance liquid chromatography (NP-HPLC) was performed by injecting phospholipid preparations (resuspended in chloroform-methanol) on to a silica column (Superspher Si 60, 100 A, 250x4.0 mm, 5µm; E. Merck Darmstadt) and eluting isocratically with a mobile solvent of acetonitryle/methanol/85% phosphoric acid (130:5:1.5; v/v/v) at a flow rate of 1 ml/min. UV absorbance was detected with a diode array detector (L-4500, Hitachi, Merck) scanning from 200 to 350 nm at 214 nm resolution. The time required for each HPLC analysis was about 30 min. Under the chromatographic conditions tested, a linear relationship was verified in the range 50-250 µg/ml for phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylethanolamine (PE), and phosphatidylcholine (PC) of standard solutions, by analysis of variance of the regression (r^2). For all these compounds, the r^2 values were >0.999.

Electrochemical Methods

Tissue was disintegrated into 2-3 mm pieces and was washed in a physiological salt solution and then placed in 1% trypsin solution in the proportion 10 cm^3 solution per 1g solution. The tissue was again placed in 1% trypsin solution at 37°C for 20 minutes. The operation was repeated, extending the action time to 40 minutes. Several drops of calf serum were added to the resulting supernatant liquid to neutralize trypsin. The suspension was centrifuged at 1300 × g for 15 minutes.

In order to determine surface charge density of the cell membrane, liver cells were put into the measuring vessel, then electrophoretic mobility was measured using a DTS5300 Zetasizer 3000 apparatus from Malvern Instruments.

The surface charge density has been determined using equation: $\sigma=\eta u/d$; where u – electrophoretic mobility, η – viscosity of solution, d- diffuse layer thickness [19]. The diffuse layer thickness was determined from the formula [20]:

$$d = \sqrt{\frac{\varepsilon \cdot \varepsilon_0 \cdot R \cdot T}{2 \cdot F^2 \cdot I}}$$

where R is the gas constant, T is the temperature, F is the Faraday number and I is the ionic strength of 0.9% NaCl, $\varepsilon\varepsilon_o$ – permeability electric medium.

On the basis of surface charge density as a function of pH, acidic (C_{TA}) and basic (C_{TB}) functional group concentrations were evaluated in the given studies [21].

Results

Typical separation of four phospholipid classes (PI-phosphatidylinositol, PS-phosphatidylserine, PE-phosphatidylethanolamine, and PC-phosphatidylcholine) in liver cell membranes is presented in Fig.1. PI is eluted first and it is followed by PS, PE, and PC. Amounts of separated phospholipids per unit surface area were determined from peak area.

Fig. 2 shows changes in content of phospholipids in liver cell membrane from control rats (2-, 12-and 24-months) and animals that received ethanol or ethanol and green tea. Ethanol intoxication caused an increase in phospholipid content in the liver cell membrane, in comparison with control group. The content individual phospholipids PI, PS, PE and PC increased by about 80%, 35%, 50%, and 65%, respectively, for rats aged 2 months, by about 90%, 60%, 60%, and 70%, respectively, for rats aged 12 months, and by about 100%, 40%, 75%, and 70%, respectively, for rats aged 24 months. Administration of alcohol to green tea rats causes less increase in the cited parameters characterizing PI, PS, PE, and PC than administration of ethanol only. The lowest increase in the amount of these phospholipids for the rats aged 24 months, by about 15%, 15%, 15%, and 20%, was observed compared with the ethanol group.

With the control rats, the content of PI, PS, PE, and PC decreased with age. The most decrease for the rats aged 12 and 24 months compared with those aged 2 months was observed for PI by 30% and 80%, respectively, and for PC by 30% and 50%, respectively. In the case of rats aged 2, 12 and 24 months treated with green tea and the control group no essential differences were observed in the content of individual phospholipids in liver cell membrane.

Table 1 shows changes in acidic (C_{TA}) and basic (C_{TB}) functional group concentrations of liver cell membrane from different age control rats (2-, 12- and 24-months)

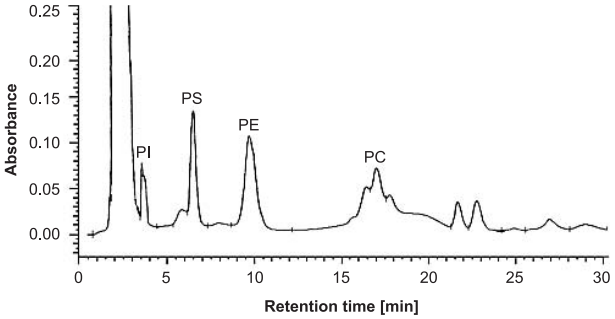

Fig.1. Typical separation of four phospholipid classes (PI-phosphatidylinositol, PS-phosphatidylserine, PE-phosphatidylethanolamine, PC-phosphatidylcholine) in liver cell membranes.

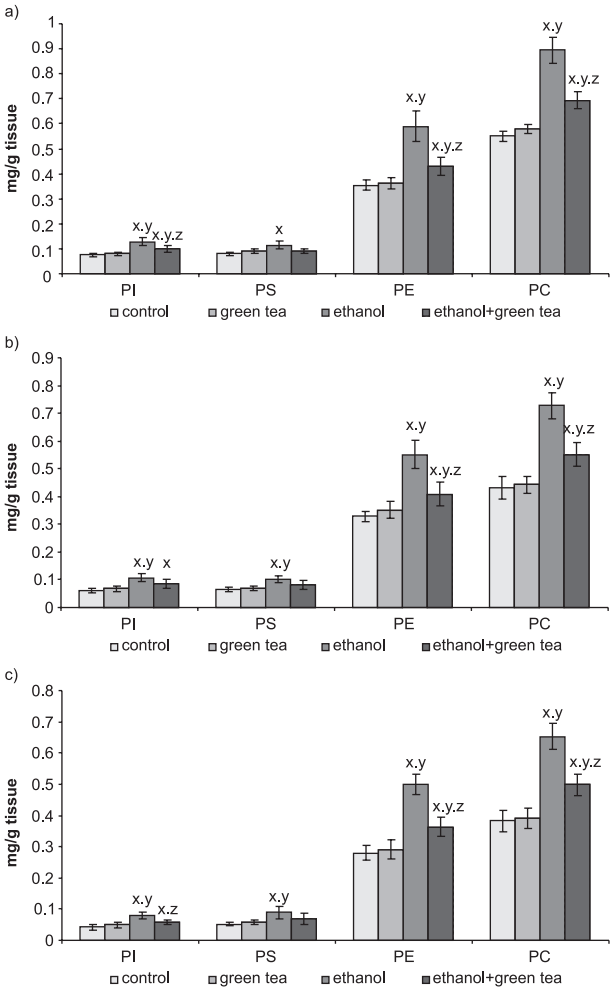

Fig. 2. Effect of green tea on content of four phospholipid classes in liver cell membranes of rats aged 2 (a), 12 (b) and 24 (c) months) receiving ethanol and/or green tea. Statistically significant differences for $p<0.05$ with respect to: x – control group; y – animals receiving green tea; z – animals receiving ethanol.

and animals that received ethanol or ethanol and green tea. The value C_{TA} of the control rats aged 12 and 24 months increases by about 12% and 24%, respectively, with respect to the rats aged 2 months. On the other hand, the positive charge value of liver cell membrane of rats aged 12 months decrease by about 17% when compared with 2-month-old ones and that of 12-month-olds increases by about 25%. No essential differences in surface charge density were observed in the case of rats of any age group watered with green tea and of the control groups. Ethanol intoxication of the rats aged 2, 12, and 24 months caused increase in the value C_{TA} and in the value C_{TB} with respect to the control group animals: the acidic functional group concentration increased by 35%, 34%, and 44%, respectively, and the basic functional group concentration increased by 46%, 77%, and 49%, respectively. Administering alcohol to the green tea drinking animals provoked lower increase both in the value C_{TA} of the liver cell membrane and in the value C_{TB} with respect to the animals which received ethanol only.

Discussion

Ethanol intoxication in rats is associated with its rapid metabolism in the liver. These processes are accompanied by ROS formation [22]. As a consequence the changes in liver cell membrane structure and function have been observed in the present study. Ethanol as well as its oxidation products may influence the structure and physicochemical properties of liver cell membranes. The results presented in this work demonstrate that ethanol intoxication brings about an increase in the amount of all phospholipids in liver cell membrane. It has been supported by the literature data [23, 24]. It is known that ethanol influences the activity of enzymes participating in phospholipids, particularly in PE and PC metabolism. Ethanol enhances the activity of acyltransferase that participates in phospholipid biosynthesis and decreases activity of phospholipase A_2 that initiates phospholipid catabolism [25, 26]. Moreover, ethanol may also increase the level of diacylglicerol that participates in phospholipid resynthesis [27]. In consequence, ethanol intoxication may be conducive to an increase in phospholipid level. The increase in amount of phospholipids provokes an increase in functional groups, both positively and negatively charged, to appear at the membrane surface. In the acid medium (low pH), the phospholipid charge mainly originates from

Table 1. Effect of green tea on the values C_{TA}, C_{TB} in liver cell membranes of rats aged 2, 12 and 24 months receiving ethanol and/or green tea. Statistically significant differences for $p<0.05$ with respect to: x, control group; y, animals receiving green tea; z, animals receiving ethanol.

Parameter	Age of rats	Group of rats			
		Control	Green tea	Alcohol	Green tea + alcohol
C_{TA} [10^{-7} mol/m^2]	2-months	2.07 ± 0.10	2.05 ± 0.12	2.80 ± 0.15^{xy}	2.36 ± 0.08^{xyz}
	12-months	2.32 ± 0.12	2.31 ± 0.11	3.12 ± 0.11^{xy}	2.58 ± 0.07^{xyz}
	24-months	2.57 ± 0.11	2.56 ± 0.08	3.71 ± 0.16^{xy}	2.99 ± 0.12^{xyz}
C_{TB} [10^{-7} mol/m^2]	2-months	0.63 ± 0.03	0.54 ± 0.06	0.92 ± 0.05^{xy}	0.74 ± 0.05^{xyz}
	12-months	0.56 ± 0.03	0.53 ± 0.04	0.99 ± 0.06^{xy}	0.91 ± 0.06^{xyz}
	24-months	0.79 ± 0.04	0.74 ± 0.08	1.18 ± 0.05^{xy}	1.04 ± 0.04^{xyz}

amino groups, whereas in basic medium (high pH) it is mainly due to carboxyl groups. It results from Fig. 2 that ethanol provokes a higher increase in phosphatidylinositol and phosphatidylcholine content in liver cell membrane of rats of various age groups than of other phospholipids. The amount of phosphatidylcholine is greatest in the outer liver membrane layer and this phenomenon can provoke a increase, both in C_{TA} value and C_{TB} value.

The ethanol molecules can penetrate into the lipid bilayer and can perturb lateral mobility of membrane lipids or they can react with hydrophobic fragments of proteins causing steric transformations [28, 29]. Changes in protein structure can markedly perturb protein-lipid interactions. In addition, penetration of ethanol into the lipid bilayer destroys their hydrogen bonding by assuming the role of water and by reducing membrane surface hydration [5]. However, its molecule can form only one hydrogen bond in which its hydrogen atom is involved, either with a protein or with a lipid. A functional group is then liberated and may provoke an increase or decrease in the cell membrane electric charge. Consequently, surface concentration of basic and/or acidic groups on the membrane can increase. Interaction of alcohol with the outer membrane surface and penetration of its molecules between phospholipid molecule heads can result in increased fluidity of the membrane [23]. In turn, acetaldehyde being very reactive can modify amino acid residues of membrane proteins by reacting with free amino and sulfhydryl groups. These reactions can be a reason for lower positive charges of proteins [30, 31].

The aging process and/or alcohol metabolism is accompanied by increasing amounts of reactive oxygen species (ROS) [32, 33]. The process of aging is characterized by changes in cell metabolism resulting in modification of the structure and function of cell membrane components. The process of aging was accompanied by a change in the total amount of phospholipids, in chain length and in unsaturation degree of their constituent fatty acids, and in location of phospholipids in the lipid bilayer [34, 35]. The results of this work confirm that the content of individual phospholipids in liver cell membranes decrease during the aging process. Such changes have also been supported in the literature [35]. The constituents of membrane phospholipids are polyunsaturated fatty acids that are particularly vulnerable to the peroxidatible action of ROS and their degree of unsaturation increases with age [36]. The enhanced level of lipid peroxidation products was observed in this study during aging as well as after ethanol intoxication [37]. Lipid peroxidation products reduce hydrophobicity of the lipid cell membrane interior and modify the bilayer organization, thereby modifying physical properties of cell membranes and influencing membrane fluidity [38, 39]. The above processes perturb the lipid asymmetry of the membrane by exposing phosphatidylserine to the outer membrane surface [40]. Physiologically it is present in the inner, cytoplasmatic half of the membrane lipid bilayer. It implies the appearance of an additional negatively charged group resulting in increased surface concentration of acidic groups of the membrane. It is supported by the results presented in Table 1. A decrease in the content of phospholipids caused by aging is accompanied by decreases in the amount of functional groups, both concentration of basic groups and concentration of acidic groups. As observed in this work (Table 1), the value C_{TB} of liver cell membrane is lower for rats aged 12 months than that for the rats aged 2 month and it can be related to lower content of phospholipids.

The ultimate consequence of oxidative modification of proteins can be their aggregation or fragmentation [41]. Protein fragmentation creates increasing functional groups, both acidic and basic. The process can also expose membrane phospholipid functional groups hitherto screened by proteins. The changes can yield higher C_{TA} value while lowering C_{TB} value, as has been confirmed by this work.

Changes in the composition and physicochemical properties of liver cell membrane appearing as the result of ethanol intoxication and the aging process are mainly due to ROS. Therefore, antioxidant preparations can be used to prevent the effects of ROS interaction with the liver cell membrane components. A natural preparation of such action is green tea. It contains large amounts of polyphenols that are strong antioxidants. Tea polyphenols comprise mainly catechins and catechin derivatives, which are absorbed, metabolized and distributed into all organs [42]. Catechins are considered to exert protective effects against oxidative stress [43]. Protective action of green tea is connected with the ability of its catechins to prevent oxygen radical formation and scavenge free radicals such as hydroxyl, peroxyl and lipid radicals and superoxide anions [42-44]. Scavenging the most active hydroxyl radical, which may initiate lipid peroxidation, protects membrane phospholipids, which has been shown in this paper. Additionally, catechins may chelate metal ions, especially iron and copper, which in turn preclude the generation of hydroxyl radicals and degradation of lipid hydroperoxides to reactive aldehydes [42]. Some changes in liver cell physico-chemical properties, which such as membrane composition may also be provoked by disturbances in cellular metabolism which may be independent from ROS. It has been shown that green tea does not effectively protect phospholipid levels against ethanol action. It may be connected with the fact that green tea administration influences the activity of enzymes participating in phospholipid metabolism, resulting in decrease in phospholipase A_2 activity [45, 46] but not changes in acyltransferase activity [47].

Green tea catechins decrease the lipid peroxidation when membrane phospholipids are exposed to oxygen radicals from the aqueous phase [48]. The oxidative attack from the aqueous phase seems to be an important reaction for initiating membrane lipid peroxidation. Perhydroxyl radicals, which are produced by the promotion of superoxide and whose concentration is enhanced during alcohol intoxication, are regarded as the most feasible radicals for initiating lipid peroxidation in vivo. Conse-

quently the slower pace of free radical reactions leads to inhibition of lipid peroxidation and in consequence to a decrease in membrane fluidity [48]. Administration of ethanol together with green tea to the rats partly reduces the effect of ethanol as it is indicated by a markedly lower increase in phospholipid content and their peroxidation and in consequence lower surface charge density.

In conclusion, green tea protects liver lipid against ethanol-induced oxidative modification and, consequently, prevents changes in their biological functions. Considering that the metabolism of ethanol and tea flavonoids is the same in rats as in humans, the results obtained here suggest that green tea may also protect human liver cells against the consequences of oxidative stress caused, for example, by ethanol intoxication. Evidence for the bioactivity of the phenolic compounds of green tea in vivo supports the notion that their antioxidant properties contribute to important role in the health protection and disease prevention.

Abbreviations

HPLC – High Performance Liquid Chromatography;
ROS – reactive oxygen species;
NP-HPLC – Normal Phase High Performance Liquid Chromatography;
PI – phosphatidylinositol,
PS – phosphatidylserine,
PE – phosphatidylethanolamine,
PC – phosphatidylcholine

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