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Resveratrol alters the lipid composition, metabolism and peroxide level in senescent rat hepatocytes

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ABSTRACT

Investigations were performed on the influence of resveratrol on the lipid composition, metabolism, fatty acid and peroxide level in plasma membranes of hepatocytes, isolated from aged rats. Hepatocytes were chosen due to the central role of the liver in lipid metabolism and homeostasis. The obtained results showed that the level of sphingomyelin (SM) and phosphatidylserine (PS) was augmented in plasma membranes of resveratrol-treated senescent hepatocytes. The saturated/unsaturated fatty acids ratio of the two most abundant membrane phospholipids, phosphatidylcholine (PC) and phosphatidylethanolamine (PE), was decreased as a result of resveratrol treatment. The neutral sphingomyelinase was found to be responsible for the increase of SM and the decrease of ceramide in plasma membranes of resveratrol-treated senescent hepatocytes. Using labeled acetate as a precursor of lipid synthesis we demonstrated, that resveratrol treatment resulted in inhibition mainly of phospholipid synthesis, followed by fatty acids synthesis. Resveratrol induced reduction of specific membrane-associated markers of apoptosis such as localization of PS in the external plasma membrane monolayer and ceramide level. Finally, the content of lipid peroxides was investigated, because the unsaturated fatty acids, which were augmented as a result of resveratrol treatment, are an excellent target of oxidative attack. The results showed that the lipid peroxide level was significantly lower, ROS were slightly reduced and GSH was almost unchanged in resveratrol-treated hepatocytes. We suggest, that one possible biochemical mechanism, underlying the reported resveratrol-induced changes, is the partial inactivation of neutral sphingomyelinase, leading to increase of SM, the latter acting as a native membrane antioxidant.

In conclusion, our studies indicate that resveratrol treatment induces beneficial alterations in the phospholipid and fatty acid composition, as well as in the ceramide and peroxide content in plasma membranes of senescent hepatocytes. Thus, the presented results imply that resveratrol could improve the functional activity of the membrane lipids in the aged liver by influencing specific membrane parameters, associated with the aging process.

1. Introduction

Oxidative damage of cellular components has been postulated to be among the factors underlying the genesis of a wide range of patho-physiological events, which accompany the aging processes [1]. Thus, the ability of cells to resist or prevent oxidative attack could possibly slow down the development of age-related changes, and so the therapies aimed at reduction of oxidative stress could as well induce anti-aging effects [2].

One of the widely studied antioxidants, which has also been associated with anti-aging effects, is the naturally occurring phytoalexin resveratrol (3,4′,5′-trihydroxystilbene) [3]. It can be found in different plants, mainly grapes, peanuts, berries, as well as in many types of red wines [4]. Resveratrol has been reported to exhibit various beneficial effects such as antioxidant, anti-inflammatory and anti-aging, among others. [4,5]. In addition, there is evidence that resveratrol has lipid-lowering effect on serum and liver lipids and also inhibits hyperlipidemia and atherosclerosis in diabetic LDL receptor-deficient mice [6].

The aim of the present study was to investigate the effect of resveratrol treatment of hepatocytes isolated form aged rats on plasma membrane lipid composition, metabolism, fatty acids and

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oxidative status. Liver cells were chosen due to the central role of the liver in lipid metabolism and lipid homeostasis. Isolated hepatocytes represent a convenient model for investigation of liver functions, which is very close to *in vivo* conditions. Studies were performed on the ability of resveratrol to reverse some parameters of the membrane lipids, which change in the process of aging, such as phospholipid and fatty acid composition, lipid synthesis, sphingomyelin metabolism, accumulation of sphingolipid metabolites, cholesterol level, phospholipid asymmetry etc.

The obtained results showed that resveratrol treatment induced beneficial alterations of the membrane lipids and peroxide content in plasma membranes of hepatocytes isolated from old rats, thus implying that this lipophilic antioxidant could partially improve the functional activity of the membrane lipids in the aged liver.

2. Materials and methods

2.1. Animals

Male Wistar rats (purchased from the Department for Laboratory Animals, Bulgarian Academy of Sciences) were kept for 20 months in laboratory conditions (in a ventilated room at ambient temperature 22 ± 2 °C) and had free access to food and water. All experiments with animals were performed in strict accordance with the national and institutional rules for use of animals for experimental purposes. The performed experiments with animals have been approved by the Ethical commission of Bulgarian Academy of Sciences.

2.2. Reagents

Trans-resveratrol (more than 99% pure) was purchased from Sigma–Aldrich. C_6-NBD-Cer{6-[N-(7-nitro-2,1,3-benzoxadiazol-4-yl) amino] hexanoylceramide}, C_6NBD-SM {6-[N-(7-nitro-2,1,3 benzoxadiazol-4-yl)amino] hexanoylsphingosyl phosphocholine}, $C_{17:0}$ ceramide (N-heptadecanoyl – D-sphingosine), palmitoyl-(NBD-hexanoyl)-phosphatidylserine (NBD-PS) and *cis*-parinaric acid were obtained from Avanti Polar Lipids. [1-^{14}C] acetate (58.9 mCi/mmol) was from Amersham Int.

2.3. Isolation of hepatocytes from old rats (senescent hepatocytes) and incubation with resveratrol

Hepatocytes were isolated by liver perfusion with collagenase [7]. The hepatocytes thus obtained were suspended in Krebs–Henseleit buffer, pH 7.4, supplemented with 10 mM glucose and 1% (w/v) defatted bovine serum albumin [8]. The viability of the isolated cells was monitored by the trypan blue test. In our experiments we used preparations, which exhibited at least 90% viability. After isolation, the hepatocytes were incubated in conditioned shaker under 95% air and 5% CO_2 in Erlenmeyer flasks, containing 10 mg of cellular protein per ml for 1 h in the presence or absence of resveratrol (50 μM). This concentration of resveratrol was chosen because the measured alterations of sphingomyelin content were linear up to 70 μM. Resveratrol was delivered from a stock solution in dimethyl sulfoxide. Control cells were incubated only with dimethyl sulfoxide.

2.4. Cell viability assay after incubation with resveratrol and [1-^{14}C] acetate incorporation

After incubation with resveratrol, cell viability was determined by tetrazolum salt measurement (MTT assay), involving assessment of succinate dehydrogenase-induced conversion of (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolum bromide into formazan crystals [9]. Formation of formazan was measured at 570 nm. The viability of the cells after incubations was estimated as percentage of the absorbance of resveratrol-treated cells compared to controls. In the experiments involving incubation with labeled acetate the incubation medium contained 10 mM glucose, 1% defatted albumin and labeled acetate at a final concentration of 20 μM in accordance with the procedure described by Gnoni and Paglialonga [10]. The reactions were stopped with 10 N NaOH after 1 h of incubation and the hepatocytes were used immediately for isolation of plasma membranes or for analysis of reactive oxygen species (ROS) and glutathione (GSH) as explained below.

2.5. Isolation of liver plasma membranes

Plasma membranes from hepatocytes were isolated according to the procedure described by Pankov et al. [11] with modifications, involving differential centrifugation. Briefly, the post-nuclear supernatant was loaded on a discontinuous sucrose gradient and centrifuged at 100,000*g* for 2.5 h. The plasma membrane fraction was obtained at a density of 8% (w/v), suspended in ice-cold 10 mM Tris buffer, pH 7.4 and used immediately for lipid analysis.

2.6. Lipid extraction and analysis

Lipid extraction was performed with chloroform/methanol according to the method of Bligh and Dyer [12]. The organic phase obtained after extraction was concentrated and analyzed by thin layer chromatography. The phospholipid fractions were separated on silica gel G 60 plates in a solvent system containing chloroform/methanol/2-propanol/triethylamine/0.25% KCl (30:9:25:18:6 v/v) [13]. The location of the separate fractions was determined by spraying the plates with 2′,7′-dichlorofluorescein. The spots were scraped and quantified by determination of the inorganic phosphorus [14]. Neutral lipids were analyzed by thin-layer chromatography in a solvent system containing hexan:dietyl ether:acetic acid (90:30:1v/v).

The incorporation of labeled acetate into the separate lipid fractions was assayed by measuring the radioactivity of the spots which were scraped and eluted.

Cholesterol content was assayed by gas chromatography using a medium polarity RTX-65 capillary column (0.32 mm internal diameter, length 30 m, thickness 0.25 μm). Calibration was achieved by a weighted standard of cholestane.

2.7. Fatty acid and ceramide analysis

The phospholipid extracts were saponified with 0.5 N methanolic KOH and methylated with boron trifluoride-methanol complex (Merck) [15]. The fatty acid methyl esters were extracted with hexane and separated by gas chromatography on a capillary column coated with Supelcowax 10-bound phase 9 (i.d. 0.32 mm, length 30 m, film thickness 0.25 μm; (Supelco, Bellafonte, PA) fitted in a Perichrom gas chromatograph. Quantification was referred to an internal standard of heptadecanoic methyl ester. The level of ceramide was determined by the fatty acid content in its molecules after separation from the total phospholipids in developing system containing diethyl ether:methanol (99:1 v/v).

2.8. Sphingomyelinase activity assay

Sphingomyelinase activity was determined by the method of Nikolova-Karakashian et al. [16] with minor modifications. Briefly, aliquots of the cell suspensions were lysed in 0.2% Triton X-100 in 100 mM Tris pH 7.4 buffer supplemented with 25 μM genestein for 10 min on ice. The samples were homogenized with three passes