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.
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. C6-NBD-Cer[6-[N-(7-nitro-2,1,3-benzoxadiazol-4-yl) amino] hexanoylceramide], C6-NBD-SM [6-[N-(7-nitro-2,1,3 benzoxadiazol-4-yl)amino] hexanoylsphingosyl phosphocholine], C17,0 ceramide (N-heptadecanoyl – D-sphingosine), palmitoyl-(NBD-hexanoyl)-phosphatidylserine (NBD-PS) and cis-parinaric acid were obtained from Avanti Polar Lipids. [1-14C] acetic acid (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% CO2 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 [1-14C] acetate incorporation

After incubation with resveratrol, cell viability was determined by tetrazolium salt measurement (MTT assay), involving assessment of succinate dehydrogenase-induced conversion of (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium 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:diethyl ether:acetetic 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 genistein for 10 min on ice. The samples were homogenized with three passes.
through a 25-gauge needle and 10 μl aliquots were taken for protein assay. NBD-sphingomyelin was added to the lysates to a final concentration of 20 μM and incubations were performed for 10 min at 4 °C. Aliquots of this mixture containing 0.1 mg protein and 3 μM substrate were added to 5 mM MgCl₂, 10 mM Tris pH 7.4 to a final volume of 0.3 ml. All buffers contained 0.2% Triton X-100. After incubation for 1 h at 37 °C the reaction was stopped by addition of 2 ml chloroform-methanol 2:1 (v/v). The samples were evaporated and separated in a solvent system containing diethyl ether:methanol (99:1v/v) and the spots corresponding to ceramide were scraped and eluted. After addition of hexane the fluorescence of the samples was measured at 455 nm (excitation) and 530 nm (emission).

2.9. Sphingomyelin synthase assay

Sphingomyelin synthase was determined by the procedure described by Tefesse et al. [17] with modifications. The incubation mixture contained 20 mM Tris pH 7.4, 0.3 mg membrane protein, 50 μg NBD-ceramide and 5 μg PC to final volume of 500 μl. After incubation for 2 h at 37 °C the reaction was stopped with 1 ml chloroform-methanol 2:1 (v/v). The lipids were separated in a system containing diethyl ether:methanol (99:1v/v) and the spots corresponding to sphingomyelin was scraped and eluted. Hexane was added to the samples and fluorescence was measured at 455 nm (excitation) and 530 nm (emission).

2.10. Ceramidase assay

Ceramidase activity was determined by the method of Nikolova-Karakashian et al. [16] with modifications. Briefly, the cells were removed from the dishes and centrifuged at 300 g for 5 min. They were lysed in 0.2% Triton X-100 in 100 mM Tris pH 7.4 buffer supplemented with 25 μM sodium vanadate for 10 min on ice. The lysed cells were homogenized with three passes through a 25-gauge needle and 10 μl aliquots were taken for protein assay. NBD-ceramide was added to the lysates to a final concentration 20 μM and incubations were performed for 10 min at 4 °C. Aliquots of this mixture containing 0.1 mg protein and 3 μM substrate were added to 0.5 M acetyl buffer pH 4.5 to a final volume of 0.3 ml. All buffers contained 0.2% Triton X-100. After incubation for 1 h at 37 °C the reaction was stopped by addition of 5 ml hexane and 4 ml 10% citric acid. The samples were evaporated, dissolved in hexane and NBD-labeled fatty acid was quantified after addition of hexane and determination of the fluorescence intensity (excitation 455 nm and emission 530 nm).

2.11. Measurement of reactive oxygen species (ROS)

The generation of ROS was assessed by spectrofluorimetric analysis, using 2′,7′-dichlorodihydrofluorescin diacetate, a non-polar compound which reacts with ROS to produce the highly fluorescent dihydrofluorescein. Control and resveratrol-treated hepatocytes were incubated with 10 μM dichlorodihydrofluorescein at 37 °C for 30 min. The cells were washed 3 times with warm PBS to remove the unincorporated dye, placed in 2 ml PBS and fluorescence was measured at 485 nm (excitation beam) and 525 nm (emission beam) [18]. The level of ROS is presented as measured fluorescence intensity per mg protein.

2.12. Measurement of the reduced glutathione (GSH)

The content of GSH was determined according to Ellman [19] using cell lysate obtained from control and resveratrol-treated hepatocytes in 0.25 M Tris, 20 mM EDTA, pH 8.2). The obtained values are expressed as nmol GSH per mg protein.

2.13. Determination of lipid peroxidation

Lipid peroxidation was determined by the procedure described by Kuypers et al. [20] and Carini et al. [21]. The lipid peroxidation was assessed by measuring the loss of fluorescence of cis-parinaric acid (PNA) (Molecular Probes, Invitrogen, UK). The hepatocytes plasma membranes were incubated with 10 μM PNA at 37 °C for 30 min in the dark. The incubation buffer was immediately removed and the plasma membranes suspensions were washed 3 times with warm PBS to remove the unincorporated dye. The membranes were transferred to fluorescence cuvettes in 2 ml 10 mM Tris pH 7.4. The emission fluorescence of wavelength 455 nm (slit width 5 nm) was measured using an excitation wavelength of 312 nm (slit width 5 nm). A blank sample containing unlabeled cells was also used and the measured values of the labeled cells were accordingly corrected by subtraction. The level of lipid peroxidation was calculated as fluorescence intensity per mg membrane protein.


The internalization of the lipid fluorescent analogs was assessed by back exchange to serum albumin as described by Pomorski et al. [22] with modifications. Exchangeable fluorescence lipids residing in the outer membrane leaflet can be removed, allowing quantitative determination of the lipid internalized into the inner monolayer. In short, cells were incubated with fatty acid-free BSA on ice for 10 min, followed by washing with HBS and centrifugation at 12,000g. The pellets were solubilized in 2% Triton X-100 and the amount of internalized lipid was determined by comparing the fluorescent intensity before and after back exchange to albumin.

2.15. Protein determination

The content of protein was determined according to Bradford [23].

2.16. Statistical analysis

Statistical processing of the data was made by one-way analysis of variance (ANOVA), using InStat software.

3. Results

The influence of resveratrol on the lipid composition of plasma membranes, isolated from hepatocytes of aged rats (referred to as senescent hepatocytes or aged hepatocytes), is presented in Table 1. The mol% of sphingomyelin (SM) and phosphatidylserine (PS) was elevated by 39% and 21% respectively as a result of resveratrol treatment. Phosphatidylcholine (PC) was decreased by about 12%,

<table>
<thead>
<tr>
<th>Lipids</th>
<th>Control</th>
<th>Resveratrol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sphingomyelin</td>
<td>8.1</td>
<td>11.4</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>37.4</td>
<td>33.1</td>
</tr>
<tr>
<td>Phosphatidylserine</td>
<td>8.2</td>
<td>10.1*</td>
</tr>
<tr>
<td>Phosphatidylinositol</td>
<td>10.6</td>
<td>9.9</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>25.3</td>
<td>25.8</td>
</tr>
<tr>
<td>Phosphatidylglycerol</td>
<td>10.4</td>
<td>9.7</td>
</tr>
<tr>
<td>Cholesterol/phospholipids</td>
<td>0.345</td>
<td>0.359</td>
</tr>
</tbody>
</table>

Results are means of three separate experiments. *P < 0.001.
whereas the changes in the rest of the membrane lipids were insignificant. The cholesterol/total phospholipids (CH/TPL) molar ratio remained almost unchanged in plasma membranes of resveratrol-treated aged hepatocytes (Table 1).

Since SM was affected most significantly by resveratrol treatment, we analyzed the possible reasons for its increase by measuring the activities of two enzymes involved in SM metabolism – neutral sphingomyelinase (nSMase) and SM synthase (Fig. 1). The results showed that the activity of nSMase was markedly lower in plasma membranes from resveratrol-treated hepatocytes, whereas the activity of SM synthase was insignificantly augmented, thus suggesting that the decreased SMase activity was likely responsible for the elevated SM content after resveratrol treatment.

In addition, the level of ceramides was altered from 236 to 174 nmol/mol phospholipid in control and resveratrol-treated cells respectively. This change was also attributed to the inhibition of nSMase activity since, as evident from Fig. 1, the activity of the membrane bound ceramidase, which catalyses the conversion of ceramide to sphingosine remained unchanged after resveratrol treatment.

Besides SM, the other plasma membrane lipid which was increased as a result of resveratrol treatment was PS (Table 1). This aminophospholipid is important in physiological aspect, because its appearance in the outer membrane monolayer is associated with apoptosis initiation [24] and aging is known to alter the asymmetrical distribution of membrane phospholipids [25]. Since under normal conditions PS is localized predominantly in the inner membrane monolayer, we analyzed its distribution between the two membrane leaflets before and after resveratrol treatment of aged hepatocytes. The obtained results indicated that resveratrol treatment affected the intramembrane distribution of PS by reducing its content in the outer plasma membrane leaflet (Fig. 2).

Since the fatty acid composition of biological membranes changes in the course of aging and is also closely related to lipid susceptibility to oxidative damage, we analyzed the acyl chain composition of the two most abundant membrane phospholipids, phosphatidylcholine (PC) and phosphatidylethanolamine (PE), before and after treatment of senescent hepatocytes with resveratrol (Table 2). The results showed that one major saturated fatty acid, palmitic acid (C16:0), was reduced in both tested phospholipids, whereas some of the polyunsaturated fatty acids (C18:3 and C20:4) were increased in membranes of resveratrol-treated hepatocytes (Table 2). The results showed that one major saturated fatty acid, palmitic acid (C16:0), was reduced in both tested phospholipids, whereas some of the polyunsaturated fatty acids (C18:3 and C20:4) were increased in membranes of resveratrol-treated hepatocytes (Table 2).

Fig. 2. Inward translocation of NBD-phosphatidylserine in membranes of control (squares) and resveratrol-treated (circles) senescent hepatocytes. Cells were labeled as described under Materials and methods. The fraction of fluorescent phospholipid in the inner membrane leaflet was determined by back exchange to albumin. The data represent means ± SD of four separate determinations.

Table 2 Fatty acid composition of phosphatidylcholine and phosphatidylethanolamine in plasma membranes of resveratrol-treated senescent hepatocytes (mol%).

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Phosphatidylcholine</th>
<th>Phosphatidylethanolamine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Resveratrol</td>
</tr>
<tr>
<td>C16:0</td>
<td>26.7</td>
<td>23.8</td>
</tr>
<tr>
<td>C16:1</td>
<td>2.1</td>
<td>2.2</td>
</tr>
<tr>
<td>C18:0</td>
<td>19.8</td>
<td>19.5</td>
</tr>
<tr>
<td>C18:1</td>
<td>10.7</td>
<td>9.8</td>
</tr>
<tr>
<td>C18:2</td>
<td>10.9</td>
<td>11.1</td>
</tr>
<tr>
<td>C18:3</td>
<td>5.6</td>
<td>7.2</td>
</tr>
<tr>
<td>C20:4</td>
<td>21.8</td>
<td>22.4</td>
</tr>
<tr>
<td>C22:5</td>
<td>0.5</td>
<td>1.2</td>
</tr>
<tr>
<td>C22:6</td>
<td>1.9</td>
<td>2.8</td>
</tr>
<tr>
<td>SAT/UNSAT</td>
<td>0.802</td>
<td>0.763&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

SAT-saturated fatty acids; UNSAT-unsaturated fatty acids. Values are means of four separate experiments.

<sup>*</sup> P < 0.001.
<sup>**</sup> P < 0.01.

and unsaturated (UNSAT) fatty acids was reduced as a result of resveratrol treatment (0.802 vs. 0.763 for PC and 0.597 vs. 0.524 for PE).

To analyze the mechanisms underlying the influence of resveratrol on liver membrane lipids, we investigated the changes in the lipid synthesis by incubating control and resveratrol-treated hepatocytes with radiolabeled acetate. We followed the distribution of the labeled precursor into the major membrane lipid fractions – fatty acids, phospholipids, diacylglycerols, triacylglycerols and cholesterol (Fig. 3). Apparently, resveratrol reduced the incorporation of acetate mainly in the total phospholipid fraction (by 21%), followed by the fatty acid fraction (by 13%). The decrease of acetate incorporation in diacylglycerols and triacylglycerols was statistically insignificant. There was no change at all in the precursor incorporation into cholesterol after resveratrol treatment. Since the reduction of acetate incorporation was most prominent in the phospholipid fraction, further studies were carried out on the label distribution among the separate phospholipid classes (Fig. 4).

Taking into consideration the reported resveratrol-induced augmentation of the fatty acid degree of unsaturation, we further analyzed the accompanying changes in the level of lipid peroxides,
parinaric acid becomes fluorescent only when incorporated in lipid peroxides, together with the level of ROS and GSH in whole hepatocytes, and were used for analysis of the lipid composition and metabolism, fatty acids and lipid peroxides.

Because unsaturated acyl chains are an excellent target of oxidative attack and resveratrol is known to act as a potent natural antioxidant [4]. Thus, the alterations of the membrane content of lipid peroxides, together with the level of ROS and GSH in whole hepatocytes, would be indicative of the degree of age-induced oxidative stress and the impact of resveratrol on the cellular oxidative status.

Peroxide formation was assessed by the decrease of cis-parinaric acid fluorescence. Due to its conjugated tetracene structure cis-parinaric acid becomes fluorescent only when incorporated in lipid environment [21]. Oxidative destruction of the double bonds is directly translated into irreversible loss of fluorescence, and this is the basis for the use of parinaric acid as a sensitive indicator of the oxidation of conjugated double bonds of membrane lipids [21]. As evident from Fig. 5 resveratrol treatment induced a marked reduction in the level of lipid peroxides. The content of ROS was also reduced in resveratrol-treated aged hepatocytes, whereas GSH remained almost unchanged (Table 3).

4. Discussion

Resveratrol is a naturally occurring phytoalexin, that has been reported to exhibit antioxidant, anti-inflammatory and anti-aging effect on cells [3]. In the present studies we used as experimental model resveratrol-treated hepatocytes isolated from old rats. Plasma membranes were isolated from control and resveratrol-treated hepatocytes, and were used for analysis of the lipid composition and metabolism, fatty acids and lipid peroxides.

The analysis of the plasma membrane lipid composition revealed augmentation of sphingomyelin and phosphatidylserine, as well as reduction of phosphatidylcholine. The effect of resveratrol on sphingomyelin content is a finding of particular interest. On one hand, SM is a major component of the membrane raft domains, which are recognized as cellular signaling platforms [26]. On the other hand, SM is the main source of ceramide, a bioactive lipid second messenger, which is reported to increase in the course of aging and is also considered as a marker of senescence [27]. The accumulation of ceramide has been correlated with the onset of aging-associated inflammation, cellular senescence, growth arrest and many aging-associated diseases [27]. Our results showed that in vitro resveratrol treatment significantly reduced ceramide content in plasma membranes of senescent hepatocytes. This observation is in contrast with the results of Scarlatti et al. [28], who reported resveratrol-induced increase of ceramide in breast cancer cells. It is possible that this discrepancy is due to the differences in the type of cells under investigation – we used normal hepatocytes, isolated from healthy aged animals, whereas Scarlatti et al. [28] used cancer cells. It is quite likely, that resveratrol exhibits diverse effects on ceramide accumulation, depending on the degree of patho-physiological changes, occurring in the corresponding cell model. One could further speculate, that in cancer cells the increase of ceramide and the consequential initiation of apoptosis

Table 3

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Resveratrol</th>
</tr>
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<tbody>
<tr>
<td>ROS</td>
<td>387</td>
<td>295</td>
</tr>
<tr>
<td>GSH</td>
<td>456</td>
<td>471</td>
</tr>
</tbody>
</table>

ROS – reactive oxygen species.
GSH – reduced glutathione.
ROS are expressed as fluorescence intensity per mg protein.
GSH is expressed as nmol per mg protein.
Values are means of three separate determinations.
* P < 0.01.
is a rational way for elimination of such pathological cells. How-
ever, in hepatocytes of old animals, resveratrol reduced the level of ceramide by inhibiting nSMase activity, which is most probably beneficial for the senescent cells.

To elucidate the biochemical mechanism, underlying the alter-
ations of ceramide, we analyzed the activities of specific sphingo-
lipid-metabolizing enzymes, that are related to SM hydrolysis and ceramide accumulation (Fig. 1). As mentioned above, the activity of neutral SMase was markedly lower in resveratrol-treated hepatocytes. What is more, membrane-bound ceramidase, which hydrolyzes ceramide to sphingosine, was not changed as a result of resveratrol treatment (Fig. 1). Thus, resveratrol-induced reduc-
tion of ceramide in aged hepatocytes was due mainly to the de-
crease of nSMase activity and not to stimulation of ceramide hydrolysis. In addition, the accumulation of SM, a lipid acting as a natural membrane antioxidant, was also a result mainly of the re-
cuced SM hydrolysis, performed by nSMase. So it seems likely, that nSMase is the key enzyme responsible for ceramide reduction and SM accumulation in resveratrol-treated aged cells. The observed effect seems to be specific for senescent cells, because similar stud-
ies, performed on hepatocytes of three month old rats did not show any statistically significant differences neither in SM content, nor in nSMase activity (data not shown). Since sphingomyelin pathway plays an important role in age-related changes occurring in liver plasma membranes [27,29], we presume, that one of the major mechanisms, underlying effect of resveratrol on membranes of aged hepatocytes, is its impact on the sphingolipid-metabolizing enzymes and more specifically on nSMase and its products.

As mentioned above PS, which was augmented in resveratrol-
treated membranes, is important in physiological and pathophys-
iological aspect, because its translocation to the outer membrane leaflet is a marker of apoptosis and serves as a signal for macrophage attack. The observed slight, but statistically significant reduction of PS exposure in the external membrane monolayer, taken together with the decrease of ceramide implies a reduction of specific apoptotic markers in the senescent cells, which could be considered as a favorable event for the hepatocytes functional activity.

The fatty acid analysis of the most abundant membrane phos-
pholipids, PC and PE, showed an increase of the polyunsaturated acids such as linolenic and docosahexaenoic, as well as a reduction of the saturated palmitic acid. The latter observation is in accord-
ance with the finding of Gnoni and Paglialonga [10], who reported resveratrol-induced short-term inhibition of palmitic acid synthe-
sis in hepatocytes of young rats. Thus, it is possible that resveratrol exerts a similar effect on this saturated fatty acid in hepatocytes of both young and old rats. It is also possible that resveratrol stimu-
lates the activities of elongase and desaturase enzymes, because we observed an elevation in the mol% of the long-chain polyunsat-
urated docosahexaenoic (in PC and PE), and docosapentaenoic (only in PE) acids.

In our previous paper we reported that SM acts as a natural membrane antioxidant, which could protect the polyunsaturated fatty acid chains against oxidative destruction [30]. Since the level of SM was higher in resveratrol-treated hepatocytes, we followed the accompanying changes in the content of lipid peroxides, ROS and GSH (Fig. 5 and Table 3). We did not observe significant changes in the GSH level, which was rather unexpected, because resveratrol is known to act as a potent antioxidant, and has been reported to increase the expression of glutathione peroxidase [31]. It is possible, that such effect takes place in young cells and is not valid for cells of aged animals, where the synthesis of GSH is hindered. However, the content of ROS was lower in resvera-

trol-treated cells (Table 3). Nevertheless, it should be noted that free radicals do not only cause molecular damage to cells, but could also participate in cell signaling and thus act as mediators of physiological processes [32]. Also, there are reports which associ-
ate increased ROS production with higher longevity. So our finding that ROS have been reduced by resveratrol treatment could be a subject of a more complex interpretation. Based on the observed decrease of lipid peroxides in plasma membranes from resvera-
trol-treated cells (Fig. 5) we presume, that the influence of resvera-
trol was most prominent on the membranes of senescent hepatocytes, possibly due to the lipophilic nature of this polyphenol. The finding that lipid peroxidation was lower and the degree of fatty acid unsaturation was elevated in membranes of resveratrol-
treated senescent hepatocytes is of particular interest. This implies that certain intrinsic antioxidant factors are responsible for the reduction of lipid peroxides in the membranes of resveratrol-trea-
ted aged hepatocytes. One possible explanation is the antioxidant effect of SM [30,33] which was elevated due to resveratrol treat-
ment. Subbaiah et al. reported that the sphingosine backbone of SM has unique structure, which not only makes SM less susceptible to free radical reactions, but also inhibits the oxidation of the neighboring unsaturated lipid molecules [33]. Another mechanism could be related to the reported lower level of ROS (Table 3). Of course, other cellular mechanisms which could be activated by res-
veratrol treatment of hepatocytes obtained from old rats should not be ruled out. In addition, the relative increase of omega-3 fatty acids, represented by docosahexaenoic acid, should also be emphasized, because the abundance of these essential fatty acids in the membrane phospholipids is considered favorable for the membrane functional activity and structural orga-
nization [34].

In conclusion, the presented results demonstrate that resvera-

trol treatment of hepatocytes obtained from old rats induces sig-
nificant changes especially on membrane level. Particular attention require the alterations in the fatty acid composition, the ceramide level, the activity of nSMase as well as the lipid per-
oxide level in plasma membranes of resveratrol-treated senescent hepatocytes. Although at this point the exact mechanisms underly-
ing the reported changes could not be specified, it is clear that res-
veratrol induces specific alterations in the membrane lipids which can be estimated as beneficial for the plasma membrane and for the whole senescent liver cell.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgement

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