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RESVERATROL ALTERS SPHINGOLIPID METABOLISM IN *RAS*-TRANSFORMED 3T3 FIBROBLASTS

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Abstract

The effect of resveratrol on the sphingolipid metabolism has been investigated in *ras*-transformed 3T3 fibroblasts. Resveratrol is a phytoalexin of a great medico-biological significance, which has been reported to exhibit significant beneficial effects such as antioxidant, lipid-lowering, anti-neoplastic, etc. Sphingolipids are functionally active lipid molecules, which are implicated in important cellular processes, such as proliferation, migration, apoptosis and transmembrane signalling, among others. Although there are many investigations devoted to the influence of resveratrol on the lipid metabolism of various types of cancer cells, the mechanism of this effect on the sphingolipid pathway remains unclear. The present studies showed that the major sphingolipid, sphingomyelin, was decreased in membranes from resveratrol-treated *ras*-transformed 3T3 fibroblasts, whereas ceramide, which is a pro-apoptotic factor, was elevated due to neutral sphingomyelinase up-regulation. In addition, the level of sphingosine, a product of ceramidase, was increased and the content of sphingosine-1-phosphate, which is related to cell proliferation, was lowered due to resveratrol treatment, which is an important finding especially for cancer cells. The balancing enzyme in the sphingolipid pathway, sphingosine kinase, which produces sphingosine-1-phosphate, was down-regulated in resveratrol-treated oncogene-transformed fibroblasts. In conclusion, the present

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results clearly show that treatment of *ras*-transformed fibroblasts with resveratrol induces changes in the major representatives of the sphingolipid metabolic pathway shifting the "sphingolipid rheostat" towards prevalence of the pro-apoptotic factor ceramide. The reported observations can be applied in the development of complex anti-tumour therapeutic strategies.

Key words: resveratrol, sphingomyelin, ceramide, sphingosine, sphingosine-1-phosphate

Introduction. Resveratrol is a widely investigated phytoalexin, which has been reported to exhibit various beneficial effects such as antioxidant, lipid-lowering and anti-neoplastic, among others [1,2]. There is evidence that resveratrol affects lipid metabolism of serum and liver lipids and also inhibits glycerophospholipid catabolism in cancer cells [3].

Sphingolipids are functionally active lipid molecules, their activity being related to cell proliferation, migration, apoptosis, etc. [4,5]. In our previous studies we reported that treatment of senescent rat hepatocytes with resveratrol induces partial inactivation of membrane-associated neutral sphingomyelinase and reduction of ceramide level, the latter being associated with apoptosis [6]. In addition, we observed that resveratrol treatment reduced the susceptibility of membrane cholesterol to oxidative damage in three-dimensional fibroblast cell cultures [7].

Although there are numerous studies devoted to the effect of resveratrol on the lipid metabolism of various types of cancer cells, the mechanism of this effect on the sphingolipid metabolic pathway remains largely unclear.

The aim of the present study was to investigate the effect of resveratrol treatment on the lipid composition of *ras*-transformed 3T3 fibroblasts, and more specifically on the sphingolipid catabolic pathway, the latter producing sphingolipid metabolites with high physiological activity.

Materials and methods. Treatment of fibroblasts with resveratrol was performed as explained elsewhere [6]. Plasma membranes from fibroblasts were isolated as described earlier [8]. Lipid extraction was performed according to Bligh and Dyer [9]. Phospholipids were determined according the procedure of Kahovcova and Odavic [10]. Neutral sphingomyelinase [11] and ceramidase [12] activities were measured exactly as described. Sphingosine level was determined using sphingosine ELISA kit Aviva Systems Biology according to the manufacturer's instructions. S1P level and sphingosine kinase activity were determined using ELISA kits Abcam according to the manufacturer's instructions.

Results. Treatment of *ras*-transformed fibroblasts with the phytoalexin resveratrol induced alteration in the level of the major representative in the sphingolipid metabolic pathway – sphingomyelin (SM). As evident from Fig. 1, the membrane level of SM was reduced by 24%, whereas the content of the next sphingolipid in this metabolic pathway, ceramide, which is basically associated with apoptosis initiation, was increased by 32% due to resveratrol treatment. These results clearly indicate that resveratrol affects sphingolipid metabolites in the

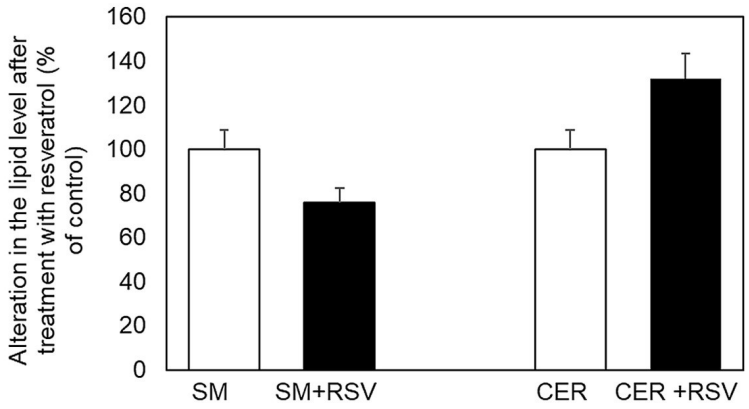

Fig. 1. Alterations in the level of sphingomyelin (SM) and ceramide (CER) in control and resveratrol (RSV)-treated *ras*-transformed 3T3 fibroblasts. Results are presented as % of control values (100%). The experiments were performed in triplicates. The changes in SM and CER are statistically significant compared to controls. $P < 0.001$

oncogene-transformed fibroblasts, which is why we measured the other physiologically important sphingolipids – sphingosine (SPH) and sphingosine-1-phosphate (S1P) (Fig. 2). The level of SPH was higher by 28% compared to untreated fibroblasts, whereas S1P was reduced by 19% as a result of resveratrol treatment. The observed alterations implied that resveratrol affected either the catabolism and/or the anabolism of the measured sphingolipids, which is why we analyzed the

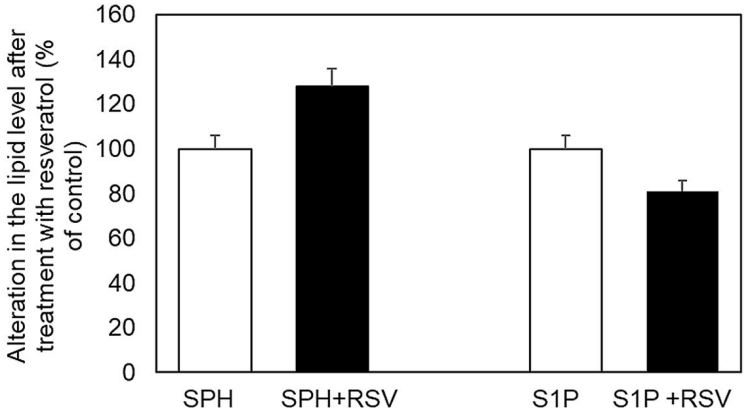

Fig. 2. Alterations in the level of sphingosine (SPH) and sphingosine-1-phosphate (S1P) in control and resveratrol (RSV)-treated *ras*-transformed 3T3 fibroblasts. Results are presented as % of control values (100%). The experiments were performed in triplicates. The changes in SM and CER are statistically significant compared to controls. $P < 0.001$

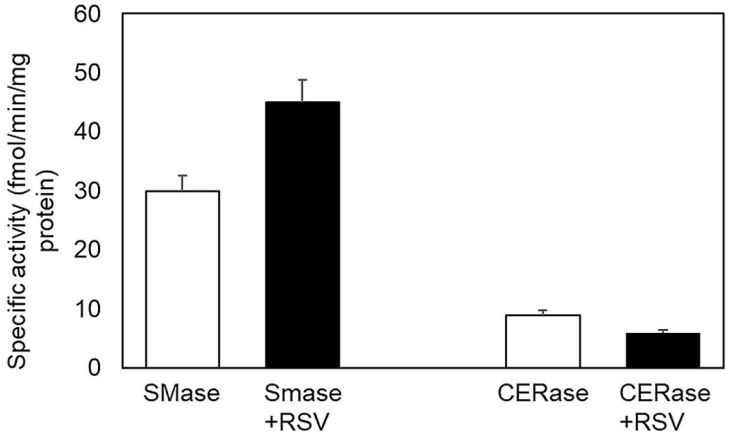

Fig. 3. Changes in the specific activity of sphingomyelinase (SMase) and ceramidase (CERase) in control and resveratrol (RSV)-treated *ras*-transformed 3T3 fibroblasts. Results are presented as fmol/min/mg membrane protein. The experiments were performed in triplicates. The changes in the two enzyme activities are statistically significant compared to control values. $P < 0.001$

major enzyme activities related to the hydrolysis of the four measured metabolites. The results showed that the enzyme responsible of SM degradation, neutral sphingomyelinase, was activated in resveratrol-treated *ras*-transformed fibroblasts (Fig. 3). In addition, ceramidase, the enzyme hydrolyzing the sphingomyelinase product – ceramide, was down-regulated due to resveratrol treatment (Fig. 3). The product of ceramidase, SPH, is basically phosphorylated by SPH kinase to yield S1P, the latter being associated with cell proliferation and survival. The results showed that SPH kinase was down-regulated due to resveratrol treatment of *ras*-transformed 3T3 fibroblasts (Fig. 4).

Discussion. Resveratrol is a naturally occurring phytoalexin that has been reported to exhibit antioxidant, anti-inflammatory and anti-cancer effect on cells [13]. In the present studies we used as experimental model *ras* oncogene-transformed 3T3 mouse fibroblasts to test the effect of resveratrol on the sphingolipid metabolism and the level of key metabolites, participating in the sphingomyelin pathway. Plasma membranes were isolated from control and resveratrol-treated *ras*-transformed fibroblasts, and were used for analysis of the lipid composition and metabolism.

The analysis of the membrane lipid composition showed that the level of SM was decreased due to resveratrol treatment (Fig. 1). In our previous studies we analyzed the influence of resveratrol on the level of membrane glycerophospholipids and sphingolipids as a result of treatment with resveratrol of rat senescent hepatocytes [6]. In these experiments we observed an elevation of SM and a decrease

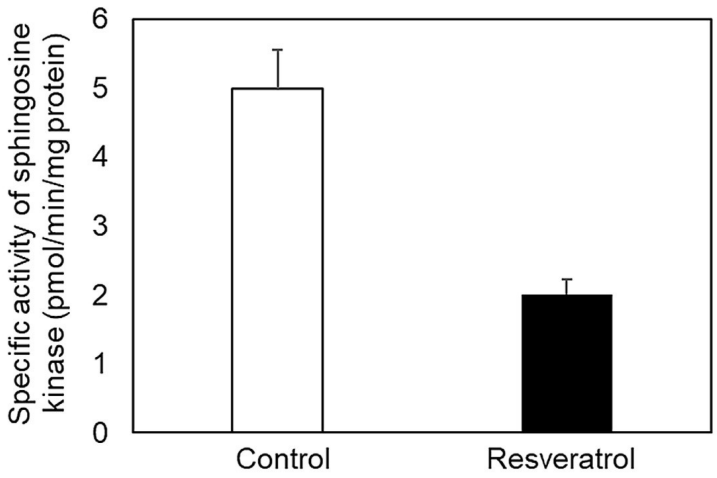

Fig. 4. Changes in the specific activity of membrane-bound sphingosine kinase in control and resveratrol (RSV)-treated *ras*-transformed 3T3 fibroblasts. Results are presented as pmol/min/mg membrane protein. The experiments were performed in triplicates. The changes in the enzyme activity are statistically significant compared to control values. $P < 0.001$

of ceramide which seem to be controversial to the data reported in the present work. We presume that these differences are due to the opposite effect displayed by resveratrol on cancer and non-cancer cells. In senescent cells resveratrol effect was related to increase of membrane SM which acts as an intrinsic antioxidant and protects the acyl chains from oxidative destruction. In addition, SM is a major component of the membrane raft domains, which are recognized as cellular signalling platforms [14]. On the other hand, SM is the main source of ceramide, a bioactive lipid second messenger, which is reported to initiate apoptotic processes [8]. So it seems likely that in non-cancerous cells resveratrol increases the membrane level of the intrinsic antioxidant SM, whereas in oncogene-expressing cells this flavonoid induces decrease of SM at the expense of elevation of the apoptosis-inducing sphingolipid ceramide. To elucidate the biochemical mechanism, underlying the alterations of SM, we analyzed the activity of the enzyme responsible for SM hydrolysis – neutral sphingomyelinase (Fig. 3). The observation that this enzyme was up-regulated in resveratrol-treated oncogene-expressing fibroblasts implied that sphingomyelinase upregulation underlies the elevation of ceramide, thus increasing the content of this pro-apoptotic factor. This is an important observation, because stimulation of apoptotic processes is crucial for cancer cells and the elucidation of the mechanism responsible for apoptosis onset could be helpful in constituting of therapeutic approaches for definite tumours.

So it seems likely, that sphingomyelinase is the key enzyme responsible for

alterations in the SM level in resveratrol-treated cells. Since sphingomyelin pathway plays an important role in regulation of the balance between proliferation and apoptosis [4], we presume that one of the major mechanisms, underlying the effect of resveratrol on membranes and cells is its impact on the sphingolipid-metabolizing enzymes.

Another important finding is the decrease of ceramidase activity, which additionally contributes for maintenance of ceramide levels (Fig. 3). The product of ceramidase, sphingosine, is also a physiologically active sphingolipid. Our studies showed that resveratrol treatment induced an increase of sphingosine in the *ras*-transformed fibroblasts. Since sphingosine acts as a precursor of a sphingolipid with significant biological importance, S1P, which is associated with cellular proliferation, the enzyme producing S1P, sphingosine-kinase, is very important for maintenance of the balance between the pro-apoptotic ceramide and the pro-proliferative factor S1P. The results showed that resveratrol treatment down-regulated sphingosine-kinase, thus maintaining a higher level of ceramide at the expense of S1P. This process is called "sphingolipid rheostat" and plays an essential role in the balance between apoptosis and proliferation of cells.

Finally, the presented results show that resveratrol treatment of *ras*-transformed 3T3 fibroblasts shifted the balance of the sphingolipid rheostat towards apoptosis at the expense of proliferation, which is important especially when it comes to dealing with cancer cells. These data help for better understanding of the effect of definite flavonoids in the regulation of sphingolipid metabolism and could help in the development of complex anti-tumour therapeutic approaches.

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