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QUERCETIN AFFECTS CERAMIDE BUT NOT SPHINGOSINE-1-PHOSPHATE IN *ras*-TRANSFORMED 3T3 FIBROBLASTS

Albena Momchilova[#], Roumen Pankov[*], Plamen Krastev[**], Tania Markovska, Borislav Arabadjiev[*], Nikolai Krastev[***], Evgenia Vassileva[****], Dimo Krastev[*****], Dimitar Tonev[******]

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Abstract

Investigations were carried out on the effect of quercetin on the level of the major sphingolipid metabolites in *ras*-transformed 3T3 fibroblasts. Quercetin is a polyphenol of a significant biomedical importance, which has been reported to show beneficial effects such as antioxidant, anti-neoplastic, anti-ageing, etc. Sphingolipids are functionally active lipid molecules, which regulate important cellular processes, like proliferation, apoptosis and transmembrane signalling, among others. Despite the numerous investigations devoted to the influence of quercetin on the lipid metabolism of various types of cancer cells, the mechanism of this influence on the major sphingolipid pathways still remains unclear. Our studies showed that sphingomyelin was decreased in *ras*-transformed 3T3 fibroblasts, whereas ceramide, which is a pro-apoptotic factor, was increased as a result of elevated neutral sphingomyelinase activity. However, the level of sphingosine-1-phosphate, which is related to cell proliferation, was not altered as a result of quercetin treatment, which is an unexpected finding when compared to our previous studies with cancer cells. In addition, the observed changes in the balancing enzyme in the sphingolipid pathway, sphingosine kinase 1, which produces sphingosine-1-phosphate, were not statistically significant in quercetin-treated oncogene-transformed fibroblasts.

[#]Corresponding author.

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The presented data are useful for better understanding of the effect of flavonoids on the regulation of sphingolipid metabolism and could help in the development of complex antitumour therapeutic approaches, involving natural antitumour agents like polyphenols.

Key words: quercetin, sphingomyelin, ceramide, sphingosine-1-phosphate, *ras*-transformed cells

Introduction. Quercetin is a widely investigated polyphenolic compound, which has been reported to exhibit various beneficial effects such as antioxidant, anti-neoplastic, anti-ageing among others [1–3]. Numerous investigations are devoted to the beneficial health effects of quercetin in various pathologies such as oxidative stress, toxicology stress after pesticide intake, neurodegenerative disorders, inflammation, neoplastic processes, age-related diseases, etc. [3].

Sphingolipids are functionally active lipid molecules, their activity being related to essential cellular processes like proliferation, migration, apoptosis, etc. [4, 5]. In our recent studies we reported that treatment of HepG2 cells with quercetin induces decrease of the level of sphingomyelin and increase of neutral sphingomyelinase activity and ceramide level, the latter being associated with apoptosis [6]. In addition, we observed that quercetin affected phospholipase A2 activity and the membrane fatty acid composition of three-dimensional tissue-like fibroblast cultures [7].

Although there are numerous studies devoted to the effect of quercetin on the lipid metabolism of cancer cells, the mechanism of this effect on the main sphingolipid metabolic pathways still remains unclear.

The aim of the present study was to investigate the effect of quercetin treatment on the major functionally active sphingolipids in *ras*-transformed 3T3 fibroblasts, because some of them like ceramide and sphingosine-1-phosphate trigger opposite cellular processes like apoptosis and proliferation.

Materials and methods. Treatment with quercetin was performed as explained elsewhere [7]. Lipid extraction was performed according to Bligh and Dyer [8]. Phospholipids were determined according to the procedure of Kahovcova and Odavic [9]. Neutral sphingomyelinase and ceramidase activities were measured exactly as described elsewhere [6]. 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 quercetin induced alteration in the level of the major representative in the sphingolipid metabolic pathway – sphingomyelin (SM). As evident from Fig. 1, the level of SM was significantly reduced, whereas the content of one of the most physiologically active sphingolipids, ceramide, which is related to apoptotic processes, was markedly increased. The presented results show that quercetin affects major sphingolipid metabolites in *ras*-transformed fibroblasts, which is why we tried to analyze the

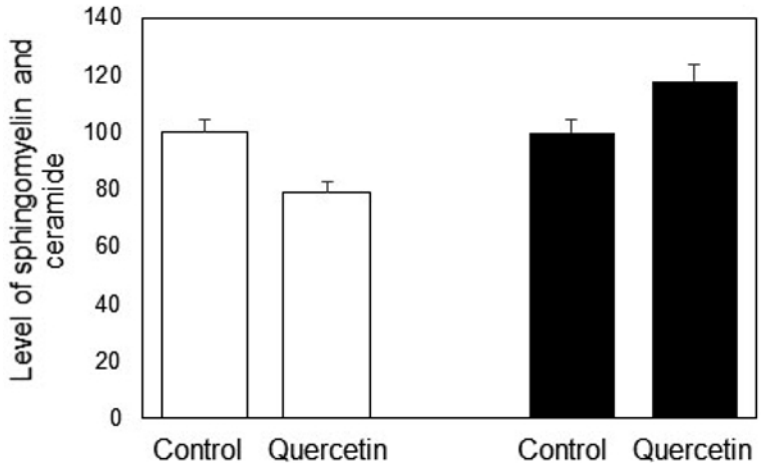

Fig. 1. Alterations in the level of sphingomyelin (white bars) and ceramide (black bars) in control and quercetin (Querc)-treated *ras*-transformed 3T3 fibroblasts. Results are presented as % of control values. The changes are statistically significant compared to controls. $P < 0.001$

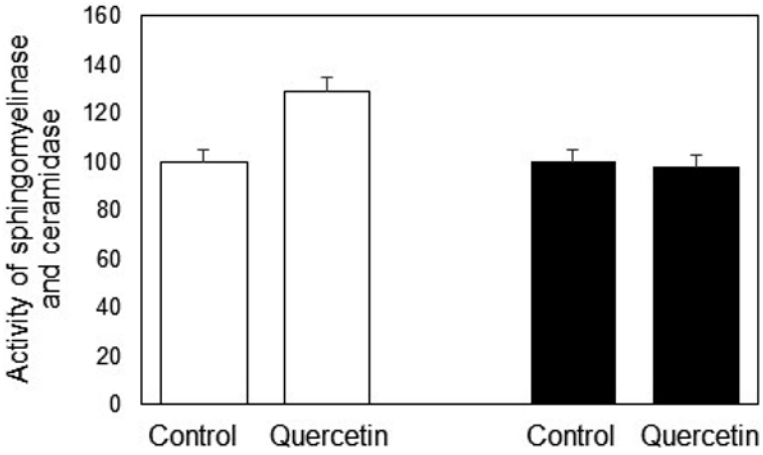

Fig. 2. Changes in the specific activity of sphingomyelinase (white bars) and ceramidase (black bars) in control and quercetin (Querc)-treated *ras*-transformed 3T3 fibroblasts. Results are presented as % of control values. Only the changes in sphingomyelinase activity are statistically significant. $P < 0.001$

biochemical mechanism underlying the reported changes by measuring the activity of the main SM hydrolyzing and ceramide-producing enzyme, neutral sphingomyelinase (Fig. 2). Also, we studied the impact of quercetin on ceramidase, the latter contributing to maintenance of ceramide level. The obtained results showed that the enzyme responsible of SM degradation, neutral sphingomyelinase, was ac-

tivated in quercetin-treated *ras*-transformed fibroblasts, whereas ceramidase was not significantly affected by quercetin treatment of *ras*-transformed fibroblasts (Fig. 2).

One sphingolipid with particular physiological significance is S1P, which is a product of the main balancing enzyme in the SM pathway, sphingosine kinase. S1P is associated with cell proliferation and survival, which underlies its specific physiological significance. The obtained results showed that the level of S1P was not affected significantly by quercetin treatment (Fig. 3). Sphingosine kinase activity showed a tendency of reduction, but this reduction was not statistically significant when compared to untreated cells (Fig. 4).

Discussion. Quercetin is a naturally occurring polyphenolic compound that has been reported to exhibit antioxidant, anti-inflammatory and anti-cancer effect on cells [10]. In the present study we used as experimental model *ras* oncogene-transformed 3T3 mouse fibroblasts to test the effect of quercetin on the level of key metabolites, participating in the sphingolipid pathways.

The analysis of the level of the major sphingolipids showed that SM was decreased and ceramide was elevated due to quercetin treatment (Fig. 1). In our previous studies we analyzed the influence of quercetin on the level of cellular sphingolipids as a result of treatment with quercetin of HepG2 cells [6]. In these experiments we also observed a decrease of SM and an increase of ceramide which are in agreement with the data reported in the present work. Thus, it seems that the effect of quercetin on the level of sphingomyelin and ceramide in cancer cells seems to be similar, resulting in reduction of the intrinsic membrane antioxidant SM and elevation of the pro-apoptotic factor ceramide.

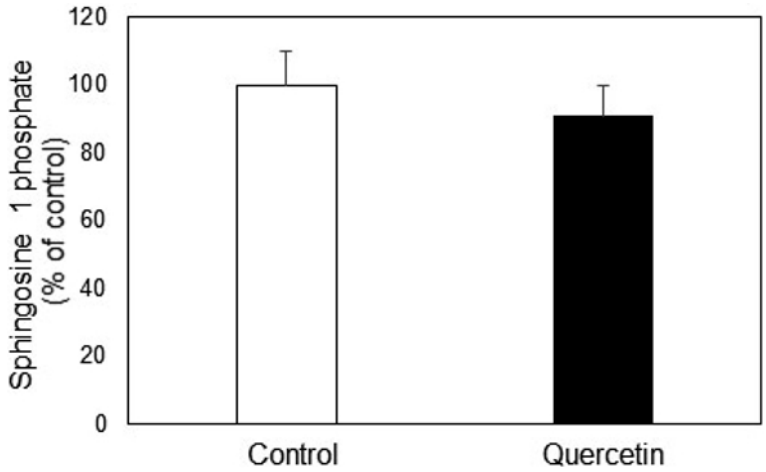

Fig. 3. Alterations in the level of sphingosine-1-phosphate (S1P) in control and quercetin-treated *ras*-transformed 3T3 fibroblasts. Results are presented as % of control values. The changes are not statistically significant compared to controls

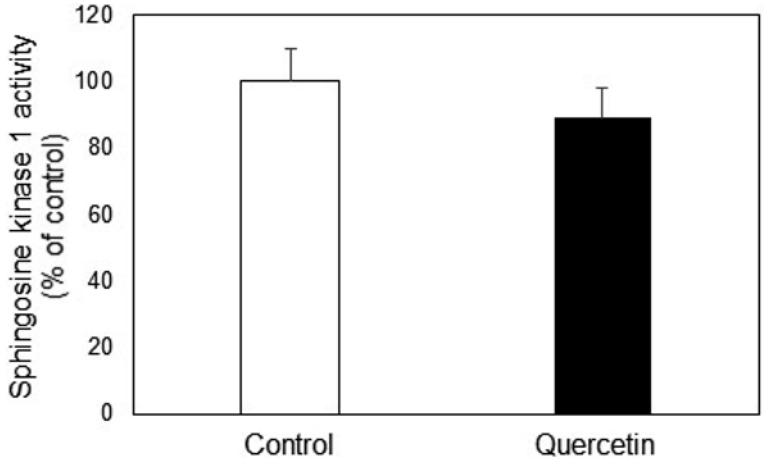

Fig. 4. Changes in the specific activity of sphingosine kinase 1 in control and quercetin-treated *ras*-transformed 3T3 fibroblasts. The changes in the enzyme activity are not statistically significant compared to control values

In addition, SM is a major component of the membrane raft domains, which are recognized as cellular signalling platforms [11]. On the other hand, SM is the main source of ceramide, a bioactive lipid second messenger, which, as mentioned above, is reported to initiate apoptotic processes in cells [12]. However, in tissue-like 3D fibroblast cultures we observed quite opposite effects – increase of SM and reduction of ceramide. So it seems likely that in non-cancerous cells quercetin 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 shed some light on the biochemical mechanism, underlying the alterations of SM, we analyzed the activity of the enzyme responsible for SM hydrolysis – neutral sphingomyelinase (Fig. 2). The observation that this enzyme activity was up-regulated in quercetin -treated oncogene-expressing fibroblasts implies that sphingomyelinase activation 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 clarification of the mechanism responsible for apoptosis onset is useful in the development of therapeutic strategies for treatment of definite tumours.

So it seems likely, that sphingomyelinase is the main enzyme responsible for alterations in the SM and ceramide level in quercetin-treated tumour cells. Thus, since the sphingomyelin pathway plays a crucial role in regulation of the balance between proliferation and apoptosis [4], it is quite likely, that one of the major mechanisms, underlying the effect of quercetin on tumour cells is its impact on the sphingolipid-metabolizing enzymes.

However, it should be noted that ceramidase activity, which contributes for maintenance of ceramide levels, remained rather unchanged in quercetin-treated cells, implying that the observed elevation of ceramide level was a result mainly form sphingomyelinase up-regulation (Fig. 2).

Interestingly, the level of a sphingolipid with significant biological importance, S1P, which is associated with cellular proliferation, was not significantly altered in quercetin-treated *ras*-transformed fibroblasts (Fig. 3). The enzyme producing S1P, sphingosine-kinase 1, is very important for maintenance of the balance between the pro-apoptotic ceramide and the pro-proliferative factor S1P. The obtained results showed that quercetin treatment down-regulated sphingosine-kinase, but the observed alteration was not statistically significant (Fig. 4). Sphingosine kinase is the main regulatory enzyme in the so-called "sphingolipid rheostat" and, as mentioned above, it plays an essential role in the balance between the processes of apoptosis and proliferation. Our findings showed that quercetin treatment of *ras*-transformed fibroblasts induced elevation in the main pro-apoptotic factor ceramide, whereas no significant changes were observed in the proliferation-supporting sphingolipid, S1P.

In conclusion, the presented results show that quercetin treatment of *ras*-transformed 3T3 fibroblasts stimulates the apoptotic processes through ceramide elevation, but it does not contribute to restriction of the proliferative processes, unlike for example resveratrol, when applied to oncogene-transformed cells [12]. It should be noted that *ras*-transformed cells are a very adequate model for studies on tumour cells, because about one third of the human tumours express *ras* oncogenes. The presented data help for better understanding of the effect of flavonoids on the regulation of sphingolipid metabolism and could help in the development of complex anti-tumour therapeutic approaches, involving natural antitumour agents like polyphenols.

REFERENCES

[1] Saito K. (1974) Possible site of flavonoid synthesis in the photosynthetic apparatus, Biochem. J., **144**(2), 431–442.

[2] Filip R., P. G. López Giberti, J. Coussio, G. Ferraro (2001) Phenolic compounds in seven South American Ilex species, Fitoterapia, **72**(7), 774–778.

[3] Zang M., S. Xu, K. Maitland-Tooland, A. Zuccollo, X. Hou et al. (2006) Polyphenols stimulate ATP-activated protein kinase, lower lipids and inhibit accelerated atherosclerosis in diabetic LDL receptor-deficient mice, Diabetes, **55**, 2180–2191.

[4] Spiegel S., A. H. Merrill (1996) Sphingolipid metabolism and cell growth regulation, FASEB J., **10**, 1388–1397.

[5] Hannun Y. A., R. M. Bell (1989) Functions of sphingolipids and sphingolipid breakdown products in cellular regulation, Science, **243**, 500–507.

[6] Momchilova A., G. Nikolaev, S. Pankov, E. Vassileva, N. Krastev et al. (2022) Effect of quercetin and fingolimod, alone and in combination, on the sphingolipid metabolism in HepG2 cells, Int. J. Mol. Sci., **23**, 13916.
[7] Nikolova B., R. Pankov, G. Staneva, N. Krastev, T. Markovska et al. (2022) Quercetin influences phospholipase A2 in A549 adenocarcinoma cells, C. R. Acad. Bulg. Sci., **75**(11), 1588–1594.
[8] Bligh E., W. Dyer (1959) A rapid method of total lipid extraction and purification, Can. J. Physiol., **37**, 911–917.
[9] Kahovcova J., R. Odavic (1969) A simple method for analysis of phospholipids separated on thin layer chromatography, J. Chromatogr., **40**, 90–95.
[10] Guo H., H. Ding, X. Tang (2021) Quercetin induces pro-apoptotic autophagy via SIRT1/AMPK signaling pathway in human lung cancer cell lines A549 and H1299 in vitro, Thorac. Cancer, **12**, 1415–1422.
[11] Simons K., W. Vaz (2004) Model systems, lipid rafts and cell membranes, Annu. Rev. Biophys. Biomol. Struct., **33**(7), 269–295.
[12] Momchilova A., G. Staneva, S. Pankov, N. Krastev, T. Markovska et al. (2022) Resveratrol alters sphingolipid metabolism in *ras*-transformed 3T3 fibroblasts, C. R. Acad. Bulg. Sci., **75**(9), 1310–1316.

Institute of Biophysics
and Biomedical Engineering
Bulgarian Academy of Sciences
Akad. G. Bonchev St, Bl. 21
1113 Sofia, Bulgaria
e-mails: albena_momchilova@abv.bg
markobska@abv.bg

[*] Faculty of Biology
Sofia University "St. Kliment Ohridski"
8 Dragan Tzankov Blvd
1164 Sofia, Bulgaria
e-mails: rpankov@biofac.uni-sofia.bg
arabadjiev.borislav@gmail.com

[**] Cardiology Clinic
University Hospital "St. Ekaterina"
52 Pencho Slaveykov Blvd
1431 Sofia, Bulgaria
e-mail: plamenkr@mail.bg

[***] Department of Anatomy,
Histology and Embryology
Medical University – Sofia
1 St. Georgi Sofiiski St
1431, Sofia, Bulgaria
e-mail: dr.krustev.dm@gmail.com

[****] Clinic of Neurology
Tsaritsa Yoanna University Hospital-ISUL
8 Byalo more St
1527 Sofia, Bulgaria
e-mail: e.vassilevva@gmail.com

[*****] Medical University – Sofia
Medical College "Y. Filaretova"
3 Yordanka Filaretova St
1606 Sofia, Bulgaria
e-mail: Dimo_krustev@mail.bg

[******] Clinic of Anesthesiology and Intensive Care
Tsaritsa Yoanna University Hospital-ISUL
8 Byalo more St
1527 Sofia, Bulgaria
e-mail: dgtsofia@abv.bg