International Journal of Biochemistry and Cell Biology 117 (2019) 105618

Contents lists available at ScienceDirect

International Journal of Biochemistry and Cell Biology

journal homepage: www.elsevier.com/locate/biocel

Quercetin inhibition of SREBPs and ChREBP expression results in reduced cholesterol and fatty acid synthesis in C6 glioma cells

Fabrizio Damiano[a], Laura Giannotti[a], Gabriele V. Gnoni[a], Luisa Siculella[a,*], Antonio Gnoni[b]

[a] Department of Biological and Environmental Sciences and Technologies, University of Salento, 73100, Lecce, Italy
[b] Department of Basic Medical Sciences, Neurosciences and Sense Organs, University of Bari "Aldo Moro", 70124, Bari, Italy

ARTICLE INFO

Keywords:
3-Hydroxy-3-methyl-glutaryl-CoA reductase
Acetyl-CoA carboxylase 1
C6 glioma cells
Cholesterol and fatty acid synthesis
ChREBP and SREBPs
Quercetin

ABSTRACT

Quercetin (Que), a widely distributed flavonoid in the human diet, exerts neuroprotective action because of its property to antagonize oxidative stress. Here, we investigated the effects of Que on lipid synthesis in C6 glioma cells. A rapid Que-induced inhibition of cholesterol and, to a lesser extent, of fatty acid synthesis from [1-^{14}C] acetate was observed. The maximum decrease was detected at the level of palmitate, the end product of *de novo* fatty acid synthesis. The effect of Que on the enzyme activities of acetyl-CoA carboxylase 1 (ACC1) and fatty acid synthase (FAS), the two enzymes of this pathway, was investigated directly *in situ* in permeabilized C6 cells. An inhibitory effect on ACC1 was observed after 4 h of 25 μM Que treatment, while FAS activity was not affected. A reduction of polar lipid biosynthesis was also detected. A remarkable decrease of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) activity, regulatory enzyme of cholesterol synthesis, was evidenced. Expression studies demonstrated that Que acts at transcriptional level, by reducing the mRNA abundance and protein amount of ACC1 and HMGCR. Deepening the molecular mechanism, we found that Que decreased the expression of SREBP-1 and SREBP-2, transcriptional factors representing the main regulators of *de novo* fatty acid and cholesterol synthesis, respectively. Que also reduced the nuclear content of ChREBP, a glucose-induced transcription factor involved in the regulation of lipogenic genes. Our results represent the first evidence that a direct and rapid downregulatory effect of Que on cholesterol and *de novo* fatty acid synthesis is elicited in C6 cells.

1. Introduction

Quercetin (3,3′,4′,5,7-pentahydroxyflavone, Que) is a flavonol abundant in several foods such as vegetables, fruit, tea and red wine. A broad spectrum of beneficial properties have been described for Que, including antioxidant and anti-inflammatory activities, which are thought to play a role in protecting against diabetes, obesity, cardiovascular and neurodegenerative diseases (Costa et al., 2016).

The neuroprotective action of Que has been correlated with its property to antagonize oxidative stress. Administration of Que protects rodents from neurotoxic insults such as heavy metals and insecticides (Costa et al., 2016). Que also antagonizes mice cognitive impairment induced by high fat diet (HFD) (Xia et al., 2015). Of relevance are also some recent findings showing that Que ameliorates Alzheimer's disease pathology (Sabogal-Guáqueta et al., 2015), and, in combination with fish oil, enhances neuroprotection in a rat model of Parkinson's disease (Denny Joseph, 2015). Que has been also described as a potential anticancer agent (Murakami et al., 2008), since it inhibits cell growth in different human cancers.

In relation to the health effects of Que on the aforementioned pathologies, an interesting aspect is the action of this natural compound on lipid metabolism. Que protects rats from HFD-induced adipose tissue accumulation and body weight gain (Rivera et al., 2008). In HFD-fed rats, Que suppresses adipogenesis by reducing the key adipogenic transcription factor C/EBPα expression and reduces lipogenesis (*i.e.de novo* fatty acid synthesis) by down-regulating the expression of the two enzymes of this pathway, acetyl-CoA carboxylase 1 (ACC1) and fatty acid synthase (FAS) (Moon et al., 2013). In hepatocytes from normal rats, Que decreases both *de novo* fatty acid and triacylglycerol (TAG) syntheses, with consequent reduction of VLDL-TAG formation (Gnoni et al., 2009). In diet-induced obese mice Que acts as hypotriacylglycerolemic agent by inducing white adipose tissue browning (Kuipers et al., 2018).

Brain is the organ with the highest lipid content after white adipose tissue, and lipids, fundamental components of neuronal membranes, are essential for brain function. Cholesterol and fatty acids are particularly present in the synaptic membranes and play a key role in the membrane fluidity and in the formation of lipid rafts, specialized microdomains

* Corresponding author.
E-mail address: luisa.siculella@unisalento.it (L. Siculella).

https://doi.org/10.1016/j.biocel.2019.105618
Received 16 July 2019; Received in revised form 13 September 2019; Accepted 18 September 2019
Available online 19 September 2019
1357-2725/

essential for synaptic transmission (Zhang and Liu, 2015). Therefore, alterations in lipid metabolism are often associated with many neurological diseases (Adibhatla and Hatcher, 2008). Brain is reported to be shielded by the blood–brain barrier (BBB) from blood lipids (Morell and Jurevics, 1996), so it may be viewed as being largely autonomous in lipid synthesis.

Sterol regulatory element-binding proteins (SREBPs) are basic helix-loop-helix leucin zipper (bHLH-LZ) transcription factors activating genes involved in lipid synthesis. The SREBP family consists of SREBP-1a, SREBP-1c and SREBP-2, which differ in their target gene selectivity (Horton, 2002). SREBP-1a is a potent activator of genes involved in the synthesis of fatty acids, triacylglycerols and cholesterol. SREBP-1c and SREBP-2 enhances the transcription of genes required for fatty acids and for cholesterol synthesis, respectively. In the brain, SREBP-2 plays a role in the cholesterol synthesis, fundamental for myelin membrane formation and integrity (Camargo et al., 2009) as well as for cholesterol supply to neurons by astrocytes (Tarr and Edwards, 2008). The function of SREBP-1 is correlated with the synthesis of fatty acids and occupies a central position in glia-neuron interactions involving fatty acids, such as neurite growth and synaptic transmission (Camargo et al., 2009).

Carbohydrate response element binding protein (ChREBP) is a bHLH-LZ transcription factor regulating glucose-responsive genes in the liver. Genes in the pathway of *de novo* lipogenesis have been identified as targets of ChREBP (Ferré and Foufelle, 2010; Jois and Sleeman, 2017). No information about regulation of ChREBP expression in the mammalian brain has been reported so far.

Aim of the present study was to investigate whether Que plays a role in fatty acid and cholesterol biosynthesis in C6 rat glioma cells, which present a large repertoire of astrocyte-expressing enzymatic activities and exhibit a prevalent astrocyte-like phenotype when cultured in serum-rich medium (Natali et al., 2007). Thus, they are considered a useful cellular model to study cerebral dysfunction (Matias et al., 2016).

Here, we present the first evidence that Que added to C6 cells determines a remarkable cholesterol, and to a lesser extent, fatty acid synthesis inhibition, occurring at the level of the activities of 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR) and ACC1, key enzymes of cholesterol and fatty acid synthesis, respectively. The mRNA abundance and protein amount of HMGCR and ACC1 are parallelly reduced. The molecular mechanism of this inhibition has been further deepened by investigating the key transcriptional factors SREBPs and ChREBP regulating these pathways.

2. Materials and methods

2.1. Cell culture

Rat C6 glioma cells were from the American Type Culture Collection. C6 cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin (Sigma-Aldrich), at 37 °C in a humidified atmosphere of 5% CO_2. C6 cells were cultured in 10% Fetal Bovine Serum (FBS)-supplemented medium. 24 h after plating, the medium was changed and, following further 24 h, Que was added to the DMEM at the concentration of 25 μM. Que (Sigma-Aldrich) stock solution was 10 mM in DMSO. For each determination, an equal volume of vehicle (DMSO) was added to control cells.

2.2. Cell viability assay

To assess cell viability, C6 cells were cultured at a density of 5×10^3 cells/well in a 96-well plate (Corning Inc.) and after 24 h, the serum-rich medium was refreshed. Following further 24 h, cells were incubated for 4 h with three Que concentrations: 25 μM, 50 μM, or 100 μM. Then, 1 mg/ml MTT was added to the medium, and after 3 h formazan crystals formed in the cells were dissolved in 100 μL DMSO. The absorbance was measured at 570 nm using a Multiskan FC ELISA reader (Thermo Fisher Scientific, Rodano-Italy). The viability is calculated as percentage of absorbance relative to control cells.

2.3. Rate of fatty acid and cholesterol synthesis

Lipogenic activity was monitored by analyzing the synthesis of lipids, through the incorporation of [1-^{14}C]acetate (16 mM, 0.96 mCi/mol) into total fatty acids and cholesterol (Natali et al., 2007). Cells were incubated for 3 h with 25 μM Que, then labelled acetate was added to the medium for 1 h. At the end of the experiment, cells were washed three times with ice-cold PBS to remove unreacted acetate, and the reaction was stopped by 1.5 ml of 0.5 N NaOH. Cells were collected into a test tube, and saponified with ethanolic KOH. Sterols and fatty acids were extracted and counted for radioactivity as reported (Natali et al., 2007).

2.4. Chromatographic analysis of lipid fractions and of newly synthesized fatty acids

Radiolabelled acetate incorporation was evaluated to follow the synthesis of complex lipids and of individual newly synthesized fatty acids. To this aim, cells were seeded and treated with Que, as described in the previous paragraph. Following two washing steps with PBS, reaction was blocked with 2 ml of KCl:CH_3OH (1:2, v/v). Total lipids were extracted as previously described (Natali et al., 2007) and resolved by thin layer chromatography on silica gel plates, using as developing system $CHCl_3$: CH_3OH: 28% NH_4OH (65:25:4) and hexane : ethyl ether : acetic acid (80:20:1) for phospholipids and neutral lipid analysis, respectively (Priore et al., 2017). Lipid spots were visualized by iodine vapor and scraped into counting vials for radioactivity measurement. Newly synthesized fatty acids were determined by HPLC analysis as reported (Priore et al., 2017). 20 μL of sample was injected into a Beckman Coulter System Gold Programmable Solvent Module 125 and furnished with a C18 ODS column (4.6 mm × 250 mm) and Diode Array Detector 168 (Beckman Coulter, Milan, IT). Eluted fractions were collected for radioactivity measurement.

2.5. Determination of de novo fatty acid synthesis enzyme activities

The activities of the lipogenic enzymes, ACC1 and FAS, were determined directly *in situ* in C6 cells permeabilized with digitonin (Natali et al., 2007). ACC1 activity was determined as the incorporation of [1-^{14}C]acetyl-CoA into fatty acids in an assay coupled with FAS activity, as reported (Natali et al., 2007). FAS activity was assayed by measuring the incorporation of radiolabelled acetyl-CoA into fatty acids essentially as described above for ACC1 activity, except that 0.2 mM malonyl-CoA was included and ATP, butyryl-CoA and FAS were omitted in the digitonin-containing assay mixture (Natali et al., 2007). After the incubation at 37 °C for 10 min, 100 μL of 10 M NaOH was added to the reaction mix and the samples were saponified by adding 5 ml of CH_3OH and boiling for 45–60 min in capped tubes. After acidification with 200 μL of 12 M HCl, fatty acids were extracted and counted for the radioactivity as in (Natali et al., 2007).

2.6. Assay of 3-hydroxy-3-methyl-glutaryl-CoA reductase activity

The activity of HMGCR, a rate limiting enzyme of cholesterol synthesis, was determined as described (Natali et al., 2007). Briefly, C6 cells were seeded at a density of 2×10^6 cells per 100 mm diameter Petri dish. Control cells and cells treated with 25 μM Que for 4 h were scraped into a buffer containing 50 mM Tris − HCl (pH 7.4) and 150 mM NaCl, collected by centrifugation at 900 × *g*, 4 °C, for 3 min, and kept at − 80 °C until use. The pellets were thawed, resuspended, and subsequently used for HMGCR activity assay. Reaction was conducted with 100–250 μg of protein extract in a buffer containing 0.1 M K_2HPO_4 (pH 7.5), 5 mM DTT, and 2.5 mM NADPH. After a preincubation for 10 min

at 37 °C, the reaction started by adding [3-^{14}C]HMG-CoA (75 mM, 1.8 Ci/mol) and kept for 120 min at 37 °C. Upon addition of 20 µl of 7 M HCl, synthesis of mevalonolactone was obtained by incubating the reaction mix for 1 h at 37 °C. Radioactive product was isolated by TLC using toluene-acetone (1:1) as the mobile phase. Silica spots were recovered and subjected to scintillation counting.

2.7. Isolation of RNA from C6 cells and real-time qPCR analysis

Total RNA was extracted from control cells and cells treated with 25 µM Que for 4 h and used for RT-qPCR analysis as described previously (Damiano et al., 2015). Quantitative gene expression analysis was performed using SYBR® Select Master Mix for CFX (Invitrogen) and 18S rRNA for normalization. The primers used for quantitative real-time PCR analysis for the determination of FAS, ACC1, HMGCR mRNA abundance have been previously reported (Gnoni et al., 2019). For the quantification of SREBP-1, SREBP-2 and ChREBP mRNA, the primers were: rSREBP1rev TAGTCGGTGGATGGGCAG, rSREBP2for AGACCAG GATCATCCAGCAG; rSREBP2rev CCATTGGCTGTCTGAGTCAA; rChREBPfor CGGGACATGTTTGATGACTATGTC; rChREBPrev AATAAA GGTCGGATGAGGATGCT.

2.8. Western blotting analysis

Cells grown in 100 mm dishes were treated with Que for 4 h. Determination of SREBP-1, SREBP-2, ACC1, FAS and HMGCR was performed on total protein extract, as previously described (Gnoni et al., 2012). Analysis of ChREBP level was performed on nuclear protein extract, obtained by lysis of purified nuclei in high-salt buffer (20 mM Tris−HCl [pH 7.9], 420 mM NaCl, 10 mM KCl, 0.1 mM Na_3VO_4, 1 mM EDTA, 1 mM EGTA, 20% [v/v] glycerol) (Gnoni et al., 2019). Samples containing an equal amount of proteins (50 µg) were loaded on 10% SDS-polyacrylamide gels. Following electrophoresis, proteins were electrophoretically transferred onto nitrocellulose membrane (BioTrace NT Nitrocellulose Transfer Membrane, Pall Life Science, Milan-IT). The filter was blocked with 5% (w/v) non-fat dried milk in buffered saline. Blots were incubated with specific primary antibodies directed against ACC1 (Merck Millipore, Milan-IT), FAS (Abnova, Taipei-Taiwan), HMGCR, SREBP-1, SREBP-2, ChREBP and β-actin (Santa Cruz Biotechnology, Dallas, TX-USA). The immune complexes were detected using peroxidase-conjugated secondary antibodies by chemiluminescence (Pierce™ ECL Plus Western Blotting Substrate, Thermo Fisher Scientific, Waltham, MA-USA). Densitometric analysis was carried out on the Western-blots using the NIH Image 1.62 software (National Institutes of Health, Bethesda, MD), normalizing to β-actin used as an internal control.

2.9. Statistical analysis

Results shown represent means ± SD of the number of experiments indicated in every case. In each experiment, determinations were carried out in triplicate. Statistical analysis was performed with Student's t-test.

3. Results

3.1. Effect of different concentrations of Que on cell viability

The effect of Que on C6 cells viability was evaluated by MTT test. After treatment with 25 µM Que for 4 h, no difference in cell viability was observed in treated with respect to untreated control cells (Fig. 1). This finding was corroborated by morphological observation, total protein content, and trypan blue exclusion (data not shown). A diminution of viability was evidenced when cells were treated with 50 µM or 100 µM of Que. Thus, all further experiments were performed on cells treated with 25 µM Que and incubated for 4 h in order to exclude putative toxic effects.

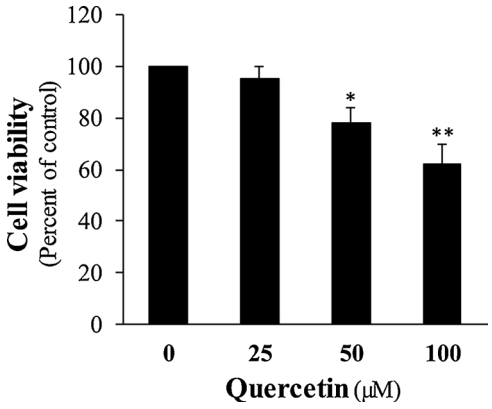

Fig. 1. Effect of Quercetin on C6 cell viability.
C6 cells were incubated for 4 h without (0 µM, control) or with 25 µM, 50 µM, and 100 µM Que in serum-rich medium. Cell viability was estimated by an MTT assay. Values, expressed as % of control, are means ± S.D., n = 5. (*$P < 0.05$ *vs.* control; **$P < 0.01$ *vs.* control).

3.2. Quercetin reduces fatty acid and cholesterol syntheses

To analyse the effects of Que on lipid synthesis, [1-^{14}C]acetate was added to cultured C6 cells, where the radiolabelled compound is transformed into acetyl-CoA, the common precursor for both fatty acid and cholesterol synthesis. As reported by histograms depicted in Fig. 2A, when compared to control cells, a significant (24%) diminution in the synthesis of radiolabelled fatty acids was observed in cells treated with 25 µM Que for 4 h. With respect to *de novo* lipogenesis, cholesterol synthesis was greater affected by Que. Actually, incubation of C6 cells with 25 µM Que caused a remarkable (55%) reduction of the radiolabelled acetate incorporation into cholesterol (Fig. 2B).

3.3. Addition of quercetin reduces radiolabelled acetate incorporation into phospholipids, neutral lipids and into newly synthesized fatty acids

As a successive step, we analysed radioactivity distribution among polar and neutral lipids, where fatty acids newly synthesised from labelled acetate were incorporated (Table 1). In Que-treated cells, a general decrease of labelled precursor incorporation into the different phospholipid fractions was observed. When results were expressed as percentage of control, a 33% decrease was detected concerning phosphatidylcholine, the most abundant phospholipid fraction in C6 glioma cells. Among neutral lipids, diacylglycerols, TAG and mainly cholesterol were the fractions showing a significant reduction in radioactivity incorporation following the Que addition to the cells.

In order to investigate the Que effect on the individual fatty acids synthesized from [1-^{14}C]acetate, HPLC analysis of total fatty acid extract was carried out. Fig. 3 shows that in control cells the incorporation of labelled acetate into the main individual fatty acids was as follows: palmitic acid (C16:0) > stearic acid (C18:0) > oleic acid (C18:1). Other longer chain and more unsaturated fatty acids were synthesized in small quantities, in agreement with previous observation that they are hardly detected in C6 cells (Robert et al., 1983). As depicted by histograms in Fig. 3, treatment with Que for 4 h caused a reduction of the radiolabelled incorporation into palmitic (which in the cell is the main product of *de novo* lipogenesis), stearic, and oleic acid.

3.4. Quercetin modulation of ACC1, FAS, and HMGCR activities

Next, to evaluate the enzymatic steps of the lipid biosynthetic pathways affected by Que, the activities of the key enzymes ACC1 and FAS for *de novo* fatty acid synthesis and of HMGCR for cholesterol

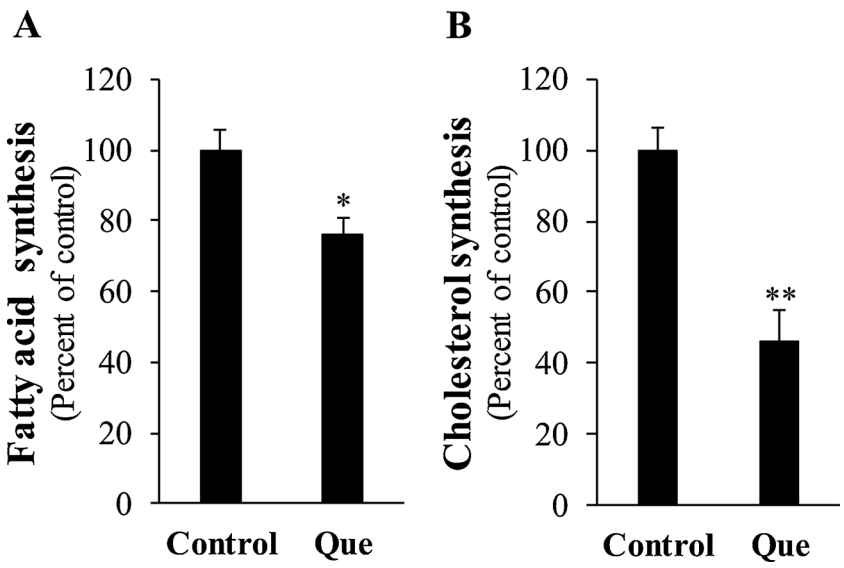

Fig. 2. Modulation of fatty acid and cholesterol syntheses by quercetin.
After an initial 48 h plating, C6 glioma cells, growing in serum-rich medium, were incubated for 4 h with 25 μM Que. During the last hour of incubation, labelled acetate was added and its incorporation into fatty acids (A) and cholesterol (B) was followed. Data, nmol [1-^{14}C]acetate inc./h/mg protein, are expressed as % of control and are means ± S.D. (n = 5). In each experiment, determinations were carried out in triplicate. In control cells, rates of cholesterol and fatty acid synthesis were 1.46 ± 0.09 and 8.44 ± 0.42 nmol [1-^{14}C] acetate inc./h/mg protein, respectively. (*P < 0.05 *vs.* control; **P < 0.01 *vs.* control).

Table 1
Effect of Que on [1-^{14}C]acetate incorporation into various lipid fractions in C6 cells.

Added effectors	None	Que	%
Polar lipids			
CL + PE	2151 ± 151	1658 ± 99*	77 %
PC	15908 ± 875	10597 ± 583*	67 %
SM	741 ± 67	634 ± 44	86 %
PS + PI	3781 ± 246	2172 ± 195**	57 %
Neutral lipids			
MAG	289 ± 20	256 ± 18	88 %
DAG	2704 ± 216	2015 ± 169*	74 %
Cholesterol	2155 ± 194	1225 ± 102**	57 %
Free fatty acids	239 ± 14	249 ± 15	104 %
TAG	1123 ± 73	772 ± 50*	69 %
CE	896 ± 63	987 ± 69	110 %

C6 cells were incubated with 25 μM Que for 4 h, and labelled acetate was added 1 h before ending the incubation. After total lipids extraction, phospholipids and neutral lipids were resolved by TLC, and the radioactivity associated with the different lipid fractions was counted. Values are expressed as cpm/mg protein ± SD, n = 5. CL: cardiolipin; PE: phosphatidylethanolamine; PC: phosphatidylcholine; SM: sphingomyelin; PS: phosphatidylserine; PI: phosphatidylinositol; MAG: monoacylglycerols; DAG: diacylglycerols; TAG: triacylglycerols; CE: cholesterol esters. (*P < 0.05 *vs.* control; **P < 0.01 *vs.* control).

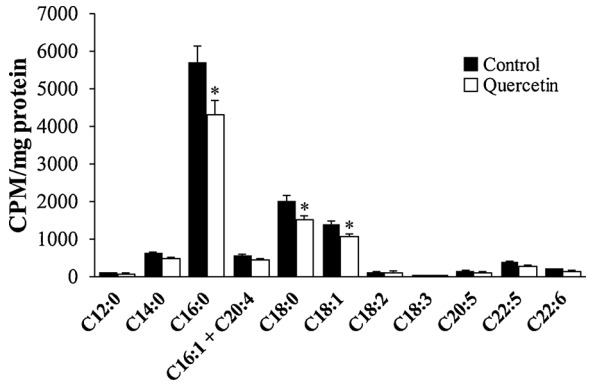

Fig. 3. Effect of quercetin on [1-^{14}C]acetate incorporation into individual fatty acids.
The effect of 25 μM Que on the incorporation of labelled acetate into different fatty acids were assayed. After 4 h of incubation, the radiolabelled neosynthesized fatty acids were extracted and separated by HPLC. Eluted fractions, corresponding to the different fatty acids, were collected for radioactivity measurement. Data, expressed as cpm/mg protein, represent means ± S.D., n = 5. Fatty acid: C12:0, lauric; C14:0, myristic; C16:0, palmitic; C16:1 palmitoleic; C20:4, arachidonic (ω-6); C18:0, stearic; C18:1, oleic; C18:2, linoleic (ω-6); C18:3, α-linolenic (ω-3); C20:5, eicosapentaenoic (ω-3); C22:6, docosahexanoic (ω-3).

synthesis were analysed.

4 h incubation of C6 cells with 25 μM Que caused a reduction of ACC1 activity by about 25% (Fig. 4). Conversely, the activity of FAS was not significantly affected by Que treatment. With respect to the untreated cells, HMGCR activity was remarkably lowered by 52% in Que-treated cells. Note that the entity of the decrease of ACC1 and HMGCR activities is in good accordance with the results of Fig. 2, regarding the reduction of total synthesis of fatty acids (Fig. 2A) and of cholesterol (Fig. 2B) starting from [1-^{14}C]acetate.

3.5. Quercetin regulation of ACC1, FAS, and HMGCR expression

The above-reported effects of Que on the changes in the enzymatic activities involved in lipid synthesis might be attributed to the modulation of gene expression. To verify this hypothesis, the abundance of mRNAs for ACC1, FAS, and HMGCR was quantified by real-time qPCR analysis.

Results depicted in Fig. 5A indicate that the treatment with Que caused a 39% diminution of HMGCR mRNA, whereas the ACC1 mRNA was approx. 20% less abundant with respect to the untreated control cells. The content of FAS mRNA resulted unaffected by Que treatment (Fig. 5A). Results obtained for HMGCR, ACC1, and FAS mRNA abundance were also confirmed by western blotting experiments carried out for the quantification of the respective protein contents (Fig. 5B).

3.6. Quercetin reduces the expression of SREBP-1, SREBP-2 and ChREBP lipogenic transcription factors

In Que-treated C6 cells, the mRNA level of SREBP-1 and SREBP-2 was significantly reduced with respect to the control (Fig. 6A). Then, we evaluated the level of SREBP-1 and SREBP-2 proteins in term of both precursor (p) and nuclear (n) forms. With respect to the untreated cells, the levels of the precursor and mature forms of both SREBP-1 and SREBP-2 proteins were reduced after Que treatment (Fig. 6B). Nuclear level of ChREBP protein has been also analyzed. Results reported in the Fig. 6 show that in nuclei from Que-treated C6 cells the ChREBP mRNA and protein levels decreased with respect to the untreated cells.

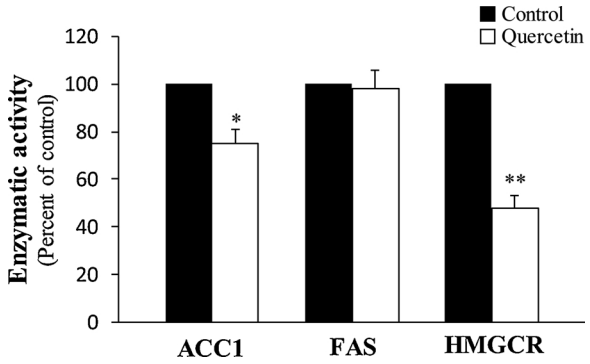

Fig. 4. Quercetin modulation of ACC1, FAS, and HMGCR activities.
After 4 h incubation with 25 μM Que, ACC1 and FAS enzyme activities were assayed in digitonin-permeabilized C6 cells. HMGCR activity was determined in C6 cell lysate. Values, expressed as percentage of control, are means ± SD of five independent experiments. Control-specific activities were: ACC1, 0.178 ± 0.011 nmol [1-^{14}C]acetyl-CoA inc./min/mg protein; FAS, 0.051 ± 0.003 nmol [1-^{14}C]acetyl-CoA inc./min/mg protein; HMGCR, 33.6 ± 1.9 pmol [3-^{14}C]HMG-CoA inc./min/mg protein. ACC1, acetyl-CoA carboxylase 1; FAS, Fatty acid synthase; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase.

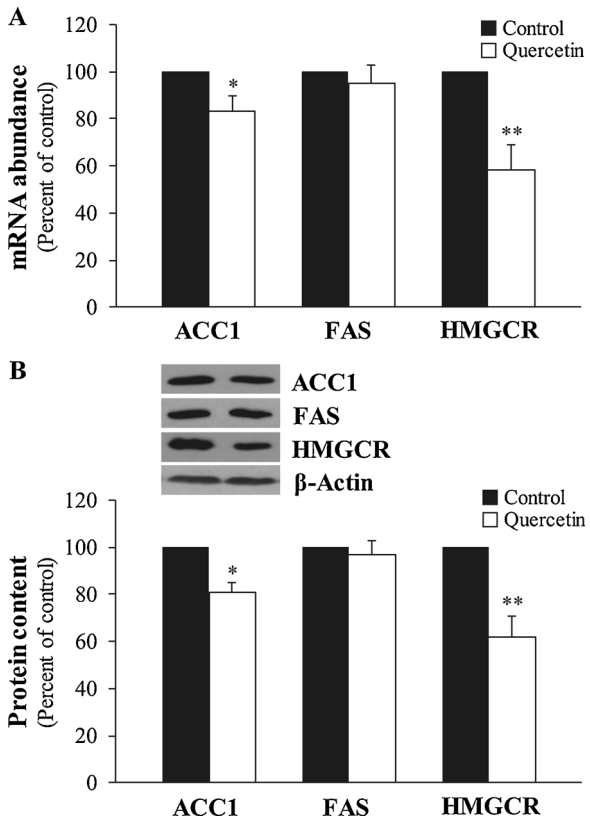

Fig. 5. Effect of quercetin on ACC1, FAS and HMGCR mRNA abundance and protein content in C6 glioma cells.
C6 cells were incubated with 25 μM Que for 4 h in serum-rich medium. (A) The abundance of ACC1, FAS, and HMGCR mRNA was determined by RT-qPCR and normalized with respect to β-actin mRNA, used as a reference. Normalized values are expressed in histograms as percentage of the control. Values are means ± S.D. of triplicate samples from each of four independent experiments. (B) C6 cells were lysed and protein content was isolated. ACC1, FAS and HMGCR protein amount were then assessed by Western blotting and quantified by densitometry. The protein contents are expressed as percentage of the control and are means ± SD, n = 3.

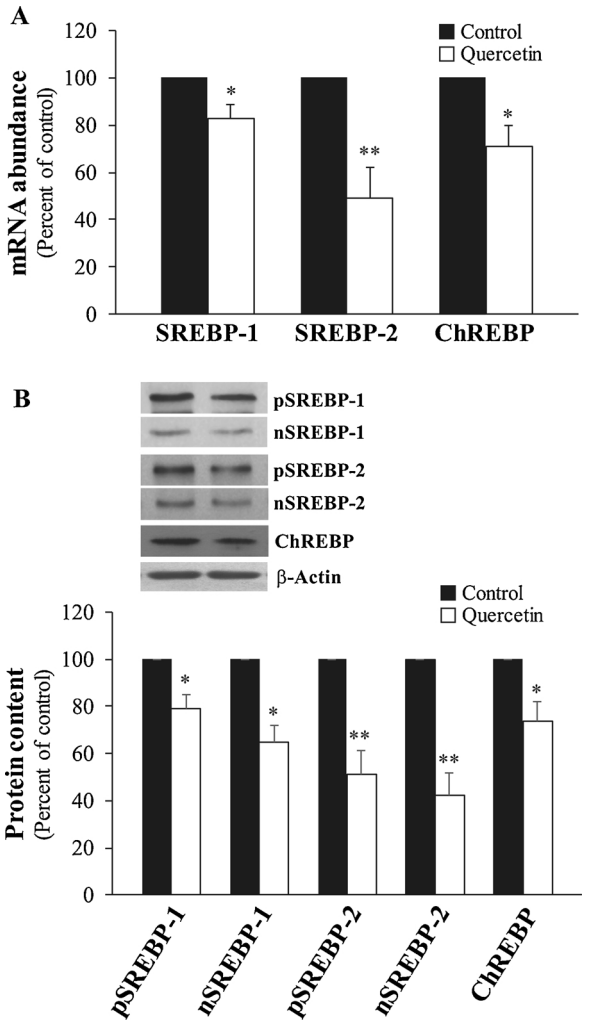

Fig. 6. Effect of quercetin on SREBP-1, SREBP-2 and ChREBP mRNA abundance and protein content in C6 glioma cells.
(A) The abundance of SREBP-1, SREBP-2, and ChREBP mRNA was determined by RT-qPCR and reported in histograms as described in Fig. 6. Values are means ± S.D. of triplicate samples from each of four independent experiments. (B) The levels of SREBP-1, SREBP2, and ChREBP in C6 lysates were assessed by Western blotting and quantified by densitometry. The protein contents are expressed as percentage of the control and are means ± SD of three independent experiments.

4. Discussion

Brain makes its own cholesterol *in situ* and very little circulating cholesterol enters into the brain owing to the impermeability of BBB (Pfrieger and Ungerer, 2011; Olsson et al., 2017), which represents a barrier also for fatty acids and complex lipids (Romano et al., 2017). *In vitro* studies have demonstrated that astrocytes, the major class of glial cells in the mammalian brain, synthesize and release lipids complexed to apolipoprotein E (Apo E)-containing lipoproteins (Zhang and Liu, 2015). Despite the great importance of lipid metabolism in brain development and homeostasis, the potential regulation of cholesterologenesis and lipogenesis by nutritional factors is poorly investigated in glial cells (Natali et al., 2007; Priore et al., 2017).

The present study represents the first information that Que, a common flavonoid of the human diet, rapidly (within 4 h) reduces cholesterol and, to a lesser extent, *de novo* fatty acid synthesis in C6 cells.

First, in agreement with previous reports (Natali et al., 2007; Priore et al., 2017), we show that in C6 cells cholesterol and fatty acid

syntheses are quite active being the [1-^{14}C] acetate incorporation into both these lipid fractions much higher than that reported in other lipogenic cells, such as isolated rat liver cells (Gnoni et al., 2009; Gnoni and Paglialonga, 2009).

The observed reduction of cholesterol synthesis by Que might be, at least partially, ascribed to the Que-induced decrease (52%) of HMGCR activity, the rate limiting and irreversible step of cholesterol synthesis.

Labelled acetate incorporation is reduced in most of lipid fractions, including CL + PE. Considering that CL is rich in unsaturated fatty acids (Acaz-Fonseca et al., 2019), its contribution to labelling should be low compared to PE. Therefore, Que should affect mainly PE synthesis.

As regards radioactivity distribution among newly synthesized fatty acids, the inhibitory effect of Que was mainly observed at the level of palmitic acid, the principal end product of *de novo* lipogenesis. This pathway is catalyzed by two enzymatic activities working in sequence, *i.e.* ACC1 and FAS.

While the activity of ACC1, the first committed step in fatty acid biosynthesis is significantly reduced, no change is observed in FAS activity. Thus, also the reduced labelled acetate incorporation into individual fatty acids and their subsequent esterification into complex lipids could be linked to the decreased ACC1 activity.

Note that the percentage of Que inhibition of HMGCR and ACC1 activities well fits with the Que-induced decrease of total fatty acid and cholesterol synthesis reported in Fig. 2.

The decrease in both mRNA abundance and protein amount of HMGCR and ACC1 we observed after Que addition to C6 cells indicates that transcriptional mechanisms are involved in the action of this flavonol. Results here reported are obtained by Que concentration of 25 μM, considered by some authors as a pharmacological dose (Casaschi et al., 2002). This concentration is effective on different metabolic pathways in other cell types (Gnoni and Paglialonga, 2009; Yang et al., 2008).

Cooperation between 25 μM Que and resveratrol in the induction of senescence-like growth arrest in rat C6 glioma cells has been described (Zamin et al., 2009). The rapid Que effect observed in the present study is corroborated by the observation that the highest plasma level of Que was detected 30 min after Que oral administration (Moon et al., 2001; Shimoi et al., 2003). The ability of Que to cross BBB following its oral administration has been reported (Ferri et al., 2015; Youdim et al., 2004). Our results also endorse the inhibitory effects on lipid biosynthesis by Que and by various phenolic compounds reported in other cell models (Gnoni and Paglialonga, 2009; Priore et al., 2015a, b). However, differently from the latter studies, the inhibitory action of Que we observed in C6 cells is more pronounced in the cholesterol synthesis with respect to the fatty acid synthesis and was greater than that observed in C6 cells treated with 25 μM hydroxythyrosol, a phenolic compound abundant in extra virgin olive oil (Priore et al., 2017).

The beneficial effects of Que *versus* several pathological states have been generally attributed to its antinflamatory and antioxidant role (Costa et al., 2016).

In the present study, it is reported that Que acts directly on lipid metabolism, by inhibiting the transcription of ACC1 and HMGCR, two regulatory enzymes along palmitate and cholesterol biosynthesis, respectively.

To further go in detail in the molecular mechanism of Que action, we also looked at the possible involvement of SREBP-1, SREBP-2 and ChREBP, the main transcription factors regulating lipogenic and cholesterologenic genes (Horton, 2002; Jois and Sleeman, 2017; Siculella et al., 2016). SREBPs are synthesized as inactive precursors (pSREBPs) bound to the endoplasmic reticulum membrane and activated in the Golgi apparatus by a two-step proteolytic cleavage, which releases the N-terminal mature (nSREBPs) transcription factors (Horton, 2002). ChREBP transactivation is controlled by a nuclei-cytoplasmic shuttling mechanism which depends on its phosphorylation status (Jois and Sleeman, 2017).

Inhibition of SREBP-2 observed in C6 cells upon Que treatment is of particular importance for the down-regulation of HMGCR expression. These data are supported by studies of chemical inhibition of HMGCR or gene silencing of SREBP-2 that highlight the importance of the SREBP-2/HMGCR axis in controlling cholesterol homeostasis in astrocytes, essential to the correct development and function of the brain (Ferris et al., 2017; Valenza et al., 2015; Tradtrantip et al., 2019). Analogously to SREBP-2, the expression of SREBP-1 and ChREBP, which participate in the regulation of lipogenic genes expression, are negatively affected by Que treatment. The transactivation of ChREBP is dependent on glucose metabolism. The Que inhibitory effect we observed on ChREBP level well agrees with the reduction of ACC1 expression (Fig. 5). To the best of our knowledge, no study has been so far reported evidencing the regulation of ChREBP in brain or in brain cell lines. Our findings are of particular importance on the basis that brain is mostly autonomous in lipid biosynthesis, which mostly comes from glucose source.

Modifications in the lipid composition or structure of cell membranes have been associated with the development of neurodegenerative diseases, such as Alzheimer (Simons et al., 1998; Kojro et al., 2001) and Parkinson (Vance, 2012). In this regard, some studies correlate the low incidence of neurodegenerative diseases with consumption of diets rich in Que (Commenges et al., 2000; Scarmeas et al., 2018). The anticancer properties of Que have been widely studied and include inhibition of the growth of cells derived from different human cancers (Zamin et al., 2014). In human malignant glial cells, compared with their normal counterparts, a very active *de novo* fatty acid and cholesterol syntheses, necessary for membranogenesis in cell proliferation (Lladó et al., 2014), have been reported (Prasanna et al., 1996).

Overall, our study shows that Que exerts on *de novo* fatty acid and cholesterol synthesis a rapid and direct inhibitory action, which occurs through transcriptional mechanisms involving the reduction of SREBP-1, SREBP-2 and ChREBP expression.

Based on the role of lipids on neuronal membrane functions and cell proliferation, our findings suggest a further mechanism by which Que elicits its beneficial effect *versus* neurodegenerative diseases (Costa et al., 2016) and cancer development (Murakami et al., 2008).

Funding

This work was in part supported by Ministero della Salute-Italy, grant: Project GR-2013-02355646.

References

Costa, L.G., Garrick, J.M., Roquè, P.J., Pellacani, C., 2016. Mechanisms of neuroprotection by quercetin: counteracting oxidative stress and more. Oxid. Med. Cell. Longev. 2016, 2986796.

Xia, S.F., Xie, Z.X., Qiao, Y., Li, L.R., Cheng, X.R., Tang, X., Shi, Y.H., Le, G.W., 2015. Differential effects of quercetin on hippocampus-dependent learning and memory in mice fed with different diets related with oxidative stress. Physiol. Behav. 138, 325–331.

Sabogal-Guáqueta, A.M., Muñoz-Manco, J.I., Ramírez-Pineda, J.R., Lamprea-Rodriguez, M., Osorio, E., Cardona-Gómez, G.P., 2015. The flavonoid quercetin ameliorates Alzheimer's disease pathology and protects cognitive and emotional function in aged triple transgenic Alzheimer's disease model mice. Neuropharmacology 93, 134–145.

Denny Joseph, K.M., 2015. Muralidhara. Combined oral supplementation of fish oil and quercetin enhances neuroprotection in a chronic rotenone rat model: relevance to Parkinson's disease. Neurochem. Res. 40, 894–905.

Murakami, A., Ashida, H., Terao, J., 2008. Multitargeted cancer prevention by quercetin. Cancer Lett. 269, 315–325.

Rivera, L., Moron, R., Sanchez, M., Zarzuelo, A., Galisteo, M., 2008. Quercetin ameliorates metabolic syndrome and improves the inflammatory status in obese Zucker rats. Obesity 16, 2081–2087 (Silver Spring).

Moon, J., Do HJ, K.O.Y., Shin, M.J., 2013. Antiobesity effects of quercetin-rich onion peel extract on the differentiation of 3T3-L1 preadipocytes and the adipogenesis in high fat-fed rats. Food Chem. Toxicol. 58, 347–354.

Gnoni, G.V., Paglialonga, G., Siculella, L., 2009. Quercetin inhibits fatty acid and triacylglycerol synthesis in rat-liver cells. Eur. J. Clin. Invest. 39, 761–768.

Kuipers, E.N., Dam, A.D.V., Held, N.M., Mol, I.M., Houtkooper, R.H., Rensen, P.C.N., Boon, M.R., 2018. Quercetin lowers plasma triglycerides accompanied by white adipose tissue browning in diet-induced obese mice. Int. J. Mol. Sci. 19, E1786.

Zhang, J., Liu, Q., 2015. Cholesterol metabolism and homeostasis in the brain. Protein

Cell 6, 254–264.
Adibhatla, R.M., Hatcher, J.F., 2008. Altered lipid metabolism in brain injury and disorders. Subcell. Biochem. 49, 241–268.
Morell, P., Jurevics, H., 1996. Origin of cholesterol in myelin. Neurochem. Res. 21, 463–470.
Horton, J.D., 2002. Sterol regulatory element-binding proteins: transcriptional activators of lipid synthesis. Biochem. Soc. Trans. 30, 1091–1095.
Camargo, N., Smit, A.B., Verheijen, M.H., 2009. SREBPs: SREBP function in glia-neuron interactions. FEBS J. 276, 628–636.
Tarr, P.T., Edwards, P.A., 2008. ABCG1 and ABCG4 are coexpressed in neurons and astrocytes of the CNS and regulate cholesterol homeostasis through SREBP-2. J. Lipid Res. 49, 169–182.
Ferré, P., Foufelle, F., 2010. Hepatic steatosis: a role for de novo lipogenesis and the transcription factor SREBP-1c. Diabetes Obes. Metab. 12, 83–92.
Jois, T., Sleeman, M.W., 2017. The regulation and role of carbohydrate response element-binding protein in metabolic homeostasis and disease. J. Neuroendocrinol. 29, e12473.
Natali, F., Siculella, L., Salvati, S., Gnoni, G.V., 2007. Oleic acid is a potent inhibitor of fatty acid and cholesterol synthesis in C6 glioma cells. J. Lipid Res. 48, 1966–1975.
Matias, I., Buosi, A.S., Gomes, F.C., 2016. Functions of flavonoids in the central nervous system: astrocytes as targets for natural compounds. Neurochem. Int. 95, 85–91.
Priore, P., Gnoni, A., Natali, F., Testini, M., Gnoni, G.V., Siculella, L., Damiano, F., 2017. Oleic acid and hydroxytyrosol inhibit cholesterol and fatty acid synthesis in C6 glioma cells. Oxid. Med. Cell. Longev. 2017, 9076052.
Damiano, F., Tocci, R., Gnoni, G.V., Siculella, L., 2015. Expression of citrate carrier gene is activated by ER stress effectors XBP1 and ATF6α, binding to an UPRE in its promoter. Biochim. Biophys. Acta 1849, 23–31.
Gnoni, A., Siculella, L., Paglialonga, G., Damiano, F., Giudetti, A.M., 2019. 3,5-diiodo-L-thyronine increases de novo lipogenesis in liver from hypothyroid rats by SREBP-1 and ChREBP-mediated transcriptional mechanisms. IUBMB Life 71, 863–872.
Gnoni, G.V., Rochira, A., Leone, A., Damiano, F., Marsigliante, S., Siculella, L., 2012. 3,5,3′triiodo-L-thyronine induces SREBP-1 expression by non-genomic actions in human HEP G2 cells. J. Cell. Physiol. 227, 2388–2397.
Robert, J., Montaudon, D., Hugues, P., 1983. Incorporation and metabolism of exogenous fatty acids by cultured normal and tumoral glial cells. Biochim. Biophys. Acta 752, 383–395.
Pfrieger, F.W., Ungerer, N., 2011. Cholesterol metabolism in neurons and astrocytes. Prog. Lipid Res. 50, 357–371.
Olsson, A.G., Angelin, B., Assmann, G., Binder, C.J., Björkhem, I., Cedazo-Minguez, A., Cohen, J., von Eckardstein, A., Farinaro, E., Müller-Wieland, D., Parhofer, K.G., Parini, P., Rosenson, R.S., Starup-Linde, J., Tikkanen, M.J., Yvan-Charvet, L., 2017. Can LDL cholesterol be too low? Possible risks of extremely low levels. J. Intern. Med. 281, 534–553.
Romano, A., Koczwara, J.B., Gallelli, C.A., Vergara, D., Micioni Di Bonaventura, M.V., Gaetani, S., Giudetti, A.M., 2017. Fats for thoughts: an update on brain fatty acid metabolism. Int. J. Biochem. Cell Biol. 84, 40–45.
Gnoni, G.V., Paglialonga, G., 2009. Resveratrol inhibits fatty acid and triacylglycerol synthesis in rat hepatocytes. Eur. J. Clin. Invest. 39, 211–218.
Acaz-Fonseca, E., Ortiz-Rodriguez, A., Garcia-Segura, L.M., Astiz, M., 2019. Sex differences and gonadal hormone regulation of brain cardiolipin, a key mitochondrial phospholipid. J. Neuroendocrinol. e12774.
Casaschi, A., Wang, Q., Dang, K., Richards, A., Theriault, A., 2002. Intestinal apolipoprotein B secretion is inhibited by the flavonoid quercetin: potential role of microsomal triglyceride transfer protein and diacylglycerol acyltransferase. Lipids 37, 647–652.
Yang, J.Y., Della-Fera, M.A., Rayalam, S., Ambati, S., Hartzell, D.L., Park, H.J., Baile, C.A., 2008. Enhanced inhibition of adipogenesis and induction of apoptosis in 3T3-L1 adipocytes with combinations of resveratrol and quercetin. Life Sci. 82, 1032–1039.
Zamin, L.L., Filippi-Chiela, E.C., Dillenburg-Pilla, P., Horn, F., Salbego, C., Lenz, G., 2009. Resveratrol and quercetin cooperate to induce senescence-like growth arrest in C6 rat glioma cells. Cancer Sci. 100, 1655–1662.
Moon, J.H., Tsushida, T., Nakahara, K., Terao, J., 2001. Identification of quercetin 3-O-beta-D-glucuronide as an antioxidative metabolite in rat plasma after oral administration of quercetin. Free Radic. Biol. Med. 30, 1274–1285.
Shimoi, K., Yoshizumi, K., Kido, T., Usui, Y., Yumoto, T., 2003. Absorption and urinary excretion of quercetin, rutin, and alphaG-rutin, a water soluble flavonoid, in rats. J. Agric. Food Chem. 51, 2785–2789.
Ferri, P., Angelino, D., Gennari, L., Benedetti, S., Ambrogini, P., Del Grande, P., Ninfali, P., 2015. Enhancement of flavonoid ability to cross the blood–brain barrier of rats by co-administration with α-tocopherol. Food Funct. 6, 394–400.
Youdim, K.A., Qaiser, M.Z., Begley, D.J., Rice-Evans, C.A., Abbott, N.J., 2004. Flavonoid permeability across an in situ model of the blood-brain barrier. Free Radic. Biol. Med. 36, 592–604.
Priore, P., Cavallo, A., Gnoni, A., Damiano, F., Gnoni, G.V., Siculella, L., 2015a. Modulation of hepatic lipid metabolism by olive oil and its phenols in nonalcoholic fatty liver disease. IUBMB Life 67, 9–17.
Priore, P., Caruso, D., Siculella, L., Gnoni, G.V., 2015b. Rapid down-regulation of hepatic lipid metabolism by phenolic fraction from extra virgin olive oil. Eur. J. Nutr. 54, 823–833.
Siculella, L., Tocci, R., Rochira, A., Testini, M., Gnoni, A., Damiano, F., 2016. Lipid accumulation stimulates the cap independent translation of SREBP-1a mRNA by promoting hnRNP A1 binding to its 5′-UTR in a cellular model of hepatic steatosis. Biochim. Biophys. Acta 1861, 471–481.
Ferris, H.A., Perry, R.J., Moreira, G.V., Shulman, G.I., Horton, J.D., Kahna, C.R., 2017. Loss of astrocyte cholesterol synthesis disrupts neuronal function and alters whole-body metabolism. Proc. Natl. Acad. Sci. U. S. A. 114, 1189–1194.
Valenza, M., Marullo, M., Di Paolo, E., Cesana, E., Zuccato, C., Biella, G., Cattaneo, E., 2015. Disruption of astrocyte-neuron cholesterol cross talk affects neuronal function in Huntington's disease. Cell Death Differ. 22, 690–702.
Tradtrantip, L., Duan, T., Yeaman, M.R., Verkman, A.S., 2019. CD55 upregulation in astrocytes by statins as potential therapy for AQP4-IgG seropositive neuromyelitis optica. J. Neuroinflammation 16 (57).
Simons, M., Keller, P., De Strooper, B., Beyreuther, K., Dotti, C.G., Simons, K., 1998. Cholesterol depletion inhibits the generation of beta-amyloid in hippocampal neurons. Proc. Natl. Acad. Sci. U.S.A. 95, 6460–6464.
Kojro, E., Gimpl, G., Lammich, S., Marz, W., Fahrenholz, F., 2001. Low cholesterol stimulates the nonamyloidogenic pathway by its effect on the alpha–secretase ADAM 10. Proc. Natl. Acad. Sci. U.S.A. 98, 5815–5820.
Vance, J.E., 2012. Dysregulation of cholesterol balance in the brain: contribution to neurodegenerative diseases. Dis. Model. Mech. 5, 746–755.
Commenges, D., Scotet, V., Renaud, S., Jacqmin-Gadda, H., Barberger-Gateau, P., Dartigues, J.F., 2000. Intake of flavonoids and risk of dementia. Eur. J. Epidemiol. 16, 357–363.
Scarmeas, N., Anastasiou, C.A., Yannakoulia, M., 2018. Nutrition and prevention of cognitive impairment. Lancet Neurol. 17, 1006–1015.
Zamin, L.L., Filippi-Chiela, E.C., Vargas, J., Demartini, D.R., Meurer, L., Souza, A.P., Bonorino, C., Salbego, C., Lenz, G., 2014. Quercetin promotes glioma growth in a rat model. Food Chem. Toxicol. 63, 205–211.
Lladó, V., López, D.J., Ibarguren, M., Alonso, M., Soriano, J.B., Escribá, P.V., Busquets, X., 2014. Regulation of the cancer cell membrane lipid composition by NaCHOleate: effects on cell signaling and therapeutical relevance in glioma. Biochim. Biophys. Acta 1838, 1619–1627.
Prasanna, P., Thibault, A., Liu, L., Samid, D., 1996. Lipid metabolism as a target for brain cancer therapy: synergistic activity of lovastatin and sodium phenylacetate against human glioma cells. J. Neurochem. 66, 710–716.