

Molecular Mechanisms of Flavonoids Regulating Cellular Processes in Human Fibroblasts

Marta Moskot

Flavonoids which are ubiquitous plant secondary products best known as the characteristic red, blue, and purple pigments of plant tissues, are essential part of daily dietary sources, like fruits and vegetables, but they are also plenty in tea, cocoa and wine. In higher plants, flavonoids are involved in UV filtration, symbiotic nitrogen fixation and floral pigmentation. They consist of over 8,000 already-identified compounds that have a common chemical structure and may be further divided on the basis of their molecular structure into subclasses like: anthocyanidins, flavanols, flavanones, flavonols, flavones and isoflavones. The best-described property of almost every group of flavonoids is their capacity to act as antioxidants and anti-inflammatory factors. They are considered as therapeutic molecules for cancer, infections and also some genetic diseases, for example cystic fibrosis and mucopolysaccharidoses. Therefore, determining mechanisms of their biological activities is very important to assess safety of these compounds and to develop optimal therapeutic procedures. Many of the biological effects of flavonoids appear to be related to their ability to modulate cell-signaling pathways, rather than their antioxidant activity. Genistein (an isoflavone), kaempferol (a flavonol) and daidzein (another isoflavone) were previously found to be able to reduce efficiency of glycosaminoglycan (GAG) synthesis in cells of patients suffering from mucopolysaccharidoses (MPSs), inherited metabolic diseases with often brain disease symptoms. It is worth to mention that these flavonoids can cross the blood-brain barrier (BBB), what makes reasonable considering these compounds as potentially useful in the optimization of treatment for neuronopathic forms certain diseases.

Among different flavonoids genistein (5,7-dihydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one) has been studied most intensively, and it was proposed that this compound can down regulate GAG production by blocking phosphorylation of the epidermal growth factor receptor (EGF receptor, EGFR), thus impairing a signal transduction pathway necessary for activation of genes coding for enzymes involved in this anabolic process. In contrary to genistein it was demonstrated that, other flavonoids were not effective in inhibiting EGFR phosphorylation, however, the exact mechanism of action of flavonoids as genetic regulators of GAG turnover remained to be elucidated.

Lysosomal storage diseases (LSDs) are a group of over 50 rare inherited metabolic

disorders that result from defects in lysosomal function, leading usually to deficiency of a single enzyme required for the metabolism of various macromolecules. LSDs are classified by the accumulated substrate and include the sphingolipidoses, oligosaccharidoses, mucolipidoses, mucopolysaccharidoses, lipoprotein storage disorders, lysosomal transport defects, neuronal ceroid lipofuscinoses, and others. Impaired hydrolysis of substrates leads to their accumulation in cells of patients, which results in a progressive damage of the affected tissues and organs, including the heart, respiratory system, bones, joints and central nervous system (CNS). One of several factors contributing to the molecular pathogenesis and clinical form of LSDs is the level of residual catabolic activity. Correlation between functional residual catabolic activity and the progression of the lipid storage disorder was basically confirmed for different clinical forms of diseases such as metachromatic leukodystrophy, GM2-gangliosidosis, Gaucher disease and Niemann-Pick type A and B diseases.

Mucopolysaccharidoses are autosomal, or X-linked (type II) recessive lysosomal storage disorders caused by the deficiency in activity of a lysosomal enzyme involved in catabolism of glycosaminoglycans (chondroitin, dermatan, heparan and keratan sulfates; CS, DS, HS and KS, respectively). Although all types of mucopolysaccharidoses (MPS I, II, III, IV, VI, VII and IX), are caused by mutations in single genes, their pathomechanisms are more complicated than just accumulation of non-degradable compounds in cells. Despite the fact that the storage is the primary effect of each MPS-causing mutation, there are various secondary and tertiary effects that lead to a complicated picture of each MPS type and subtype, as well as to a high variability of symptoms among patients suffering from the same disease.

Sphingolipidoses (SLs) are heterogeneous group of inherited disorders of lipid metabolism where lysosomal accumulation of sphingolipids occurs. Predominantly it appears in cells and organs that have the highest rates of biosynthesis or uptake of the undegradable sphingolipids and their precursors, mostly in the central nervous system. Sphingolipid accumulation also occurs in a group of SLs in which the primary issue is not a lack of a degradation enzyme. Mostly these diseases are associated with problems with trafficking and fusion in the endocytic system, leading to secondary storage of sphingolipids. Niemann-Pick type C (NPC), a disease thought to be mainly a disorder of cholesterol transport from the lysosome is an extreme example of this.

Currently, bone marrow or hematopoietic stem cell transplantations (respectively BMT, HSCT) and enzyme replacement therapy (ERT) are the only approved treatments of LSDs. However, neurological symptoms, developing due to undegraded substrate

accumulation in CNS, cannot be managed by ERT owing to an inefficient delivery of proteins through the blood-brain barrier. One of potential therapeutic method for LSDs is substrate reduction therapy (SRT). This kind of therapy is based on an assumption that inhibition of synthesis of compounds that cannot be efficiently degraded may facilitate an establishment of a new balance between their production and degradation, already lost due to a defect in a specific hydrolase. Genistein, kaempferol and daidzein were demonstrated previously to significantly inhibit synthesis and reduce levels of GAGs in cultures of fibroblasts and in MPS mice.

The overall objective of my work was to investigate the mechanism of flavonoids action on cellular processes in human dermal fibroblasts. Tested flavonoids were chosen taking on account data about inhibition of synthesis and reduced levels of GAGs in cell cultures. Genistein, kaempferol, daidzein and mixtures of genistein and kaempferol, and also genistein and daidzein were dissolved in dimethyl sulfoxide (DMSO) and added in the indicated final concentrations as determined previously to cell cultures (30, 60 and 100 µM for genistein as well as for kaempferol, 60 and 100 µM for daidzein; and 30 µM for genistein and kaempferol, and genistein and daidzein mixtures, each). Experimental model consist of *in vitro* cultures of HDFa (*Human Dermal Fibroblasts adult*) cell line, but also MPS II cells, HeLa (cervical cancer cell line) and MEF (*Mouse Embryonic Fibroblasts*). In order to investigate more extensively the effects of flavonoids in cell culture models, DNA microarrays Illumina's Human HT-12 v3 and v4 Expression BeadChips were used to assess the global effects of these compounds on gene expression in human dermal fibroblasts. In the light of the above described facts and uncertainty on mechanisms of flavonoid-mediated gene expression regulation, the activity of transcriptome of the cell line HDFa was profiled microarrays in the presence or absence of various flavonoids or their mixtures. The comparison of relative fold change of gene expression levels between flavonoid-treated and non-treated cells was performed. Microarray analysis of transcripts showed those with expression identified as different by at least 0.5 and 2 fold change ($p < 0.05$) following flavonoids' exposure in relation to control of non-treated samples. Concentration- and time-dependent effects of tested flavonoids on global gene expression in fibroblasts were observed [1,2]. The highest number of genes with modulated expression was observed for kaempferol and genistein-kaempferol mix treatment type of fibroblasts after both 24 and 48 hours [2]. In total, 698 and 362 for 24 h, and 1506 and 1328 transcripts for 48 h handling with 100 µM kaempferol and mix of genistein with kaempferol (30 µM each), respectively, were affected. In the course of the study, 436 (100 µM kaempferol), 242 (genistein and kaempferol, 30 µM

each), 41 (100 μ M daidzein) and 24 (genistein and daidzein, 30 μ M each) transcripts, which levels were affected after both 24 and 48 hour treatment time period was observed while 263 for 100 μ M genistein [1,2]. Analysis of selected genes, which signals in the microarray experiments suggested a significant influence of tested compounds on their expression, were repeated using real-time qRT-PCR [1-4]. Conducted experiments involved not only HDFa cells, but also fibroblasts derived from a patient suffering from MPS II [2]. Importantly, both microarray and real-time qRT-PCR analyses gave similar results, indicating accuracy of both methods [1-4].

Moreover modulated metabolism pathways were defined according to the KEGG annotation (*Kyoto Encyclopedia of Genes and Genomes*) and AmiGO. Gene Ontology analysis and data visualization were performed on the up-regulated and down-regulated gene lists using the web tools GOrilla (*Gene Ontology enRICHment anaLysis and visuaLizAtion tool*) and REViGO (*REduce + VIsualize Gene Ontology*) restricting the output to biological process and cell compartment. Interestingly, the analysis of ‘Cellular Compartment’ as well as ‘Biological Processes’ terms showed that enrichments for categories linked to lysosome were among the significant ones with positively modulated expression in case of genistein, kaempferol and their mixture treatment [1,2]. Taking on account negative modulation – cell cycle and DNA metabolism were mostly represented ones. Gene Set Enrichment Analysis (GSEA) performed on the up-regulated gene lists has shown lysosomal metabolism pathway to be most enriched in genistein, kaempferol and genistein and kaempferol mixture conditions. Analysis done for down-regulated genes has confirmed dominant effect on cell cycle and replication. No evident results were seen both for daidzein and genistein and daidzein mixture. The comparison of relative fold change of gene expression levels between flavonoid-treated and non-treated cells was performed for genes of fibroblasts treated with genistein, kaempferol, and genistein and kaempferol mixture with selected metabolism pathways defined according to the KEGG annotation and AmiGO [2].

Both, microarray and real-time qRT-PCR analyses indicated that selected flavonoids influence expression of several genes involved in GAG metabolism [1]. Although the gene expression changes observed were rather subtle, they appeared to be relevant in cellular GAG level normalization. Among genes coding for enzymes involved in GAG biosynthesis pathway and required for production of chondroitin, dermatan, heparan and keratan sulfates, expressions of *EXT1*, *ST3GAL2* and *XYLT1* were significantly impaired in the presence of genistein. *EXT1* and *XYLT1* code for enzymes acting at very early stages of production of heparan and chondroitin/dermatan sulfates, while the enzyme encoded by *ST3GAL2* catalyzes

the initial phase of keratan sulfate II synthesis pathway [1,2]. These results were mostly observed in genistein treated fibroblasts. Therefore, the final effect of the action of genistein is a decreased efficiency of production of CS, DS, HS and KS. The results suggested that tested flavonoids may differentially influence GAG metabolism, indeed. Moreover, changes in expressions of selected genes in flavonoid-treated cells, relative to controls, were similar in both wild-type and MPS II fibroblasts [2]. Interestingly, effects of genistein were more similar to those of kaempferol than daidzein [2]. One might suppose that enhanced synthesis of products of genes coding for lysosomal hydrolysis in the presence of genistein could result in an increased residual activity of the particular deficient enzyme. By monitoring particular mRNA levels in human fibroblasts and mouse embryonic fibroblasts, with the use of real-time qRT-PCR, high content imaging and western blot, respectively, it was found that genistein stimulates expression of the transcription factor EB (TFEB) demonstrated previously to act as a master positive regulator of lysosomal biogenesis [1]. The necessary TFEB shuttling from cytoplasm to the nucleus was also induced by genistein [1]. In addition to genistein-mediated induction of TFEB target genes, an increase in lysosomal amount was also observed [1]. Despite differences in gene expression modulation kaempferol, and also genistein and kaempferol mixture stimulated expression of transcription factor EB significantly [2].

When looking at particular studied conditions, numerous of transcripts with altered expression (59 among 121 genes) of glycosphingolipid (GSL) metabolism were regulated at least in one experimental condition [3]. Interestingly, 16 genes from them are associated with well-known sphingolipids disorders: *ARSA* (metachromatic leukodystrophy, MLD), *ASAHI* (Farber lipogranulomatosis), *CLN8* (neuronal ceroid lipofuscinosis, late-Infantile Neuronal Ceroid Lipofuscinosis variant, CLN8), *GALC* (Krabbe disease), *GBA1* (Gaucher disease; Lewy body dementia, LBD), *GLA* (Fabry disease), *GM2A* (Tay-Sachs disease AB variant), *HEXA* (Tay-Sachs disease), *HEXB* (Sandhoff disease), *NAGA* (Schindler/ Kanzaki disease), *NEU1* (sialidosis, salactosialidosis, mucolipidosis I), *NPC1* (Niemann-Pick disease type C), *NPC2* (Niemann-Pick disease type C), *PPT1* (Neuronal ceroid lipofuscinosis, Infantile Neuronal Ceroid Lipofuscinosis, INCL, CLN1), *SMPD1* (Niemann-Pick disease NPD type A and B) and *SUMF1*(Multiple sulfatase deficiency, MSD) [2,3].

As it was observed previously, flavonoids can influence cell cycle and DNA metabolism, but detailed mechanisms of their actions was not completely understood, especially in untransformed human cell lines. To address these issues, methods like: transcriptomic approach, i.e. a microarray gene profiling and a real-time qRT-PCR to examine gene expression, as well as cell viability and cell cycle progression tests to study

development of fibroblasts exposed to different concentrations of tested compound, were utilized [4]. Both microarray and real-time qRT-PCR analyses indicated that flavonoids significantly influence expression of many genes involved in cell cycle control at its various stages and DNA replication regulation [4]. Among them, genistein was found as the most significantly modulating, in a time- and dose-dependent manner, compound of activity of studied genes, whose products are involved in different phases of the cell cycle and/or in regulatory processes important for DNA replication and cell growth [4]. It considerably reduced the efficiency of expression of genes coding for MCM2-7 and MCM10 proteins, as well as some other proteins involved in the S phase control [4]. Moreover, genistein caused cell cycle arrest in the G2/M phase, which was accompanied by activation of *CDKN1A*, *CDKN1C*, *CDKN2A*, *CDKN2B*, *CDKN2C* and *GADD45A* genes, as well as down-regulation of several mRNAs specific for this stage, demonstrated by transcriptomic assessments [4]. Addition of genistein to HDFa fibroblasts culture resulted in observation that in cells treated with 60 and 100 µM of this isoflavone, the percentage of G0/G1 cells initially (at 24 h) increased in respect to DMSO and 30 µM genistein and thereafter gradually decreased after 48 and 72 h [4]. As to the S phase, genistein slightly decreased the population of HDFa fibroblasts in a dose-dependent manner, while it induced cell number in the G2/M phase, especially at 72 h [4]. The concentrations greater than 30 µM genistein were necessary to alter cell cycle progression, with maximal accumulation of G2/M cells observed at 100 µM [4]. Furthermore, the analysis of genistein effect on fibroblast migration by *in vitro* scratch assay showed acceleration of movement of cells treated with this compound when coupled with control untreated fibroblasts, with time- and dose-dependent alterations, thus revealing *in vitro* wound healing properties of genistein [4].

Current findings comprising my PhD thesis allowed to learn about a putative flavonoid targetome responsible for significant molecular processes, such as impairment of production and enhancement of degradation of glycosaminoglycans and sphingolipids [1,2,3] or cell cycle and DNA replication regulation [4]. Model of genistein-directed lysosomal biogenesis modulation was described for the first time demonstrating directly that this naturally occurring compound alters expression of genes involved in lysosomal metabolism [1]. It is tempting to speculate that because of upregulation of lysosomal hydrolases transcripts, genistein and other tested flavonoids may be considered as potential drugs in treatment of LSD, especially mucopolysaccharidoses and sphingolipidoses [1,2,3]. Results of transcriptomic analysis supported by *in vitro* proliferation and cell cycle studies, obtained with the human fibroblasts model, indicate that flavonoids may regulate those processes in

human cells due to modulation of expression of a relatively large group of genes whose products are involved in these processes [4]. All these findings included in my dissertation enabled to elucidate flavonoids' impact on human fibroblasts' cellular processes making a great input on nature of this compounds and most of all revealing a new field to be explored.

Literature references:

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