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CROATICA CHEMICA ACTA
CCACAA **78** (3) 337–342 (2005)

ISSN-0011-1643
CCA-3017
Original Scientific Paper

Monitoring Flavonoid Metabolism in Human Cells by Exploiting Fluorescence Elicited upon Quercetin/Protein Interactions*

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RECEIVED NOVEMBER 26, 2004; REVISED MARCH 2, 2005; ACCEPTED MARCH 15, 2005

Despite the wealth of information concerning biological effects of flavonoids, a systematic approach to analyzing the molecular targets is still lacking and, for this reason, a rational evaluation of the risks or benefits of flavonoid-containing foods or of possible pharmaceutical applications is difficult. We have exploited the property of quercetin to elicit fluorescence when bound to specific target proteins and assayed several flavonoids with different modifications (methylation, hydroxylation, glycosylation). Quercetin target proteins can be visualized in living cells, but in vital human leukaemia cells (HL-60) the fluorescence decreases rapidly after labelling, while metabolically inactive apoptotic cells retain the fluorescence. These cytological differences were apparent under the fluorescent microscope and were quantified using flow cytometry. Metabolic conversion of quercetin in vital cells was confirmed and quantified by HPLC analysis. While apoptotic cells still contained considerable amounts of quercetin, vital cells rapidly metabolized the flavonoid (*e.g.,* by methylation or glycosylation). Biochemical results are consistent with the cytological observations and support the conclusion that quercetin becomes rapidly converted to non-fluorogenic metabolites in vital cells. Loss of fluorescence in vital cells allows convenient monitoring and quantifying of the dynamics of quercetin metabolism in human cells.

Keywords
quercetin
protein targets
flavonoid/protein fluorescence
flavonoid metabolism
leukaemia cells

INTRODUCTION

Flavonoids are common plant intermediary products present in human food and they are generally considered to be beneficial, an assumption that is largely based on the anti-oxidative properties of at least some members of this very heterogeneous family of substances. Only in a few cases have the interactions of flavonoids with defined cellular proteins been studied in greater detail. Estrogenic properties of some isoflavonoids such as genistein are good examples. Its interaction with the estrogen receptor (as agonist or antagonist) has nourished the hope that compounds that possess therapeutical potential might be identified.[1] However, genistein inhibits tyrosine kinase and several other enzymes, so complex biological reactions must be expected.[2] Apparently, flavonoids bind

* Dedicated to Professor Željko Kućan on the occasion of his 70th birthday. Presented at the Congress of the Croatian Society of Biochemistry and Molecular Biology, HDBMB2004, Bjelolasica, Croatia, September 30 – October 2, 2004.

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to several – perhaps numerous – target proteins, resulting in complex biological effects that are difficult to interpret. This also holds for the flavonol quercetin, which is known to inhibit several signal transduction targets, and which has been tested in phase I clinical trials because of its cytostatic properties.[3]

A further complication in the analysis of flavonoid biology is the rapid metabolism in the vertebrate body and the generation of metabolites which also possess biological activity and will presumably bind to another set of target proteins. As a consequence, *in vitro* and *in vivo* experiments might give conflicting results, an observation that has been made in studies on the inhibitory activity of quercetin on steroidogenic enzymes.[4] When related flavonoids are compared with respect to their biological effects, it is apparent that small structural differences like, for example, hydroxyl or prenyl groups alter the biological activity significantly.[5,6,7] These problems are by no means peculiar to flavonoids. The target specificity and metabolic conversion of a drug of interest is a common pharmacological problem, which is reflected in unwanted »side effects« and poses a formidable challenge to pharmaceutical industry.

We have chosen the flavonol quercetin, which is present in many vegetables and fruits such as oranges, apples, onions, and lettuce,[8] as the model substance to address the above-mentioned problems since quercetin elicits fluorescence upon binding to target proteins.[6,9–12] Fluorescence can be measured *in vitro* using, for example, human serum albumin as quercetin-binding protein. In addition, quercetin target proteins can be visualized in living cells, as it has been demonstrated on the example of *Drosophila* follicles.[6] The respective target molecules in living cells are not known yet, but the elicitation of fluorescence offers the unique possibility of biochemically isolating the target proteins and identifying the binding motives. Another experimental option is to monitor the presence of quercetin in the cells and its disappearance due to metabolic conversion by attenuation of the fluorescent signal. The present communication elaborates this experimental strategy.

EXPERIMENTAL

Cell Cultures

Acute myeloid leukaemia cells (HL-60, DSMZ, Germany) were maintained in RPMI 1640 medium (Biochrom, Germany) with 10 % heat-inactivated foetal calf serum (FCS; Gibco, France). Cells were grown at 37 °C in a humidified 5 % CO_2 atmosphere and maintained at a density of 2×10^5 to 1×10^6 cells/ml by re-suspending the cells in fresh culture medium every 2 days. In order to study quercetin metabolism in apoptotic cells, HL-60 cell cultures were exposed to heat shock (HS, 45 °C, 60 min) 24 h before incubation with a quercetin-containing medium. The chosen stress-condition was previously shown to efficiently induce apoptosis in HL-60 cells.[13] The cultures were agitated under constant gentle shaking (ST3 Shaker, ELMI, Germany). Under these conditions, 90 ± 5 % of the cells were apoptotic. The percent of vital and apoptotic cells in the control and HS-treated cultures was determined by the flow cytometric analysis of the forward (FSC) and side scatter (SSC) parameters.[14] The cells were centrifuged for 10 min at 100 *g* and the cell pellet was suspended in RPMI-1640 medium without FCS. The cells were washed twice in the same medium and cultured in 6-well plates (5 ml/well). Heat shocked cells and cells cultured at normal temperature (37 °C) were exposed to quercetin (100 μmol dm^{-3}) for 10, 20, 30, 60, 120, 180 and 240 minutes at 37 °C under constant gentle shaking as described above. Since the quercetin stock solutions (dilution 1:1000) were prepared in DMSO, all cultures including the control samples contained 0.1 % of DMSO. Finally, the cells were centrifuged for 10 min at 100 *g* and analyzed as described below.

Flow Cytometry

The HL-60 cell cultures were analyzed by flow cytometry (CyFlow, Partec, Germany) and the level of quercetin-specific fluorescence was quantified in vital and apoptotic cells. Between $(0.5–1.0) \times 10^6$ cells per sample were analyzed with respect to the parameters FSC, SSC and the green fluorescence signal emitted by quercetin bound to cellular proteins (excitation 473 nm, emission 520 nm). For each variable (exposure conditions, culture periods, *etc.*) a minimum of 5 samples was quantified. The flow cytometer was calibrated using 2.5 mm polyfluorescent AlignFlow beads (Molecular Probes, Eugene, OR) before each series of measurements. The fraction of cells present in different cell populations and the level of quercetin fluorescence were calculated using the CyFlow software (Partec, Germany).

Biochemical Analysis of Quercetin and Metabolites

HL-60 cells and the corresponding culture supernatant were prepared as described above. To each cell pellet, 100 ml H_2O containing 1 mmol dm^{-3} ascorbic acid was added. Proteins were precipitated and the flavonoids were extracted by adding 2.5 vol. acetonitrile. The mixture was continuously shaken at room temperature for 10 min. It was then centrifuged for 10 min at 10 000 *g* at room temperature. The supernatant was collected and evaporated to 50 μl under a stream of nitrogen. The final volume of 100 μl was filled up with methanol. The total extract was subjected to HPLC analysis (see below). Ascorbic acid was added directly to the cell culture supernatant to make a final concentration of 1 mmol dm^{-3}, followed by addition of 2.5 vol. acetonitrile. The extraction procedure was as described for the cell pellet, only the solvent was removed by rotary evaporation to dryness. The residue was taken up in 50 % aqueous methanol and analyzed by HPLC.

For hydrolysis of conjugated flavonoids, the extracts obtained by the procedure described above were re-suspended directly in a solution of 50 % methanol in 2 M HCl, con-

TABLE I. List of flavonoids tested for elicited fluorescence in combination with BSA[a]

No.	Systematic name	Generic name (OH groups)
1	3-hydroxyflavone	flavonol (1)
2	3,7-dihydroxyflavone	(2)
3	3,6-dihydroxyflavone	(2)
4	3,5,7-trihydroxyflavone	galangin (3)
5	3,4',5,7-tetrahydroxyflavone	kaempferol (4)
6	3,3',4',7-tetrahydroxyflavone	fisetin (4)
7	2',3,4',5,7-pentahydroxyflavone	morin (5)
8	3,3',4',5,7-pentahydroxyflavone	quercetin (5)
9	3,3',4',5,5',7-hexahydroxyflavone	myricetin (6)
10	3'-methoxy-3,4',5,7-tetrahydroxyflavone	isorhamnetin (4)
11	3,3',4',5,7-pentahydroxyflavone 3-*O*-glucoside	quercetin 3-*O*-glucoside (4)
12	3'-methoxy-3,4',5,7-tetrahydroxyflavone 3-*O*-glucoside	isorhamnetin 3-*O*-glucoside (3)
13	3,3',4',5,5',7-hexahydroxyflavone 3-*O*-glucoside	myricetin 3-*O*-glucoside (5)

[a] See Figure 1.

taining 1 % butylhydroxytoluene. The mixture was incubated in sealed vials for 2 h at 85 °C under continuously shaking. The efficiency of the method was checked with a solution of rutin, which gave rise to complete hydrolysis, as evidenced by the only peak corresponding to quercetin after HPLC analysis (see below).

Total extract (100 μl) was subjected to HPLC (Jasco BT 8100 pumps, Germany) coupled to an autosampler (Jasco AS-1550, Germany), equipped with a 4 mm × 125 mm Lichrosorb-100 C_{18} 5μ, reverse phase column and a Multiwavelength Diode Array detector (Jasco MC-919, Germany) set between 200 and 600 nm. 2.5 % aqueous acetic acid (solvent A) and 100 % acetonitrile (solvent B) were used as solvents. The acetonitrile concentration (solvent B) was 3 % at the beginning of the analysis and changed as follows: 9 % (5 min), 16 % (10 min), 50 % (30 min), 50 % (5 min), followed by an equilibration step to the initial analysis conditions (3 %). The flow rate was 1 ml min^{-1}. The BORWIN chromatography software (JMBS Developments Software for Scientists, Germany) was used. Identification of substances was achieved by comparing the retention times and respective peak spectra with authentic flavonoid standards. For flavonoid detection, substances were monitored at 370 nm. The relative concentrations were calculated on the basis of peak areas.

Quantification of Fluorescence

To identify fluorogenic flavonols and natural metabolites of quercetin, we screened a number of substances with the test protein BSA. Table I lists the test compounds (Sigma, Germany) with increasing number of hydroxyl groups (number in brackets). The position of hydroxyl groups of the different compounds is specified by their respective systematic names. Both BSA and flavonoids were used in equimolar concentrations (25 μmol dm^{-3}). Fluorescence of BSA/flavonoid interactions was determined in black microtiter plates (Fluoro Nunc, PolySorp 96-well plates, Nunc, Denmark). The reaction mixture (200 μl/well) was made up in PBS (pH = 7.5) and contained 0.1 % DMSO. The solution was incubated for 15 min at 37 °C before the fluorescence was quantified in a Microtiterplate Reader (FLUOstar Galaxy, BMG, Germany) suitable for fluorescence determinations (emission 545 nm, excitation 485 nm). For each combination of reactants, 3 experiments with 5 determinations (*i.e.,* a total of 15) were made.

Statistics

The experimental data are expressed as the mean ± s.d. of several independent experiments (see Results for details). Significance of the recorded effects was assessed by the analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Quercetin Target Proteins Can Be Identified by Elicited Fluorescence

Flavonoids are useful model substances for spectroscopic studies of their interactions with relevant target proteins due to excited-state intramolecular proton transfer and dual fluorescence behaviour[11] and the high intensity of fluorescence in low-polar solvents.[15] Quercetin was shown recently to bind to specific proteins *in vitro* and to elicit fluorescence upon binding.[6] Binding is saturable at about equimolar concentrations, so the protein apparently contains only one binding site. Biophysical binding properties have been explored in some detail using human serum albumin (HSA) and quercetin as binding partners.[9,10] Other quercetin binding proteins like bovine serum albumin (BSA) or insulin have been used as model proteins to quantify the elicited fluorescence. Quercetin is weakly fluorescent in aqueous media. Binding to BSA leads to drastic enhancement of fluorescence

intensity.[6] Fluorescence is strongly pH dependent and the largest signal is obtained at about pH = 8, depending on the particular protein under study (data not shown).

The interaction between proteins and quercetin can be visualized in living cells, as demonstrated for *Drosophila* follicles.[6] However, in human HL-60 cells only apoptotic cells showed strong fluorescence while vital cells stained weakly. This observation gave a first hint that quercetin metabolism in vital HL-60 cells is rapid and prompted further studies of the metabolic activity and properties of the generated metabolites.

Fluorogenic Properties of Quercetin Metabolites and Related Flavonols

Fluorogenic properties of different flavonoids have been studied before.[6,9–12] Table I lists a selection of different flavonols that differ only in the degree of hydroxylation. In addition, some flavonol conjugates were analyzed with respect to their fluorogenic properties using BSA as test protein. The results are displayed in Figure 1. There is a clear tendency to fluorescence increasing with increasing the degree of hydroxylation. At least 4 OH-groups seem to be necessary to elicit a strong fluorescent signal. Methylation (*e.g.,* Isorhamnetin, Figure 1) did not prevent fluorescence but glycosylation efficiently eliminated fluorogenic properties in the three tested compounds (Table I and Figure 1, substances 11–13).

Quercetin Metabolism in Vital and Apoptotic Cells

Since quercetin passes quickly through cell membranes, the distribution of target proteins can be studied in living cells (see above). In the case of human cells, such an analysis is complicated by the fact that quercetin seems to be metabolized quickly and the fluorescence is week in vital cells. In cytologically apoptotic cells, however, a bright staining is seen in the fragmented nuclei of HL-60 cells (unpublished observation). The cytological observation was confirmed by the analysis of vital and apoptotic cells by flow cytometry. While in exponentially growing HL-60 cell cultures at most 10 % of the cells undergo apoptosis (Figure 2a), the fraction of apoptotic cells increases dramatically after heat shock (Figure 2b). Under the chosen conditions, about 90 % of the cells could be classified as apoptotic according to the characteristic parameters SSC/ FSC. Four hours after exposure to quercetin (100 μmol dm^{-3}), vital and apoptotic cell populations could be easily distinguished by their level of fluorescence in the control (Figure 2c) and heat shocked cell cultures (Figure 2d). When the time course of these differences was monitored, it turned out that the level of fluorescence increased rapidly during the first few minutes of incubation (*e.g.,* up to 10 min), showing that the cell membrane presents no barrier to the flavonoid. For 30 min no difference in fluorescence between vital and apoptotic cells was detected (Figure 2e) but after that the picture changed:

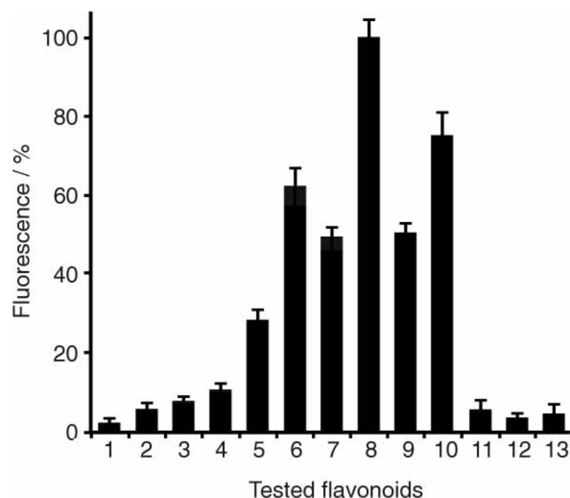

Figure 1. Quantification of the fluorescence of different BSA/ flavonoid combinations (see Table I for specification of test compounds). Fluorescence was quantified in a microtiter plate reader at 545 nm (excitation 485 nm) relative to the fluorescence of BSA/quercetin (set to 100 %). Both BSA and flavonoids were used in equimolar concentrations (25 μmol dm^{-3}). The data represent the means (± s.d.) of 15 determinations.

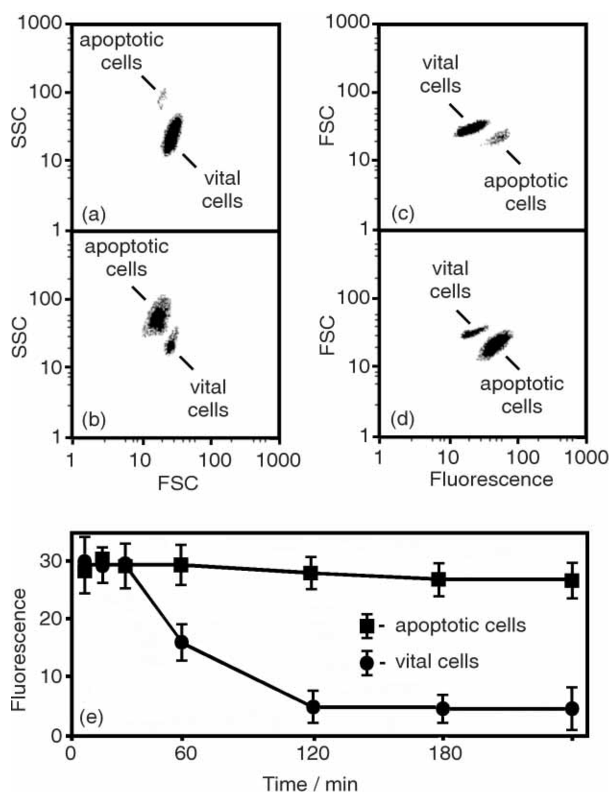

Figure 2. Apoptotic and vital cells were characterized by flow cytometry according to size (FSC) and their granular content (SSC). In asynchronously growing HL-60 cell cultures (control), the fraction of apoptotic cells is small (a). In heat shocked cells (45 °C for 60 min), the ratio of vital to apoptotic cells is reversed after 1 day of culture (b). After exposure to quercetin (100 μmol dm^{-3} for 4 hours), fluorescence was quantified in control (c) and heat-shocked cell cultures (d) *versus* FSC. The initial high level of fluorescence decreased rapidly and selectively in vital cells while the fluorescence in apoptotic cells remained at a high level (e). Standard deviations (± s.d.) are indicated.

in vital cells the level of quercetin fluorescence decreased rapidly with time while the level of apoptotic cells fluorescence remained comparatively high. When the cells were exposed to quercetin for 4 hours, the apoptotic cells showed 3.8 ± 0.5 times higher level of fluorescence than the vital cells ($P<0.05$).

Biochemical Quantification of Quercetin Metabolism

If the observed, rapidly decreasing fluorescence is indeed due to metabolic conversion to non-fluorogenic quercetin metabolites or complete degradation, this should be reflected in a decrease of quercetin concentration in vital cells and a comparatively high concentration in apoptotic cells. A biochemical analysis using HPLC showed that this was indeed the case (Figure 3). Relative concentrations of quercetin and metabolites were calculated on the basis of their peak areas in μg according to known concentrations of standard substances (Figure 3a) and the total amount of metabolites in the cell extracts before and after hydrolysis was set as 100 %. Based on these values, the percentage of quercetin, isorhamnetin and putative metabolites was calculated (Figure 3b). After 4 hours incubation, the concentration of quercetin in apoptotic cells remained high while in vital cell cultures the quercetin concentration dropped to about 20 % (Figure 3b). Of the two possible fluorogenic quercetin metabolites (Figure 1) only isorhamnetin was detected but its concentration in vital and apoptotic cells remained very low (5±2 %, Figure 3b) and could not contribute much to the fluorescence in cell cultures when viewed under a microscope or analyzed by flow cytometry. Hydroxylation of quercetin may give rise to fluorogenic myricetin (Figure 1) but this compound was not detected in our cultures and hence could not contribute to the observed fluorescence in cells. None of the tested glycosylated metabolites were fluorogenic (Figure 1) and since these compounds seem to be present in considerable amounts (collectively labelled as »rest«, Figure 3b), it seems likely that glycosylation is a major metabolic pathway in our cultures and, at the same time, an important reason for the loss of fluorescence observed in vital cells. In addition, glycosylated metabolites were also found in the medium (data not shown), indicating export of metabolites across the cell membrane.

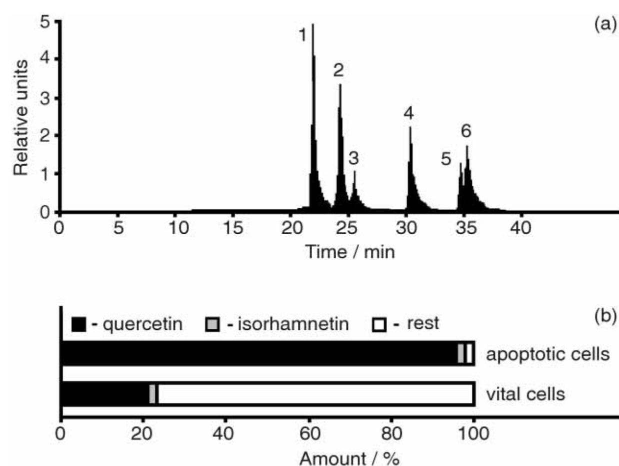

Figure 3. HPLC analysis of quercetin metabolites: (a) Analysis of selected flavonoid standards with HPLC: 1 – isoquercitrin (quercetin-3-β-D-glucoside); 2 – quercitrin (quercetin-3-D-rhamnoside); 3 – myricetin; 4 – quercetin; 5 – kaempferol; 6 – isorhamnetin (3'-methylquercetin). (b) Percentage of free quercetin, isorhamnetin and other metabolites (rest) after incubation of control and apoptotic HL-60 cells with quercetin (100 μmol dm^{-3}) for 4 hours.

Major Pathways of Quercetin Metabolism

Major pathways of quercetin metabolism are shown in Figure 4. Methylation or hydroxylation of quercetin gives rise to isorhamnetin and myricetin, respectively. Both flavonols elicit fluorescence upon binding to BSA (Fig-

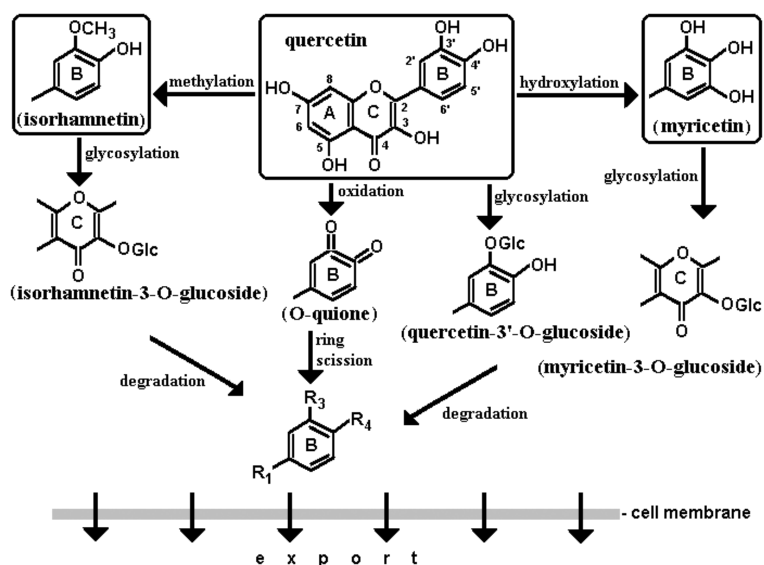

Figure 4. Pathways of quercetin metabolism/degradation (based on Refs. 16, 17). Fluorogenic flavonoids are labelled (frame). Ring scission and degradation may form phenolic compounds. Four alternative substitution patterns are: 3,4-dihydroxybenzoic acid (R_1 = COOH, R_3 = OH, R_4 = OH), 3-methoxy-4-hydroxybenzoic acid (R_1 = COOH, R_3 = OCH_3, R_4 = OH), 3,4-dihydroxyphenylacetic acid (R_1 = CH_2COOH, R_3 = OH, R_4 = OH), 3-methoxy-4-hydroxyphenylacetic acid (R_1 = CH_2COOH, R_3 = OCH_3, R_4 = OH). Arrows across the cell membrane symbolize export of different flavonoid metabolites, including glucosides (Glc).

ure 1) but quantitatively do not play any role as metabolites in our cell cultures. Not all compounds in the illustrated pathways were experimentally tested with respect to their fluorogenic properties. However, it has become clear that the structural preconditions for fluorogenic properties are very stringent and any alteration of the flavonol structure[5] or glycosylation inevitably led to the loss of fluorescence when tested with BSA or other model proteins (Figure 1). Based on the present evidence, we suggest that the major reason for the fast attenuation of fluorescence in vital cells is the oxidative degradation and glycosylation of flavonols with subsequent export of the conjugates from the cells.

CONCLUSION

The fluorogenic property of quercetin allows identifying cellular target proteins and this offers the opportunity to systematically study and predict biologically and pharmacologically interesting effects. Furthermore, by monitoring decreasing fluorescence in living cells, an estimation of the metabolic dynamics can be obtained. Target specificity and metabolic stability and availability are crucial factors in the evaluation of pharmacologically interesting compounds such as quercetin. The methods described in this communication will greatly facilitate this process.

Acknowledgements. – We acknowledge the excellent technical assistance of Yvonne Henker. We are grateful for the financial support of the Federal Ministry of Education and Research of Germany (BMBF) and the Ministry of Science and Technology of Croatia (project No. HRV 01/025).

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SAŽETAK

Praćenje metabolizma flavonoida u humanim stanicama na temelju fluorescencije izazvane interakcijom kvercetina s proteinima

Herwig O. Gutzeit, Sergey V. Tokalov, Jutta Ludwig-Müller i Gordana Rusak

Unatoč mnoštvu informacija koje se odnose na biološke učinke flavonoida, sustavni pristup analizi njihovih ciljnih molekula još uvijek nedostaje. Iz toga razloga vrlo je teško racionalno vrednovati opasnosti ili koristi koje donosi hrana koja sadrži flavonoide kao i njihovu moguću farmakološku primjenu. Iskoristili smo svojstvo kvercetina da izazove fluorescenciju kada se veže za specifične ciljne proteine i analizirali nekoliko različito modificiranih flavonoida (metilacija, hidroksilacija, glikozilacija). Ciljni proteini za koje se kvercetin veže u živim stanicama mogu se vizualizirati na temelju fluorescencije. U živim stanicama humane leukemije (HL-60) fluorescencija naglo pada nakon označavanja flavonoidima, dok metabolički inaktivne apoptotične stanice zadržavaju fluorescenciju. Te su citološke razlike jasno zapažene pod fluorescencijskim mikroskopom, a kvantificirane su pomoću protočne citometrije. Metabolička pretvorba kvercetina u živim stanicama potvrđena je i kvantificirana pomoću HPLC analiza. Dok apoptotične stanice zadržavaju značajnu količinu kvercetina, žive ga stanice brzo metaboliziraju (npr. metilacijom ili glikozilacijom). Ti su biokemijski rezultati u skladu s citološkim promatranjima i podupiru zaključak da se kvercetin u živim stanicama brzo pretvara u nefluorogene metabolite. Gubitak fluorescencije u živim stanicama omogućava praćenje i kvantifikaciju dinamike metabolizma kvercetina u humanim stanicama.