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Phosphoprotein levels, MAPK activities and NFκB expression are affected by fisetin

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Abstract

Flavonoids, polyphenolic phytochemicals, are ubiquitous in plants and are commonly present in the human diet. They may exert diverse beneficial effects, including antioxidant and anticarcinogenic activities. The present study was designed to evaluate three biomolecules that play important roles in the apoptotic process: mitogen-activated protein kinases, protein phosphatases and NFκB, using HL60 cells treated with fisetin as an experimental model. Our results demonstrated that cells treated with fisetin presented high expression of NFκB, activation of MAPK p38 and an increase of phosphoprotein levels; inhibition of enzymes involved in redox status maintenance were also observed. Our findings reinforce the hypothesis that fisetin is likely to exert beneficial and/or toxic actions on cells not through its potential as antioxidant but rather through its modulation of protein kinase and phosphatase signaling cascades. Additionally, our results also indicate that the cellular effects of fisetin will ultimately depend on the cell type and on the extent to which they associate with the cells, either by interactions at the membrane or by uptake into the cytosol.

Keywords: *Fisetin, flavonoids, HL60, NFκB, MAPK, phosphatases*

Introduction

Flavonoids are polyphenolic compounds widely found in plants [1]. Components of fruits, vegetables and beverages, such as wine and tea, many flavonoids are present in a regular diet [2]. Flavonoids exhibit a variety of effects such as inhibition of malignant cell growth [1], regulation of lymphocyte activation, cell proliferation and differentiation [2–4]. These biological effects of flavonoids on cells can be due to the inhibition of different key enzymes. For these reasons, the flavonoids can be considered potential compounds in the selective blocking of signal transduction pathways and in the design of more potent analogues for use in proliferative disease therapies.

Several studies have demonstrated that, depending on their structures, flavonoids can be potent inhibitors of several kinases involved in signal transduction, mainly protein kinase C (PKC) [5] and tyrosine kinases [6]. On the other hand, some flavonoids can activate cell differentiation through activation of the Ras-ERK cascade [7].

Fisetin is a common dietary component found in several fruits and vegetables [8]. Some authors have demonstrated different biological activities for this flavonoid: inhibition of topoisomerase II, an essential nuclear enzyme for DNA replication [9,10], neuroprotective, cardioprotective and anti-carcinogenic activities, which have been attributed to its antioxidant properties [7,11–13], inhibition of cellular proliferation

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and *in vitro* angiogenesis [14], induction of apoptosis in leukemic cells [13]. Recently, Haddad et al. [15] have demonstrated that fisetin caused cell cycle arrest (G2/M) in a prostate cancer human cell line (PC3). In addition, fisetin inhibited glucose uptake in a competitive manner in a myeloid cell (U937), which indicated that this flavonoid could be used as an alternative blocker of glucose uptake *in vitro* [16].

The present study was designed to evaluate three biomolecules that play important roles in the apoptotic process: mitogen-activated protein kinases (MAPKs), protein phosphatases and NFκB, using HL60 cells treated with fisetin as an experimental model. Our results demonstrated that cells treated with fisetin presented high expression of NFκB, activation of MAPK p38 and an increase of phosphoprotein levels; inhibition of enzymes involved in redox status maintenance was also observed.

Materials and methods

Materials

HL60 cells was from ATCC (Rockville, MD) and fisetin (Figure 1) was from Sigma Chemical Co. (St Louis, MO). The polyclonal antibodies against antiphospho-p38 mitogen-activated protein kinase (p38), antiphospho-p42/44 (ERK 1/2), antiphospho-c-jun NH_2-terminal protein kinase (JNK), antiphospho-MAP-K/ERK kinase 1 (MEK1), antirabbit and antimouse peroxidase conjugated antibodies were obtained from Cell Signaling Technology (Beverly, MA).

Cell culture

HL60 cells were routinely grown in suspension in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% penicillin–streptomycin (10,000 U/mL penicillin and 10 mg/mL streptomycin) and 1% glutamine, grown at 37°C under a humidified 5% CO_2 atmosphere. In all experiments 3×10^5 cells/mL were seeded, and after 72 h the cells were treated with fisetin for 24 h. Fisetin dissolved in dimethyl sulfoxide (DMSO) was added to the culture medium and adjusted to a final DMSO concentration of 0.1%.

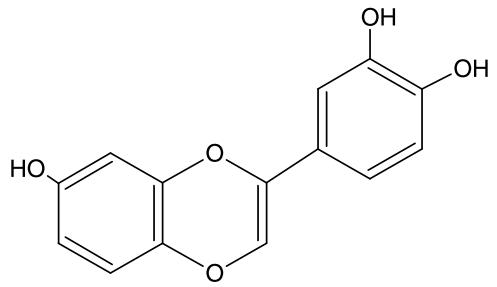

Figure 1. Molecular structure of fisetin.

Cell viability

Cell viability was assessed by the trypan blue dye exclusion and the MTT reduction assays as previously described [17].

Western blotting

Cells (3×10^7) were lysed in 200 μL cell lysis buffer (50 mM Tris-HCl pH 7.4, 1% Tween 20, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM *o*-vanadate, 1 mM sodium fluoride, and protease inhibitors (1 μg/mL aprotinin, 10 μg/mL leupeptin, and 1 mM phenylmethylsulfonyl fluoride (PMSF)) for 2 h on ice. Protein extracts were cleared by centrifugation, and the protein concentration was determined using the Lowry method [18]. Twice the volume of sodium dodecyl sulfate (SDS) gel loading buffer (100 mM Tris-HCl, pH 6.8, 200 mM dithiothreitol, 4% SDS, 0.1% bromophenol blue, and 20% glycerol) was added to the samples and the mixture boiled for 10 min. Cell extracts, corresponding to 3×10^5 cells, were resolved by SDS-polyacrylamide gel (12%) electrophoresis (PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked for 1 h in 1% fat-free dried milk or bovine serum albumin (2%) in Tris-buffered saline (TBS) - Tween 20 (0.05%) and incubated overnight at 4°C with appropriate primary antibody at 1:1000 dilution. After washing in TBS-Tween 20 (0.05%), membranes were incubated with antirabbit or antimouse horseradish peroxidase-conjugated secondary antibodies, at 1:2000 dilutions (in all Western blotting assays), in blocking buffer for 1 h. Detection was performed by using enhanced chemiluminescence (ECL).

Antioxidant enzyme activities

Total superoxide dismutase (SOD) activity was determined from the rate of inhibition of ferrocytochrome c oxidation, at 550 nm, in a standard reaction medium [19]. The manganese superoxide dismutase (MnSOD) activity was measured after inhibition of the Cu/Zn isoenzyme by addition of 1 mM KCN [20]. Catalase activity was determined by measuring the decrease in absorption of H_2O_2 at 240 nm [21]. Glutathione peroxidase (GPX) activity was determined by measuring the NADPH oxidation rate in the presence of GSH and GSH reductase [22].

Marker enzyme activities for oxidative stress

Aconitase activity was measured at 25°C by following the change in the absorption at 340 nm, due to $NADP^+$ reduction [23]. Fumarase activity was measured at 25°C by following the increase in absorbance at 240 nm at 25°C in a standard reaction mixture [24]. All the measurements were carried out in a UV-VIS spectrophotometer (Hitachi, model U-2001).

Statistical evaluation

The Western blots represent three independent experiments. Cell viability was expressed as the mean ± standard error of three independent experiments run in triplicate. Data for each assay were statistically evaluated by analysis of variance (ANOVA).

Results and discussion

Differential effect of fisetin on HL60 cells and normal human lymphocyte viabilities

We have previously described cytotoxic effects and mechanism of action of different compounds on cancer cells [25–27]. Other natural products have also been reported as important sources of potential chemotherapeutic agents [28–30]. Flavonoids, widely distributed in vegetables, fruits, and wine, have been shown to exert anticarcinogenic effects [10,13,15,31]. However, the molecular mechanisms by which flavonoids can act against cancer cells need to be elucidated. To establish the specificity of fisetin action on HL60 cells we checked, in parallel, the effect of this compound on normal human lymphocytes viability using the MTT assay. It was observed that after 24 hours of fisetin-treated HL60 cells, the mitochondrial activity was decreased, displaying an IC_{50} value around 30 μM (Figure 2). In agreement with other authors [13], it was also observed that fisetin induced HL60 cells death by apoptosis. Interestingly, human lymphocyte viability remained unchanged, even in the presence of fisetin at concentrations up to 200 μM. These results suggest that fisetin can be an interesting candidate for cancer treatment with a cellular-specific mechanism of action.

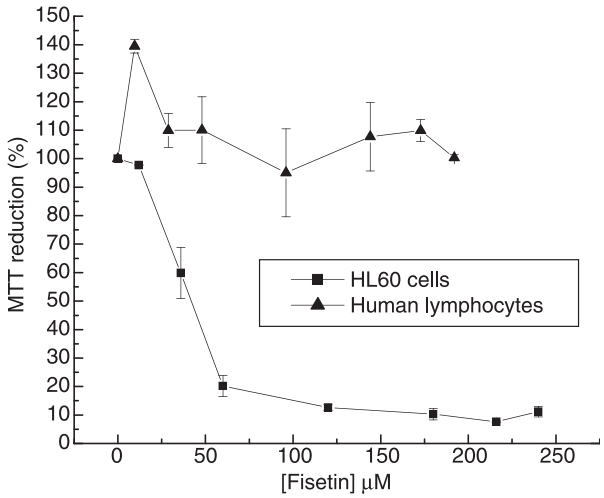

Figure 2. Cytotoxicity of fisetin in leukemic cells and normal human lymphocytes. HL60 cells (■) and normal human lymphocytes (▲) were treated with different concentrations of fisetin for 24 h. In the absence of fisetin, the MTT reduction was considered as 100%. The experiment was performed in a 24-well plate. Results represent the means ± standard error of three experiments run in triplicate ($P < 0.05$).

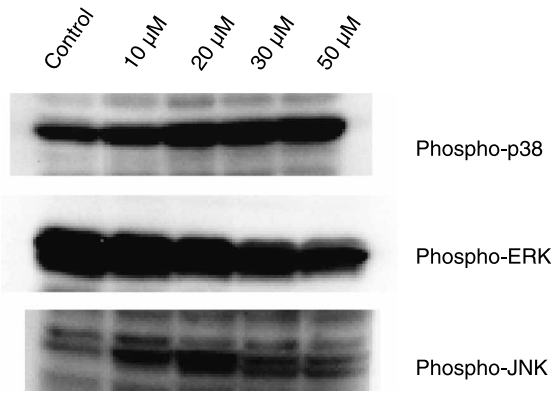

Figure 3. Effect of fisetin on MAPKs phosphorylation in HL60 cells. Cells were treated with different concentrations of fisetin (10, 20, 30 and 50 μM). Soluble lysates were matched for protein content and analyzed by Western blot. One representative immunoblot of three independent experiments is presented.

Effect of fisetin on MAPKs phosphorylation and NFκB expression in HL60 cells

To obtain more insight into the molecular mechanisms mediated by fisetin on HL60 cells, we examined the phosphorylation state of total proteins and MAPKs, in response to fisetin at concentrations up to 50 μM. Fisetin caused activation of p38 and JNK MAPKs, while ERK was inhibited (Figure 3). Williams et al. [32] have demonstrated that flavonoids and their metabolites differentially acted on PI3-kinase, Akt/protein kinase B (Akt/PKB), tyrosine kinases, PKC, and MAPK signaling cascades. Inhibitory or stimulatory actions at these pathways are likely to profoundly affect cellular function by altering the phosphorylation state of target molecules and/or by modulating gene expression.

In addition, we also observed that cells treated with fisetin presented high expression of NFκB (Figure 4). Decrease in MAPKs phosphorylation (Figure 3) and in the expression of NFκB p65 (Figure 4) in HL60 cells at fisetin concentrations higher than 20 μM could be ascribed to different steps of apoptosis. Our results indicate that depending on the fisetin concentration two steps of apoptosis can be reached: early and late apoptosis. Apparently, fisetin concentrations

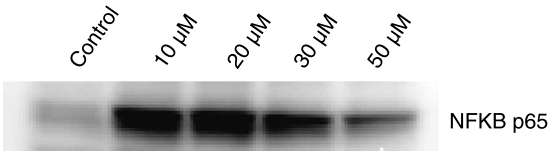

Figure 4. Fisetin changes the expression of NFκB p65. After treatment of HL60 cells with different concentrations of fisetin for 24 h, equal amounts of protein (50 μg) of total lysates were subjected to immunoblot analysis with NFκB (p65) antibodies.

up to 20 μM caused early apoptosis, that was reinforced by the overexpression of NFκB. Recently, Kanno et al. [33] demonstrated that the overexpression of NFκB is a pivotal event for apoptosis in HL60 cells induced by the flavonoid naringenin. It has been shown that the transcription factor NFκB participated in cell growth, differentiation, inflammatory responses induced by different signals related to the regulation of apoptosis and neoplastic transformation [34,35]. The pro- and antiapoptotic regulatory functions of NFκB have been shown to depend on the cell type, the differentiation state of the cell, and the nature of the apoptotic stimulus [35]. Our data provided evidence that the overexpression of the subunit NFκB p65 in cell death was associated with ROS generation. Some authors observed that ROS *per se* were potent inducers of apoptosis [36] and that the hydrogen peroxide-induced apoptosis required the release of mitochondria-derived ROS and the activation of NFκB [37]. Our results demonstrating the ability of NFκB p65 overexpression to induce apoptosis, are in agreement with published data implicating NFκB to the induction of cell death in certain cells such as neurons, Schwann cells, prostate carcinoma, and embryonic kidney cells [38–42].

Effects of fisetin on protein phosphorylation in HL60 cells

In order to analyze the phosphorylation state in HL60 cells treated with fisetin, we examined the tyrosine and threonine phosphorylation on the cellular proteins. Phosphorylation of both residues increased in the cells treated with fisetin, except for a decrease in tyrosine phosphorylation at 50 μM fisetin (Figure 5). Our results indicated that the fisetin action in HL60 cells was accompanied by an increase in tyrosine and threonine phosphorylations. We have observed that fisetin inhibited cytosolic phosphatase activities in HL60 cells (not shown).

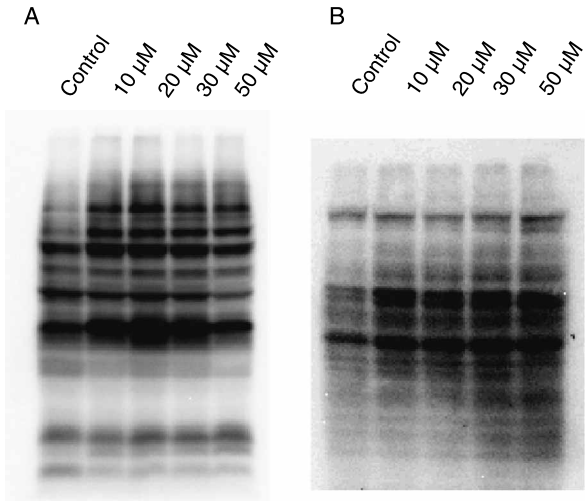

Figure 5. Effect of fisetin on protein phosphorylation levels. Cells were treated with fisetin (10, 20, 30 and 50 μM), and the tyrosine (A) and threonine (B) phosphorylations were evaluated by immunoblotting. One representative immunoblot of three independent experiments is presented.

Fisetin induces oxidative stress and decrease in antioxidant enzymes activities in HL60 cells

In order to analyze the cellular redox status after treatment of HL60 cells with fisetin we quantified the activities of aconitase, fumarase, catalase, glutathione peroxidase and two isoforms of superoxide dismutase (SOD), i.e. the MnSOD (mitochondrial isoform), and the CuZnSOD (cytosolic isoform). Treatment of HL60 cells with fisetin resulted in inactivation of mitochondrial aconitase, an enzyme sensitive to oxidative stress, but not fumarase, a mitochondrial enzyme sensitive to oxidative stress (Table I). Fisetin caused also an expressive decrease in the antioxidant enzymes catalase, MnSOD, CuZnSOD and GPX. Our results suggest that fisetin can induce oxidative stress through ROS production. ROS can lead to cell death through inactivation of mitochondrial aconitase, an iron-sulfur (Fe-S) protein [43]. Recent studies showed that ROS are emerging as obligatory mediators of cell death signaling in response to stimulation of TNF receptors and induction of JNK and p38 signaling [44–47]. A MAPK phosphatase (MKP) was identified as a critical molecular target of ROS during TNFα-induced apoptosis, due to oxidation of an essential cysteine residue to sulfenic acid [46]. MKP plays a critical role in the regulation of the activity of MAPKs [48,49]. Thus, ROS-dependent inhibition of MKPs caused persistent activation of JNK by TNFα, and, ultimately, programmed cell death *via* either a necrotic or an apoptotic pathway [47]. These findings are in agreement with our results since, besides activating JNK and p38, fisetin also caused an increase of phosphoprotein levels which can be due to either inactivation of protein phosphatases or activation of protein kinases, activities which are highly sensitives to oxidant agents.

Conclusion

In summary, our results have reinforced the hypothesis that fisetin was likely to exert beneficial and/or

Table I. Effects of fisetin on antioxidant enzymes activities of HL60 cells. Cells were treated with fisetin (100 μM) and enzyme activities were determined as described in Materials and methods.

	Specific activities	
Enzyme	Control	+Fisetin
Aconitase	2.32 mU/mg	0.11 mU/mg
Fumarase	0.029 mU/mg	0.029 mU/mg
Catalase	0.23 pmoles/mg	0.11 pmoles/mg
GPX	0.66 mU/mg	0.22 mU/mg
MnSOD	6.22 U/mg	2.46 U/mg
CuZnSOD	11.74 U/mg	3.33 U/mg

toxic actions on cells not through its potential to act as antioxidant but rather through its modulation of protein kinase and phosphatase signaling cascades. Additionally, our results also indicated that the cellular effects of fisetin ultimately depended on the cell type, and on the extent to which it associated with the cells, either by interactions with the membrane or by uptake into the cytosol.

Acknowledgements

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