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Pro-Oxidant Activity of Flavonoids Induces EpRE-Mediated Gene Expression

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Flavonoids are important bioactive dietary compounds. They induce electrophile-responsive element (EpRE)-mediated expression of enzymes, such as NAD(P)H−quinone oxidoreductase (NQO1) and glutathione *S*-transferases (GSTs), which are major defense enzymes against electrophilic toxicants and oxidative stress. The induction of EpRE-mediated gene transcription involves the release of the transcription factor Nrf2 from a complex with Keap1, either by a direct interaction of the inducer with Keap1 or by protein kinase C (PKC)-mediated phosphorylation of Nrf2. The inhibition of PKC in Hepa1c1c7 cells, stably transfected with human NQO1-EpRE-controlled luciferase revealed that PKC is not involved in flavonoid-induced EpRE-mediated gene transcription. However, the ability of flavonoids to activate an EpRE-mediated response correlates with their redox properties characterized by quantum mechanical calculations. Flavonoids with a higher intrinsic potential to generate oxidative stress and redox cycling are the most potent inducers of EpRE-mediated gene expression. Modulation of the intracellular glutathione (GSH) level showed that the EpRE-activation by flavonoids increased with decreasing GSH and vice versa, supporting an oxidative mechanism. In conclusion, the pro-oxidant activity of flavonoids can contribute to their health-promoting activity by inducing important detoxifying enzymes, pointing to a beneficial effect of a supposed toxic chemical reaction.

Introduction

Fruit- and vegetable-rich diets are associated with reduced incidence of various cancer types, and flavonoids are important key compounds in these food items, considered to be health-protecting (*1*). The estimated daily intake of flavonoids ranges up to 1 g/day (*2*). Flavonoids have been reported to protect against coronary heart disease, stroke and certain cancer types through their antioxidant, anti-inflammatory, anti-allergic, and antiviral activities (*3*). However, the pro-oxidant activity of flavonoids has also been reported (*4*−*5*). The cancer-preventive activity of flavonoids has been attributed to multiple parallel mechanisms. One important mechanism is the induction of detoxifying enzymes by flavonoids, such as glutathione *S*-transferases, UDP-glucuronosyltransferases, γ-glutamylcysteine synthetase, NAD(P)H−quinone oxidoreductase 1, heme oxygenase-1, epoxide hydrolase, leukotriene B4 dehydrogenase and aldehyde dehydrogenase (*6*−*7*). These enzymes play a central role in the defense system of cells, being able to detoxify reactive genotoxic substances and to contribute significantly to the cellular protection against redox cycling and oxidative stress (*6*).

Regulation of this protective gene expression by dietary chemopreventive compounds can be mediated by the electrophile-responsive element (EpRE), initially referred to as the antioxidant-responsive element (ARE) (*8*). The EpRE is a regulatory sequence involved in the coordinated transcriptional activation of genes associated with phase 2 biotransformation, protection against oxidative stress, and other cancer-chemoprotective mechanisms (*9*). The key regulator of EpRE-mediated gene expression is the transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2) and, to a lesser extent, Nrf1, both of which are members of the nuclear basic leucine zipper transcription factors (*10*). The major regulator of Nrf2 is identified to be Keap1 (Kelch-like erythroid-cell-derived protein with CNC homology-associating protein 1), a dimeric cytoplasmic actin-binding protein (*11*), which represses Nrf2 transcription activation by cytoplasmic sequestration and mediation of the degradation of Nrf2 (*12*). Several mechanisms of Nrf2 activation resulting in the release of Nrf2 from Keap1 have been proposed. A suggested pathway of increased EpRE-mediated gene induction by nuclear Nrf2 accumulation is through the phosphorylation of Nrf2 by protein kinase C (PKC), leading to the dissociation of Nrf2 from the complex (*13*−*15*).

Another proposed mechanism is the direct reaction of oxidative compounds with the Keap1−Nrf2 complex (*16*). The fact that the dimeric Keap1 contains multiple cysteine residues in each monomer, many of which are potential sites of oxidative attack by inducers of the EpRE-mediated gene expression, has led to the suggestion that the Keap1−Nrf2 interaction constitutes a sensor of oxidative stress involved in triggering EpRE-controlled responses to restore the physiological redox status in cells (*16*−*18*). The release of Nrf2 from Keap1, leading to the activation of EpRE-mediated gene transcription, is reported to be a redox-dependent process (*19*) and activated by ROS and/or electrophiles (*9*−*20*). It is suggested that these inducers can interact with reactive thiol groups of the Keap1 protomers, resulting in intermolecular disulfide formation and conformational changes ultimately resulting in Nrf2 release (*18*−*21*).

The molecular mechanism by which flavonoids are able to induce detoxifying enzymes is not yet known. Although it was shown that flavonoids induce detoxifying enzymes via an EpRE-

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mediated response (*6*, *22*, *24*), flavonoids as such do not have electrophilic activity but are commonly known to have electron-donating antioxidant properties (*25*). However, we recently showed that flavonoid metabolites do have electrophilic activity and can covalently bind to GSH and DNA (*26*). Therefore, the objective of this study is to elucidate the mechanism by which flavonoids are able to induce EpRE-mediated induction of detoxifying enzymes.

We investigated the EpRE-mediated gene expression induced by a series of flavonoids in Hepa1c1c7 cells, stably transfected with a luciferase reporter gene under the control of the EpRE derived from the human NQO1 gene (EpRE-LUX cells). The induction potential of flavonoids in EpRE-LUX cells was studied in the presence of the PKC inhibitor staurosporine and correlated with the redox properties of the inducers as quantified by molecular orbital calculations. In addition, the induction potential of flavonoids was studied on EpRE-LUX cells with modified intracellular GSH levels. The results obtained indicate a role for flavonoid pro-oxidant chemistry in their mechanism of EpRE-mediated gene expression control.

Materials and Methods

Materials. Alpha-Modified Eagle's Medium, Hanks' balanced salt solution (HBSS), trypsin, fetal calf serum (FCS), phosphate-buffered saline (PBS), gentamicin, and G418 were purchased from Gibco Invitrogen Corporation (Breda, The Netherlands). Dimethyl sulfoxide (DMSO) was obtained from Acros Organics (New Jersey). All tested flavonoids were purchased from Extrasynthese (Genay Cedex, France). For each experiment, a fresh stock solution in DMSO of all standards and substrates was prepared.

Cell Lines. The Hepa-1c1c7 mouse hepatoma cells were a kind gift from Dr. M. S. Denison, (University of California, Davis) and were cultured in Alpha-Modified Eagle's Medium, supplemented with 10% FCS and 50 μg/mL gentamicin. The cells were maintained in a humidified atmosphere with 5% CO_2 at 37 °C. Hepa-1c1c7 cells were stably transfected with the reporter vector pTI(hNQO1-EpRE)Luc+ carrying the EpRE from the human NQO1 gene regulatory region between −470 to −448 (5′-AGT CAC AGT GAC TCA GCA GAA TC-3′) coupled to a luciferase reporter gene, as described previously (*24*). The culture medium of the transfected Hepa-1c1c7 cells was the same as that for the wild-type Hepa-1c1c7 cells, containing in addition 0.5 mg/mL G418. These transfected Hepa-1c1c7 cells, containing the luciferase gene under expression regulation of the EpRE from the human NQO1 gene will further be addressed as EpRE-LUX cells.

EpRE-LUX Assay. EpRE-mediated induction of gene expression by flavonoids was tested using the EpRE-LUX luciferase reporter gene assay as described previously (*24*). Briefly, EpRE-LUX cells were cultivated as described above. To investigate the effect of the inducers of EpRE-mediated gene expression, cell suspensions (2×10^5 cells/mL) were plated in culture medium in 96-well view plates (Corning, 100 μL/well) and incubated for 24 h to allow attachment of the cells to the bottom of the wells and the formation of a confluent monolayer. Next, the culture medium was removed, and the cells were treated with 200 μL of the medium containing the flavonoid of interest. The DMSO concentration in the culture medium was kept constant at 0.5%. After 24 h of exposure cells were washed with 0.5 × PBS, harvested, and homogenized in Low Salt Buffer (10 mM Tris, 2 mM DTT, and 2 mM *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetra-acetic acid monohydrate; pH 7.8). Luciferase reagent (20 mM tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 2 mM DTT, 0.47 mM D-luciferin, and 5 mM ATP; pH 7.8) was added and luciferase activity was measured using a Luminoskan RS (Labsystems) luminometer.

Role of PKC in the Induction of EpRE-Mediated Gene Expression by Flavonoids. The effect of PKC inhibition on EpRE-mediated luciferase induction by flavonoids was investigated using the PKC inhibitor staurosporine. EpRE-LUX cells were cultured as described above, and cell suspensions (2×10^5 cells/mL) were plated in culture medium in 96-well view plates (Corning, 100 μL/well) and incubated for 24 h to allow the formation of a confluent monolayer. Next, the culture medium was removed, and the cells were treated with staurosporine concentrations ranging from 0.5−10 nM in the culture medium without FCS supplementation. After 3 h of pretreatment of the cells with staurosporine, the medium was removed, and 200 μL of the medium without FCS supplementation, containing the same amount of staurosporine and the inducer of EpRE-mediated gene expression to be tested, was added to the cells. The DMSO concentration in the culture medium was kept constant at 0.5%. After 24 h of exposure, the cells were washed with 0.5 × PBS, harvested, and homogenized in low salt buffer. Luciferase reagent was added, and luciferase activity was measured as described above.

Quantum Mechanical Calculations. The quantum mechanical calculations were carried out with Spartan 04 for Windows, version 1.0.3 (Wavefun, CA). All possible geometrical conformers of each flavonoid were used as input for the semiempirical molecular orbital calculations. Austin Model 1 (AM1), the E_{HOMO} energy (eV), and the van der Waals volume (Å^3) of the most probable conformer, the one with the lowest heat of formation, were chosen to correlate with the induction factor observed for EpRE-mediated gene expression.

Statistical Analysis. The Statistical Package for Social Scientists (SPSS) 10.1 for Windows (SPSS, Chicago, IL) was used to correlate the experimental data with the values derived from quantum mechanical calculations.

Cross-validation was performed using the leave-out-many method, with 20% of the calibration compounds left out at each step (*27*). To reduce bias, the validation groups were created using the method of unsupervised stratification and the data were ranked according to increasing E_{HOMO} values. The internal cross-validated coefficient of determination (q^2) was calculated using the following equation.

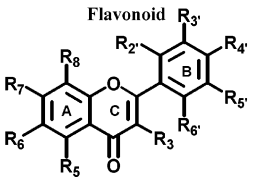
$$q^2 = 1 - (PRESS/SSD)^2$$

where the predictive sum of squares (PRESS) is the sum of the squared differences between actual and predicted induction factors, and *SSD* is the sum-of-squares deviation for each actual induction factor from the mean induction factor of all of the compounds. The correlation is acceptable when $q^2 > 0.5$ and $r^2 - q^2 < 0.3$ (*27*), with r^2 being the correlation coefficient.

Effect of Oxidative Stress on EpRE-Mediated Gene Induction by Flavonoids. To monitor the ability of flavonoids to induce EpRE-mediated gene expression of phase 2 enzymes through their pro-oxidant properties, the intracellular GSH level was modulated by the addition of *N*-acetyl-L-cysteine (NAC), a precursor of GSH able to generate high levels of GSH in cells (*28*), and by the addition of BSO to decrease the intracellular level of GSH (*29*). Cells were cultured and plated as described above. The culture medium was removed after 24 h of incubation, and the cell monolayers were treated with a different concentration of NAC in the medium ranging from 0.01−40 mM or BSO in the medium ranging from 5−100 μM. After 4 h of preincubation with NAC or 24 h with BSO, to allow for the increase or decrease in GSH inside the cells, the medium was removed, and 200 μL of medium containing NAC or BSO and the inducers of interest were added to the cells. The DMSO concentration in the culture medium was kept constant at 0.5%. After 24 h of exposure, the cells were washed with 0.5 × PBS, harvested, and homogenized in a low salt buffer. Luciferase reagent was added, and luciferase activity was measured as described above.

Cytotoxicity. The cytotoxicity of test compounds was determined using the lactate dehydrogenase (LDH) assay with minor adaptations for 96-well-plates (*30*). Briefly, cells were plated 24 h before exposure at a density of 10^4 cells per well in a 96-well plate. Subsequently, for testing the cytotoxicity of the flavonoids, staurosporine, BSO, or NAC, 200 μL of culture medium containing different concentrations of the specific test substance was added.

Table 1. Structure, Induction Factor (IF) of EpRE-Mediated Gene Transcription, Calculated E_{HOMO} Energy, and Van der Waals Volume (VdW) of Each Tested Flavonoid



	flavonoid	OH	OCH_3	IF	E_{HOMO} (eV)	VdW (Å^3)
1	flavone			2.8	−9.27	232.75
2	5-OH flavone	R5		2.5	−9.12	239.45
3	7-OH flavone	R7		2.5	−9.36	240.12
4	chrysin	R5, R7		2.1	−9.25	246.61
5	3,7-OH flavone	R3, R7		3	−8.89	246.87
6	6,7-OH flavone	R6, R7		3	−9.10	247.21
7	3,3′-OH flavone	R3, R3′		5.8	−8.88	246.88
8	7,8-OH flavone	R7, R8		2.2	−9.23	247.18
9	galangin	R3, R5, R7		4.0	−8.90	253.37
10	resokaempferol	R3, R7, R4′		6.5	−8.72	254.06
11	baicalein	R5, R6, R7		2.5	−8.99	253.59
12	apigenin	R5, R7, R4′		2.0	−9.15	253.80
13	kaempferol	R3, R5, R7, R4′		7.1	−8.74	260.54
14	luteolin	R5, R7, R3, R4′		3.0	−9.09	260.89
15	fisetin	R3, R7, R3′. R4′		8.0	−8.69	261.14
16	quercetin	R3, R5, R7, R3′, R4′		10	−8.72	267.65
17	morin	R3, R5, R7, R2′, R4′		8.4	−8.81	267.39
18	myricetin	R3, R5, R7, R3′, R4′, R5′		9	−8.80	274.75
19	tectochrysin	R5	R7	4.4	−9.16	266.76
20	genkwanin	R5, R4′	R7	5	−9.09	273.80
21	isorhamnetin	R3, R5, R7, R4′	R3′	7.8	−8.65	287.80

The DMSO concentration in the culture medium was kept constant at 0.5%. After 24 h of exposure, the culture medium was collected, cells were lysed, and LDH activity was measured in the culture medium and in the cell lysate. Cytotoxicity was expressed as the ratio of extracellular to total LDH activity found inside and outside the cells. Under the experimental conditions used, no cytotoxicity was observed with any of the tested compounds.

Results

Activation of EpRE-Controlled Gene Expression by Flavonoids. The chemical structures of the tested flavonoids are shown in Table 1. To measure the potential of flavonoids to induce EpRE-mediated gene expression, Hepa-1c1c7 cells containing a firefly luciferase reporter gene under the expression regulation of an EpRE from the human NQO1 gene (EpRE-LUX cells) were used (*24*). The induction factor is defined as the potency of each flavonoid to increase luciferase expression compared to that in cells incubated with only the control medium. All flavonoids tested showed, as shown in the examples with quercetin, kaempferol, fisetin, and apigenin (Figure 1), a concentration-dependent luciferase induction. The concentrations tested ranged between 0.5 and 60 μM flavonoid. Table 1 shows the maximal level of induction (IF) observed for each flavonoid, which was reached at a concentration of 10−20 μM. Generally, flavonoids bearing a hydroxyl group at the 3-position are the best inducers of EpRE-mediated luciferase induction. Induction factors with these compounds ranged from 3-fold for 3,7-OH-flavone up to 10-fold for quercetin, whereas flavonoids without a hydroxyl group at the 3-position only show a low luciferase induction. In addition, three methylated flavonoid derivatives were included in this study. Isorhamnetin, the 3′-*O*-methylated metabolite of quercetin, shows a lower EpRE-mediated response of 7.8-fold, compared to that of quercetin with a 10-fold induction. In contrast, tectochrysin, the 7-*O*-methyl derivative of chrysin, with 4.4-fold induction and genkwanin, the 7-*O*-methyl derivative of apigenin, with an induction factor of 5-fold

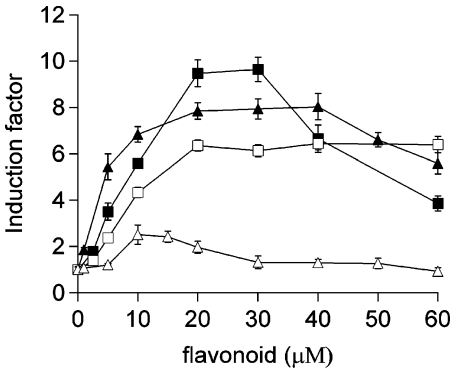


Figure 1. Induction of EpRE-mediated gene transcription by flavonoids. Effect of quercetin (-■-), fisetin (-▲-), kaempferol (-□-), and apigenin (-△-) on EpRE-mediated luciferase induction. Data are presented as means with standard error based on six independent measurements.

show a higher EpRE-mediated response compared to that of chrysin with 2.1-fold induction and apigenin with 2-fold induction. There is no significant correlation between luciferase induction and the degree of hydroxylation of flavonoids.

Involvement of PKC in EpRE-Mediated Gene Transcription Activation. To investigate whether EpRE-mediated transcription activation by flavonoids requires PKC activity, luciferase induction in the EpRE-LUX reporter cells by tBHQ, a standard inducer of the EpRE-mediated gene transcription, and two main dietary flavonoids with high inducing activity of EpRE-mediated gene expression, quercetin, and kaempferol, was studied in the presence of staurosporine, a standard inhibitor of PKC. Figure 2A shows the effect of increasing staurosporine concentration on the tBHQ-mediated luciferase induction in EpRE-LUX cells. Significant inhibition of the luciferase induction by tBHQ was already visible at 1 nM staurosporine. The tBHQ-mediated luciferase induction decreased from 12- to 5.5-fold (60% reduction) and remained nearly constant between 1 and 10 nM staurosporine (Figure 2A). Staurosporine showed no luciferase-inducing activity by itself up to a concentration of 10 nM (data not shown).

In contrast to the inhibition of the luciferase induction response to tBHQ, the induction of luciferase by quercetin and kaempferol in EpRE-LUX cells was not inhibited by the PKC inhibitor staurosporine up to a concentration of 10 nM (Figure 2B).

Correlation of the Redox Properties of Flavonoids with Their Induction of EpRE-Mediated Gene Expression. The E_{HOMO} values of the tested flavonoids are presented in Table 1, listing the E_{HOMO} values of the conformer with the lowest heat of formation, representing the most probable conformation. Figure 3 shows the relationship between the experimental induction factors (IF) of the EpRE-mediated gene transcription and the E_{HOMO} values of the tested flavonoids. A linear correlation with $r^2 = 0.701$ is obtained ($n = 21$). Because steric parameters can be important for the interaction of inducers with the Keap1−Nrf2 complex, it was investigated as to whether the Van der Waals volumes (VdW) of the flavonoids would provide a suitable second descriptor for a quantitative structure−activity relationship. The Van der Waals volumes of the flavonoids are presented in Table 1. Using these Van der Waals volumes, a two parameters quantitative structure−activity relationship could be obtained (eq 1) as follows.

$$\text{Predicted } IF = 7.83 \cdot E_{HOMO} + 0.059 \cdot \text{VdW} + 59.97$$

Figure 4 shows the relationship between the experimental induction factors (IF) of the EpRE-mediated gene transcription

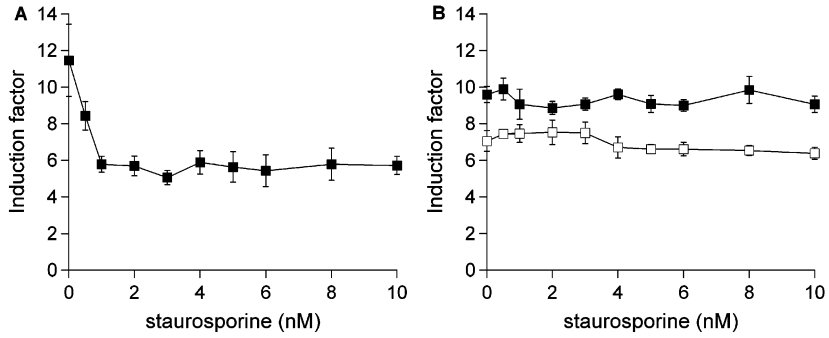


Figure 2. Effect of PKC inhibition by staurosporine on tBHQ- and flavonoid-induced EpRE-mediated gene transcription. (A) Effect of staurosporine on EpRE-mediated luciferase expression induced by 15 μM tBHQ. The effect of staurosporine was found to be significant ($p < 0.05$) starting from 1 nM staurosporine (Student's *t*-test). (B) The effect of stauropsorine on EpRE-mediated luciferase expression induced by 20 μM of quercetin (-■-) or kaempferol (-□-), respectively. All data are presented as means with standard error based on six independent measurements.

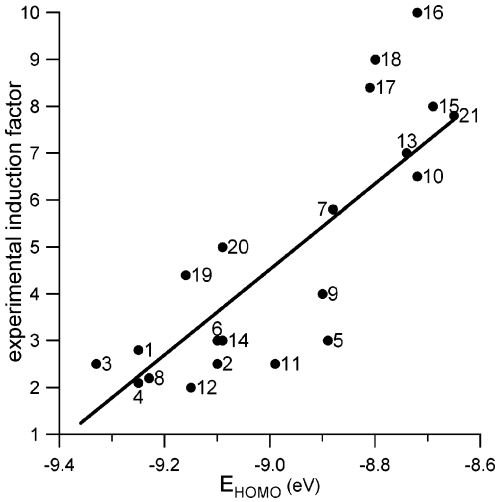


Figure 3. Correlation ($r^2 = 0.701$, $n = 21$) as observed for the tested flavonoids between the induction factor for EpRE-mediated gene transcription and redox properties quantified by their E_{HOMO} (eV) values. The numbers correspond to the numbers in Table 1.

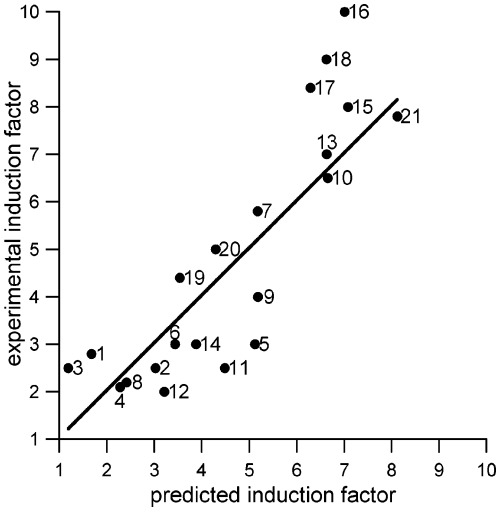


Figure 4. Correlation ($r^2 = 0.760$, $n = 21$) between the observed EpRE-mediated gene transcription induced by flavonoids and the induction factors predicted by eq 1 using E_{HOMO} and VdW as descriptors. The numbers correspond to the numbers in Table 1.

and the induction factors predicted by eq 1. A linear correlation is obtained with $r^2 = 0.760$ ($n = 21$). The internal cross-validated coefficient of the correlation between the experimental induction factor and the predicted induction factor is $q^2 = 0.638$,

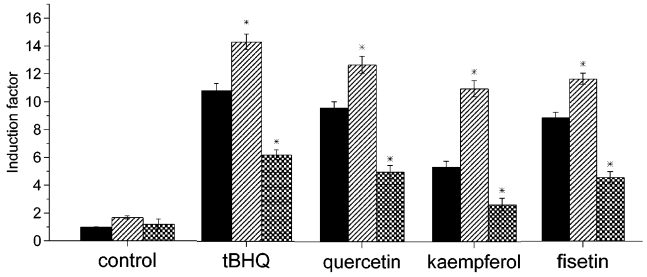


Figure 5. Effect of changes in intracellular GSH levels on tBHQ and flavonoid-induced EpRE activation. GSH levels were decreased by BSO and increased by NAC. Luciferase induction by 15 μM tBHQ, 20 μM quercetin, 20 μM kaempferol, 30 μM fisetin, and the control with only the medium in EpRE(hNQO1)-LUX cells in the absence (solid bar) or presence of 100 μM BSO (striped bar) or 40 mM NAC (criss-cross bar). All data are presented as means with standard error based on six independent measurements and (*) indicates a response significantly different from that of inducers alone ($p < 0.05$, Student's *t*-test,).

showing the validity of this correlation. Together, these data indicate that the E_{HOMO} value of each flavonoid appears to be an important factor, with the Van der Waals volume being a minor determinant, for predicting its EpRE-mediated response inducing capacity.

Role of the Pro-Oxidant Activity of Flavonoids in EpRE-Mediated Gene Transcription Activation. Three flavonoids with high EpRE-mediated response (quercetin, fisetin, and kaempferol) were selected to investigate whether flavonoids induce EpRE-mediated gene transcription by generating oxidative stress. The luciferase induction mediated by these three flavonoids was investigated in EpRE-LUX cells in which the intracellular concentration of GSH, a major compound present in cells for protection against oxidative stress, was varied. Figure 5 shows the effect of the modulation of the intracellular GSH concentration on the induction of EpRE-controlled luciferase expression as mediated by tBHQ, quercetin, kaempferol, and fisetin. The addition of 40 mM NAC, which increases GSH levels in cells (*28*), resulted in a significant decrease in luciferase induction with all tested compounds. The induction factor of tBHQ decreased from 11.0- to 6.3-fold (43% decrease, Figure 5), quercetin from 9.8- to 5.1-fold (48% decrease), and fisetin from 9.1- to 4.7-fold (48% decrease), and the kaempferol-mediated luciferase induction decreased from 5.5- to 2.7-fold (51% decrease) (Figure 5). NAC showed no significant effect on luciferase expression in non-induced (control) EpRE-LUX cells up to a concentration of at least 40 mM (Figure 5), indicating that an increase in intracellular GSH levels by itself does not affect the luciferase activity of EpRE-LUX cells.

The addition of BSO, which decreases the intracellular cytosolic GSH level (*29*), resulted in an increase in the EpRE-controlled luciferase induction by the test compounds (Figure 5). In the presence of 100 μM BSO, tBHQ-, quercetin- and fisetin-mediated luciferase induction increased up to 14.6-fold (33% increase), 13-fold (33% increase), and 12-fold (33% increase), respectively. For kaempferol, an increase in luciferase induction from 5.5- to 11.3-fold (105% increase) was observed. BSO alone also stimulated luciferase inductions by a factor of 1.6-fold in EpRE-LUX cells in a concentration-dependent manner (Figure 5). However, the response attained by flavonoid treatment in the presence of BSO is always considerably higher than the sum of the responses induced by BSO and flavonoid individually. This proves that the depletion of GSH with BSO leads to a more than additive enhancement of the EpRE-mediated gene expression activation by the inducers tested.

Discussion

Flavonoids are reported to induce phase 2 enzymes, which are important detoxifying enzymes in cells that are suggested to play a role in the prevention against cancer (*6*). The induction mechanism of detoxifying enzymes has been extensively studied, and activation of gene expression through EpRE has been described for various flavonoids. Among these compounds, flavones are found to be the most potent inducers of EpRE-mediated gene expression (*22*−*24*). Therefore, we tested 21 flavones, including some methylated derivatives, on their ability to induce EpRE-mediated gene expression. For this purpose, our newly developed EpRE-LUX cell assay (*24*) was used, which provides a powerful tool to measure EpRE-mediated transcription activation.

The results of the flavonoid-induced EpRE-mediated gene transcription show that hydroxylation at the 3-position of the flavonoids is important for the EpRE-controlled gene induction (Table 1). This finding is in line with other studies of flavonoid-mediated induction of NQO1-activity (*31*). Furthermore, our findings suggest that the degree of hydroxylation of the flavonoids do not seem to be important for the EpRE-mediated gene transcription (Table 1).

The bioavailability of flavones is relatively low. Plasma concentration of quercetin, including their phase II metabolites, in humans can reach up to several micromolars (*32*). The concentrations tested in this study ranged from 0.5 to 60 μM and relate to the unconjugated parent compounds. Because the physiological plasma concentrations will be in the lower range of the concentrations tested in the present study and because the effect of phase II conjugation on the flavonoid-induced activation of EpRE-mediated gene expression remains to be quantified, it can be concluded that the potential maximal level of EpRE-mediated gene induction *in vivo* may not be reached. However, intestinal concentrations of flavonoids are estimated to reach levels of 50 μM upon consumption of the average daily flavonoid intake (*33*). Therefore, regular consumption levels, and certainly the use of flavonoid supplements, may result in significant, up to maximal levels of flavonoid-induced activation of EpRE-mediated gene expression in the intestinal epithelium (Figure 1).

The release of Nrf2 from the Keap1−Nrf2 complex is a crucial step in the EpRE-mediated gene induction of detoxifying enzymes, and at least two important mechanisms for this event have been proposed. One proposed mechanism concerns an indirect effect of the inducer involving the activation of protein kinase C (PKC), resulting in the release of Nrf2 from Keap1 through the phosphorylation of Nrf2 (*14*). Besides PKC, kinases such as MAPK (mitogen-activated protein kinase) and PI3K (phosphatidylinositol 3-kinase) might also play a role in EpRE-mediated gene transcription (*34*−*35*). To test the involvement of PKC in the EpRE-mediated gene transcription activation by flavonoids, we used staurosporine as a specific inhibitor of PKC to study the inhibition of the luciferase induction response by tBHQ and flavonoids. Besides being a strong inhibitor of PKC (*36*), staurosporine is reported to inhibit most protein kinases with IC50 values in the range of 1−20 nM (*37*). Therefore, the experiments of the present study also give an indication on the involvement of MAPK and PI3K in the flavonoid-mediated transcription of detoxifying enzymes. In line with other studies (*38*), the EpRE-mediated gene transcription by tBHQ, which is generally used as a standard inducer of the EpRE-mediated gene transcription, was partially inhibited by staurosporine and is, thus, partially mediated by PKC (Figure 2A). Other pathways are apparently also contributing to this transcriptional activation, explaining why PKC inhibitors do not completely inhibit tBHQ-induced luciferase expression. Our findings suggest that PKC is not involved in the EpRE-mediated luciferase induction by flavonoids. This is concluded from the fact that 10 nM staurosporine, which is sufficient to inhibit 60% of the tBHQ-mediated luciferase activation, did not affect EpRE-mediated luciferase induction by quercetin or kaempferol.

Another proposed mechanism for Nrf2 release from Keap1 suggests a direct oxidative modification of Keap1 by inducers of EpRE-mediated gene transcription. Keap1 contains several reactive cysteine residues, and disulfide bridge formation between two neighboring Keap1 monomers holding Nrf2 in complex is proposed to result in the release of Nrf2 (*18*). In this way, Keap1 might act as a direct sensor for oxidative stress. Consistently, EpRE-mediated gene expression is reported to be responsive to oxidative type inducers such as ROS and electrophiles. Because flavonoids tend to act as antioxidants rather than oxidants, at first glance, an oxidative stress mechanism does not seem relevant to explain the induction of Nrf2 release from Keap1 by flavonoids. However, flavonoids have been described to display pro-oxidant activity after donating electrons by antioxidant action, enzymatic oxidation, or autoxidation (*4*, *39*−*40*). Flavonoids, especially the ones with a catechol moiety, have the potential to oxidize to quinones or semiquinones resulting in redox cycling and ROS production as well as in thiol, DNA, and protein alkylation (*4*−*5*, *41*−*43*). Although the pro-oxidant action of flavonoids is generally considered as unfavorable, the results of the present study indicate that the pro-oxidant action of flavonoids is actually of importance for their inducing activity of an EpRE-mediated response, an effect that can be considered beneficial. This could be concluded from the fact that the E_{HOMO} of the 21 tested flavonoids correlates with their induction factor of the EpRE-mediated gene transcription (Figure 3). E_{HOMO} models the ease of a molecule to donate an electron and has been shown to correlate with the reduction potential, which characterizes the ease of oxidation of a compound. Our result is in line with a study reporting the E_{HOMO} of 34 inducers, belonging to 9 different classes but not including flavonoids, to correlate with the induction of NQO1 enzyme activity in Hepa1c1c7 cells upon 24 h exposure to these inducers (*44*).

The observation that the inducing activity of flavonoids correlates with their redox properties explains the importance of the presence of a 3-hydroxyl group for their EpRE-mediated response (Table 1). Hydroxylation at the 3-position strongly increases the E_{HOMO}, and thus the ease of a flavonoid to donate electrons, whereas the overall degree of hydroxylation only marginally influences its redox behavior.

Another line of evidence provided in the present study that supports the conclusion that the pro-oxidant action of flavonoids is essential for their inducing effect of EpRE-mediated response results from studies characterizing the consequences of modulating the intracellular GSH levels for flavonoid-induced EpRE-mediated gene induction. GSH is essential for maintaining reducing conditions and the reduced state of protein thiols by scavenging reactive free radicals and electrophiles and/or by the regeneration of thiol molecules upon their oxidation (*45*). Preincubation of the EpRE-LUX cells with NAC to generate increased intracellular GSH levels resulted in a decrease in the EpRE-mediated gene transcription by quercetin, kaempferol, fisetin, and also by the standard inducer tBHQ (Figure 5). In contrast, reducing the cellular GSH levels by BSO significantly increased the flavonoid-mediated response (Figure 5). Taken together, these data provide evidence that EpRE-mediated gene transcription by flavonoids is based on their pro-oxidant activity. This finding is in line with other literature reports, suggesting that the pro-oxidant action of flavonoids rather than their antioxidant activity is important for their anticancer properties (*46*−*47*).

The precise mechanism by which the pro-oxidant chemistry of flavonoids mediates the EpRE induction is not known but may be related to either ROS production and/or direct alkylation by the flavonoid (semi)quinones resulting in the disruption of the Keap1−Nrf2 complex and release of Nrf2 (*9*, *18*, *48*). In the latter case, flavonoid quinones might, similar to triterpenoids (*21*), directly react with cysteine residues of Keap1, leading to conformational changes and Nrf2 release. A direct flavonoid quinone Keap1 interaction could be dependent on steric constraints and, thus, be dependent on steric parameters of the flavonoid such as its Van der Waals volume. In line with this suggestion, the use of the Van der Waals volume as a second descriptor for the quantitative structure−activity relationship for the prediction of the induction factors of EpRE-mediated gene transcription improves the correlation with the experimental data (Figure 4).

However, the fact that tBHQ can induce EpRE-mediated gene expression through PKC but also by its pro-oxidant activity indicates that an inducer may operate via more than one route for EpRE-mediated gene expression. Whether this also holds for the flavonoid-mediated induction remains to be investigated, but the present study clearly eliminates PKC-dependent pathways as an important mechanism for flavonoid-mediated activation of EpRE-controlled gene transcription.

Another possible pathway for the flavonoid-induced activation of EpRE-mediated gene transcription is the Ah receptor (AhR)-signaling pathway. Recently it was reported that Nrf2 is regulated by the AhR (*49*), and interacting networks between Nrf2 and AhR are increasingly discovered (*50*). Although quercetin and kaempferol are reported to be monofunctional inducers (*51*), which means that they only induce EpRE-mediated gene expression without the involvement of AhR signaling, other studies report flavonoids to be bifunctional inducers (*52*−*53*). A structure-based study should be performed to define which flavonoids are inducing EpRE-mediated gene transcription without the involvement of the AhR and which flavonoids require AhR signaling, for example, to (i) generate metabolites or (ii) to generate ROS, which in turn induces EpRE-mediated gene transcription.

Altogether, this study demonstrates that the pro-oxidant activity of flavonoids can contribute to their health-promoting activity by inducing important detoxifying enzymes, pointing to a beneficial effect of a supposed toxic chemical reaction.

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