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A comparison of the neurotrophic activities of the flavonoid fisetin and some of its derivatives

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Abstract
Neurotrophic factors promote the development, maintenance and regeneration of nerve cells. Classical neurotrophic factors are proteins and thus not well-suited for therapeutic purposes. Recently, we showed that specific flavonoids such as fisetin (3, 7, 3′, 4′ tetrahydroxyflavone) promote the differentiation of nerve cells in culture through the activation of extracellular signal-regulated kinase (ERK) suggesting that flavonoids could substitute for neurotrophic factors. It has also been shown that fisetin promotes nerve cell survival following exposure to toxic oxidative insults. To determine whether or not this is unique to fisetin, a series of related compounds were assayed for neurotrophic activities. Many of these related compounds also promote nerve cell differentiation and are neuroprotective against toxic oxidative insults. However, the mechanisms underlying these neurotrophic effects differ among the compounds.

Keywords: *Flavonoids, differentiation, neuroprotection, oxidative stress, glutathione, extracellular signal-regulated kinase (ERK)*

Introduction

Neurotrophic factors support the survival, differentiation and functional maintenance of nerve cells. Because of these properties, neurotrophic factors have the potential to treat a variety of chronic and acute disorders of the central nervous system (CNS). However, due to their large size and proteinaceous nature, classical neurotrophic factors, such as nerve growth factor, are not well-suited for therapeutic purposes [1]. Thus, the identification of small molecules which can mimic some or all of the properties of neurotrophic factors could have great potential for treating CNS disorders. Recently, we described the ability of the flavonoid fisetin (3, 7, 3′, 4′ tetrahydroxyflavone) to promote the differentiation of nerve cells [2]. Although a wide range of flavonoids were tested in this study, most failed to induce differentiation. Of the few that did, fisetin was by far the best, showing significantly greater efficacy than any of the other flavonoids. The induction of differentiation by fisetin is dependent on the activation of the Ras-ERK cascade because inhibitors of this cascade block differentiation. Not only does fisetin promote nerve cell differentiation but in earlier studies it was shown to protect nerve cells from oxidative stress-induced death [3]. Thus, fisetin has several of the properties of classical neurotrophic factors. In the present study, the relationship between the structure and activity of fisetin was examined using derivatives of the flavonoid which lack one or more hydroxyl groups with the goal of determining if fisetin is unique or if closely related flavonoids have similar or enhanced properties. All of the derivatives tested could promote nerve cell differentiation and protect nerve cells from toxic oxidative insults and several of the derivatives were better than fisetin in both assays.

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Surprisingly, however the mechanisms underlying these neurotrophic activities varied among the different derivatives.

Materials and methods

Chemicals

Fisetin was from Sigma/Aldrich (St Louis, MO). Other flavonoids were from Indofine Chemical Co. (Hillsborough, NJ). PD98059 was from Promega (Madison, WI). All other chemicals were from Sigma.

Cell culture

Fetal calf serum (FCS), dialyzed FCS (DFCS) and horse serum were from Hyclone (Logan, UT). Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Gibco (Carlsbad, CA). HT-22 cells [4,5] were grown in DMEM supplemented with 10% FBS and antibiotics. PC12 cells [6] were maintained in DMEM supplemented with 10% horse serum (Hyclone), 5% FCS and antibiotics. To examine the effects of flavonoids and other agents on the PC12 cells, the culture medium was removed and replaced by the chemically defined N2 medium (Gibco) 18 h prior to the start of the experiment.

Differentiation assay

PC12 cells in N2 medium were treated with flavonoids for 24 h at which time the cells were scored for the presence of neurites. PC12 cells produce neurites much more rapidly when treated in N2 medium than when treated in regular growth medium [7]. For each treatment, 100 cells in each of three separate fields were counted. Cells were scored positive if one or more neurites >1 cell body diameter in length were observed.

Cytotoxicity assay

Cell viability was determined by a modified version of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay based on the standard procedure [8]. Briefly, cells were seeded onto 96-well microtiter plates at a density of 5×10^3 cells per well. The next day, the medium was replaced with DMEM supplemented with 7.5% DFCS and the cells were treated with the different flavonoids alone or in the presence of 5 mM glutamate. Twenty four hours later, the medium in each well was aspirated and replaced with fresh medium containing 2.5 μg/ml MTT. After 4 h of incubation at 37°C, cells were solubilized with 100 μl of a solution containing 50% dimethylformamide and 20% SDS (pH 4.7). The absorbance at 570 nm was measured on the following day with a microplate reader (Molecular Devices). Results obtained from the MTT assay correlated directly with the extent of cell death as confirmed visually. Controls employing wells without cells were used to determine the effects of the agents upon the assay chemistry.

SDS-PAGE and immunoblotting

PC12 cells in N2 medium were treated with the flavonoids as described in the figure legends and, after the indicated time periods, the cells were solubilized in SDS-sample buffer containing 0.1 mM Na_3VO_4 and 1 mM phenylmethylsulfonyl fluoride (PMSF), boiled for 5 min and either analyzed immediately or stored frozen at -70°C. Proteins were separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose. Equal loading and transfer of the samples was confirmed by staining the nitrocellulose with Ponceau-S. Transfers were blocked for 2 h at room temperature with 5% nonfat milk in TBS/0.1% Tween 20 and then incubated overnight at 4°C in the primary antibody diluted in 5% BSA in TBS/0.05% Tween 20. The primary antibodies used were: phospho-p44/42 MAP kinase antibody (#9101, 1/1000) from Cell Signaling (Beverly, MA) and pan ERK antibody (#E17120, 1/10000) from Transduction Laboratories (San Diego, CA). The transfers were rinsed with TBS/0.05% Tween 20 and incubated for 1 h at room temperature in horseradish peroxidase-goat anti-rabbit or goat anti-mouse (Biorad, Hercules, CA) diluted 1/5000 in 5% nonfat milk in TBS/0.1% Tween 20. The immunoblots were developed with the Super Signal reagent (Pierce, Rockford, IL).

For immunoblotting of HO-1, untreated and flavonoid-treated HT22 cells from the same density cultures as used for the cell death assays were washed twice in cold phosphate-buffered saline (PBS) then scraped into lysis buffer containing 50 mM HEPES, pH 7.4, 150 mM NaCl, 50 mM NaF, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 10 mM sodium pyrophosphate, 1 mM Na_3VO_4, 1 mM PMSF, 15 μg/ml aprotinin, 1 μg/ml pepstatin, and 5 μg/ml leupeptin. Lysates were incubated at 4°C for 30 min, then cleared by centrifugation at 14,000 rpm for 10 min. For immunoblotting of Nrf2, nuclear extracts were prepared as described [9] from untreated and flavonoid-treated cells. For each flavonoid, the concentration which was most effective at preventing cell death was used. Protein concentrations were determined using the BCA protein assay (Pierce). Equal amounts of protein were solubilized in 2.5X SDS-sample buffer, separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose. The primary antibodies used were: anti-Nrf2 (#SC13032; 1/1000) from Santa Cruz Biotechnology (Santa Cruz, CA) and anti-heme oxygenase-1 (HO-1) (#SPA-896; 1/5000) from Stressgen (Victoria, BC Canada).

Total intracellular GSH/GSSG

Total intracellular GSH/GSSG was determined using whole cell lysates from untreated, glutamate treated, flavonoid treated and glutamate plus flavonoid treated cells as described [10] and normalized to total cellular protein. For each flavonoid, the concentration which was most effective at preventing cell death was used.

Determination of the Trolox equivalent activity concentration (TEAC)

TEAC values for the flavonoids were determined according to [11] but modified for a plate reader. Briefly, 250 μl of 2,2′-azinobis(3-ethylbenzothiazoline 6-sulfonate)(ABTS) treated overnight with potassium persulfate and diluted to an OD of ~0.7 at 734 nm was added to 2.5 μl of a flavonoid solution in ethanol. The change in absorbance due to the reduction of the ABTS radical cation was measured at 734 nm for 4 min. To calculate the TEAC, the gradient of the plot of the percentage inhibition of absorbance vs. concentration for the flavonoid in question is divided by the gradient of the plot for Trolox.

Statistical analysis

Experiments presented were repeated at least three times. The data are presented as the mean ± SD. An unpaired Student's *t*-test was used to compare the data obtained.

Results

To determine the structure-activity relationship between fisetin (3, 7, 3′, 4′ tetrahydroxyflavone) and nerve cell differentiation, four trihydroxyflavones (THF) and three dihydroxyflavones (DHF) which lack one or two, respectively, of the hydroxyl groups found in fisetin were tested. Their ability to induce the differentiation of PC12 cells was assayed by scoring neurite outgrowth [2]. As shown in Table I, all of these fisetin derivatives exhibited some ability to induce differentiation although there was a good deal of variation in both their potency and efficacy. By far the best was 3, 3′, 4′ THF, which induced the differentiation of >80% of the PC12 cells at a concentration of 5 μM. Fisetin activates ERK and fisetin-induced differentiation is dependent upon the activation of this kinase [2]. To determine if the fisetin derivatives could also activate ERK, PC12 cells were treated with the optimal concentrations for differentiation of the different derivatives for 10 min–6 h and then analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting with an antibody to phospho-ERK. All of the fisetin derivatives induced ERK activation with a delayed time course similar to that of fisetin (Figure 1A and data not shown). ERK activation by the different flavonoids was blocked by pretreatment with PD98059, an inhibitor of MEK, the kinase which phosphorylates ERK (Figure 1B). To determine if the differentiation seen following treatment with the fisetin derivatives was dependent upon ERK activation, PC12 cells were pre-treated with 50 μM PD98059 before the addition of the fisetin derivatives and neurite outgrowth was scored the next day. As shown in Figure 2A, inhibition of ERK activation significantly reduced the differentiation induced by all of the fisetin derivatives.

Table I. Induction of PC12 cell differentiation.

Flavonoid	EC_{50} (μM)	Maximal efficacy (%)
3, 3′ DHF	10	40–50
3, 4′ DHF	20	50–60
3′, 4′ DHF	10	40–50
3, 3,′ 4′ THF	2.5	80–90
3, 7, 3′ THF	15	40–50
3, 7, 4′ THF	10	70–80
7, 3′, 4′ THF	10	50–60
3, 7, 3,′ 4′ THF (fisetin)	5	70–80

Half maximal effective concentrations (EC_{50}) and maximal efficacy for differentiation were determined by exposing PC12 cells to different concentrations of each flavonoid and assessing differentiation as described in Materials and methods.

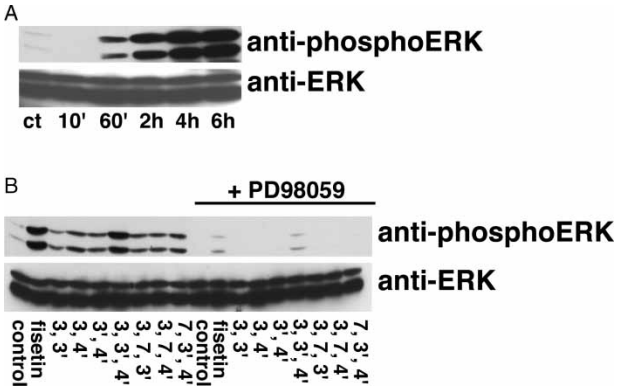

Figure 1. A. Time course of 3, 3′, 4′ THF-induced ERK activation. PC12 cells in N2 medium were untreated (Ct) or treated with 5 μM 3, 3′, 4′ THF for 10 min–6 h. The cells were scraped into sample buffer and analyzed by SDS-PAGE and immunoblotting with an antibody specific for phosphorylated ERKs (anti-phospho ERK) and an antibody that detects phosphorylated and unphosphorylated ERKs (anti-ERK). Similar results were obtained in three independent experiments. B. MEK inhibitors block ERK activation induced by fisetin derivatives. PC12 cells in N2 medium were untreated (control) or treated with 10 μM fisetin, 25 μM 3, 3′ DHF, 25 μM 3, 4′ DHF, 25 μM 3′, 4′ DHF, 5 μM 3, 3′, 4′ THF, 25 μM 3, 7, 3′ THF, 25 μM 3, 7, 4′ THF or 10 μM 7, 3′, 4′ THF for 4 h in the absence or presence of 50 μM PD98059 (30 min pretreatment). The cells were scraped into sample buffer and analyzed by SDS-PAGE and immunoblotting with an antibody specific for phosphorylated ERKs (anti-phospho ERK) and an antibody that detects phosphorylated and unphosphorylated ERKs (anti-ERK). Similar results were obtained in two independent experiments.

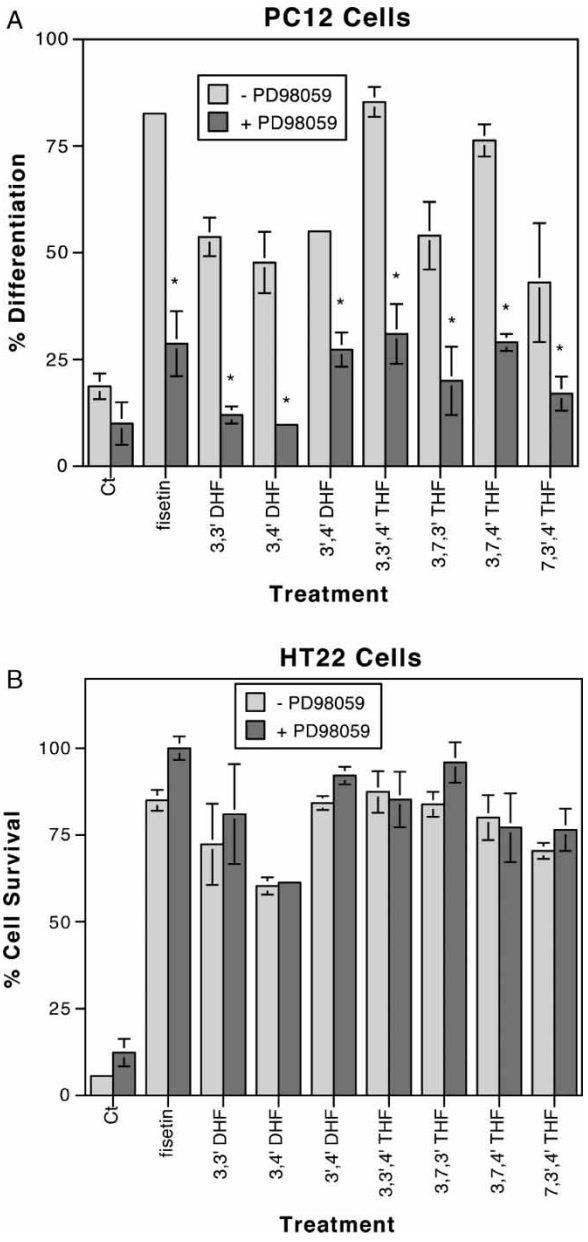

Figure 2. A. MEK inhibitors block fisetin derivative-induced PC12 cell differentiation. PC12 cells in N2 medium were untreated (Ct) or treated with 10 μM fisetin, 10 μM 3, 3′ DHF, 25 μM 3, 4′ DHF, 10 μM 3′, 4′ DHF, 5 μM 3, 3′, 4′ THF, 25 μM 3, 7, 3′ THF, 25 μM 3, 7, 4′ THF or 10 μM 7, 3′, 4′ THF in the absence or presence of 50 μM PD98059 (30 min pretreatment). Twenty-four hours later, the cells were scored for the presence of neurites. For each treatment, 100 cells in each of three separate fields were counted. Cells were scored positive if one or more neurites >1 cell body diameter in length was observed. The results presented are the average ± SD of 2–3 independent experiments. ⋆ indicates a significant difference between the cells treated with PD98059 and those treated without PD98059. B. MEK inhibitors do not block fisetin derivative-induced HT22 cell protection from oxidative glutamate toxicity. HT22 cells were untreated (Ct) or treated with 10 μM fisetin, 25 μM 3, 3′ DHF, 25 μM 3, 4′ DHF, 2.5 μM 3′, 4′ DHF, 2.5 μM 3, 3′, 4′ THF, 25 μM 3, 7, 3′ THF, 25 μM 3, 7, 4′ THF or 10 μM 7, 3′, 4′ THF in the presence of 5 mM glutamate ± 50 μM PD98059. Twenty-four hours later cell survival was measured by the MTT assay. The results presented are the average ± SD of two independent experiments.

Fisetin is also very effective at protecting nerve cells from oxidative stress-induced death [3]. To determine the structure-activity relationship between fisetin and neuroprotection, the fisetin derivatives were tested in the model of oxidative glutamate toxicity using mouse HT22 cells. This pathway of programmed cell death is initiated by the addition of glutamate to the extracellular medium. Glutamate inhibits the uptake of cystine which is required for glutathione (GSH) biosynthesis, resulting in the depletion of GSH in neurons [for review see 12]. Subsequently, this decrease in cellular GSH results in the production of reactive oxygen species (ROS) by mitochondria. The ROS accumulation then causes Ca^{+2} influx from the extracellular medium which leads to cell death. Fisetin was shown to both act as an antioxidant and to maintain the intracellular levels of GSH [3]. All of the fisetin derivatives were effective at preventing cell death in this model of neuronal oxidative stress (Table II) although, as with the differentiation studies, there was a good deal of variability in their efficacy and potency. Similar to the results with the differentiation assay, 3, 3′, 4′ THF was found to be more effective than fisetin with an EC_{50} of 1 μM and $>85\%$ protection seen at 2.5 μM. However, unlike the differentiation assay, 3′, 4′ DHF was also more effective than fisetin. Furthermore, in contrast to the differentiation assay, the protection against oxidative stress was not dependent upon ERK activation since treatment with PD98059 had no significant effect on the ability of fisetin or its derivatives to prevent cell death (Figure 2B).

To determine if the protection seen with the different fisetin derivatives correlated with their antioxidant properties, their TEAC values were determined. In this procedure, a compound is compared with Trolox, a water-soluble vitamin E analog, for its ability to reduce the radical cation of ABTS in aqueous solution. As reported previously, fisetin has a TEAC value of ~3 (Table II). None of the derivatives had TEAC values in this range despite, in some cases, showing better protection against oxidative stress-induced death. Thus, there was no correlation with antioxidant activity as defined by TEAC value and protection in this system.

In addition to being a relatively good antioxidant, part of the protection against oxidative stress provided by fisetin is due to its ability to maintain GSH levels [3]. To determine if the ability to maintain GSH levels also plays a role in the protection mediated by the fisetin derivatives, the effect of buthionine sulfoximine (BSO) on protection was examined. BSO inhibits glutamate cysteine ligase, the rate limiting enzyme in GSH biosynthesis, leading to a time-dependent decrease in GSH levels. As shown in Figure 3A, low levels of BSO significantly reduced protection by 3, 3′, 4′ THF. Higher levels reduced protection by fisetin, 3, 4′ DHF, 3′, 4′ DHF and 7, 3′, 4′ THF. Protection by

Table II. Protection from oxidative-stress induced death.

Flavonoid	EC_{50} (μM)	Maximal efficacy (%)	TEAC
3, 3′ DHF	12	>80	1.01 ± 0.01
3, 4′ DHF	10	>70	0.62 ± 0.06
3′, 4′ DHF	0.9	>90	1.24 ± 0.08
3, 3,′ 4′ THF	0.75	>90	2.07 ± 0.06
3, 7, 3′ THF	17	>90	1.55 ± 0.34
3, 7, 4′ THF	12.5	>90	1.01 ± 0.10
7, 3′, 4′ THF	5	>80	0.89 ± 0.04
3, 7, 3,′ 4′ THF (fisetin)	5	>90	3.11 ± 0.20

Half maximal effective concentrations (EC_{50}) and maximal efficacy for protection were determined by exposing HT22 cells to different concentrations of each flavonoid in the presence of 5 mM glutamate. Cell viability was determined after 24 h by the MTT assay. TEAC values were determined using the $ABTS^{\cdot+}$ decolorization assay with Trolox as a reference as described in Materials and methods.

all of the other flavonoids was unaffected by BSO. Consistent with these observations, among the fisetin derivatives, only 3, 3′, 4′ THF was able to maintain GSH levels in the presence of oxidative stress (Figure 3B) while both 3′, 4′ DHF and 7, 3′, 4′ THF raised the basal levels of GSH but could not maintain GSH levels in the presence of oxidative stress. These results suggest that all of the fisetin derivatives except 3, 3′, 4′ THF protect nerve cells from toxic oxidative insults by GSH-independent mechanisms whereas protection by 3, 3′, 4′ THF is dependent on its ability to enhance GSH levels in cells.

Cells possess a number of different endogenous antioxidant defense mechanisms. Induction of phase II detoxification proteins can provide long-term protection of cells against oxidative stress. Several studies have shown that fisetin as well as other flavonoids can induce the activity and expression of phase II detoxification proteins [13–15]. The phase II detoxification proteins include enzymes associated with glutathione biosynthesis and metabolism and redox sensitive proteins such as heme oxygenase 1 (HO-1) [16]. The transcriptional activation of these and other genes encoding phase II detoxification proteins is mediated by a *cis*-acting enhancer termed the antioxidant response element (ARE). Transcriptional activation of the ARE is dependent upon the transcription factor NF-E2-related factor 2 (Nrf2), a member of the Cap'n'Collar family of bZIP proteins [17]. Since the ability to activate the ARE could be important for some of the beneficial of flavonoids such as fisetin, it was asked which, if any, of the fisetin derivatives could activate the ARE and induce the synthesis of downstream proteins such as heme oxygenase-1 (HO-1). In contrast to the other assays, fisetin was the best of the group at increasing the levels of Nrf2 and inducing HO-1 (Figure 4A) synthesis. Several of the fisetin derivatives were fairly effective, including 3, 4′ DHF, 3, 3′, 4′ THF and 3, 7, 4′ THF, and 3′, 4′ DHF was weakly active while the rest of the derivatives were inactive in this assay. ERK activation is implicated in the induction of Nrf2 by certain stimuli in some cell types. To determine if ERK activation plays a role in the induction of Nrf2 and HO-1 by fisetin and its derivatives, cells were pretreated with PD98059 prior to the addition of the flavonoids to the cells. As shown in Figure 4B, inhibition of ERK activation reduced Nrf2 and HO-1 induction by 3, 7, 4′ THF but had no effect on the induction by fisetin or any of the other active derivatives.

Discussion

Twenty eight different flavonoids, including representatives of all of the six different flavonoid classes, were previously assayed for their ability to promote neurite outgrowth in PC12 cells, a well-established model of nerve cell differentiation [for review see 18]. Among the flavonoids tested, only four were found to promote differentiation and of these, fisetin was by far the most effective. Fisetin has an EC_{50} for differentiation of 5 μM and at 10 μM routinely induces the differentiation of 75–80% of the cells. The other three flavonoids that induced differentiation, luteolin, quercetin and isorhamnetin, had EC_{50}s of 10 μM and at best induced the differentiation of only 50% of the cells. Since both luteolin (5, 7, 3′, 4′ tetrahydroxyflavone) and quercetin (3, 5, 7, 3′, 4′ pentahydroxyflavone) are closely related to fisetin, these results suggested that there was something unique about the structure of fisetin that made it particularly effective at inducing differentiation.

The studies reported here address the structure-activity relationship between fisetin and differentiation by examining the differentiation-promoting capabilities of a variety of fisetin derivatives which lack one or two of the four hydroxyl groups found in fisetin. Among these derivatives are two, 3, 3′, 4′ THF and 3, 7, 4′ THF, that, like fisetin, promote the differentiation of 75–80% of the cells. Although 3, 7, 4′ THF is somewhat less potent than fisetin with an EC_{50} of 10 μM, 3, 3′, 4′ THF is more potent with an EC_{50} of 2.5 μM. 3, 4′ DHF is slightly less efficacious and potent than fisetin but still better than all of the other derivatives tested. Taken together these data suggest several requirements for the differentiation promoting activity of flavonoids. First, three hydroxyl

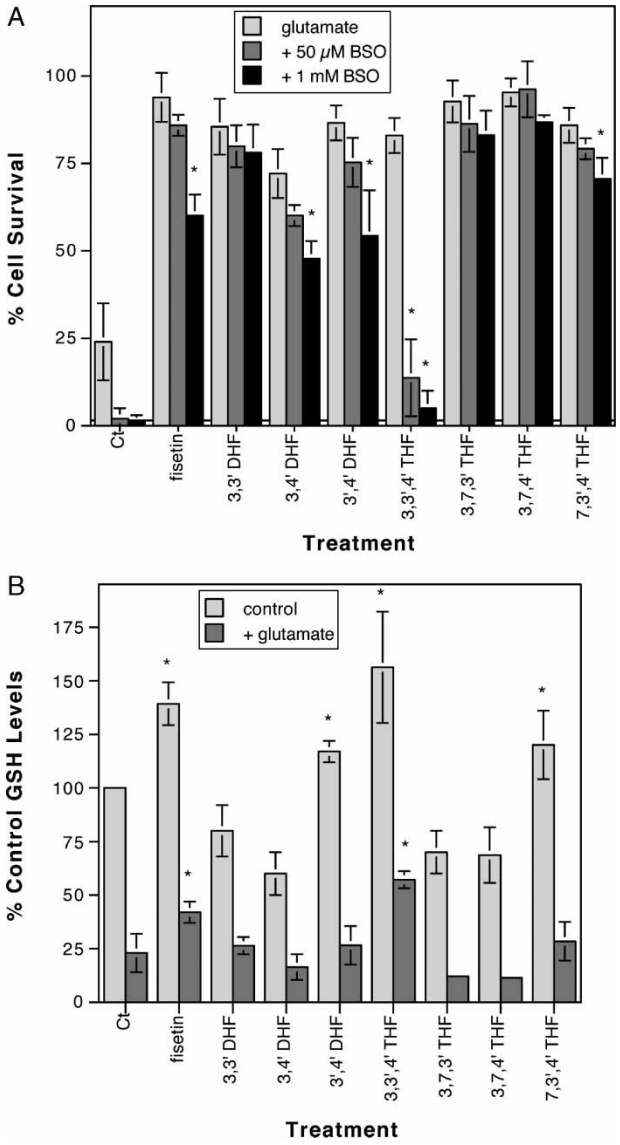

Figure 3. A. Protection by fisetin derivatives is differentially affected by BSO. HT22 cells were untreated (Ct) or treated with 10 μM fisetin, 25 μM 3, 3′ DHF, 25 μM 3, 4′ DHF, 2.5 μM 3′, 4′ DHF, 2.5 μM 3, 3′, 4′ THF, 25 μM 3, 7, 3′ THF, 25 μM 3, 7, 4′ THF or 10 μM 7, 3′, 4′ THF in the presence of 5 mM glutamate ± 50 μM BSO or 1 mM BSO. Twenty-four hours later cell survival was measured by the MTT assay. The results presented are the average ± SD of five independent experiments. ⋆ indicates a significant difference between the cells treated with BSO and those treated without BSO. B. Effects of fisetin derivatives on cellular GSH levels. HT22 cells were untreated (Ct) or treated with 10 μM fisetin, 25 μM 3, 3′ DHF, 25 μM 3, 4′ DHF, 2.5 μM 3′, 4′ DHF, 2.5 μM 3, 3′, 4′ THF, 25 μM 3, 7, 3′ THF, 25 μM 3, 7, 4′ THF or 10 μM 7, 3′, 4′ THF alone or in the presence of 5 mM glutamate. After 8 h cellular levels of total GSH were determined as described in Methods. The GSH level of the control sample (31.5 ± 10 nmoles/mg protein) was taken as 100%. The results are the means ± SD of duplicate determinations from 3 to 5 independent experiments. ⋆ indicates a significant difference between the control and the sample treated with a fisetin derivative.

groups appears to be optimal for the induction of differentiation. Both the removal or addition of hydroxyl groups to 3, 3′, 4′ THF reduces its differentiation promoting activity. Fisetin, with one additional hydroxyl (7), is less effective than 3, 3′, 4′ THF but much better than quercetin, with two additional hydroxyls (7 and 5) while myricetin, with three additional hydroxyls (7, 5 and 5′), is completely ineffective. Second, of the three hydroxyl groups present in 3, 3′, 4′ THF, the 4′ hydroxyl appears to be most important for good differentiation-promoting activity since derivatives without this group are much less effective.

Similar to fisetin, all of the fisetin derivatives induce ERK activation (Figure 1) and this effect is required for the promotion of differentiation since the MEK inhibitor PD98059 significantly reduces the differentiation induced by all of the derivatives. All of the fisetin derivatives also show the delayed time course of ERK activation that is observed with fisetin further suggesting that they are all using a similar pathway for activation. However, since the efficacy of ERK activation is not in precise agreement with the ability of the different derivatives to promote differentiation and PD98059 does not completely block differentiation in all cases, other signaling pathways may contribute to the differentiation-inducing activity of some of the derivatives.

Earlier studies demonstrated that fisetin could protect nerve cells from oxidative stress-induced death [3]. This observation led to the testing of the fisetin derivatives in this cell death assay. Unlike with the differentiation assay, a number of the fisetin derivatives were found to be equal to or more effective than fisetin in the protection assay. These include 3′, 4′ DHF, 3, 3′, 4′ THF and 7, 3′, 4′ THF. However, unlike differentiation, protection from oxidative stress was not dependent upon ERK activation. Structure-activity analysis shows a clear need for the 3′, 4′ catechol group for maximal protection. However, the catechol group is not essential for protection since all of the other fisetin derivatives were reasonably effective at preventing oxidative stress-induced death. Surprisingly, even among the most effective fisetin derivatives, the mechanisms underlying protection appeared to differ. Only protection by 3, 3′, 4′ THF was strongly dependent upon its ability to maintain GSH levels. Interestingly, antioxidant activity, as determined by measuring TEAC values, does not appear to play a major role in the protection by any of the fisetin derivatives. Thus, as demonstrated previously [3], similar levels of potency and efficacy with respect to protection from oxidative stress-induced death can be achieved by different means, even among closely related molecules.

In contrast to the results for protection, the ability to activate the ARE shows a reasonable correlation with the ability to induce differentiation. This suggests that the same pathways could be involved in the two actions of the flavonoids. However, except for 3, 7, 4′ THF, pretreatment with the MEK inhibitor PD98059 had little effect on Nrf2 induction or enhancement of

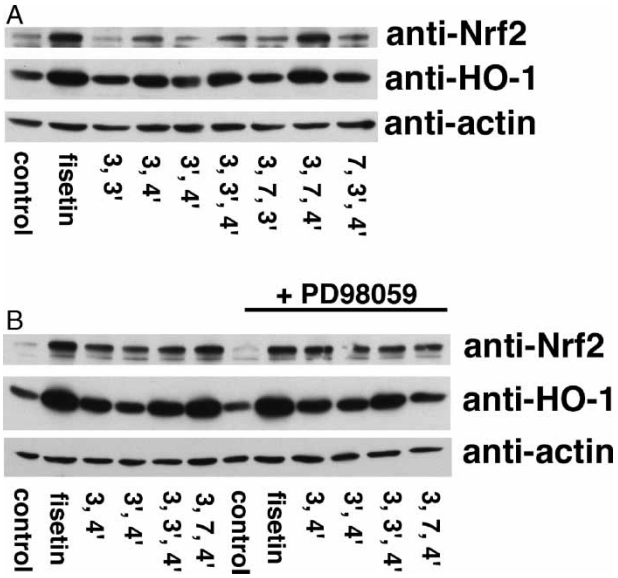

Figure 4. Fisetin derivatives induce the expression of Nrf2, the ARE-specific transcription factor and HO-1, a phase II detoxification protein. (A) HT22 cells were untreated (control) or treated with 10 μM fisetin, 25 μM 3, 3′ DHF, 25 μM 3, 4′ DHF, 10 μM 3′, 4′ DHF, 10 μM 3, 3′, 4′ THF, 25 μM 3, 7, 3′ THF, 25 μM 3, 7, 4′ THF or 10 μM 7, 3′, 4′ THF for 4 h. Nuclei were prepared and equal amounts of protein were analyzed by SDS-PAGE and immunoblotting with anti-Nrf-2, anti-HO-1 and anti-actin antibodies. Similar results were obtained in 2–3 independent experiments. (B) HT22 cells were untreated (control) or treated for 24 h with 10 μM fisetin, 25 μM 3, 3′ DHF, 25 μM 3, 4′ DHF, 10 μM 3′, 4′ DHF, 10 μM 3, 3′, 4′ THF, 25 μM 3, 7, 3′ THF, 25 μM 3, 7, 4′ THF or 10 μM 7, 3′, 4′ THF. Cell lysates were prepared and equal amounts of cellular protein were analyzed by SDS-PAGE and immunoblotting with anti-Nrf2 and anti-HO-1 antibodies. Immunoblotting with anti-actin is shown as a loading control. Similar results were obtained in 2–3 independent experiments.

HO-1 synthesis as compared with the effect on differentiation. This result indicates that even closely related molecules can utilize distinct signaling pathways in order to achieve the same outcome, and supports the idea that it is difficult to predict the mechanism of action based on structure alone.

In summary, with the long-term goal of identifying small molecules which can act as neurotrophic factors, derivatives of fisetin were tested for their ability to promote nerve cell differentiation and survival. Among the derivatives, 3, 3′, 4′ THF is better than fisetin in both assays and stands out as potentially useful for the treatment of CNS disorders. In particular, this derivative is very effective at maintaining GSH levels in the presence of oxidative stress and so might be especially effective for promoting neuronal survival and recovery in situations where GSH loss plays a significant role such as Parkinson's disease [19].

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