Genes Nutr (2009) 4:297–307
DOI 10.1007/s12263-009-0142-5

RESEARCH PAPER

Modulation of multiple pathways involved in the maintenance of neuronal function during aging by fisetin

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Received: 17 July 2009 / Accepted: 3 August 2009 / Published online: 10 September 2009

Abstract Multiple factors have been implicated in the age-related declines in brain function. Thus, it is unlikely that modulating only a single factor will be effective at slowing this decline. A better approach is to identify small molecules that have multiple biological activities relevant to the maintenance of brain function. Over the last few years, we have identified an orally active, novel neuroprotective and cognition-enhancing molecule, the flavonoid fisetin. Fisetin not only has direct antioxidant activity but it can also increase the intracellular levels of glutathione, the major intracellular antioxidant. Fisetin can also maintain mitochondrial function in the presence of oxidative stress. In addition, it has anti-inflammatory activity against microglial cells and inhibits the activity of 5-lipoxygenase, thereby reducing the production of lipid peroxides and their pro-inflammatory by-products. This wide range of actions suggests that fisetin has the ability to reduce the age-related decline in brain function.

Keywords Oxidative stress · Glutathione · Nrf2 · Memory · Microglia

Introduction

Similar to other organs, brain function declines with age. Indeed, a decline in both cognitive and motor functions is one of the characteristics of normal aging, resulting in changes in learning and memory as well as deficits in balance and coordination. Furthermore, age is the single greatest risk factor for a variety of neurological disorders including Alzheimer's disease (AD). Since the average age in many Western countries is increasing, identifying approaches for reducing the effects of aging on brain function is taking on a new urgency. However, in order to choose among possible approaches, it is first necessary to identify the factors that contribute to the decrease in brain function with age. Among the factors that have been proposed are alterations in redox homeostasis, gene transcription, protein modification and processing, neurotrophic factor signaling, mitochondrial function and the immune response. Given this multiplicity of factors and the strong possibility that the relative importance of these factors will vary among individuals, approaches that are directed against a single target are unlikely to be generally useful. A better approach is to identify small molecules that have multiple biological activities that can impact the multiplicity of factors that are associated with the age-related decrease in brain function.

One excellent source for these small molecules is the original pharmacopeia, plants. The polyphenolic flavonoids are widely distributed in fruits and vegetables and therefore regularly consumed in the human diet (for reviews see [32, 62, 75]). Flavonoids were historically characterized on the basis of their antioxidant and free radical scavenging effects. However, more recent studies have shown that flavonoids have a wide range of activities that could make them particularly effective for reducing the age-related deficits in brain function. In this article, I will discuss the multiple factors that are associated with the decline in brain function with age and show how one flavonoid, the flavone fisetin (Fig. 1), is able to impact each of these factors and therefore might hold promise for slowing or preventing this decline.

This article belongs to a special issue on Nutrients and Brain Health.

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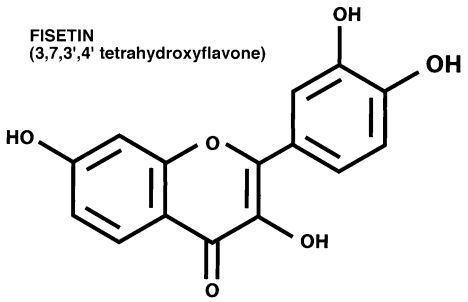

Fig. 1 Structure of fisetin

Background

Fisetin was originally identified in a screen for compounds that could prevent oxidative stress-induced nerve cell death [36]. Of the ~30 flavonoids tested in this study, only two, fisetin and quercetin, were able to maintain GSH levels in the presence of oxidative stress, indicating that this is not a common property of flavonoids. Further studies showed that fisetin also possessed neurotrophic activity, promoting the differentiation of PC12 cells via activation of the Ras-ERK cascade [76]. Again, this was a property that distinguished fisetin from almost all of the other ~30 flavonoids tested. Only quercetin, isorhamnetin and luteolin showed some differentiation-inducing activity and they were all much less effective than fisetin. Together, these observations suggested that fisetin had multiple properties that might be able to contribute to the maintenance of nerve cell function.

Unlike many of the better studied flavonoids such as quercetin and luteolin, fisetin is not particularly abundant in fruits and vegetables. The highest levels (160 μg/g) are found in strawberries [2] with five to tenfold lower levels in apples and persimmons. Small amounts are also found in kiwi fruit, peaches, grapes, tomatoes, onions and cucumbers [33]. The bioavailability of fisetin from these sources has not been studied.

Maintenance of redox homeostasis by fisetin

Antioxidant activity

Over the years, a number of theories have been put forth to explain the mechanisms underlying the process of aging. One of the theories that has received the most attention and research support is the free radical theory of aging (for reviews see [7, 31, 64] #1674). The current version of this theory proposes that there is an increase in the imbalance between pro-oxidants and antioxidants and, as a consequence, oxidative damage with aging which is the primary cause of the age-related declines in physiological function. The oxidants arise from several sources and include both reactive oxygen (ROS) and reactive nitrogen (RNS) species. The main sources of ROS include mitochondrial respiration, lipid peroxidation and NADPH oxidase activity. Fortunately, cells contain an arsenal of antioxidant defenses including both antioxidant enzymes and small antioxidant molecules such as the endogenous antioxidant glutathione (GSH), as well as antioxidants derived from fruits and vegetables which can normally remove the ROS/RNS generated by basic physiological functions. However, increasing age is associated with an imbalance between the production and removal of ROS/RNS resulting in oxidative stress and subsequent oxidative damage to proteins, lipids and DNA.

Although it is not entirely clear how relevant the antioxidant activity of flavonoids as measured in test tube assays is to their effects in vivo, fisetin is a relatively good antioxidant with a Trolox equivalent antioxidant capacity (TEAC) value of ~3 [36, 54]. Furthermore, fisetin was also shown to be very good at inhibiting lipid peroxidation and chelating iron [90]. An age-related increase in iron was found in human, rat and mouse brains [25] and was associated with markers of oxidant-mediated damage. All of these properties could contribute to the beneficial effects of fisetin on CNS cells.

Maintenance of GSH

GSH plays a central role in maintaining cellular redox homeostasis. A fairly large number of studies have shown age-dependent decreases in total GSH and/or reduced GSH levels in the brain (for reviews see [3, 25, 53]). In addition, age-dependent increases in glutathione disulfide (GSSG), the oxidized form of GSH have been observed in both mouse [72] and rat [67] brains. This, in turn, leads to an age-dependent decrease in the GSH/GSSG ratio, suggesting a significant alteration in the redox environment of the brain with age.

The age-related decreases in total GSH that are seen in many studies could be due to increased GSH consumption, decreased GSH production or some combination of the two. Increased consumption would be consistent with an increase in ROS production with age. However, recent studies suggest that decreased production also plays an important role in the decline of brain GSH with age. These studies have all shown a good correlation between decreases in the level of glutamate cysteine ligase (GCL) activity, the rate limiting enzyme in GSH biosynthesis, and decreases in GSH levels [50, 51, 63, 71, 77]. In addition, the decreases in GCL activity are correlated with decreases in the levels of GCL protein and/or mRNA of at least one of the two subunits [50, 51]. A recent paper showed that the decrease in GCL levels, at least in liver, is due to a

decrease in the level of Nrf2, the transcription factor involved in the induction of the genes encoding both chains of GCL [84]. Moreover, the low levels of Nrf2 in the livers of 24–28-month-old rats could be restored by treatment with lipoic acid, resulting in a restoration of both GCL activity and GSH levels. This suggests that the basic transcriptional response mechanism is still present in the old rats, but that for some reason the basal set point is turned down during aging. Although further research is clearly needed to determine if decreases in Nrf2 levels are also seen in the brain with aging, these findings suggest that compounds which can increase Nrf2 might be useful for maintaining redox homeostasis in the brain and thereby helping to maintain brain function.

Over the last few years, we have developed several in vitro models that can be used to identify small molecules that are able to maintain redox homeostasis in the presence of oxidative stress. Fisetin was first identified as a potential neuroprotective compound [36] using one of these models, oxidative glutamate toxicity (for review see [86]).

More recently, we have tested the effect of fisetin against peroxynitrite toxicity. Peroxynitrite levels increase during aging and may contribute to some of the nerve cell damage associated with normal aging [27]. In addition, peroxynitrite-mediated toxicity has been implicated in many age-related neurological disorders including ischemic stroke [26], AD, Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) (reviewed in [87]). Although peroxynitrite itself is not a free radical, it is a uniquely damaging molecule because it can initiate strong oxidation reactions through decomposition into a hydroxyl radical and nitrogen dioxide [5]. It can also form a highly reactive nitroderivative in the presence of transition metals [6, 35] and interact directly with protein and non-protein thiol groups, leading to the depletion of cellular antioxidant defenses including GSH [70].

Using primary cultures of cortical neurons, in combination with the peroxynitrite generator SIN-1, we found an ~50% decrease in both intracellular GSH levels and cellular viability that could be prevented by treatment with 10 μM fisetin [12]. Similar results with fisetin were obtained when authentic peroxynitrite was used as the toxic insult. The protection by fisetin, as well as its ability to maintain GSH levels, was inhibited by treatment with buthionine sulfoximine (BSO), an inhibitor of GCL [30]. In contrast, BSO had no effect on the ability of glutathione monoethyl ester, a cell permeable form of GSH, to maintain GSH levels and protect the neurons from peroxynitrite toxicity. These data showed that BSO was not blocking the neuroprotective effect of fisetin through a toxic effect unrelated to GSH.

Mechanism of action

How does fisetin maintain GSH levels? In general, intracellular GSH levels are regulated by a complex series of mechanisms that include substrate availability and transport, rates of synthesis and regeneration, GSH utilization and GSH efflux to extracellular compartments (for review see [61]). Because glutamate and glycine occur at relatively high intracellular concentrations, cysteine is limiting for GSH synthesis in many types of cells, including nerve cells. In the extracellular environment, cysteine is readily oxidized to form cystine, so for most cell types, cystine transport mechanisms are essential to provide them with the cysteine needed for GSH synthesis. Cystine uptake in many types of cells is mediated by system X_c^-, a Na^+-independent cystine/glutamate antiporter [78]. System X_c^- is a member of the disulfide-linked heteromeric amino acid transporter family and consists of a light chain (xCT) that confers substrate specificity and a heavy chain (4F2hc) that is shared among a number of different amino acid transporters. The results with BSO suggested that fisetin increases GSH levels by either increasing cystine import and/or enhancing GCL activity.

Interestingly, both xCT [37] and GCL [15] are regulated at the transcriptional level by the transcription factor NF-E2-related factor 2 (Nrf2). Nrf2 binds to the antioxidant response element (ARE; also EpRE,) within the promoter of various genes, thereby regulating the inducible production of a variety of proteins involved in the protection of cells from oxidative stress as well as in the maintenance of redox homeostasis (for reviews see [16, 41, 66]). Treatment of cells with an Nrf2 inducer results in the accumulation and translocation of Nrf2 to the nucleus where it heterodimerizes with Maf family proteins to induce gene transcription. We have found that fisetin can increase the nuclear levels of Nrf2 in a variety of nerve cells including HT22 cells [54], retinal ganglion cells [56] and primary cortical neurons (Fig. 2). In primary cortical neurons treated with peroxynitrite [12], Nrf2 levels decreased significantly but treatment with fisetin was able to prevent this decrease. The peroxynitrite-mediated decreases in Nrf2 levels correlated with a decrease in the levels of both subunits of GCL, and GCL loss was also prevented by fisetin treatment. These findings are in agreement with earlier studies which showed that fisetin could increase the expression of various ARE-dependent genes in multiple non-neuronal cell lines [34, 65, 89] as well as rat C6 glioma cells [17]. The precise mechanisms whereby fisetin increases Nrf2 levels are still under investigation.

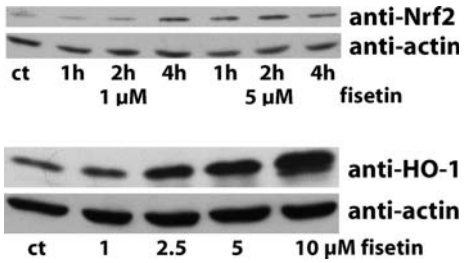

Fig. 2 Fisetin induces the expression of Nrf2, the ARE-specific transcription factor, and HO-1, an Nrf-2-dependent protein. Rat primary cortical neurons (7 days in culture) were untreated (*ct*) or treated with 1 or 5 μM fisetin for 1, 2 or 4 h (*Nrf2*) or with 1–10 μM fisetin for 24 h (HO-1). Nuclei were prepared (*Nrf2*) and equal amounts of protein were analyzed by SDS-PAGE and immunoblotting with Nrf2 antibodies or cell lysates were prepared and equal amounts of protein were analyzed by SDS-PAGE and immunoblotting with HO-1 antibodies. Immunoblotting with anti-actin is shown as a loading control. Similar results were obtained in three independent experiments

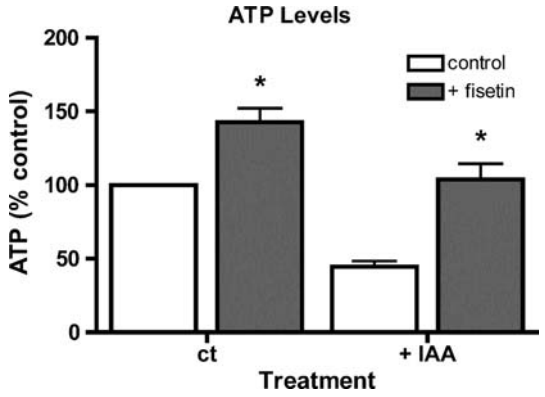

Fig. 3 Fisetin increases cellular ATP levels. HT22 cells were untreated (*ct*) or treated with 20 μM iodoacetic acid (+ *IAA*) to simulate ischemia for 2 h followed by 2 h in fresh medium alone in the absence (*control*) or presence (+ *fisetin*) of 10 μM fisetin. Cell lysates were prepared and total ATP levels were measured using a chemiluminescent assay. Adapted from [58]

Fisetin can maintain mitochondrial function

Age-dependent changes in mitochondrial function are of particular interest as mitochondria are thought to play a key role in the aging process, for mitochondria are both a major source of intracellular oxidants as well as a target for the damaging effects of oxidants. In mammalian cells, mitochondria are the major source of energy in the form of ATP.

We have recently shown that fisetin is able to enhance basal ATP levels as well as maintain ATP levels in the presence of oxidative stress (Fig. 3). For these studies we used an in vitro ischemia model that we developed for the testing of compounds that might have therapeutic value for the treatment of stroke [58]. This model utilizes the toxin iodoacetic acid (IAA), a well known, irreversible inhibitor of the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase, in combination with the HT22 nerve cell line. The changes seen following IAA treatment of nerve cells are very similar to changes that have been seen in animal models of ischemic stroke, and include alterations in membrane potential, breakdown of phospholipids, loss of ATP and GSH, and an increase in ROS. Using this model, we tested a variety of flavonoids and found that fisetin was highly effective at protecting the HT22 cells from IAA toxicity. Not only did fisetin prevent cell death but it stood out among the flavonoids tested as being able to maintain ATP levels in the presence of IAA. Further studies in the rabbit small clot embolism model of stroke demonstrated that fisetin could significantly improve the behavioral outcome when administered 5 min after the initiation of an embolic stroke [58]. Consistent with our animal data, fisetin was also shown to reduce ischemic damage and infarct volume in the permanent focal middle cerebral artery occlusion model of stroke in rats [73].

Fisetin can enhance cognitive function

Normal human aging is associated with specific memory deficits including delayed recall of verbal information and declines in working memory, short-term recall, processing speed and spatial memory (for review see [94]). Although these deficits are distinct from those seen in neurological disorders such as AD, they can still significantly impact the quality of life. Transcriptional profiling of the human frontal cortex [52] and rat hippocampus [10] showed age-related decreases in genes involved in learning and memory. The transcription factor cAMP-response element binding protein (CREB) interacts with the cAMP-response element (CRE) in the promoter region of genes that encode proteins involved in the regulation of learning and memory (for reviews see [13, 60]). A number of studies in a wide range of animal species have shown that CREB plays a key role in the formation of long-term memory (LTM) (for review see [88]). CREB is a constitutively nuclear protein whose activity is regulated by phosphorylation of both subunits of the homodimer [38]. Phosphorylation promotes the interaction of CREB with the transcriptional co-activator CREB binding protein (CBP) or its homolog p300, which stimulates the transcriptional activity of CREB. Several different kinases can phosphorylate CREB on Ser133 and positively regulate its transcriptional activity. These include protein kinase A (PKA), calmodulin dependent kinase IV, MNK1 and 2 and MSK1. The latter three kinases are all substrates of the MAP kinase ERK. Therefore, CREB activity can be regulated by the ERK signaling pathway. Using rat hippocampal slices, we

demonstrated that 1 μM fisetin could induce the rapid phosphorylation of CREB and that this phosphorylation was dependent on ERK activation since inhibitors of ERK activation also blocked CREB phosphorylation [57].

Given these results and the known associations between CREB and learning and memory, it was next asked whether fisetin could affect long-term potentiation (LTP) in the hippocampal slices. LTP is an in vitro assay that is considered to be a good model of how memory is formed at the cellular level [11]. Furthermore, age-related changes in cognitive function have been shown to correlate with impaired induction and maintenance of LTP [94]. Although fisetin had no effect on basal synaptic responses in the CA1 area of rat hippocampal slices [57], it induced LTP in slices exposed to a weak tetanic stimulus (15 pulses at 100 Hz) that by itself failed to induce LTP. The facilitation of LTP by fisetin was dose dependent, with a maximal effect seen at 1 μM and it persisted for at least 60 min. Importantly, the facilitation of LTP by fisetin was blocked by two inhibitors of ERK activation, PD98059 and U0126. Together these data strongly support the hypothesis that ERK-dependent CREB activation by fisetin is responsible for the facilitation of LTP by fisetin. Further support for this hypothesis comes from studies with the phosphodiesterase 4 inhibitor rolipram that enhances CREB phosphorylation by preventing the breakdown of cAMP. Rolipram also had no effect on basal synaptic responses in rat hippocampal slices but facilitated LTP induced by a weak tetanic stimulus in a manner very similar to fisetin [4]. However, in contrast to rolipram, fisetin did not increase cAMP levels in the hippocampal slices [57].

To determine if the biochemical and electrophysiological effects of fisetin seen in hippocampal slices could be translated into alterations in LTM in animals, we tested fisetin in mice using the object recognition task [9]. We chose this specific memory assay as our first test of fisetin on cognitive function because it had proven very effective for measuring CREB-dependent functions. In this test, during the training period, mice are presented with two identical objects, which they explore for a fixed time period. To test for LTM, the mice are presented 1 day later with two different objects, one of which was presented previously during the training and is thus familiar to the mice; the other object is new to them. The better the mice remember the familiar object, the more time they will spend exploring the novel object. To test the effects of fisetin in this memory task, it was administered orally to the mice before the start of the training period [57]. Rolipram, administered by injection, was used as a positive control. As shown in Fig. 4, fisetin significantly increased the time the mice spent exploring the novel object indicating a significant effect on LTM consistent with our cell- and tissue-based results.

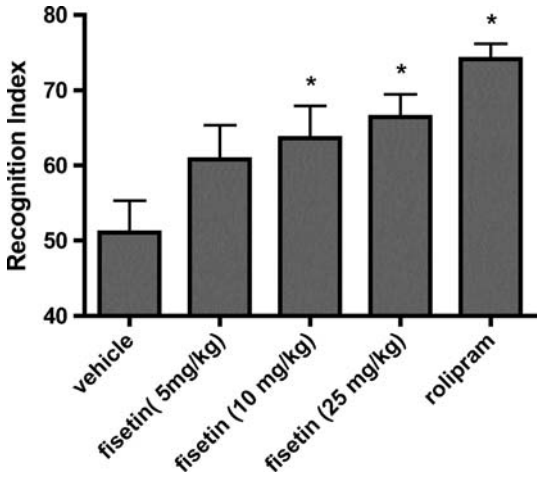

Fig. 4 Fisetin enhances long-term memory in young mice. The effect of different oral doses of fisetin on object recognition over a 10-min test period. Rolipram, injected intraperitoneally at 0.1 mg/kg, served as a positive control. Data represent the mean ± SEM of 10 mice/treatment group. Data were analyzed by one-way ANOVA followed by post hoc comparisons with Fisher's test. *Asterisk* indicates significantly different from vehicle control ($P < 0.02$). Similar results were obtained in two independent, blinded experiments done by Psychogenics. From [57] © 2006 by The National Academy of Sciences of the USA

These studies were done with young animals so it was unclear whether fisetin would have similar effects in older animals. To begin to address this question, we fed mice fisetin for 10 months and tested their learning and memory using the morris water maze (MWM) at 12 months of age. The MWM is a test of spatial learning and is strongly correlated with hippocampal synaptic plasticity [91]. It can be used to assess both learning and memory deficits. In the standard test, the mice are required to find a submerged platform in a circular pool of opaque liquid (usually water with non-toxic white paint added) by relying on distal visual cues. Typically, they are given 4 trials/day over a period of 5–7 days and the time required for the mouse to find the platform (latency) is recorded. Twenty-four hours after the end of this learning phase, the mice are placed back in the pool but in this case the platform is no longer present. They are given a single, one min trial and the amount of time that they spend in the quadrant in which the platform was previously located relative to the time that they spend in the other three quadrants is recorded. As shown in Fig. 5, fisetin feeding slightly but non-significantly improved the learning and significantly improved the memory of older mice in the MWM relative to age-matched controls fed a fisetin-free diet.

Among the fruits and vegetables where fisetin can be detected, the highest levels are found in strawberries [2]. Interestingly, supplementation of the diet of 19-month-old rats with a strawberry extract for 8 weeks resulted in

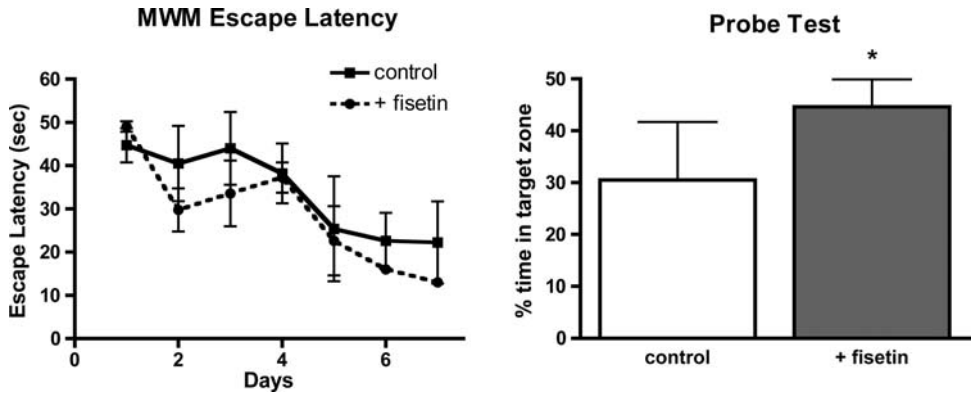

Fig. 5 Fisetin enhances long-term memory in older mice. Mice were fed fisetin (500 mg/kg of food) for 10 months beginning at 2 months of age. Learning and memory were tested using the morris water maze (*MWM*) [91]. Fisetin slightly but non-significantly enhanced learning in the mice as measured by the time taken to find a submerged platform over 7 days of training (*left graph*) and significantly enhanced memory as determined in the probe trial performed 24 h after the last training trial (*right graph*). Data represent the mean ± SD of 4–6 mice/treatment group. Data for the latency trials were analyzed by ANOVA and data from the probe trial were analyzed by *t* test. *Asterisk* indicates significantly different from control ($P < 0.05$)

enhanced performance in the MWM relative to rats fed a control diet [39]. Dietary supplementation with a strawberry extract also improved the performance of rats in the MWM in a rodent model of accelerated aging [81]. These results support our data with fisetin, and suggest that this flavonol could be useful for reducing at least some of the learning and memory deficits that accumulate with age.

Anti-inflammatory effects of fisetin

Microglia are the resident immune cell population of the CNS, comprising 10–15% of the total cell population (for reviews see [24, 29, 74]). They play important, protective roles in the CNS such as removing pathogens and promoting tissue regeneration after injury. However, activated microglia also produce a wide array of pro-inflammatory and cytotoxic factors including cytokines, ROS, excitatory neurotransmitters and eicosanoids that can promote nerve cell damage as well as impact cognitive function. For example, the cytokine IL1-β can impair LTP [22]. Microglia are implicated in the pathogenesis of a variety of age-associated chronic neurological disorders including AD and PD. Importantly, microglial activation is also seen in the brains of healthy, aged animals [22] and is thought to play a role in the exaggerated immune response that is typical of the aged brain [22, 79]. This can result in cognitive impairment and other behavioral deficits in response to stimuli that have little or no effect on these parameters in young animals. Thus, compounds that can modulate the activation of microglia in the aged brain and/or dampen their response to stimuli might have a significant benefit on brain function in the elderly.

A recent paper demonstrated that fisetin could reduce bacterial lipopolysaccharide (LPS)-induced microglial activation and neurotoxicity [96]. Using the BV-2 microglial cell line, it was shown that fisetin was very effective at blocking LPS-induced nitric oxide production, measured as accumulation of nitrite in the culture medium, with an EC_{50} of $\sim$7 μM. The same dose of fisetin also reduced LPS-induced increases in extracellular PGE2 levels as well as increases in the expression of the COX2, iNOS and interleukin-1β genes. Similar to the results obtained with other types of cells treated with fisetin and LPS [85, 92], the effects of fisetin on microglial activation appeared to be mediated by inhibition of LPS-stimulated NF-κB activation. Similar data were obtained with primary microglial cells isolated from the cerebral cortices of 1 day old mice. Importantly, these authors also showed neuroprotective effects of fisetin in a nerve/microglia co-culture system. In this assay, neuroblastoma cells were co-cultured with LPS-activated microglia with or without pre-treatment with fisetin. In the absence of fisetin, the LPS-activated microglia reduced the viability of the neuroblastoma cells by $\sim$50%. However, following pre-treatment of the microglia with $\sim$7 μM fisetin, the viability of the neuroblastoma cells was only reduced by $\sim$10%. These results indicate that fisetin has anti-inflammatory activity and therefore might be effective in a variety of conditions involving the dysregulation of the immune system in the brain, including normal aging.

5-Lipoxygenase (5-LOX) metabolizes 20-carbon unsaturated fatty acids such as arachidonic acid, which are produced from membrane phospholipids by the action of phospholipases, to 5-hydroxyperoxyeicosatetraenoic acid (5-HPETE) followed by formation of the unstable intermediate leukotriene A4 (LTA4). LTA4 is then metabolized to leukotriene B4 or conjugated with GSH to form cysteinyl leukotrienes (for review see [68]). Via their binding

to specific G-protein coupled receptors on target cells, leukotrienes can have pro-inflammatory effects. 5-LOX is expressed in the brain where its expression and activity is specifically increased in the hippocampus with age [18]. In addition, loss of 5-LOX was associated with a reduced beta amyloid peptide burden in a transgenic mouse model of AD [28] suggesting that inhibition of 5-LOX might have benefits in both normal aging and AD.

In earlier studies it was shown that fisetin is an effective inhibitor of 5-LOX activity in stimulated peritoneal leukocytes [49]. As shown in Fig. 6, we have confirmed and extended these studies. Fisetin inhibits 5-LOX from human peripheral blood mononuclear leukocytes (PBML) with an IC_{50} of 0.585 μM, a value which is as good or better than that of many known LOX inhibitors. Thus, these results suggest that an important activity of fisetin in the brain is the inhibition of 5-LOX activity. This action may contribute to both the neuroprotective and anti-inflammatory effects of fisetin and thereby promote the functional maintenance of the CNS during aging.

Fisetin can enhance proteasome activity

The ubiquitin–proteasome pathway mediates the majority of the proteolysis seen in the cytoplasm and nucleus of mammalian cells. As such it plays an important role in the regulation of a variety of physiological and pathophysiological processes (for reviews see [20, 43]). Several studies have shown that there is a specific decrease in proteasome activity in the hippocampus, cortex, striatum, globus pallidus and substantia nigra with aging in rodents [40, 95]. In contrast, little or no change in proteasome activity is seen in the cerebellum and brain stem. These findings are consistent with studies that have shown that proteasome activity is decreased in a variety of age-associated neurological disorders including AD, PD and ALS [8, 19, 21] and may contribute to disease progression. Interestingly, the age-related decreases in proteasome activity are generally not associated with decreased levels of overall proteasome immunoreactivity [23] suggesting that post-translational modifications to the proteasome are responsible for the decrease in activity. This decrease in proteasome activity is thought to play a critical role in the accumulation of abnormal and oxidized proteins. Indeed, microglia isolated from aged rodents show decreased proteasome activity and an impaired ability to degrade oxidized and glycated proteins [83].

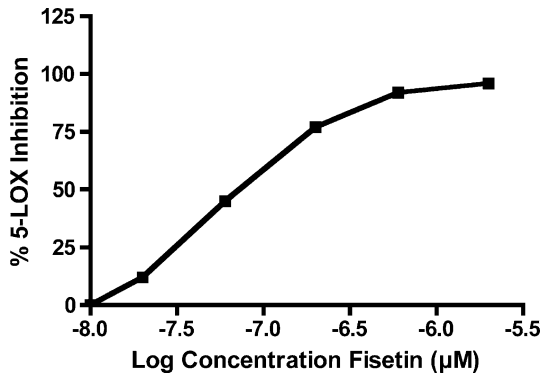

Fig. 6 Fisetin inhibits 5-LOX activity. Peripheral blood mononuclear leukocytes (*PBML*) were pre-incubated with the indicated doses of fisetin prior to the addition of arachidonic acid for 10–15 min. 5-LOX activity was determined from EIA quantitation of the 5-LOX product leukotriene B4 [14]. NDGA was used as a positive control for 5-LOX inhibition ($IC_{50} = 0.076$)

Recently, we found that fisetin was able to modestly increase the chymotrypsin-like activity of the proteasome in primary cultures of cortical neurons and this contributed to the survival promoting effects of fisetin on these cells [55]. In further studies with the HT22 nerve cell line we have found that fisetin also increases the trypsin-like and caspase-like activities of the proteasome in a time- and dose-dependent manner (Fig. 7). Although it is not clear at this time how fisetin increases proteasome activity in nerve cells, the time- and substrate-dependence of its effects suggest that fisetin may have both direct and indirect actions on proteasome activity. Many of the protein components of the proteasome are transcriptionally regulated by Nrf2 [47] and Nrf2 inducers can increase proteasome activity in several different cell types [44–46]. However, preliminary data suggest that the regulation of proteasome activity by fisetin is likely to be more complex.

Enhancing proteasome activity by compounds such as fisetin could prove beneficial for reducing the CNS consequences of normal aging as well as treating neurological disorders. Although the increase in proteasome activity brought about by fisetin is modest, it is similar to the increases seen with several other compounds such as dithiolethione, 3-methylcholanthrene and β-napthoflavone [44, 46]. Furthermore, dramatic increases in activity may not be compatible with the maintenance of normal cell function.

Consistent with the ability of fisetin to alter protein stability are two recent reports showing that fisetin can inhibit beta amyloid peptide fibril formation in a cell-free assay system [1, 42]. One of these studies [42] also showed that fisetin prevents extracellular beta amyloid peptide toxicity in the HT22 cells. Since beta amyloid peptide is thought to play a key role in the nerve cell loss that is the hallmark of AD, these results suggest that fisetin may be able to reduce the burden of beta amyloid peptide through multiple mechanisms, including inhibition of aggregation and enhancement of degradation.

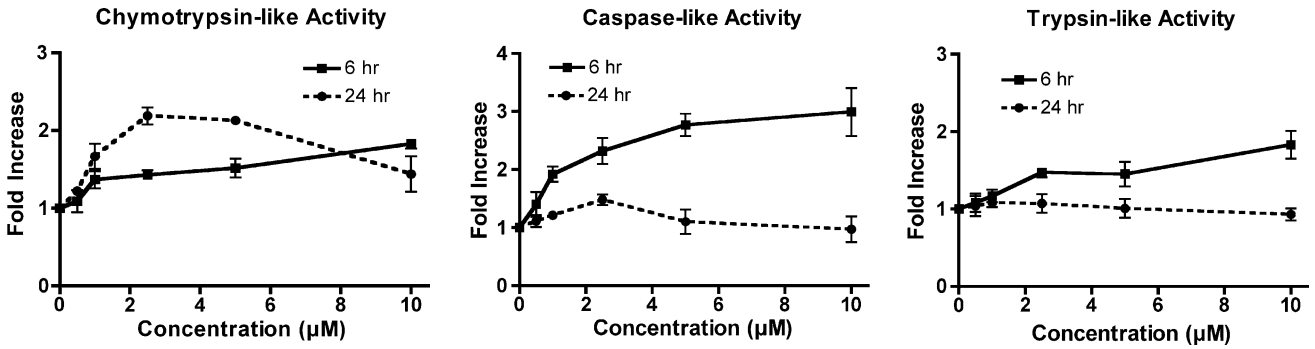

Fig. 7 Fisetin enhances proteasome activity in HT22 cells. HT22 cells were plated at 3×10^5 cells/dish in 60 mm dishes and grown for 24 h. The cells were then left untreated or treated with the indicated concentrations of fisetin for 6 or 24 h. The cells were harvested and assayed for proteasome activity as described [55] using fluorescent substrates for the chymotrypsin-like (Suc-LLVY-AMC), caspase-like (Z-LLG-AMC) and trypsin-like (Z-ARR-AMC) activity of the proteasome. Results are presented as mean ± SD and are the average of four to five independent experiments

Metabolism of fisetin

Flavonoids are known to be extensively metabolized following oral consumption resulting in glucuronidated, sulfated and methylated metabolites (for reviews see [59, 69]). The metabolism of fisetin was recently characterized in rats following intravenous and oral administration [80]. Following both types of administration, the levels of free fisetin in serum declined rapidly while the levels of sulfated/glucuronidated fisetin increased. Following oral administration at 50 mg/kg, the serum concentration of fisetin sulfates/glucuronides was maintained at ~10 μM for >24 h. These results are in sharp contrast to those obtained for 5 OH and 7 OH flavone [80] and baicalein [48] where the levels of free and conjugated flavonoid never exceeded 1 μM following oral administration. When tested in an assay for antioxidant activity, the fisetin sulfates/glucuronides showed somewhat reduced but still significant activity as compared to free fisetin [80]. This result is consistent with a recent study on the effects of glucuronidation on the ability of several different flavonoids to protect nerve and lymphoid cells from oxidative stress-induced death [82]. Although the flavonoid glucuronides had generally higher EC_{50}s for protection as compared to their unmodified parent, they still showed excellent activity. Furthermore, circulating flavonoid sulfates/glucuronides can be cleaved to the free form in a tissue-specific manner if there is a local release of β-glucouronidases and/or sulfatases [69].

There is an ongoing debate about whether flavonoids such as fisetin can reach levels in the brain that are sufficient to affect neuronal function. Recently, using an in vitro model for blood brain barrier penetration [93], we found that fisetin exhibited high brain uptake potential (Table 1). Furthermore, following a single intraperitoneal injection, fisetin was detected in the brains of rats and this correlated with a significant reduction in cerebral damage in a stroke model [73]. Similarly, we have seen significant protection in a rabbit stroke model following intravenous injection of fisetin [58] and we found that oral administration of fisetin could enhance learning and memory in mice [57]. Thus, fisetin is able to affect neuronal function in vivo. Whether this is a direct effect of the unmodified fisetin molecule remains to be determined.

Table 1 Blood brain barrier penetration potential of fisetin

Compound	A–B	B–A	Efflux	BBB penetration potential
Fisetin	18.0	1.33	0.1	High
Caffeine[a]	20.2	22.2	1.1	High
Atenolol[a]	0.13	0.25	1.9	Low

The potential for blood brain barrier penetration was determined using MDR1-MDCK cell monolayers as described [93]. MDR-MDCK cells plated at 60,000 cells/cm^2 on collagen-coated, microporous polycarbonate membranes in 12-well transwell plates were grown for 7–11 days. The quality of the monolayer was verified by TEER measurement (>1,400). The test compounds at a final concentration of 5 μM in HBSS were added to the apical or basolateral side and aliquots removed for analysis from the opposite side at 30 min intervals over 2 h. The concentrations of the compounds in the samples were determined by LC/MS/MS. The potential for BBB penetration is viewed as high if Papp $A \rightarrow B \geq 3.0 \times 10^{-6}$ cm/s and efflux < 3.0, moderate if Papp $A \rightarrow B \geq 3.0 \times 10^{-6}$ cm/s and 10 > efflux ≥ 3.0 and low if Papp $A \rightarrow B \geq 3.0 \times 10^{-6}$ cm/s and efflux ≥ 10 or Papp $A \rightarrow B < 3.0 \times 10^{-6}$ cm/s. Results for caffeine and atenolol, CNS positive and negative compounds, respectively, are shown for comparison

[a] From Wang et al. [93]

Summary

As discussed above, fisetin has positive effects on a number of common pathways associated with brain aging (Fig. 8). While the precise relationships among the

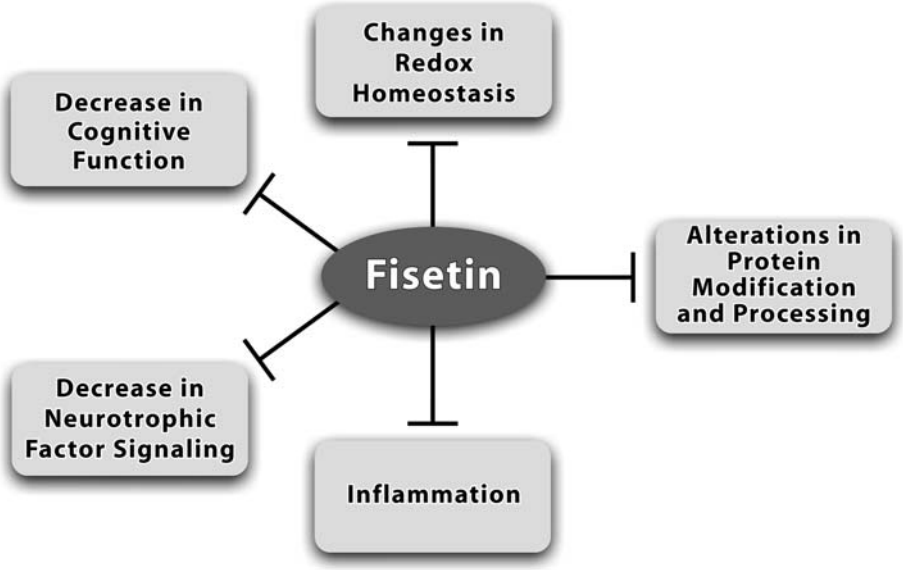

Fig. 8 Fisetin affects multiple pathways implicated in brain aging. As discussed in this article or elsewhere, fisetin can increase neuronal function and survival through maintenance of redox homeostasis, activating neurotrophic factor signaling pathways [76], regulating protein homeostasis through effects on proteasome activity and protein aggregation and inhibiting inflammatory responses. Fisetin can also enhance cognitive function. Therefore, it has the potential to act as a multi-factorial drug for reducing the age-associated decline in brain function

pleiotropic effects of fisetin on nerve cells are currently under investigation, this combination of actions suggests that fisetin has the potential to maintain neuronal function even in the presence of the diverse factors that contribute to the decline in brain function with age. Therefore, it might have therapeutic value as a multifactorial treatment to delay the decline in both cognitive and motor functions that is one of the characteristics of normal aging. These results also suggest that fisetin has the potential to reduce the impact of age-related neurodegenerative diseases, especially those associated with cognitive deficits such as AD. We are currently testing this hypothesis in feeding studies on control and AD transgenic mice.

Acknowledgments This work was supported by the Michael J. Fox Foundation and the Alzheimer's Association.

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