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Luteolin protects rat PC12 and C6 cells against MPP$^+$ induced toxicity via an ERK dependent Keap1-Nrf2-ARE pathway

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Summary Oxidative stress is central to neuronal damage in neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease. In consequence, activation of the cerebral oxidative stress defence is considered as a promising strategy of therapeutic intervention. Here we demonstrate that the flavone luteolin confers neuroprotection against oxidative stress via activation of the *nuclear factor erythroid-2-related factor 2* (Nrf2), a transcription factor central to the maintenance of the cellular redox homeostasis. Luteolin protects rat neural PC12 and glial C6 cells from N-methyl-4-phenyl-pyridinium (MPP$^+$) induced toxicity *in vitro* and effectively activates Nrf2 as shown by ARE-reporter gene assays. This protection critically depends on the activation of Nrf2 since downregulation of Nrf2 by shRNA completely abrogates the protection of luteolin *in vitro*. Furthermore, the neuroprotective effect of luteolin is abolished by the inhibition of the luteolin-induced ERK1/2-activation. Our results highlight the relevance of Nrf2 for neural cell survival conferred by flavones. In particular, we identified luteolin as a promising lead for the search of orally available, blood brain barrier permeable compounds to support the therapy of neurodegenerative disorders.

Keywords: Luteolin, flavonoids, MPP$^+$, Nrf2, nuclear factor, Parkinson's disease

Nonstandard abbreviations

ARE	Antioxidant response element
C6	rat glioblastoma cells
GCS	gamma-glutamyl-cysteine-synthase
EpRE	electrophile response element
Keap1	Kelch-like ECH associating protein
MPP$^+$	1-methyl-4-phenyl-pyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NQO1	NAD(P)H-quinone-oxidoreductase 1
Nrf2	nuclear factor erythroid 2-related factor 2
PC12	rat phaeochromocytoma cells
PI3K	phosphoinositol-3-kinase
shRNA	short hairpin RNA
siRNA	short interference RNA
tBHQ	tert-butyl hydroquinone

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Introduction

Oxidative stress and mitochondrial dysfunction are considered to be central factors for neuronal degeneration in aging as well as Alzheimer's disease, Huntington's disease, Amyotrophic lateral sclerosis or Parkinson's disease (PD) (Andersen, 2004; Götz et al., 1994; Manfredi and Xu, 2005; Mariani et al., 2005; Wright et al., 2004). Multiple lines of evidence implicate the increased formation of reactive biological intermediates including reactive oxygen species, reactive nitrogen species, and electrophiles as aggravating factors in disease progression (Moore et al., 2005). In PD defects in complex I of the mitochondrial respiratory chain (Lestienne et al., 1990; Reichmann et al., 1990; Schapira et al., 1990) and increased levels of biomarkers of oxidative stress such as increased concentrations of iron and lipid peroxidation products in the substantia nigra were discussed (Dexter et al., 1989; Riederer et al., 1989; Götz et al., 2004; Zecca et al., 2004). PD is also associated with exposure to pesticides, many of which are either oxidants or mitochondrial toxicants (Tanner, 1989; Tanner et al., 1999). Rapid onset of parkinsonism in man, primate, and mouse following administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Langston et al., 1983; Gerlach and Riederer, 1996) occurs through its active metabolite, 1-methyl-4-phenylpyridinium (MPP$^+$) (Langston et al., 1984), a reversible inhibitor of mitochondrial complex I, which leads to the depletion of energy stores and the induction of oxidative stress *in vivo* (Nicklas et al., 1987; Przedborski et al., 2004; Przedborski and Ischiropoulos, 2005). Therapeutic intervention with antioxidants, however, have failed to attenuate disease progression in PD and other neurodegenerative disorders (Shoulson, 1998).

An alternative strategy is the administration of compounds that enable the upregulation of endogenous antioxidative defence systems in the brain such as the *nuclear factor erythroid 2-related factor 2* (Nrf2). The activation of the Nrf2 transcription factor regulates the transcription of phase-II detoxifying enzymes and subsequently the redox homeostasis in numerous cell types including glia and neurons (Nguyen et al., 2000, 2003; Lee et al., 2003a, b). Nrf2 activity renders neural cells more resistant to oxidative and electrophilic stress particularly with regard to MPTP (Lee et al., 2005a; Burton et al., 2006). Binding of Nrf2 to the antioxidant response element (ARE) initiates the transcription of cytoprotective enzymes such as glutathione transferases (GST-α4, -μ1 and -μ3, π2), NADPH-quinone-oxidoreductase 1 (NQO1) as well as γ-glutamyl-cysteine-synthetase (GCS). Although the precise mechanisms of Nrf2 activation are controversially discussed, it is generally accepted that electrophiles disrupt the inhibitory Nrf2-Keap1 interaction, and stabilise Nrf2 which in turn activates the transcription of *ARE* responsive genes (Wakabayashi et al., 2004; Nguyen et al., 2005).

Flavonoids can attenuate death of neural cells (Datla et al., 2001; Dajas et al., 2003; Abdel-Wahab, 2005; Burton et al., 2006), but the underlying mechanisms of protection are not clarified and cannot be solely attributed to their intrinsic antioxidative properties. Here we have investigated the hypothesis that flavonoids that bear an electrophilic α-β-unsaturated carbonyl moiety may confer neuroprotection via activation of Nrf2. Our analysis included two flavones (luteolin and baicalein) and two isoflavones (genistein and daidzein) with intermediate polarity and solubility in DMSO. Luteolin is a natural flavone from esculent plants such as celery (Manach et al., 2004). The flavone baicalein purified from *Scutellaria baicalensis Georgi* is used as a traditional chinese herbal medicine and can protect from 6-hydroxydopamine-induced neurotoxicity (Im et al., 2005; Lee et al., 2005b). Genistein and daidzein are the aglycones of two isoflavones originating from leguminous plants such as soya and are considered as cancer chemopreventive agents (Kawanishi et al., 2005).

Here we show that selected flavonoids are potent activators of Nrf2, and that Nrf2 mediates the neuroprotection of flavonoids *in vitro*. These findings further elucidate the neuroprotective potential of Nrf2 activation.

Material and methods

Reagents

Luteolin, genistein, baicalein, daidzein, resveratrol, tBHQ, trolox, sulforaphane, PD98059, SP600125, SB203580, UO126 and wortmannin were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture and cell viability assay

Rat phaeochromocytoma cells (PC12) and rat glioblastoma cells (C6) were grown in DMEM-Ham's F12 1:1 medium with 2 mM glutamine (PAA-Laboratories, Pasching, Austria) and N2-supplement containing putrescine, IGF-1, transferrin, progesterone and selenite (Invitrogen). 5000 PC12 cells per well were plated on BIOCOAT Collagen I 96-well-plates (VWR-International, Hamburg, Germany) in 100 μl serum-free medium and allowed to attach for 24 h.

For 4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST) assay, 10 μl/well WST were added to the media (Roche Diagnostics GmbH, Penzberg, Germany) 2 h before spectrophotometric evaluation. Conversion of WST to formazan was measured at 450 nm by microplate spectrophotometry (Model680, Bio-Rad, Hercules, CA) and this reaction reflects the reductive capacity of the cell.

Plasmid construction

Both strands of *ARE1* of the rat NQO1 gene

5′-CAGTCTAGAGTCACAGTGACTTGGCAAAATCG-3′
5′ CTAGCGATTTTGCCAAGTCACTGTGACTCTAGACTGGTAC

with *Kpn*I and *Nhe*I ends were synthesized, annealed, and cloned at the *Kpn*I and *Nhe*I site of the pGL3-Promoter (Promega) to produce the reporter construct pNQO1-rARE.

Luciferase assays

1.5 μg of the NQO1-ARE reporter plasmids containing the firefly luciferase reporter gene, and 0.5 μg of the pRL-TK plasmid, containing the *Renilla* luciferase gene under the control of the herpes simplex virus thymidine kinase promoter as an internal control, were cotransfected into cells in a 10 cm plate by the lipotransfection method (Lipofectamine 2000, Invitrogen) according to the manufacturer's recommendation. 24 h after transfection, the cells were transferred into a 96-well plate. The activities of both, *Firefly* and *Renilla* luciferases were determined 48 h after transfection with the dual luciferase reporter assay system (Promega, Madison, Wis.). The luciferase activities were normalized to the corresponding *Renilla* luciferase activities.

Small interference RNA (siRNA)

The mammalian expression vector pGE1 (Stratagene) was used for the expression of siRNA in PC12 cells. The gene-specific insert which is specified by a 29-nucleotide sequence 5′-GTCTTCAGCATGTTACGTGAT GAGGATGG-3′ of the rat Nrf2 was separated by a 8-nucleotide non-complementary spacer (GAAGCTTG) from the reverse complement of the same 29-nucleotide sequence. This construct was inserted into the pGE1 using *Bam*HI and *Xba*I restriction sides, and referred to as pGE1-rNrf2. A control vector (pGE1-negative) served as a non-silencing control (Stratagene).

Protein analysis

PC12 cells were washed in ice cold PBS, harvested in 200 μl TNE lysis buffer containing 50 mM TRIS, 150 mM NaCl, 1% Nonidet P-40, 2 mM EDTA (pH 8), and centrifuged for 15 min at 4°C at 14.000 *g*. The cytosols were deep frozen at −20°C. The nuclear pellets were dissolved in 50 μl buffer containing 10 mM HEPES, 400 mM NaCl, 1 mM DTT, and 0.2 mM EDTA by sonication with 10 pulses at 10% performance using a Bandelin electronic sonicator (Berlin, Germany) and stored at −20°C. Twenty microgram of total proteins were separated on 12% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride transfer membranes (Immobilin-P, Millipore Bedford MA, U.S.A.). The membranes were blocked for 1 h with

4% non-fat dry milk and incubated with the primary antibodies against ERK1/2 and phosphorylated ERK1/2 (each 1:2000, rabbit polyclonal IgG, Cell Signaling Technology, Beverly, U.S.A.), Nrf2 (1:1000, rabbit polyclonal IgG, Santa Cruz CA, U.S.A.).

After three washing steps with TBST, the membranes were incubated with the appropriate HRP-conjugated secondary antibody for 30 min. The membranes were developed using the ECL chemiluminescence system and Hyperfilm ECL (Amersham, Piscataway, U.S.A.). For reprobing, blots were stripped in 2% SDS, 62.5 mM Tris and 100 mM mercaptoethanol for 30 min at 50°C, washed with TBST, and blocked again. All measurements of dual-phosphorylated kinase (p-ERK1/2) levels were normalized by hybridization with antibodies against total kinase protein (total ERK1/2).

Results

Cytotoxicity of flavonoids

Initially we defined the dose-dependent cytotoxicity of the selected flavonoids in WST-assays. In naive PC12 cells, the threshold dose for cytotoxicity is 10 μM for tBHQ, sulforaphane, luteolin, baicalein and genistein. Interestingly, 5 μM luteolin as well as 1–5 μM tBHQ significantly increase the viability of PC12 cells compared with solvent controls (DMSO 0.5%; Fig. 1) suggesting a stabilizing mode of energy metabolism even in the absence of stressful stimuli. As oxidative stressor we used the toxicant MPP^+ that provoked a dose-dependent death of PC12 cells (Figs. 5 and 6). If not otherwise mentioned, flavonoids were used at 5 μM, the highest non-toxic concentration; MPP^+ was used at 100 μM, a dose which reduced cell viablility by around 50% (Fig. 6); the final concentration of the solvent DMSO was limited to 0.5% (64 mM), a dose which did not affect cell viability (data not shown).

Activation of Nrf2 through the measurement of NQO1-ARE response

For the investigation of the flavonoids' potency to activate Nrf2 we conducted dual luciferase reporter gene assays. The Nrf2-reporter gene contained a classical binding site for Nrf2, the *cis*-acting antioxidant response element (*ARE*) of the NQO1-gene, and an increase in the firefly luciferase expression indicates the binding of Nrf2 to the NQO1-*ARE* element. Sulforaphane, an isothiocyanate from broccoli, and the synthetic tBHQ are well defined activators of Nrf2 and therefore were used as positive controls.

All the flavonoids investigated as well as sulforaphane and tBHQ activated the luciferase gene expression in the absence of any intentional stimulus (Fig. 2A). The activation potency differed between the compounds and the rank order of significant NQO1-*ARE* response compared with the solvent 0.5% DMSO was luteolin > sulforaphane > genistein = tBHQ = baicalein = daidzein (ANOVA and post hoc Duncan's multiple range test). To prove the direct activation of Nrf2, we investigated the nuclear presence of Nrf2 following luteolin on Western blots. Indeed, Nrf2

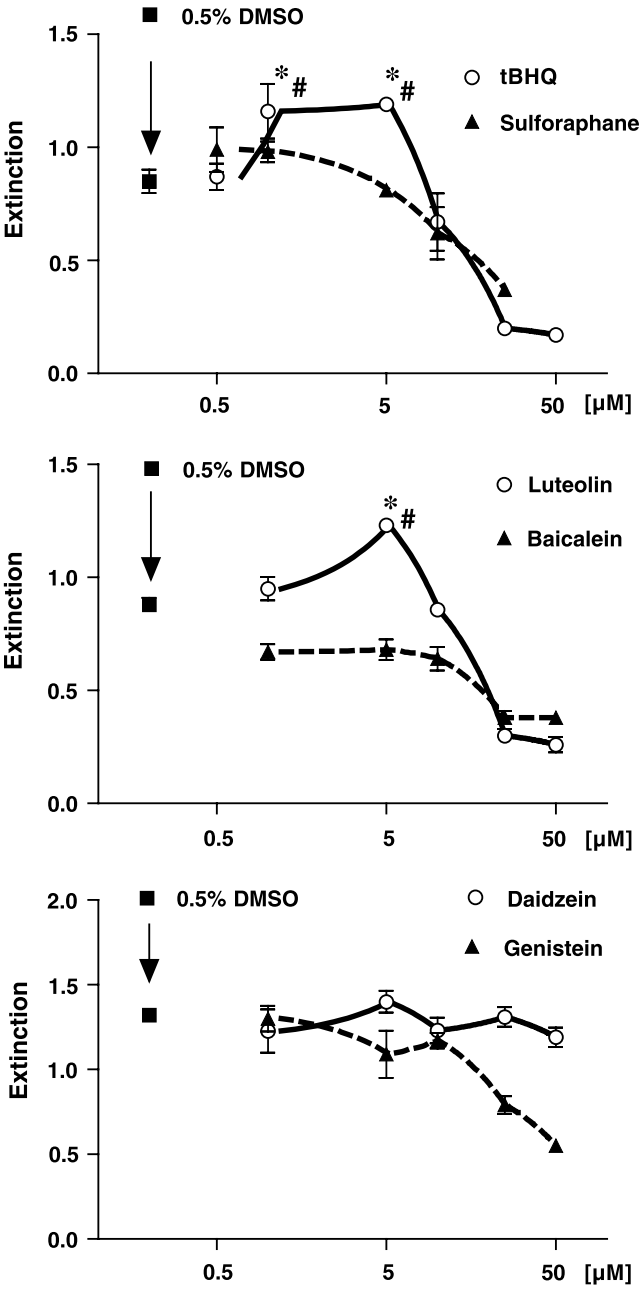


Fig. 1. Viability of rat PC12 cells as determined by the WST assay (Extinction). tBHQ, sulforaphane, luteolin, baicalein, daidzein, and genistein were administered at all doses with 0.5% DMSO as the solvent. Values are expressed as means ± SD of eight independent determinations ($n = 8$). Statistical differences ($p < 0.05$) between groups were evaluated using ANOVA and multiple range test. *Significant difference versus control, #significant difference versus 1 and 5 μM sulforaphane or versus 5 μM baicalein

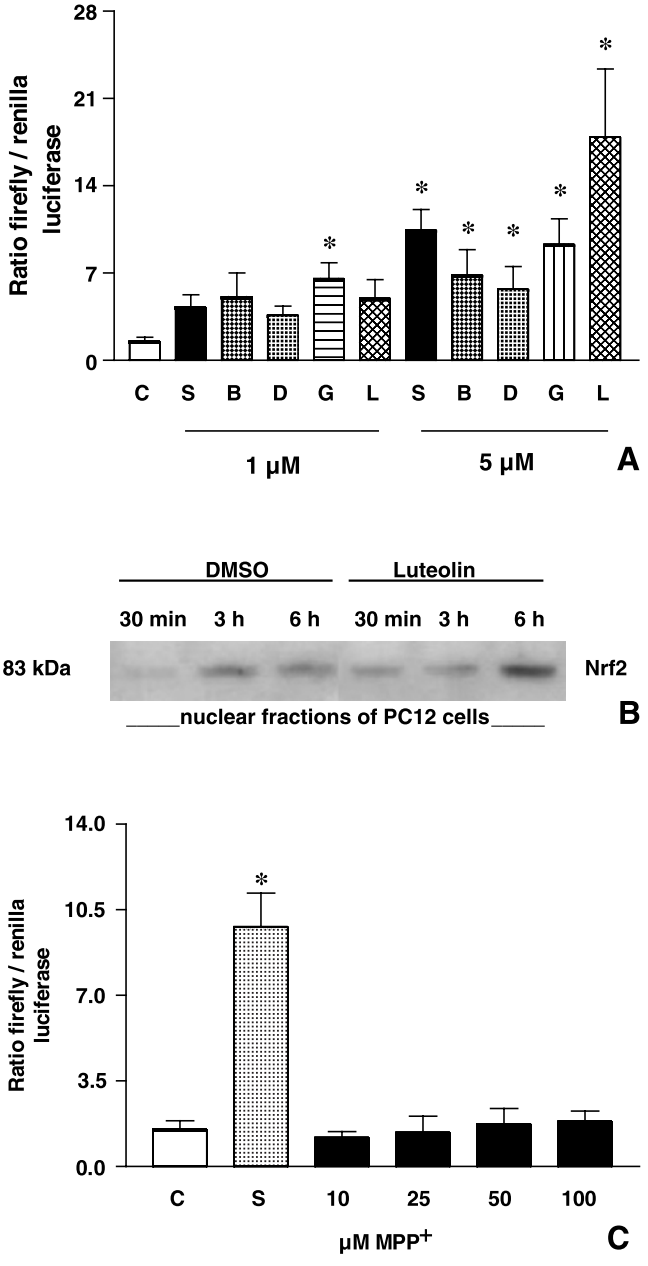


Fig. 2 **A** Ratio of firefly luciferase expression to renilla luciferase expression in a dual luciferase reporter gene assay for the determination of the NQO1-*ARE* response in rat PC12 cells. Sulforaphane (*S*), baicalein (*B*), daidzein (*D*), genistein (*G*), and luteolin (*L*) activate NQO1-*ARE* response in a dose dependent manner. Values are expressed as means ± SD of eight independent determinations ($n = 8$). Statistical differences $p < 0.05$ between groups were evaluated using ANOVA and multiple range test; *significant difference versus control (*C*). **B** Western blots of PC12 cell extracts following DMSO or luteolin. Nrf2 was detected in the nuclear fractions following incubation times indicated. Each lane was loaded with 20 µg protein. **C** Ratio of firefly luciferase expression to renilla luciferase expression in a dual luciferase reporter gene assay for the determination of the NQO1-*ARE* response in PC12 cells in the presence of sulforaphane (S) or 1-methyl-4-phenylpyridinium iodide (MPP^+). Values are expressed as means ± SD of eight independent determinations ($n = 8$). Statistical differences ($p < 0.05$) between groups were evaluated using ANOVA and multiple range test: *significant difference versus control (C = 0.5% DMSO)

increased in the nuclear compartment within 6 h after exposure to luteolin (Fig. 2B).

Furthermore, we analysed the impact of MPP^+, the pathogenic stimulus used for the following experiments, on NQO1-*ARE* response. The neurotoxicant MPP^+ alone did not affect the expression of firefly luciferase (Fig. 2C).

Finally we wanted to know whether the induction of ARE merely depends on the antioxidative properties or requires defined structural properties e.g. electrophilic α,β-unsaturated carbonyls. We investigated the NQO1-*ARE* response following exposure to the well known and

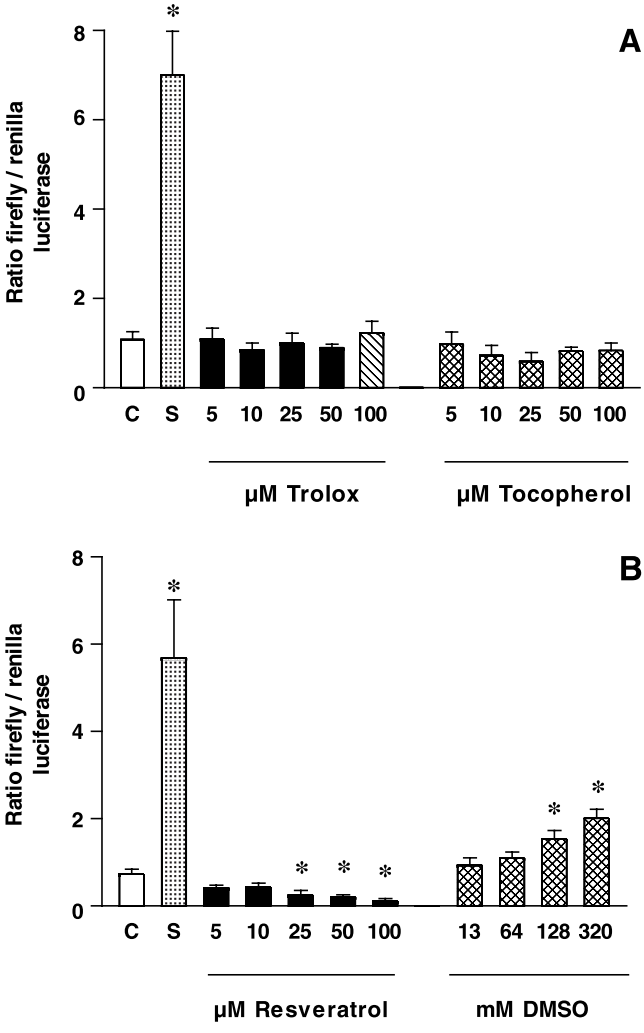
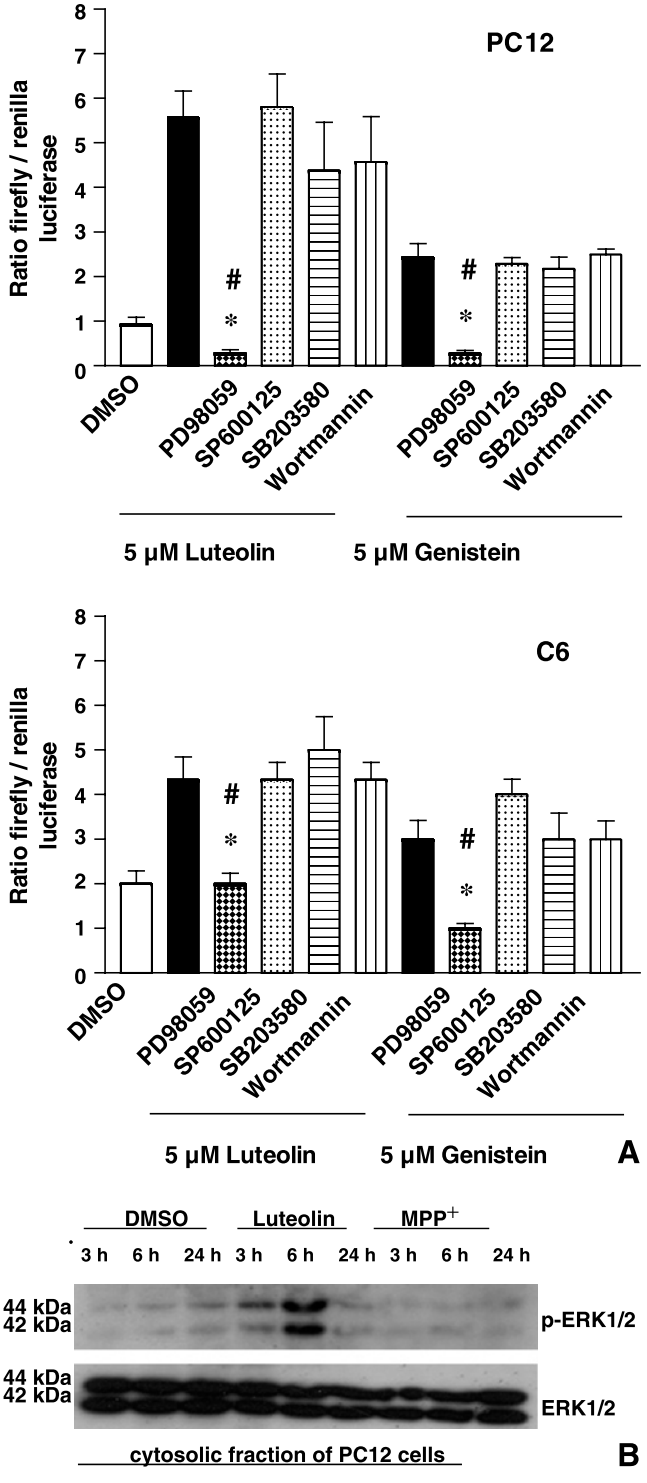


Fig. 3. Ratio of firefly luciferase expression to renilla luciferase expression in a dual luciferase reporter gene assay for the determination of the NQO1-*ARE* response in rat PC12 cells. **A** Cells were dose dependently stimulated for 24 h with trolox or tocopherolacetate in 0.5% DMSO. **B** Cells were dose dependently stimulated for 24 h with resveratrol in 0.5% DMSO or with different concentrations of solvent DMSO (0.1% = 12.8 mM; 0.5% = 64 mM; 1% = 128 mM; 2.5% = 320 mM). Values are expressed as means ± SD of eight independent determinations ($n = 8$). Statistical differences ($p < 0.05$) between groups were evaluated using ANOVA and multiple range test. *Significant difference versus control group (*C* = 0.5% DMSO = 64 mM); (*S*) sulforaphane 5 µM

potent phenolic antioxidants resveratrol and trolox that do not belong to the flavonoid family but bear antioxidative phenolic moieties resembling that of flavonoids. Interestingly, neither resveratrol nor α-tocopherol nor trolox, a water soluble analogue to tocopherol, activate the NQO1-*ARE*-response in PC12 cells (Fig. 3). This finding suggests



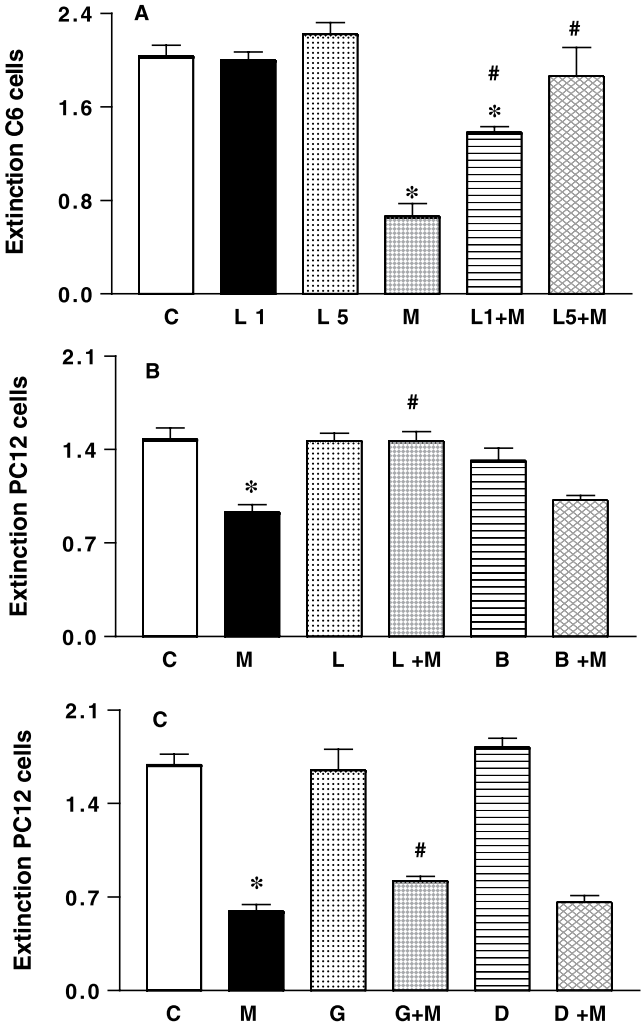


Fig. 5. Viability of PC12 cells and C6 cells as determined by the WST assay 24 h following 100 µM MPP^+ (*M*). **A** C6 glioblastoma cells were preincubated for 16 h with 1 µM luteolin (*L1*) or 5 µM luteolin (*L5*). PC12 cells were incubated with **B** 5 µM luteolin (*L*), or 5 µM baicalein (*B*); or **C** 5 µM genistein (*G*) or 5 µM daidzein (*D*). Values were collected 24 h following the exposure to 100 µM MPP^+ (*M*) and are expressed as means ± SD of eight independent determinations ($n = 8$). Statistical differences ($p < 0.05$) between groups were evaluated using ANOVA and multiple range test. *Significant difference versus control (*C*), #significant difference versus MPP^+ (*M*)

Fig. 4. **A** Ratio of firefly luciferase expression to renilla luciferase expression in a dual luciferase reporter gene assay for the determination of the NQO1-*ARE* response in the absence or presence of kinases inhibitors in rat PC12 cells and in rat C6 cells. Values are expressed as means ± SD of eight independent determinations ($n = 8$). Statistical differences ($p < 0.05$) between groups were evaluated using ANOVA and multiple range test. *Significant difference versus control (0.5% DMSO), #significant difference versus luteolin and genistein without kinase inhibitor (solid bars). ERK1/2 inhibitor PD98059 (20 µM), JNK inhibitor SP600125 (2 µM), p38 inhibitor SB203580 (5 µM), PI3K inhibitor wortmannin (1 µM). **B** Western blots of PC12 cell extracts following 3, 6, or 24 h incubation with 5 µM luteolin or 100 µM MPP^+. Phospho-ERK1/2 and total ERK1/2 were determined in the cytosolic fractions. Phospho-ERK1/2 signal appears selectively increased 6 h following 5 µM luteolin. Each lane contains 20 µg of protein

that the NQO1-*ARE* response is not activated by the mere antioxidant action of flavonoids.

Activation of NQO1-ARE-response by luteolin involves the ERK1/2 pathway

The following experiments addressed the signaling pathway underlying the activation of Nrf2 by flavonoids. As visualised by the dual luciferase assay, flavonoid-mediated Nrf2 activation was almost completely abolished in PC12 and C6 cells by PD98059, an inhibitor of MEK1, the upstream kinase of ERK1/2 (Fig. 4A). In striking contrast, inhibition of c-Jun N-terminal kinases by SP600125, inhibition of p38 by SB203580 and inhibition of phospho-inositol-3-kinase (PI3K) by wortmannin did not affect the NQO1-*ARE* response in PC12 or in C6 cells. ERK1/2 are also directly activated by flavonoids (Fig. 4B). Between 3 and 6 h after exposure, luteolin evoked a strong phosphorylation of ERK1/2 which vanished after 24 h, whereas neither DMSO nor MPP^+ showed any effect on ERK1/2 phosphorylation. The pool of total ERK1/2 did not change in all specimens.

Cytoprotection by flavonoids

In the next experiments we investigated whether flavonoids protect PC12 cells from toxicity exerted by the mitochondrial complex I inhibitor MPP^+. Non-differentiated, i.e. mitotic C6 and PC12 cells were incubated with the flavonoids 16 h before the exposure to 100 μM MPP^+. Cell viability was measured after further 24 h incubation without medium exchange. 5 μM luteolin significantly enhanced viability of C6 cells following MPP^+ mediated toxicity (Fig. 5A). In PC12 cells, luteolin completely reversed the MPP^+ induced death, whereas genistein showed only moderate effect. Neither baicalein nor daidzein counteracted the MPP^+ toxicity (Fig. 5B, C).

Does luteolin also confer protection from MPP^+ toxicity in post-mitotic neuron-like PC12 cells which were differentiated for 6 days with NGF (50 ng/ml) prior to exposure? The complete protection of luteolin was similar to the

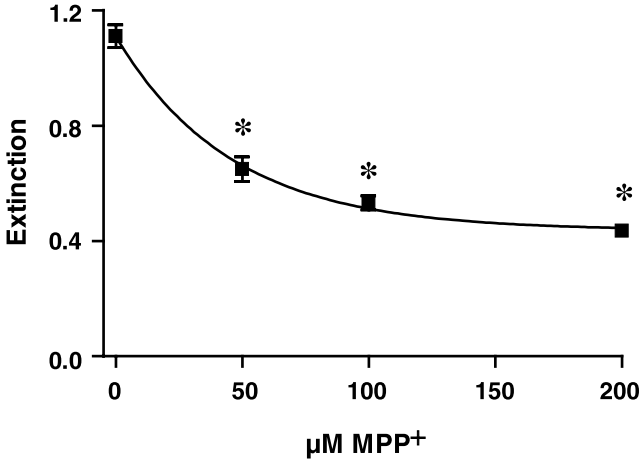


Fig. 6. PC12 cells differentiated with 50 ng/ml NGF for 6 days were exposed to various concentrations of MPP^+. Cell viability is quantified by the measurement of WST. Values were collected 24 h following the exposure to MPP^+ and are expressed as means ± SD of six independent determinations ($n = 6$). Statistical differences ($p < 0.05$) between groups were evaluated using ANOVA and multiple range test. *Significant difference versus 0.2% DMSO as control

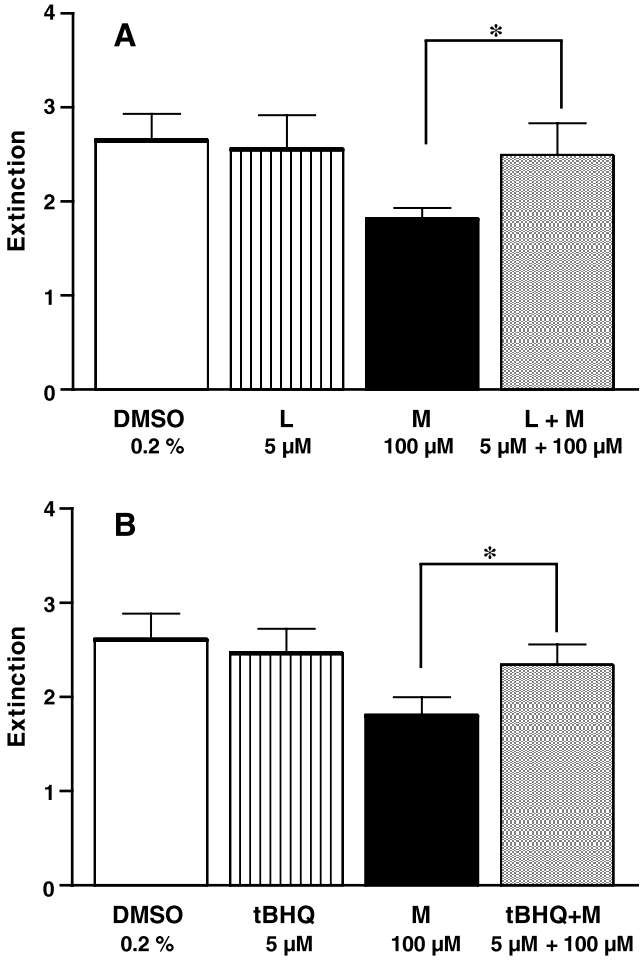


Fig. 7. **A** Luteolin and **B** tBHQ maintain the chemical reductive capacity of differentiated PC12 cells in the presence of 100 μM MPP^+. 5 μM luteolin or 5 μM tBHQ were incubated for 16 h prior to the addition of 100 μM MPP^+ and further incubation for 24 h without a medium exchange at a final concentration of 0.2% DMSO. Values were collected 24 h following the exposure to MPP^+ and are expressed as means ± SD of six independent determinations ($n = 6$). Statistical differences ($p < 0.05$) between groups were evaluated using ANOVA and multiple range test. *Significant difference versus MPP^+

protection of tBHQ (Fig. 7A, B), a strong synthetic activator of Nrf2, which was used as gold standard.

Inhibition of Nrf2 prevents neuroprotection by luteolin

So far we have shown that flavonoids – in particular luteolin – enhance the reportergene transcription driven by NQO1-*ARE* activation, increase the nuclear amount of Nrf2, activate ERK1/2 and protect against MPP^+ triggered neural death. The crucial question arose whether Nrf2 mediates the neuroprotection of luteolin. To clarify this issue, PC12 cells were stably transfected with a vector construct that expresses short hairpin RNA (*sh*RNA) against *Nrf2* mRNA which prevents the expression of Nrf2. In PC12 cells transfected with Nrf2-*sh*RNA, 5 μM luteolin did not increase the viability of untreated PC12 cells, and the oxidative stressor MPP^+ enhanced the death of Nrf2-*sh*RNA transfected PC12 cells (Fig. 8) compared to control-transfected PC12 cells. Importantly, 5 μM luteolin lost all its neuroprotective effect in MPP^+ stressed PC12 cells transfected with Nrf2-*sh*RNA, whereas 5 μM luteolin was perfectly protective in control-transfected PC12 cells (Fig. 8).

Intracellular signaling of luteolin involved in cytoprotection

As shown in the preceding experiments, luteolin induced the phosphorylation of ERK1/2 (Fig. 2B), and the Nrf2 activation by luteolin was antagonised by the MEK1 inhibitor PD98059 (Fig. 4). These findings imply that ERK1/2 triggers the cytoprotection of luteolin. Indeed, cytoprotection by luteolin was significantly attenuated in the presence of UO126, a direct inhibitor of MEK1/2 and indirect inhibitor of ERK1/2 (Fig. 9). Thus, the MEK1/2-ERK1/2 signaling also mediates the luteolin-induced cytoprotection against MPP^+.

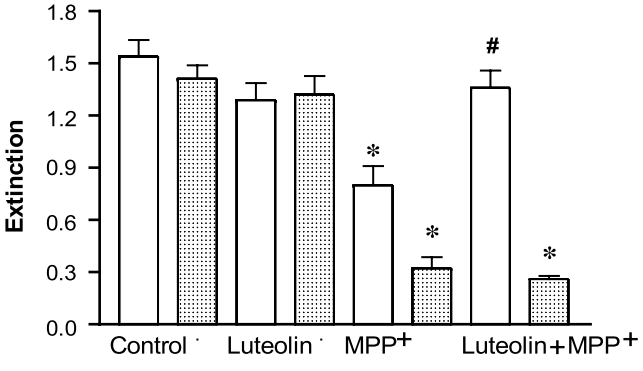


Fig. 8. Stable expression of shRNA against Nrf2 abrogates protection from toxicity of MPP^+ by luteolin. Luteolin was preincubated for 16 h with PC12 cells stably transfected with a control vector (open bars), or with cells stably expressing shRNA against Nrf2 thus downregulating Nrf2 expression (hatched bars). Values were collected 24 h following the exposure to MPP^+ and are expressed as means ± SD of eight independent determinations ($n = 8$). Statistical differences ($p < 0.05$) between groups were evaluated using ANOVA and multiple range test. *Significant difference versus DMSO or luteolin controls, respectively; #significant difference versus MPP^+

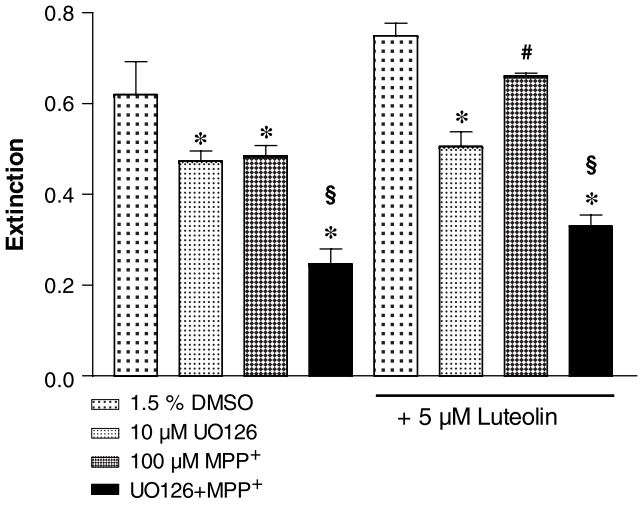


Fig. 9. Luteolin maintains the reductive capacity of differentiated PC12 cells in the presence of 100 μM MPP^+, but not in the presence of the MEK inhibitor UO126 (10 μM) that was given 1 h before luteolin. Luteolin was incubated for 16 h prior to the addition of 100 μM MPP^+ and further incubation for 24 h without a medium exchange at a final concentration of 1.5% DMSO given as three times 0.5% at the respective time points. Values are expressed as means ± SD of three independent determinations ($n = 3$). Statistical differences ($p < 0.05$) between groups were evaluated using ANOVA and multiple range test. *Significant difference versus DMSO and DMSO + luteolin; #significant difference versus MPP^+; §significant difference versus UO126

Discussion

In the present study, we have provided novel insights into the mode of neuroprotection of flavonoids. The flavones luteolin and baicalein as well as the isoflavones genistein and daidzein activate the NQO1-*ARE*, a classical consensus element of the Nrf2 transcription factor. This implicates that these flavonoids enhance the neuronal defence via Nrf2 activation. Of these flavonoids only genistein and luteolin elicit protection from MPP^+ toxicity; in particular luteolin, that shows the strongest Nrf2-activation, completely protects from MPP^+ toxicity. Indeed, inhibition of Nrf2 by shRNA abrogates the neuroprotection of luteolin which triggers neuroprotection in a MEK-ERK1/2 dependent manner.

Activation of Nrf2

Oxidative stress and mitochondrial dysfunction are considered as central factors for neuronal damage in neurodegenerative diseases such as Parkinson's disease (PD), Alzheimer's disease, and amyotrophic lateral sclerosis (ALS). Consequently, activation of the endogenous cerebral oxidative stress defence mechanisms appears as a valid strategy for therapeutic intervention (Lee and Johnson, 2004; van Muiswinkel and Kuiperij, 2005). *In vivo* and *in vitro* studies have demonstrated that polyphenolic flavonoids have neuroprotective potential (for a review, see Mandel et al., 2004), but the underlying mechanisms are still under intense investigation. We have demonstrated that the flavonoids luteolin, genistein, baicalein and daidzein potently activate Nrf2 at a concentration of 5 μM in PC12 and C6 cells. This activation depends on ERK1/2 signaling which is blocked by the MEK1/2 inhibitors PD98059 as well as UO126. This strongly indicates that ERK1/2 activation is a prerequisite for Nrf2 activation by the flavonoids investigated (Fig. 10). However, Nrf2 might not directly be a substrate of ERK1/2. Instead, it is discussed that ERK1/2 phosphorylates the nuclear transcription coactivator CREB-binding protein (CBP), and that CBP enhances Nrf2 transcriptional response (Shen et al., 2004).

Cytotoxicity of MPP^+ and flavonoids

Rapid onset of parkinsonism in man, primate, and mouse, following administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (Langston et al., 1983; Gerlach and Riederer, 1996) occurs through its active metabolite, 1-methyl-4-phenylpyridinium (MPP^+) (Langston et al., 1984), a reversible inhibitor of mitochondrial complex I. MPP^+ leads to the depletion of energy stores and the induction of oxidative stress *in vivo* (Nicklas et al., 1987; Przedborski et al., 2004). The flavones luteolin and baicalein, as well as the isoflavones genistein and daidzein are characterized by their intermediate lipophilicity and solubility in DMSO as well as by their electrophilic α,β-unsaturated carbonyl groups. This structural property is in contrast to many other flavones, isoflavones, flavonols, flavanols, flavanones,

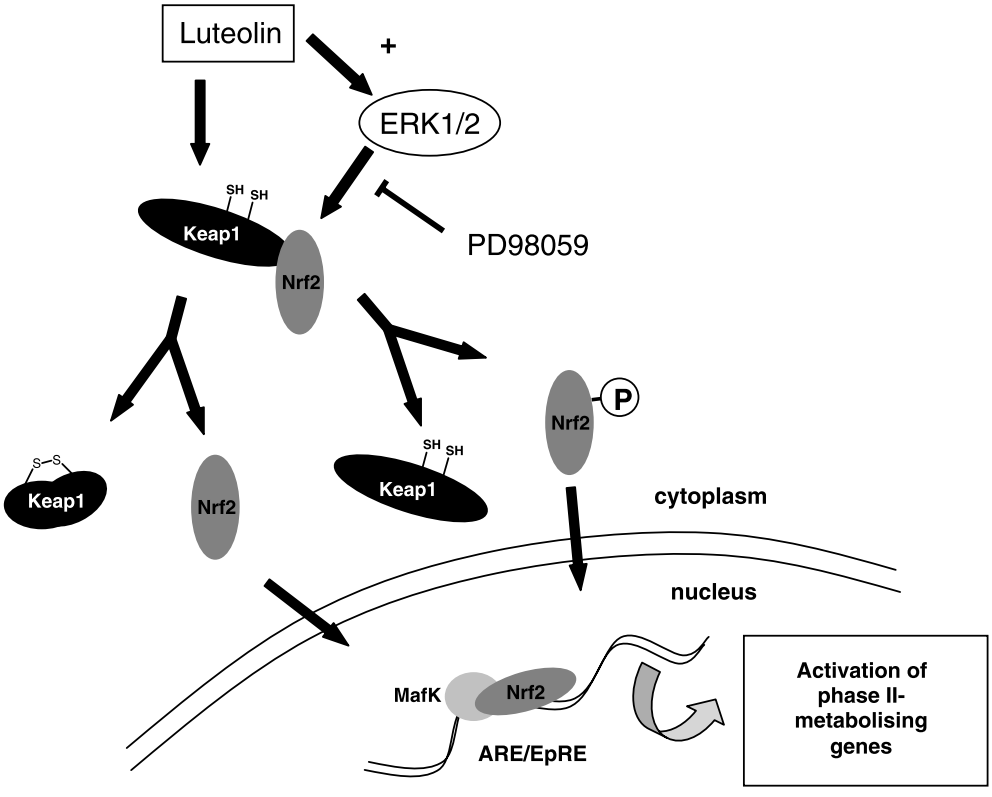


Fig. 10. A model of the mechanism postulated to be involved in the protection of PC12 cells from MPP^+ toxicity is schematically presented. ERK1/2 phosphorylation probably leads to Nrf2 phosphorylation that is consequently liberated from Keap1 to enable nuclear transcription of cytoprotective genes with the help of small Maf proteins. Alternatively, a direct interaction with nucleophilic thiol groups of Keap1 with luteolin or luteolin oxidation products such as o-quinones from catechol moieties may be postulated, but remains to be experimentally proven. PD98059 is a MEK1/ERK1/2 inhibitor

flavanonols, anthocyanins and chalcones (Nagao et al., 1999). Up to 5 μM, genistein, baicalein, and luteolin are not cytotoxic in PC12 cells. Daidzein is not cytotoxic up to 50 μM in PC12 cell culture, since above 10 μM daidzein starts to precipitate and cannot passively diffuse into cells anymore.

Interestingly, 5 μM luteolin, but not baicalein, significantly increases PC12 cell viability. This bell-shaped dose response curve holds also true for tBHQ, but not for sulforaphane, two well described Nrf2-activators, pinpointing a specific mode of action of luteolin and tBHQ within a narrow concentration range and highlighting the necessity to establish narrow dose-toxicity relationships prior to cytoprotection studies.

Cytoprotection by flavonoids

Some of the first studies on cytoprotection with Nrf2 activators at low micro Molar concentrations were undertaken with tBHQ which easily crosses lipid bilayers. tBHQ protects from 6-OHDA-induced oxidative stress in neural cell lines (Lee et al., 2001; Hara et al., 2003; Jakel et al., 2005). In addition to tBHQ, triterpene electrophiles isolated from acacia victoriae (Haridas et al., 2004) activate the innate stress response of Hep G2 cells by redox regulation of a set of target genes driven by the activation of Nrf2.

Oral administration of tBHQ (100 mg/kg in mice) prior to 2′CH3-MPTP for 7 days normalized the GSH content and SOD activity, and ameliorated several indices of lipid peroxidation (Abdel-Wahab, 2005). In a very recent second *in vivo* study, Burton et al., 2006 demonstrated that MPTP-induced neurotoxicity is diminished by oral administration of 3H-1,2-dithiole-3-thione depending on Nrf2 activation. On the other hand, tBHQ attenuates the neuronal death following stroke in $Nrf2^{+/+}$, but not in $Nrf2^{-/-}$ mice (Shih et al., 2005), demonstrating Nrf2-specific actions of tBHQ mediated neuroprotection in the nervous system.

As well in our hands, tBHQ activates Nrf2 in PC12 cells and protects from MPP^+ toxicity. Thus, we used tBHQ as the gold standard in screening experiments that aimed to identify novel Nrf2 activating compounds with neuroprotective potential.

We identified luteolin as one of the most effective compounds in protecting PC12 cells from MPP^+ toxicity in PC12 and C6 cells. Genistein was modestly cytoprotective, whereas baicalein and daidzein failed to do so. The Nrf2-activation by luteolin, is a precondition of protection since luteolin does no longer protect against MPP^+ in PC12 cells stably transfected with *sh*RNA targeting Nrf2.

The Nrf2 activation by luteolin is mediated by ERK1/2 with MEK1 as upstream activator. Since the MEK1/2 inhibnhibitors PD98059 and UO126 potently inhibit Nrf2 activation, ERK1/2 must be involved in the maintenance of Nrf2 signaling.

The pronounced protection by luteolin may be based on its superior free radical scavenging and metal chelating properties (Arora et al., 1998; Ishige et al., 2001). Luteolin is the only compound that we investigated bearing a catechol moiety in the phenyl group and therefore may be oxidized to an ortho-quinone. This electrophilic quinone could directly react with thiol groups in Keap1 with consequent liberation of Nrf2. Very recently Lee-Hilz et al. (2006) identified planar flavones that have a high intrinsic potential to generate oxidative stress and for redox cycling as potent activators of hNQO1-ARE response in Hepa1c1c7 cells. This result favours the scientific view that the pro-oxidant activity of flavonoids-induces ARE-mediated gene expression. PKC was not involved in flavonoid-induced *ARE*-mediated gene transcription in Hepa1c1c7 cells. We instead identified ERK1/2 pathway as an important additional element in Nrf2 activation in neural cells.

In conclusion we demonstrate that the flavones luteolin and baicalein and the isoflavones genistein and daidzein activate NQO1-*ARE* in a dose dependent manner. Further, dose dependent cytoprotection against MPP^+ is best conferred by luteolin and is critically depending on ERK1/2 activation, and on the subsequent activation of Nrf2. In addition to antioxidative capacity, luteolin exhibits another very important protective property, i.e. the induction of the phase 2 response. The mere antioxidant action of these compounds, however, is apparently not sufficient for neuroprotection. Based on our results we extend previous models established for the explanation of the cytoprotective effects of the green tea polyphenol epigallocatechingallate and other flavonoids (Mandel et al., 2004; Boerboom et al., 2006; Lee-Hilz et al., 2006), in which we conclude that the flavone luteolin, and the synthetic compound tBHQ activate the MAPK pathway via an electrophilic-mediated stress response, leading to the transcription activation by Nrf2/Maf heterodimers on ARE enhancers. This may induce the expression of cellular defence/detoxifying genes including conjugating enzymes which protect the cells from toxic environmental insults and thereby prolong cell survival (Fig. 10).

Thus, it is tempting to assume that the long-term activation of Nrf2 in vulnerable neural cells might become a promising therapeutic strategy to halt the progression of neuronal demise in aging and neurodegenerative diseases.

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