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Kavalactones Protect Neural Cells against Amyloid β Peptide-Induced Neurotoxicity via Extracellular Signal-Regulated Kinase 1/2-Dependent Nuclear Factor Erythroid 2-Related Factor 2 Activation

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ABSTRACT

One hallmark of Alzheimer's disease is the accumulation of amyloid β-peptide (AP), which can initiate a cascade of oxidative events that may result in neuronal death. Because nuclear factor erythroid 2-related factor 2 (Nrf2) is the major regulator for a battery of genes encoding detoxifying and antioxidative enzymes via binding to the antioxidant response element (ARE), it is of great interest to find nontoxic activators of Nrf2 rendering neuronal cells more resistant to AP toxicity. Using ARE-luciferase assay and Western blot, we provide evidence that the kavalactones methysticin, kavain, and yangonin activate Nrf2 time- and dose-dependently in neural PC-12 and astroglial C6 cells and thereby up-regulate cytoprotective genes. Viability and cytotoxicity assays demonstrate that Nrf2 activation is able to protect neural cells from amyloid β-(1-42) induced neurotoxicity. Down-regulation of Nrf2 by small hairpin RNA as well as extracellular signal-regulated kinase 1/2 inhibition abolishes cytoprotection. We further give evidence that kavalactone-mediated Nrf2 activation is not dependent on oxidative stress production. Our results demonstrate that kavalactones attenuate amyloid β-peptide toxicity by inducing protective gene expression mediated by Nrf2 activation in vitro. These findings indicate that the use of purified kavalactones might be considered as an adjunct therapeutic strategy to combat neural demise in Alzheimer disease and other oxidative stress-related diseases.

There is significant evidence that oxidative stress is a critical event in the pathogenesis of Alzheimer's disease (AD). This hypothesis is supported by studies that used postmortem brain tissue from patients with AD (Gotz et al., 1994; Butterfield et al., 2001) and by in vitro studies (Behl et al., 1994). Oxidative stress can cause cell death by damaging cardinal cellular components, such as lipids, proteins, or DNA and RNA. The brain is especially sensitive to oxidative stress because of its high concentration of readily oxidized fatty acids and high oxygen consumption. In AD, oxidative stress is suspected to be generated by the amyloid β peptide (Behl et al., 1994; Butterfield et al., 2001).

Therefore, treatment with antioxidants might theoretically act to retard spreading of neuronal damage and to improve neurological outcome. Indeed, several studies investigated whether dietary intake of antioxidants, especially vitamins, might prevent or reduce the progression of AD. Although a few of the antioxidants showed some efficacy in these trials, no answer is yet available as to whether antioxidants are truly protective in AD (Boothby and Doering, 2005).

Another way to render neuronal cells more resistant to oxidative stress is to up-regulate the endogenous protection

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ABBREVIATIONS: AD, Alzheimer's disease; Nrf2, nuclear factor erythroid 2-related factor 2; ARE, antioxidant response element; DDT, dithiothreitol; NAC, *N*-acetylcysteine; GSH, glutathione; GSH-GEE, glutathione monoethyl ester; PD98059, 2′-amino-3′-methoxyflavone; SP600125, anthra[1,9-*cd*]pyrazol-6(2*H*)-one-1,9-pyrazoloanthrone; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole; LDH, lactate dehydrogenase; WST, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene disulfonate; p-, phospho-; ERK, extracellular signal-regulated kinase; HO-1, heme oxygenase-1; γ-GCS, γ-glutamylcysteine synthetase; DMSO, dimethyl sulfoxide; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(methylthio)butadiene; Aβ, amyloid β-peptide; ANOVA, analysis of variance.

system. During evolution, cells have developed complex mechanisms to defend from oxidative and electrophilic stress. A battery of genes encoding detoxifying and antioxidative enzymes is orchestrated on exposure to electrophiles and reactive oxygen species. This coordinated response is regulated through a *cis*-acting element, the antioxidant response element (ARE) within the regulatory regions of this "safeguard" gene. Activation of nuclear factor erythroid 2-related factor 2 (Nrf2) and resultant binding to the ARE initiates or enhances the transcription of these genes, such as NAD(P)H:quinone oxidoreductase-1, thioredoxin reductase, glutathione peroxidase, and hemeoxygenase-1 (Jaiswal, 2004; Lee and Johnson, 2004). Like other "stress response" transcription factors (e.g., hypoxia inducible factor-1α), Nrf2 is expressed in a constitutive manner, and it subsequently degraded within minutes. An essential step in the stabilization and activation of Nrf2 is the liberation of the Nrf2 inhibitor Keap1, which binds Nrf2 and promotes its proteasomal degradation. Oxidative stress or electrophiles but also Nrf2 phosphorylation by kinases disrupt the Keap1-Nrf2 complex, leading to stabilization and activation of Nrf2 (Tong et al., 2006).

The relevance of Nrf2 in neuronal protection could be shown using transgenic techniques. Neural cells from these Nrf2 knockout mice were more vulnerable to oxidative stress compared with those from Nrf2 wild-type mice (Lee et al., 2003a,b). In addition, overexpression of Nrf2 dramatically increased the resistance of neurons to oxidative cell death (Shih et al., 2003).

In search of agents that activate Nrf2, three analytically pure kavalactones—methysticin, yangonin, and kavain—were under examination. These kavalactones are the main components of the rhizome and roots of kava (*Piper methysticum* G. Forst), a Piperaceae common on some Pacific Ocean islands (Bilia et al., 2004). Anthropological evidence suggests that kava has been cultivated and consumed for more than 3000 years. It is still used today by a wide range of Pacific societies for spiritual, medicinal, and recreational purposes. Kavalactones showed only moderate antioxidant activities (Wu et al., 2002), but they have well known psychopharmacological properties. The most observed effects of kava extract at low doses are mood relaxation or euphoric behavior, depending on the circumstances of ingestion, whereas higher doses cause sleepiness and skeletal muscle relaxation (Singh, 1992; Singh and Singh, 2002). Several other effects have been reported, such as antithrombotic action (Gleitz et al., 1997), anticonvulsant properties (Jamieson et al., 1989), inhibition of nuclear factor-κB (Folmer et al., 2006), and cyclooxygenases (Wu et al., 2002) as well as neuroprotection in focal cerebral ischemia in mice and rats (Backhauss and Krieglstein, 1992). No interactions with neuroreceptors have yet been found that would explain the various pharmacological effects.

Concerns have recently been raised about the safety of kava consumption. There have been several reports of rare but severe liver toxicity, including liver failure in some people who have used dietary supplements containing kava extract. In this context, Nerurkar et al. (2004) showed toxicity for the kava alkaloid pipermethystine but not for kavalactones such as methysticin and yangonin. This study suggests that pipermethystine, rather than kavalactones, is responsible for the hepatotoxic reactions to kava. On this account, we examine the kavalactones methysticin, yangonin, and kavain for their properties to activate Nrf2 and evolve neuroprotective effects.

The present study provides the first documentation of the neuroprotective properties of kavalactones from amyloid β-toxicity. We could further show that the investigated kavalactones exert their protective effects by inducing the expression of cytoprotective genes through Nrf2 activation in an ERK1/2-dependent manner.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium-Ham's F-12 (1:1) with 2 mM glutamine was obtained from PAA Laboratories GmbH (Pasching, Austria), and N2-Supplement was from Invitrogen (Karlsruhe, Germany). The tested kavalactone yangonin (purity 98%) was purchased from Phytolab (Hamburg, Germany), methysticin (purity 99.36%) was purchased from LKT Laboratories (St. Paul, MN), and kavain (purity 99%) was from Sigma Chemie (Deisenhofen, Germany). Curcumin, dithiothreitol (DTT), Trolox, ascorbic acid, *N*-acetylcysteine (NAC), GSH monoethyl ester (GSH-MEE), PD98059, SP600125, SB203580, and wortmannin were obtained from Sigma Chemie. 4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene disulfonate (WST) assay was obtained from Roche Diagnostics GmbH (Penzberg, Germany). The antibodies directed against p-ERK and total ERK1/2 were obtained from Cell Signaling Technology Inc. (Danvers, MA), and Nrf2, HO-1, γ-GCS, and β-actin antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Amyloid β-(1-42) was purchased from Tocris Cookson Inc. (Bristol, UK). All other chemicals were of the highest quality commercially available.

Cell Culture

The pheochromocytoma cell line PC-12 and rat astrocytoma cell line C6 (both purchased from LGC Promochem, Wesel, Germany) were grown in Dulbecco's modified Eagle's medium-Ham's F-12 (1:1) with 2 mM glutamine and N2-supplement containing putrescine, insulin-like growth factor-1, transferrin, progesterone, and selenite. PC-12 cells were differentiated for 6 days with 50 ng/ml nerve growth factor. For WST, lactate dehydrogenase (LDH), and luciferase assay, 5000 cells were plated per well on BIOCOAT Collagen I 96-well plates (VWR International, Hamburg, Germany) in 200 μl of serum-free medium.

Toxicity Assays

WST Assay. For WST assay, media were supplemented with 10 μl/well WST 2 h before spectrophotometric evaluation. Conversion of WST to formazan was measured at 450 nm by microplate spectrophotometry (model 680; Bio-Rad Laboratories, Hercules, CA). This reaction reflects the reductive capacity of the cells, which represented the viability of the cells, and it is expressed relative to the value of 100 ± S.E.M., which represented the reductive capacity of the untreated control.

LDH Assay. LDH is a stable cytoplasmic enzyme present in all cells, including neurons. It is rapidly released into the cell culture supernatant when the cell plasma membrane is damaged. Thus, the LDH level in the culture medium is a reliable biochemical index for neuronal plasma membrane damage. In this study, LDH release from the cytosol of damaged PC-12 cells into the culture medium after amyloid β-(1-42) exposure was measured using a cytotoxicity detection assay (Roche Diagnostics, Mannheim, Germany), which determines the LDH activity in the culture medium to enzymatically convert the lactate and NAD^+ to pyruvate and NADH. The tetrazolium salt produced in the enzymatic reaction was then reduced to red formazan in the presence of protons, thereby allowing colorimetric detection of neuronal membrane integrity. LDH release is expressed relative to the value of 100 ± S.E.M., which represented the maximum LDH release that occurred after freezing overnight at −70°C and subsequent rapid thawing of each culture, which induced nearly complete cell damage, established from n = 8 wells per one experiment from three separate experiments.

Luciferase Assay

Both strands of the rat *NQO1* gene ARE1 5′-CAGTCTAGAGTCACAGTGACTTGGCAAAATCG-3′ and 5′-CTAGCGATTTTGCCAAGTCACTGTGACTCTAGACTGGTAC-3′ with Kpn1 and Nhe1 ends were synthesized (Tib Molbiol, Berlin, Germany), annealed, and cloned at the Kpn1 and Nhe1 site of the pGL3-Promoter (Promega, Madison, WI) to produce the reporter construct pNQO1-rARE. Then, 1.5 μg of the NQO1-ARE reporter plasmid containing the firefly luciferase reporter gene, and 0.5 μg of the pRL-TK plasmid, containing the *Renilla reniformis* 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. Twenty-four hours after transfection the cells were seeded to a 96-well plate. The activities of both firefly and *R. reniformis* luciferases were determined 48 h after transfection with the Dual-Luciferase Reporter Assay system (Promega). The luciferase activities were normalized to the *R. reniformis* luciferase activity of the internal control.

RNA Interference

The mammalian expression vector pGE1 (Stratagene, La Jolla, CA) was used for the stable expression of shRNA against Nrf2-mRNA in PC-12 cells. The gene-specific insert, which is specified by a 29-nucleotide sequence 5′-GTCTTCAGCATGTTACGTGATGAGGATGG-3′ of the rat Nrf2, is separated by an eight-nucleotide noncomplementary spacer (GAAGCTTG) from the reverse complement of the same 29-nucleotide sequence. The oligonucleotides were synthesized by Tib Molbiol. This construct was inserted into the pGE1 using BamHI and XbaI restriction sides, and it is referred to as

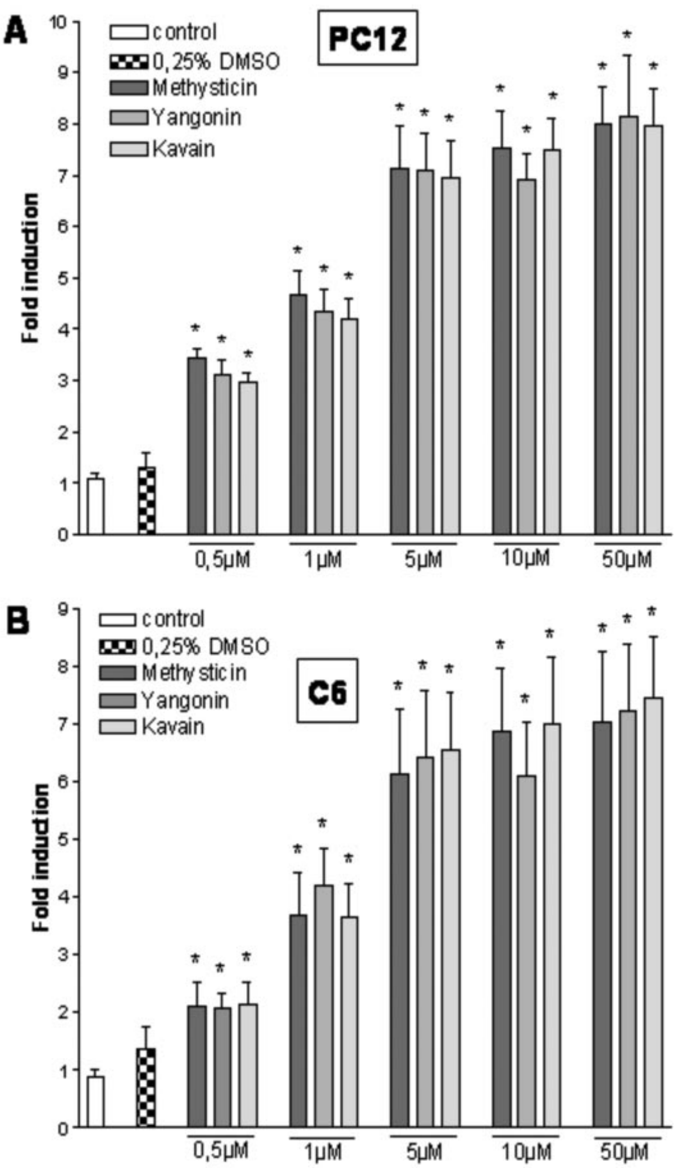


Fig. 1. Dose-dependent changes in ARE activity of PC-12 and C6 cells treated with kavalactones. PC-12 (A) and C6 (B) cells were transfected with NQO1-ARE-luciferase plasmid, and luciferase activity was measured as described under *Materials and Methods*. Dose response of ARE activation was assayed using 0 to 50 μM kavalactones for 12 h. Data are presented as -fold induction after treatment with kavalactones compared with vehicle control. The concentration of vehicle (0.25% DMSO) was unchanged in all experiments. All experiments were performed with n = 8, and error bars indicate S.E.M.. *, significant difference versus DMSO control.

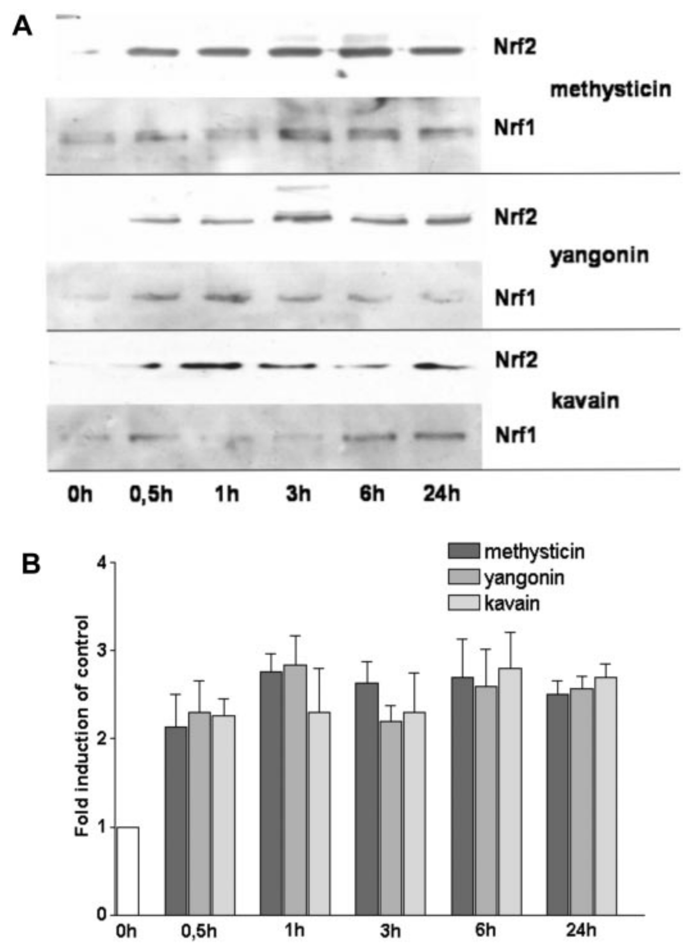


Fig. 2. Western blot analysis to measure the effects of kavalactones on Nrf2 in PC-12 cells. A, Nrf2 response to kavalactones. PC-12 cells were stimulated with 5 μM methysticin, yangonin, or kavain for various times, and nuclear fractions were prepared as described under *Materials and Methods*. Nuclear proteins (20 μg) were separated on SDS-polyacrylamide gel electrophoresis, Western blotted, and probed with anti-Nrf2 and reprobed with anti-Nrf1 (as loading control) antibodies (one representative Western blot was shown; n = 3). B, density of corresponding bands, Nrf2 and Nrf1, were measured, and the ratio of Nrf2/Nrf1 was calculated. The median of three independent experiments is shown. Data are presented as -fold induction after treatment compared with vehicle control (control = 1).

pGE1-rNrf2. A control vector (pGE1-negative) serves as a nonsilencing control (Stratagene).

Cloning of Nrf2

The rat full-length Nrf2 cDNA was amplified from PC-12 mRNA by polymerase chain reaction (sense primer, 5′-CACCGCTAGCCAGCATGATGGACTTGGAGTTGCCACC-3′; antisense, 5′-CCCTGGTACCCCGTTTTTCTTTGTATCTGGCTTCTTGCTT-3′) and the polymerase chain reaction product was cloned into the mammalian expression vector pcDNA 3.1 (Invitrogen) to make the expression plasmids pcDNA-rNrf2.

Western Immunoblotting

HO-1 and γ-GCS protein levels, ERK1/2 phosphorylation in whole-cell lysates, and Nrf2 in nuclear extracts were measured by Western immunoblotting, using protocols described previously (Varoga et al., 2006). For the Nrf2 detection in the nucleus, nuclear and cytoplasmic fractions of PC-12 cells were separated with the NE-PER kit purchased from Pierce Chemical (Rockford, IL). To monitor potential artifacts in loading and transfer among samples in different lanes, the blots for phospho-ERK1/2 were stripped and reprobed with antibodies against total ERK1/2. The digitized images were quantitated with the PCBAS program (Raytest Isotopen Meßgeräte GmbH, Straubenhardt, Germany).

Results

Tolerability of Kavalactones. The first experiments defined the cytotoxicity of kavalactones as well as of the Nrf2 activator curcumin (Balogun et al., 2003). Therefore, the viability of PC-12 and C6 cells was measured by the use of WST assay after incubation with kavalactones and curcumin in different concentrations for 24 h. These studies reveal a significant cytotoxicity in PC-12 as well as C6 cells beginning at 5 μM for curcumin. The kavalactones showed no cytotoxic effects up to 100 μM in PC-12 and C6 cells (data not shown).

Induction of ARE-Mediated Reporter Activity by Kavalactones. To investigate the efficacy of kavalactones to activate the *cis*-acting element ARE, we carried out a dual-

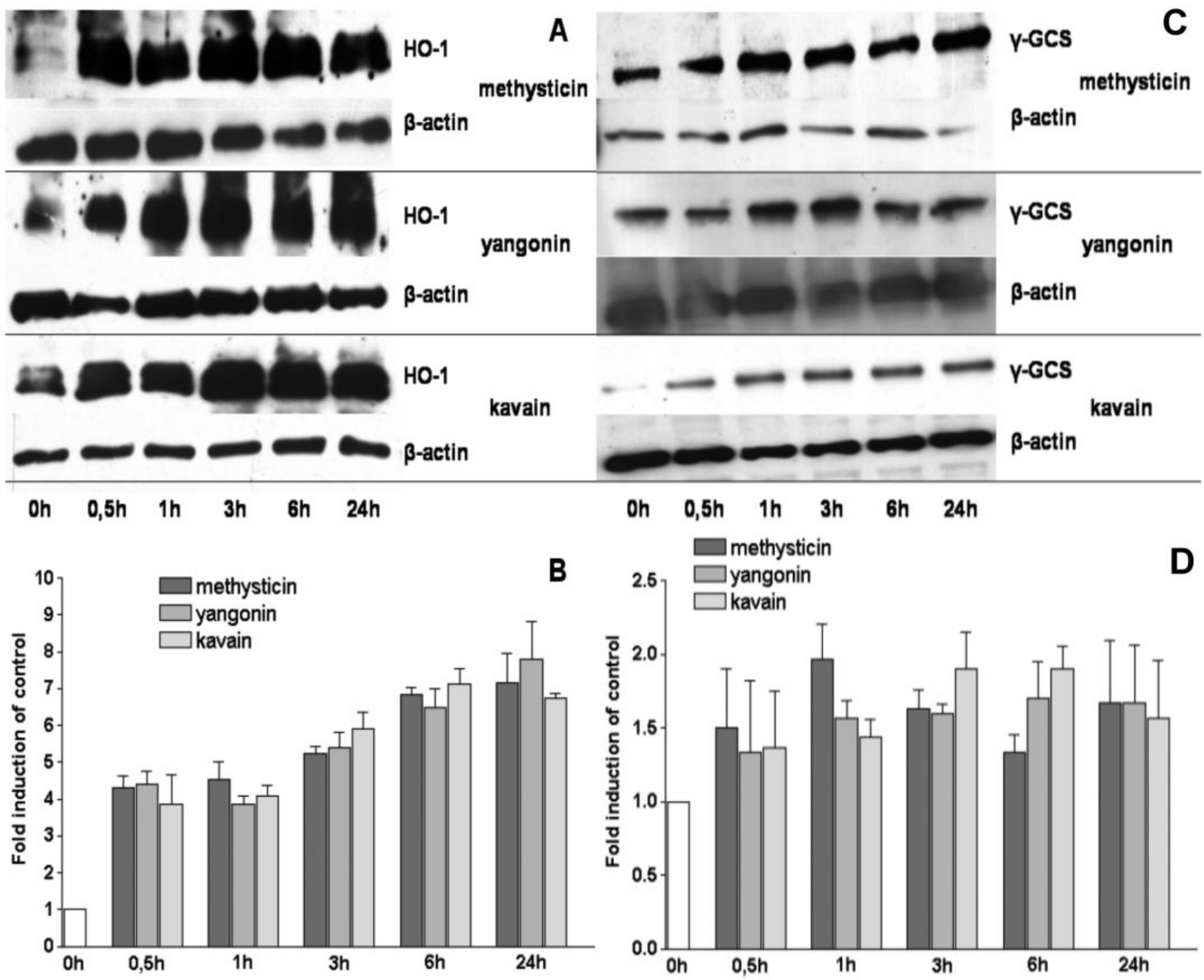


Fig. 3. Western blot analysis to measure the effects of kavalactones on the expression of antioxidative stress genes PC-12 cells. A and B, Western blot show the time dependent induction of HO-1 (A) and γ-GCS (C) expression in PC-12 cells treated with 5 μM kavalactones. The blots were probed with anti-HO-1 or anti-γ-GCS antibody and reprobed with anti-β-actin (as loading control) antibodies. The densities of corresponding bands were measured, and the ratio was calculated. The median of three independent experiments is shown (B and D). Data are presented as fold induction after treatment compared with vehicle control (control = 1).

luciferase reporter gene assay with the ARE of the rat NQO1-gene. ARE activation was determined in a dose-response assay up to 50 μM for kavalactones in PC-12 and C6 cells for 12-h incubation time. Kavalactones (0.5 μM) activate the luciferase gene expression 3-fold in PC-12 cells (Fig. 1A) and 2-fold in C6 cells (Fig. 1B). The activation reached a plateau at 5 μM with a 7-fold induction over control in PC-12 cells (Fig. 1A) and 6-fold in C6 cells (Fig. 1B). Higher concentrations of kavalactones yielded no further increase in the luciferase gene expression. Therefore, 5 μM kavalactones were used in all further experiments. DMSO used as vehicle in a concentration of 0.25% showed no significant effect on ARE activation (Fig. 1, A and B).

Kavalactones Induced Nrf2 Stabilization and HO-1 and γ-GCS Expression. PC-12 cells were exposed to methysticin, yangonin, or kavain at the final concentration of 5 μM to examine its effect on Nrf2 protein stability over time via Western blot of the nuclear fraction. As shown in Fig. 2A, treatment with kavalactones caused a significant time-dependent increase in Nrf2 protein stabilization in nuclear extracts. We measured Nrf2 induction within 30 min, pictured by occurrence of Nrf2 in the nuclear fraction. The induction is still measurable after 24 h. As loading control, we reprobed the blots for Nrf1, a transcription factor with constant expression. The density of both bands, Nrf2 and Nrf1, were measured and the ratio of Nrf2/Nrf1 was calculated. The median of three independent experiments is shown in Fig. 2B.

To further confirm that kavalactones are activators of the Nrf2-ARE system, we studied the effect of kavalactones on the expression of two well known Nrf2 target genes, HO-1 and γ-GCS, via Western blot of the whole extract. Both HO-1 and γ-GCS were up-regulated over time by incubation of PC-12 cells with 5 μM kavalactones (Fig. 3, A–D). As loading control, we reprobed the blots for β-actin. The densities of both bands were measured, and the ratio was calculated. The medians of three independent experiments are shown in Fig. 3, B and D.

Kavalactone-Mediated ARE Activation Was ERK1/2-Dependent. To address the role of individual MAPK pathways in ARE gene regulation by kavalactones, we examined the effects of various kinase inhibitors. We observed the activation of ERK1/2 to be a prerequisite for the activation of Nrf2 by all kavalactones investigated because kavalactone-mediated Nrf2 activation is exclusively inhibited by the MEK1 inhibitor PD98059 at 20 μM as well as the MEK1/2

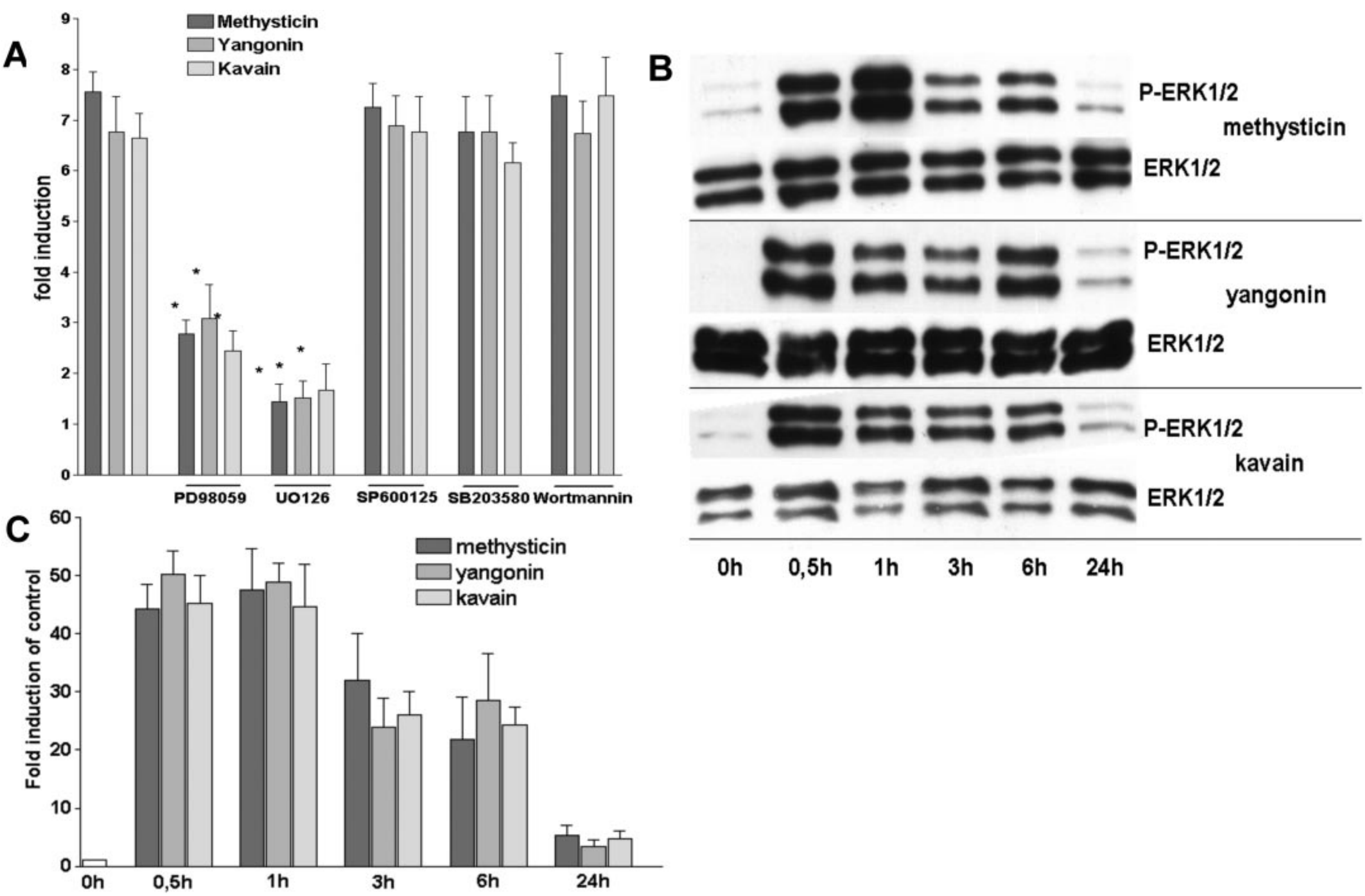


Fig. 4. Kavalactones activate Nrf2 via ERK1/2 activation. A, dual-luciferase assay to analyze the effect of kinase inhibitors on kavalactone-mediated NQO1-ARE activation; 5 μM concentrations of each kavalactone was added to the culture 1 h after addition of the kinase inhibitors (20 μM PD98059, 10 μM, U0126, 2 μM SP600125, 5 μM SB203580, and 1 μM wortmannin). Data are presented as -fold induction after treatment compared with vehicle control (control = 1). All experiments were performed with $n = 8$, and error bars indicate S.E.M. Statistical differences ($p < 0.001$) between groups were evaluated using ANOVA and multiple range test. *, significant difference versus solely kavalactone-stimulated cells. B, methysticin stimulates ERK1/2 phosphorylation. PC-12 cells were exposed to 5 μM methysticin for various times, and total cell lysates (5 μg) were used for immunoblot analysis using phospho-specific anti-ERK1/2 antibodies (p-ERK1/2). The membrane was stripped and probed with anti-ERK1/2 antibodies for loading control (ERK1/2). C, densities of corresponding bands were measured, and the ratio was calculated. The median of three independent experiments is shown. Data are presented as -fold induction after treatment compared with vehicle control (control = 1).

inhibitor U0126 (10 μM) in PC-12 cells (Fig. 4A). The c-Jun NH_2-terminal kinase inhibitor SP600125 at 2 μM, the inhibitor of p38-MAPK SB203580 at 5 μM, and wortmannin at 1 μM, an inhibitor of phosphoinositol-3-kinase, did not diminish the Nrf2 activation in PC-12 cells (Fig. 4A).

Kavalactones Activated ERK1/2. To determine the role of ERK1/2 in kavalactone-mediated Nrf2 activation, we examined its effect on ERK1/2 activity. ERK1/2 are activated by dual phosphorylation of threonine and tyrosine residues located in the "activation lip" of the conserved core kinase sequence, and the activated species can be detected by antibodies directed against phosphorylated peptides encompassing these residues. PC-12 cells were treated with 5 μM methysticin, yangonin, or kavain in a time course, and cell extracts were analyzed for phosphorylated and total ERK1/2 by Western blotting. All three kavalactones stimulated the activation of ERK1/2 in a time-dependent manner (Fig. 4B). A strong increase of ERK1/2 phosphorylation was detected within 30 min after treatment with methysticin, followed by a period of weaker activation for up to 6 h. After 24 h, the phosphorylation status was back nearly to control level. The loading control via total ERK1/2 Western blot showed insignificant differences (Fig. 4B). The densities of both bands, phosphorylated and total ERK1/2, were measured, and the ratio was calculated. The medians of three independent experiments are shown in Fig. 4C.

Kavalactone-Mediated Nrf2 Activation Was Not Dependent on Oxidative Stress Production or Glutathi-

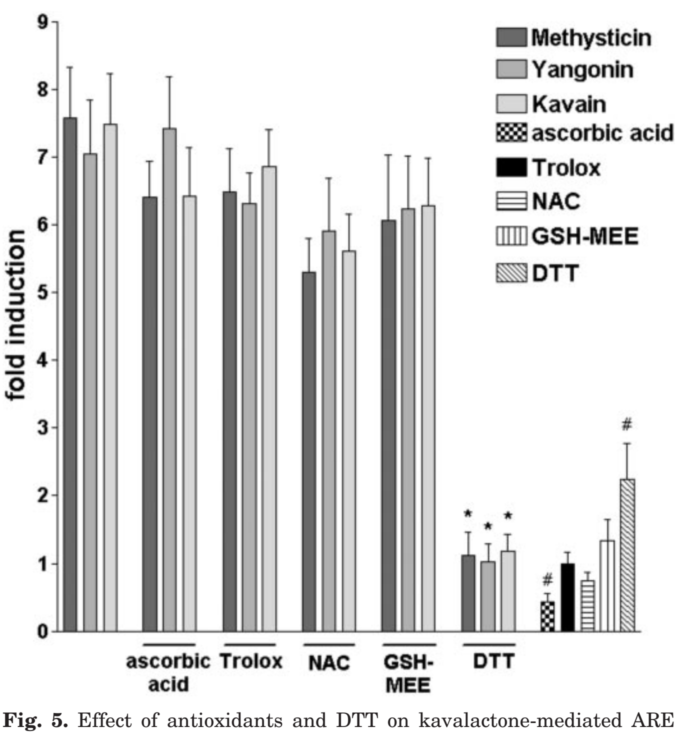


Fig. 5. Effect of antioxidants and DTT on kavalactone-mediated ARE activity. To analyze the effect of antioxidants and DTT on kavalactone-mediated ARE activation, 5 μM concentrations of each kavalactone was added to the culture 1 h after addition of 50 μM ascorbic acid, 50 μM Trolox, 5 μM NAC, and 2 mM GSH GSH-MEE as well as 10 μM DTT. PC-12 cells were transfected with NQO1-ARE-luciferase plasmid, and luciferase activity was measured as described under *Materials and Methods*. Data are presented as -fold induction after treatment compared with vehicle control (control = 1). All experiments were performed with $n = 8$, and error bars indicate S.E.M. Statistical differences ($p < 0.001$) between groups were evaluated using ANOVA and multiple range test. *, significant difference versus solely kavalactone-stimulated cells; #, significant difference versus control.

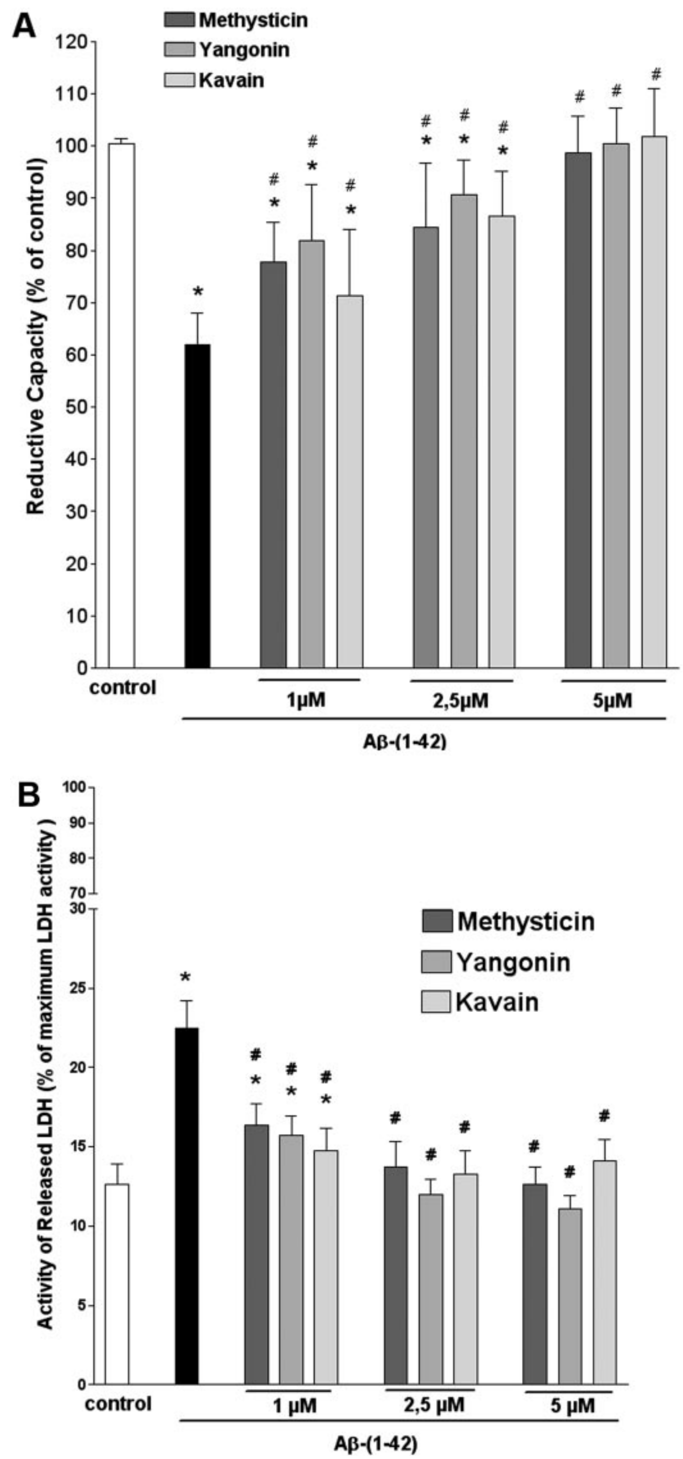
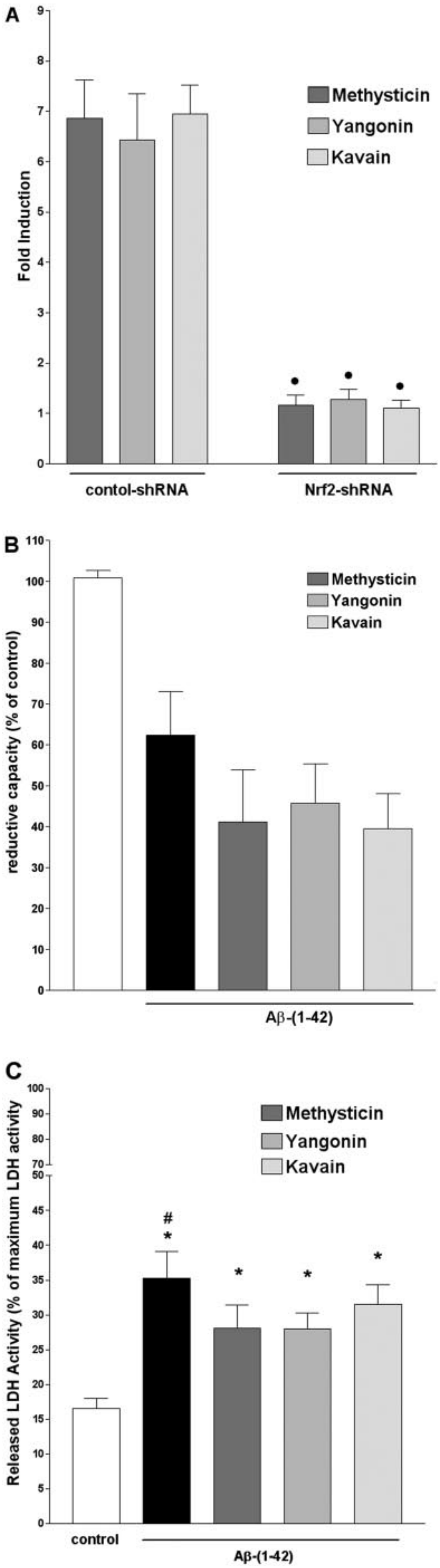


Fig. 6. Protective effect of kavalactones on Aβ-(1-42)-treated PC-12 cells. We added 1, 2.5, or 5 μM concentrations of each kavalactone to the culture 6 h before addition of 10 μM Aβ-(1-42), which was then incubated for 24 h. A, cell viability was measured using WST assay. B, cell death was measured using LDH release assay. Data were normalized to the activity of mitochondrial activity or LDH release from vehicle-treated cells (100%), and they are expressed as a percentage of the control ± S.E.M. established from $n = 8$ wells per one experiment from three separate experiments Statistical differences ($p < 0.001$) between groups were evaluated using ANOVA and multiple range test. *, significant difference versus control. #, significant difference versus Aβ-(1−42)-treated cells.



one Depletion. To exclude the possibility that kavalactones activate Nrf2 via production of oxidative stress or glutathione depletion, PC-12 cells were pretreated with the antioxidants ascorbic acid at 50 μM, Trolox at 50 μM, and NAC at 5 μM, and GSH-MEE at 2 mM. None of these antioxidants had an inhibitory effect of the kavalactone-induced Nrf2 activation, providing evidence that activation is not dependent on oxidative stress production or glutathione depletion. Although DTT by itself induced a small increase in ARE activation (Haridas et al., 2004), pretreatment of cells with 10 μM DDT almost completely blocked the effects of incubations with 5 μM kavalactones for 24 h in PC-12 cells. Unlike NAC and GSH-MEE, DTT seems to have a mechanism of protein thiol reduction independent of GSH (Rafeiro et al., 1994), but the exact mechanism has not been shown. These observations suggest a protection of critical thiol groups of Keap1 by DTT, whereas replenishment of GSH content via NAC and GSH-MEE does not. Moreover, treatment of PC-12 cells for 24 h with 50 μM ascorbic acid down-regulates Nrf2 activation significantly compared with control cells (Fig. 5).

Cytoprotection by Kavalactones. We tested the hypothesis that preactivation of Nrf2 could protect from lesions caused by Aβ-(1-42). Therefore, differentiated PC-12 cells were incubated with 1, 2.5, or 5 μM methysticin, yangonin, or kavain 16 h before exposure to 10 μM Aβ-(1-42). Cell viability was measured via WST assay, and cell death via LDH release assay 24 h after Aβ-(1-42) administration. These assays revealed that preincubation of PC-12 cells with 5 μM concentrations of each kavalactone tested effectively protects from Aβ-(1-42) induced toxicity in both the WST (Fig. 6A) as well as in the LDH-release assay (Fig. 6B).

Nrf2 Activation Conferred Cytoprotection. We further investigated a causal relationship between Nrf2 activation and cytoprotection mediated by kavalactones. Therefore, we designed a shRNA against mRNA coding for rat Nrf2 (pGE1-rNrf2), and we produced a PC-12 cell line (PC-12-shNrf2) by stable transfection of this shRNA construct. In a NQO1-ARE luciferase assay, PC-12-shNrf2 cells are not responsible for ARE-dependent reporter gene induction mediated by kavalactones, demonstrating the efficacy of pGE1-rNrf2 (Fig. 7A). We used these PC-12-shNrf2 cells for cytoprotection assays against Aβ-(1-42) toxicity conferred by kavalactones. As shown in Fig. 7, B and C, kavalactones are not capable to protect PC-12-shNrf2 cells from Aβ-(1-42) toxicity (Fig. 7, B and C). Furthermore, PC-shNrf2 cells are more vulnerable to Aβ-(1-42) toxicity. After incubation with 10 μM Aβ-(1-42) the cell viability of PC-12-shNrf2 cells de-

Fig. 7. Effect of Nrf2-shRNA on the protective effects of kavalactones on Aβ-(1-42) toxicity in stable Nrf2-shRNA-transfected PC-12 cells. A, to test the efficiency of Nrf2-shRNA, PC-12 cells were as well stably transfected with shRNA against Nrf2-mRNA or control-RNA, and ARE inductions were measured after 24-h incubation with 5 μM kavalactone. B and C, 5 μM kavalactone was added to the cultures 6 h before the addition of 10 μM Aβ-(1-42), which were then incubated for 24 h. Cell viability (B) and cell death (C) were measured using WST and LDH release assays, respectively. Results are presented as -fold induction after treatment compared with vehicle control (control = 1; A). All experiments were performed with n = 8, and error bars indicate S.E.M. Statistical differences ($p < 0.001$) between groups were evaluated using ANOVA and multiple range test. ●, significant differences of Nrf2-shRNA-transfected cells versus control shRNA-transfected cells, stimulated with the same kavalactone. *, significant differences of shRNA-Nrf2-transfected cells versus control-shRNA transfected cells, stimulated with the same kavalactone. #, significant difference versus kavalactone-stimulated cells.

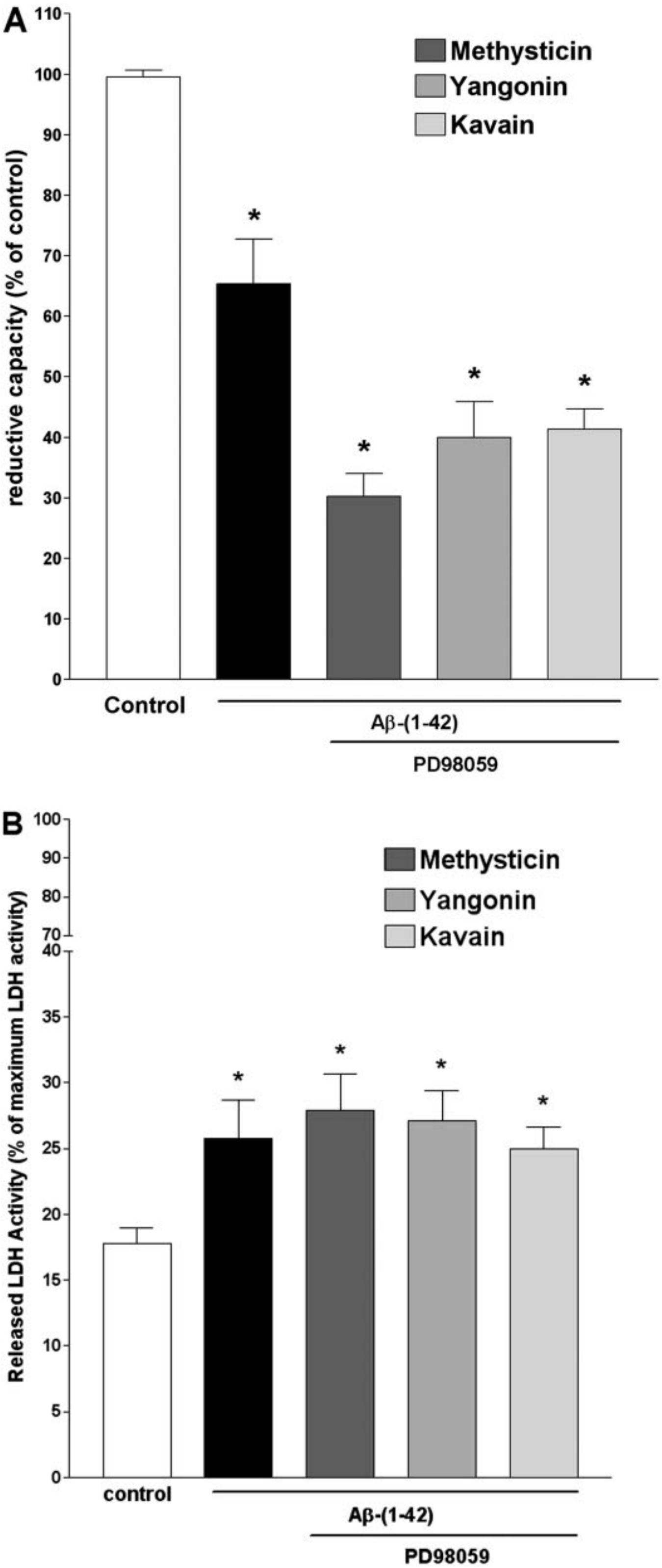


Fig. 8. Effect of the ERK1/2 kinase inhibitor PD98059 on the protective effect of kavalactones with respect to cell death and reduction of cell viability induced by Aβ-(1-42) in PC-12 cells. Kavalactone (5 μM) was added after 1-h preincubation with 20 μM PD98059 to the cultures 6 h before the addition of 10 μM Aβ-(1-42), which were then incubated for 24 h. A, cell viability was measured using WST assay. B, cell death was measured using LDH release assay. Data were normalized to the activity of mitochondrial activity or LDH release from vehicle-treated cells (100%) and expressed as a percentage of the control ± S.E.M. established from $n = 8$ wells per one experiment from three separate experiments. Statistical differences ($p < 0.001$) between groups were evaluated using ANOVA and multiple range test. *, significant difference versus control.

clined by 75% (Fig. 7B) versus the 30% decline, which was found with naive PC-12 cells (Fig. 6A). PC-12-shNrf2 cells consistently showed a greater increase of released LDH activity after Aβ-(1-42) treatment than naive PC-12 cells, because PC-12-shNrf2 showed an 18% increase from 17% (control) to 35% [Aβ-(1-42)-treated] (Fig. 7C), whereas naive PC-12 showed an 10% increase from 13% (control) to 23% [Aβ-(1-42)-treated] (Fig. 6B). Data in percentage of maximum LDH activity equal 100%. The higher vulnerability of PC-12-shNrf2 cells emphasizes the important role of Nrf2 in the defense from Aβ-(1-42) toxicity.

Signaling of Cytoprotection by Kavalactones. Having established that kavalactones are protective against Aβ-(1-42) toxicity through Nrf2 activation, studies were conducted to determine whether ERK1/2 activation would be essential for the cytoprotective effect. As shown in Fig. 8, A and B, cytoprotection of methysticin, yangonin, and kavain were abrogated in the presence of 20 μM PD98059, an inhibitor of the upstream kinases of ERK1/2 MEK1/2. Thus, kavalactones activate Nrf2 through an ERK1/2-dependent mechanism, and the concerted action of Nrf2 and ERK1/2 is critical for PC-12 cell survival in the presence of Aβ-(1-42).

Discussion

It is generally accepted that Nrf2 plays a key role in the adaptive response to oxidative and electrophilic stress, maintaining the cellular self-defense. This study was designed to discover nontoxic substances able to activate the Nrf2-mediated adaptive response and enhance the cell defense. These substances should further be tested for protective effects against Aβ-(1-42) inducted toxicity, making them potentially useful for the treatment of Alzheimer disease.

Kavalactones Activate Nrf2 at Nontoxic Concentration in Vitro. The first criterion was low toxicity of the tested substances. In a viability assay, the kavalactones methysticin, yangonin, and kavain showed no toxicity to differentiated PC-12 and C6 cells up to 100 μM (Fig. 1). These results are consistent with the literature describing kava-kava and particularly purified kavalactones as virtually nontoxic substances (Clouatre, 2004; Nerurkar et al., 2004; Sorrentino et al., 2006).

Next, we examined the potential of kavalactones to activate Nrf2 in neural PC-12 and glial C6 cells. We used glial in addition to neural cells because Murphy et al. (2001) showed that within the brain the protection against oxidants is mainly supported by astrocytes. We established a dual luciferase assay with an ARE of the rat NQO-1 gene transfected in PC-12 and C6 cells. In these systems, exposure of kavalactones activated the Nrf2-ARE system dose-dependently (Fig. 2, A and B). The induction of the ARE system could be confirmed by Western blot showing the time-dependent stabilization of Nrf2 in the nucleus (Fig. 3, A and B) and upregulation of the Nrf2-target genes γ-GCS and HO-1 in PC-12 cells (Fig. 3, C–F).

In contrast to most other Nrf2 activators like flavonoides, sulforaphane, or curcumin, which are already toxic at low concentration, kavalactones showed no toxicity in our assays in vitro, and they are thereby able to activate Nrf2 at extremely low concentrations.

Kavalactones Mediate Nrf2 Activation via ERK1/2. Several studies have shown that MAPKs are involved in the activation of Nrf2 (Owuor and Kong, 2002). To examine whether MAPKs are involved in the signal transduction of kavalactones, we tested various MAPK inhibitors with respect to Nrf2 activation. From all tested inhibitors, only the MEK1 inhibitor PD98059 and the MEK1/2 inhibitor U0126 showed an inhibitory effect on the Nrf2 activation (Fig. 4A). This identifies ERK1/2 activation to be a prerequisite for Nrf2 activation by kavalactones. 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, and that CREB-binding protein enhances Nrf2 transcriptional response (Katoh et al., 2001; Shen et al., 2004). In addition, we stimulated PC-12 cells with kavalactones, and we analyzed the phosphorylation status of ERK1/2 to back-reference the activation status of the kinases. All three tested kavalactones showed a strong but transient activation of ERK1/2 after 30 min up to 1 h, followed by a period of weaker activation for a minimum 6 h. After 24 h, the phosphorylation status renormalized similar to control (Fig. 4B).

ERK1/2 are traditionally viewed as the survival factors of the MAPK family. In contrast, there are suggestions that ERK1/2 activation may also be associated with neuronal cell death in various neurodegeneration models, especially if occurring coincidentally with oxidative stress (Zhu et al., 2004). However, activation of ERK1/2 in situations devoid of prevailing oxidative stress, as may be the case with kavalactones, could be neuroprotective (see below; Culmsee et al., 2005).

Kavalactone-Mediated Nrf2 Activation Is Not Dependent on Oxidative Stress Production or Glutathione Depletion. The Keap1-Nrf2 complex serves as a cytoplasmic sensor enabling the cell to respond to electrophiles and oxidative stress. Many Nrf2 activators, also those considered to act as protective substances such as curcumin, sulforaphane, flavonoids, or epigallocatechin gallate, activate Nrf2 via oxidative stress (Gong et al., 2004; Jakubíková et al., 2006; Lee-Hilz et al., 2006; Wu et al., 2006). The protective effect of these substances may by based upon a phenomenon referred to as preconditioning, an effect well established for hypoxia via hypoxia inducible factor-1α or hyperthermia via heat shock transcription factor 1.

To test whether kavalactones also activate Nrf2 via oxidative stress, PC-12 cells were pretreated with the various

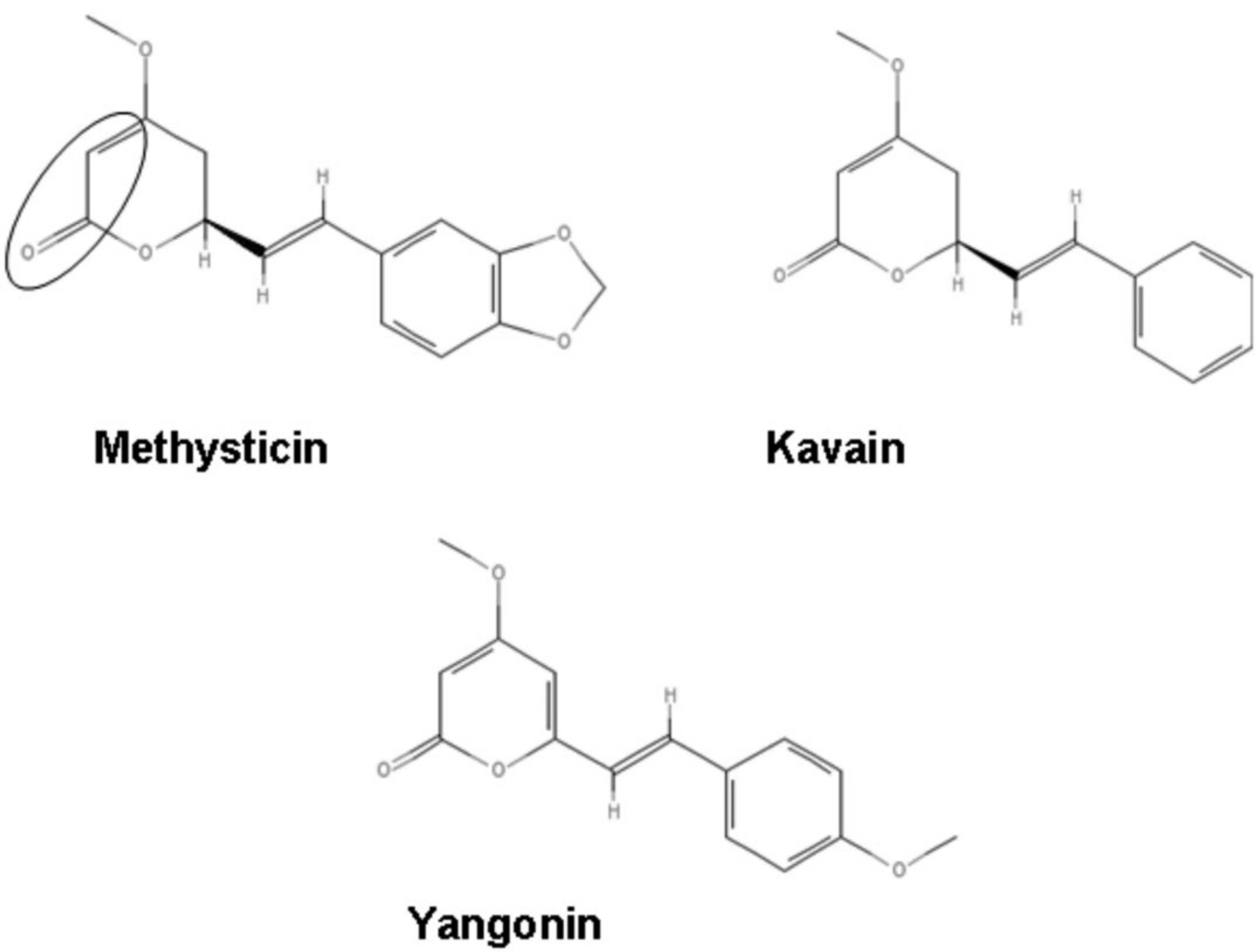


Fig. 9. Structures of kavalactones used in this study show the presence of a lactone ring that contained an α,β-unsaturated carbonyl group (exemplarily encircled by methysticin).

antioxidants. But none of the tested antioxidants had an inhibitory effect on kavalactone-induced Nrf2 activation (Fig. 4), suggesting another mechanism of Nrf2 activation than oxidative stress production. To address whether kavalactones activate Nrf2 via decline of the GSH/oxidized glutathione ratio, we pretreated the cells with GSH monoethyl ester. This enhancement of the GSH pool did not decrease the Nrf2 activation, giving evidence that glutathione depletion is not the way kavalactones activate Nrf2. It is noteworthy that treatment of PC-12 cells with 50 μM ascorbic acid but not Trolox down-regulates the Nrf2 activation significantly compared with control cells (Fig. 4), indicating an adaptive effect on the reduced oxidative burden of the cells.

We further tested the effect of DTT, a reagent commonly used in biochemical studies as an agent to prevent the oxidation of thiol groups and for reducing disulfides to dithiols. Although DTT by itself induced a small increase in ARE activation, pretreatment of cells with DTT almost completely blocked the effects of kavalactones in PC-12 cells (Fig. 4). Because of the α,β-unsaturated carbonyl group present in its lactone ring (Fig. 9), kavalactones may act as a Michael reaction acceptor and readily interact with critical cellular nucleophiles, such as cysteine thiol groups in proteins such as Keap1, but the exact mechanism has not been shown. Therefore, the inhibition of kavalactone-induced Nrf2 activation by DTT might suggest that kavalactones regulate critical redox-sensitive thiol groups of Keap1. Whether kavalactones interact directly with cysteine residues of Keap1 has to be elucidated in further studies.

According to these results, we presumed that kavalactones do not activate Nrf2 via oxidative stress production or glutathione depletion. This may explain the low toxicity of kavalactones, compared with other Nrf2 activating substances. This is of great interest, particularly in reference to treatment of neurodegenerative conditions, in which an additional increase of oxidative stress could have destructive consequences.

Kavalactones Protect against Amyloid β-(1-42) Toxicity. Our hypothesis was that Nrf2 activation with a nontoxic substance would render neuronal cells more resistant to Aβ-(1-42)-induced oxidative stress and toxicity. To test this hypothesis, we pretreated differentiated PC-12 cells with kavalactones for 16 h to allow a complete up-regulation of genes encoding detoxifying and antioxidative enzymes, and we confronted them with Aβ-(1-42). Indeed, kavalactone-pretreated cells were more resistant to Aβ-(1-42) toxicity than untreated cells as shown in cell viability and cytotoxicity assays (Fig. 6, A and B). Furthermore, the protective doses tested showed a dose-response relationship and correlation with those of Nrf2 induction (Fig. 1).

A neuroprotective effect of kava extract and its constituents kavain, dihydrokavain, methysticin, dihydromethysticin, and yangonin on ischemic brain damage in mice and rats was first shown by Backhauss and Krieglstein (1992). The molecular mechanisms of these effects were not further elucidated. Because the production of reactive oxygen species has been implicated in reperfusion injury after cerebral ischemia, it is likely that Nrf2 also plays a role in cerebral ischemia (Love, 1999). Indeed, Zhao et al. (2006) give the first evidence that this hypothesis holds true.

To elucidate the role of Nrf2 in the protective effect of kavalactones, we used a PC-12 cell line (PC-12-shNrf2) that carried a stable transfected shRNA against Nrf2-mRNA. This cell line was no longer able to activate the Nrf2-ARE system (Fig. 7A). We used shRNA technology rather than dominant-negative Nrf2 overexpression, because shRNA against Nrf2 knocks down solely Nrf2, whereas dominant-negative Nrf2 blocks the binding to ARE by competitive inhibition and thereby blocks all factors with affinity to ARE.

In these Nrf2 deficient PC-12 cells, kavalactones are no longer protective against Aβ-(1-42) toxicity (Fig. 7, B and C), supporting the pivotal function of Nrf2 in cytoprotection mediated by kavalactones; in addition, Nrf2-deficient cells are more vulnerable to Aβ-(1-42) toxicity.

We further examined whether the ERK1/2 activation is required for the protective effects of kavalactones by treating ERK1/2 inhibitor-preincubated PC-12 cells with kavalactones and testing these cells for their Aβ-(1-42) vulnerability. In fact, the ERK1/2 inhibitor abolished the protective effects of kavalactones in toxicity assays (Fig. 8, A and B). Supposing that kavalactone-mediated ERK1/2 activation occurred also in PC-12-shNrf2 cells, ERK1/2 activation without Nrf2 induction would not be sufficient for cytoprotection (Fig. 7, B and C).

According to these results, we propose that kavalactones activate Nrf2 and thereby elevate cytoprotective gene expression as exemplified by γ-GCS and HO-1 up-regulation. Other Nrf2 target genes that were not under examination here surely contribute to the described cytoprotection.

Conclusions

At present, patients with AD have access to two common treatments, the glutamate receptor antagonist memantine and several acetylcholinesterase inhibitors, but none of them halt the progression of neuronal demise. Several experimental approaches are under investigation with the objective to decelerate neurodegeneration in AD, including Aβ vaccines, metal chelators, derivatives of the Congo red dye that bind Aβ, and antioxidants; however, until now, these approaches have been without clear success (Klafki et al., 2006). Consequently efforts to identify and promote new therapeutic strategies for patients with AD are still of great interest.

We found that a beneficial effect can be induced in neuronal as well as glial cells via kavalactones. Thus, kavalactones are able to activate ERK1/2 and Nrf2 at nontoxic concentrations and thereby mediate an up-regulation of a battery of genes encoding detoxifying and antioxidative enzymes, effective in protecting neurons against amyloid β-(1-42) toxicity in vitro. If studies using kavalactones in an in vivo model of Alzheimer's disease prove this beneficial effect, the use of kavalactones might be considered as an adjunct therapeutic strategy to combat neural demise in Alzheimer's disease and other oxidative stress-related diseases.

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