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Neuroprotective effects of resveratrol against β-amyloid-induced neurotoxicity in rat hippocampal neurons: involvement of protein kinase C

[1]Ying-Shan Han, [1]Wen-Hua Zheng, [1]Stéphane Bastianetto, [1]Jean-Guy Chabot & *,[1]Rémi Quirion

[1]Department of Psychiatry, Douglas Hospital Research Centre, McGill University, 6875 Boulevard LaSalle, Montreal, Québec, Canada H4H 1R3

1 Resveratrol, an active ingredient of red wine extracts, has been shown to exhibit neuroprotective effects in several experimental models.

2 The present study evaluated the neuroprotective effects of resveratrol against amyloid β(Aβ)-induced toxicity in cultured rat hippocampal cells and examined the role of the protein kinase C (PKC) pathway in this effect.

3 Pre-, co- and post-treatment with resveratrol significantly attenuated Aβ-induced cell death in a concentration-dependent manner, with a concentration of 25 μM being maximally effective.

4 Pretreatment (1 h) of hippocampal cells with phorbol-12-myristate-13-acetate, a PKC activator, at increasing concentrations (1–100 ng ml^{-1}), resulted in a dose-dependent reduction in Aβ-induced toxicity, whereas the inactive 4α-phorbol had no effect.

5 Pretreatment (30 min) of hippocampal cells with GF 109203X (1 μM), a general PKC inhibitor, significantly attenuated the neuroprotective effect of resveratrol against Aβ-induced cell death.

6 Treatment of hippocampal cells with resveratrol (20 μM) also induced the phosphorylation of various isoforms of PKC leading to activation.

7 Taken together, the present results indicate that PKC is involved in the neuroprotective action of resveratrol against Aβ-induced toxicity.
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Keywords: Resveratrol; hippocampus; β-amyloid; signal transduction pathway; neuroprotection; red wine extracts

Abbreviations: AD, Alzheimer's disease; Aβ, amyloid β-peptide; DMSO, dimethyl sulfoxide; ERK, extracellular signal-regulated kinases; HBSS, Hanks' balanced salt solution; LDH, lactate dehydrogenase; LY294002, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one; MAPK, mitogen-activated protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PI3-K, phosphatidylinositol 3′-kinase; PCD, programmed cell death; PD98059, 2(2′-amino-3′-methoxyphenyl; PKC, protein kinase C; PMA, phorbol-12-myristate-13-acetate; SDS, sodium dodecylsulfate

Introduction

Resveratrol, a polyphenolic compound found in juice and wine from dark-skinned grape cultivars, has been shown to have a neuroprotective role in various models, *in vitro* and *in vivo* (Chanvitayapongs *et al*., 1997; Draczynska-Lusiak *et al*., 1998; Virgili & Contestabile, 2000; Bastianetto *et al*., 2000; Karlsson *et al*., 2000; Huang *et al*., 2001; Jang & Surh, 2001; 2003; Gelinas & Martinoli, 2002; Sharma & Gupta, 2002; Sinha *et al*., 2002; Wang *et al*., 2002; Nicolini *et al*., 2003; Russo *et al*., 2003). In particular, resveratrol has been shown to protect cultured neurons against amyloid β-peptide (Aβ) (Jang & Surh, 2003), a neurotoxic peptide that likely plays a critical role in the neuropathology of Alzheimer's disease (AD) (for a review, see Butterfield, 2002). However, the mechanism(s) involved in these neuroprotective effects remains to be fully established. Resveratrol is known to possess potent antioxidant activity (Miller & Rice-Evans, 1995) and it is generally assumed that its neuroprotective action is principally associated with this property (Bastianetto *et al*., 2000; Karlsson *et al*., 2000; Ishige *et al*., 2001; Sinha *et al*., 2002; Jang & Surh, 2003). In addition to its antioxidant effects, resveratrol may be acting by modulating intracellular signaling pathways (Miloso *et al*., 1999; Della Ragione *et al*., 2002; Nicolini *et al*., 2003; Jang & Surh, 2003).

One important intracellular signaling system is protein kinase C (PKC), a family of 12 serine/threonine kinases. Since PKC has been found to modulate cell viability resulting in the protection of various neuronal cells (Behrens *et al*., 1999; Dore *et al*., 1999; Xie *et al*., 2000; Maher 2001; Cordey *et al*., 2003), we investigated here if this pathway could be involved in the neuroprotective effects of resveratrol against Aβ-induced neurotoxicity.

Methods

Chemicals

Materials used for cell cultures were obtained from Gibco BRL (Burlington, Ontario, Canada). PKC sampler kit was

*Author for correspondence; E-mail: quirem@douglas.mcgill.ca

purchased from Transduction Laboratories (Lexington, KY, U.S.A.). Anti-phospho-PKC (pan) antibody, phospho-PKC antibody sampler kit, p42 MAP kinase (ERK2) antibody, p44 MAP kinase (ERK1) antibody and anti-phospho-Akt (Ser473) antibody were obtained from Cell Signaling Technology (Beverly, MA, U.S.A.). Donkey anti-rabbit horseradish peroxidase-conjugated secondary antibody and anti-mouse horseradish peroxidase-conjugated secondary antibody were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.). Hybond-C nitrocellulose membrane and enhanced chemiluminescence reagents were from Amersham Biosciences (Baie d'Urfé, Québec, Canada). Resveratrol, phorbol-12-myristate-13-acetate (PMA), poly-D-lysine and β-actin antibody (clone AC15) were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). Aβ_{25-35} was kindly provided by P. Gaudreau (CHUM, University of Montreal, Montreal, Canada). Aβ_{1-40} and Aβ_{1-42} were obtained from American Peptide Co. (Sunnyvale, CA, U.S.A.). 4α-Phorbol-12,13-didecanoate (4α-phorbol) and GF 109203X were purchased from Calbiochem (La Jolla, CA, U.S.A.).

Primary hippocampal cell cultures

Pregnant Sprague–Dawley rats were obtained from Charles River Laboratories (St-Constant, Quebec, Canada). Animal care was according to protocols and guidelines of the McGill University Animal Care Committee in accordance with regulations of the Canadian Council for Animal Care. Hippocampal neuronal cell cultures were prepared from hippocampi of fetuses at embryonic day 19–20 as described by Bastianetto *et al*. (1999). Briefly, hippocampi were dissected in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (HBSS) supplemented with 15 mM HEPES, 10 U ml^{-1} penicillin and 10 μg ml^{-1} streptomycin. Tissues were collected and washed in HBSS four to five times and 0.25% (v v^{-1}) trypsin was added for digestion at 37°C for 10 min. The digestion was stopped by the addition of fetal bovine serum at a final concentration of 10% (v v^{-1}). After rinsing four to five times with HBSS, a cell suspension was obtained by repeated aspiration with a Pasteur pipette. Cells were collected by centrifugation at 800 $\times$ *g* for 10 min to remove the HBSS, and were resuspended in neurobasal medium supplemented with B27 (2%, v v^{-1}), L-glutamate (25 μM), 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin.

Cells were plated onto poly-D-lysine (10 μg ml^{-1}) precoated 96-well plates (about 1×10^5 well^{-1}) for cytotoxicity analysis and six-well plates at a density of 1×10^6 cells well^{-1} for Western blot analysis. Cells were cultured at 37°C in 95% humidified atmosphere with 5% CO_2 until use. The initial medium was removed at day 3 and replaced with fresh medium. Cell cultures were routinely observed under phase-contrast inverted microscope.

Cell treatments

Cells grown in 96-well plates at day 6 were washed, and the medium was replaced with neurobasal medium containing no B27 for 2 h before treatment. Cultured cells were pre- (2 h), co- or post-treated (2 h) with resveratrol at various concentrations (0–40 μM), followed by exposure to various Aβ peptides (Aβ_{25-35}, Aβ_{1-40}, Aβ_{1-42}) for 24 h. Prior to use, Aβ_{25-35}, Aβ_{1-40}, Aβ_{1-42} were dissolved in sterilized distilled water and were aggregated at 37°C for 1, 72 and 72 h, respectively. To determine whether a signaling pathway is involved in the neuroprotective effect of resveratrol, a few signaling pathway-specific inhibitors were used, including GF 109203X, a general PKC inhibitor, PD98059, a selective mitogen-activated protein kinase (MEK) inhibitor, and LY294002, a selective phosphatidylinositol 3′-kinase (PI3-K) inhibitor. In addition, PMA, an activator of PKC (Nishizuka, 1992), and 4α-phorbol, as a negative control compared to PMA, were also used. These pathway-specific inhibitors, PMA and 4α-phorbol, were first dissolved in dimethylsulphoxide (DMSO) and then diluted in neurobasal medium as a 20 $\times$ working solution. Each pathway-specific inhibitors (5 μl) was added to cell culture 30 min before treatment with resveratrol at 20 μM and coadministrated with Aβ_{25-35} for a further 24 h. PMA at various concentrations and 4α-phorbol (100 ng ml^{-1}) were added 1 h before administration of Aβ_{25-35}. Control conditions were treated with the appropriate amount of vehicle, DMSO, at a final concentration of 0.1%, which had no effect on cell viability (data not shown). After these treatments, cell viability was assessed as described below.

For Western blot analysis, cells were grown to confluence in six-well plates. At day 6, the media was removed, and cells were washed twice in neurobasal medium containing no B27 and were then placed in the same medium 2 h before treatment. Resveratrol was dissolved in DMSO as a 1000 $\times$ stock solution and then diluted 10 times with neurobasal media as 100 $\times$ working solutions. Cell cultures in 1 ml medium in six-well plates in triplicates were added with 10 μl of the final solution containing resveratrol to give concentrations ranging from 5 to 40 μM. Synthetic Aβ_{25-35} was dissolved in distilled water at 2 mM and incubated for 1 h at 37°C before use. Aβ_{1-40} and Aβ_{1-42} were dissolved in neurobasal medium and incubated for 4 days at 37°C before use. For time course experiments, cells were grown in the presence of resveratrol at a concentration of 20 μM for 5 to 60 min.

Cell viability assays

After these various treatments, cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as described by Bastianetto *et al*. (2000). Briefly, MTT was dissolved in HBSS at 5 mg ml^{-1}, and 10 μl of the solution was added to each well of 96-well plates. After incubation for 3 h at 37°C, the medium was removed and 100 μl of 0.1 N HCl in isopropanol was added to solubilize the reaction product formazan by shaking for 5 min. Absorbance at 570 nm was measured with a microplate reader (Bio-Tek Instruments Inc., Ville St-Laurent, Québec, Canada). Cell viability of vehicle-treated control groups not exposed to either Aβ_{31-35} or resveratrol was defined as 100%. In addition, cell viability was evaluated by measuring the amount of cytoplasmic lactate dehydrogenase (LDH) released into the medium. Culture supernatants (50 μl) were collected from each well, and LDH activities were measured at 490 nm using a CytoTox96 nonradioactive assay kit (Promega, Madison, U.S.A.), according to the manufacturer's instructions. LDH activity in each experiment was calculated as percentage of control LDH in the culture medium exposed to Aβ-peptides in the absence of resveratrol, which was defined as 100%. Assays were

repeated in three independent experiments, each performed in triplicates. The qualitative assessment of neuronal injury was examined by phase-contrast microscopy.

Western blot

Following treatments, media were removed and cells were rinsed twice with ice-cold HBSS. Cells were then put into 200 μl of lysis buffer that contained 50 mM Tris/HCl (pH 7.5), 2 mM phenylmethylsulfonyl fluoride, 100 μM sodium orthovanadate, 10 μg ml^{-1} aprotinin and 10 μg ml^{-1} leupeptin. In order to maximally inhibit protein tyrosine phosphatases, the lysis buffer was prepared with an activated sodium orthovanadate. The stock solution of sodium orthovanadate (200 mM) was prepared and its pH was adjusted to 10.0. At pH 10.0, the solution turned yellow and was boiled until it became colorless. The solution was cooled to room temperature and its pH readjusted to 10.0. The solution was boiled again until it remained colorless and the pH stabilized at 10.0. After cooling to room temperature, the solution was stored as aliquots at -20°C. The activated sodium orthovanadate, aprotinin and leupeptin were added to the lysis buffer just prior to use. Cells were lysed on ice for 30 min with vortexing for 1 min at 10 min intervals. Cell lysates were centrifuged at 13,000 $\times$ *g* for 15 min at 4°C to remove debris. The protein concentration of the supernatant was measured using bicinchoninic acid (Pierce, Rockford, IL, U.S.A.), with bovine serum albumin used as standards. After adjusting the concentrations, equal amounts of samples were added to appropriate amount of 6 $\times$ sodium dodecylsulfate (SDS) sample buffer and shaken for 15 min. Samples were boiled for 5 min, and 10 μl of the samples was loaded onto NuPAGE 4–12% bis-tris electrophoresis gel (Invitrogen, Burlington, Ontario, Canada). Electrophoresis was run on a constant voltage at 160 V for $\sim$1.2 h and proteins were then transferred to nitrocellulose membranes. Blots were analyzed as described in Zheng *et al.* (2002) with the appropriate antibodies: anti-phospho-PKC (pan) antibody (to detect PKC-α, -$\beta_{\rm I}$, -$\beta_{\rm II}$, -ζ, -ε, -η and -δ, phosphorylated at a carboxy-terminal residue homologous to Ser-660 of PKC-$\beta_{\rm II}$), PKC sampler kit (to detect PKC -α, -β, -γ, -δ, -ε, -η, -θ, -ι, -λ) and phospho-PKC antibody sampler kit (to detect phospho-PKC-$\alpha/\beta_{\rm II}$, -μ, -δ, -ξ/λ, -θ). Appropriate peroxidase-conjugated secondary antibodies (1 : 10,000) were used to detect the proteins of interest by enhanced chemiluminescnce. An aliquot of samples were loaded and probed with anti-β-actin antibody for detection of β-actin as a loading control. Films were scanned at 600 d.p.i. in transmittance mode using a UMAX scanner and the levels of PKC phosphorylation and PKC isoenzymes were assessed by densities of bands, which were quantified using a MCID program (Imaging Research Inc., St Catharines, Ontario, Canada) or the NIH Image program available at their web site (http://rsb.info.nih.gov/nih-image). The values of the band intensities were normalized with that of β-actin as internal standards. The levels of PKC phosphorylation, PKC isozymes of treated cultures were compared with those of untreated control cultures.

Statistical analyses

Data are expressed as mean $\pm$ standard error of the mean (s.e.m) from three or more independent experiments, each performed in triplicate. Statistical significance was established by ANOVA followed by Student *t*-test using SPSS (v.10.1) (SPSS Inc., Chicago, IL, U.S.A.) or Microsoft Excel software. Statistical significance was established at $P<0.05$.

Results

Neuroprotective/neurorescuing effect of resveratrol against Aβ*-induced cytotoxicity in rat hippocampal cell cultures*

To establish that Aβ causes neuronal cell death, we performed cytotoxicity experiments using three different Aβ fragments namely Aβ_{1-42}, Aβ_{1-40} and Aβ_{25-35}, a synthetic toxic fragment of the amyloid protein. In initial experiments, rat hippocampal cells grown in serum-free neurobasal media containing B27 supplements at day 6 were treated with different concentrations of Aβ_{1-42} or Aβ_{1-40} for 24 h and then the cell viability was measured by colorimetric MTT assay. Aggregated Aβ_{1-42} and Aβ_{1-40} caused up to 40–60% cell death at concentrations ranging from 5 to 20 μM. Both soluble and aggregated Aβ_{25-35} (20 μM) induced similar toxic effects that were comparable to Aβ_{1-42} at 5 μM. Since it has been shown that Aβ_{25-35} and full-length Aβ_{1-42} can induce neuronal apoptosis by similar mechanisms (for a review, see Mattson, 1997), Aβ_{25-35} was used in all subsequent experiments.

Based on recent literature (Miloso *et al.*, 1999; Bastianetto *et al.*, 2000; Nicolini *et al.*, 2001), resveratrol is highly effective in maintaining cell viability at concentrations ranging between 1 and 50 μM. To examine the neuroprotective effects of resveratrol against Aβ-induced toxicity, rat cultured hippocampal cells were pre- (2 h), co- or post-treated (2 h) with resveratrol in the presence or absence of Aβ_{25-35} (20 μM) for 24 h. As shown in Figure 1a, viability of hippocampal neuronal cells exposed to Aβ_{25-35} (20 μM) for 24 h was reduced to 63.5 $\pm$ 3.9% compared to that of vehicle-treated control groups. A pretreatment of hippocampal neuronal cells with resveratrol (15–40 μM) significantly reduced Aβ_{25-35}-induced cell death in a dose-dependent manner, with a maximal effect (93%) obtained at 25 μM. The neuroprotective effect of resveratrol was also confirmed using the LDH assay (Figure 1b). Co- and post-treatment with resveratrol was neuroprotective but with a somewhat lower potency than seen following a pretreatment of the cells (Figure 1c and d). Similarly, a cotreatment with resveratrol showed similar neuroprotective effects against Aβ_{1-40} (Figure 2a) and Aβ_{1-42} (Figure 2b) toxicity in cultured hippocampal cells. Interestingly, resveratrol, in a dose-dependent manner, was also able to rescue hippocampal cells pre-exposed (0–12 h) to Aβ_{25-35} (Figure 2c). Resveratrol at the concentrations of up to 30 μM was tested alone for possible intrinsic cytotoxicity activity and showed no significant differences in cell survival compared to untreated control cells.

Changes in cell morphology were assessed by microscopic examination (Figure 3). Cultured hippocampal cells were treated without or with resveratrol in the absence or presence of Aβ_{25-35} at 20 μM for 24 h. Cells treated with vehicle (control) (Figure 3a) exhibited large vacuole-free cell bodies with elaborate networks of neurites. The synapse connections between neurons could be clearly seen. Exposure of cells to Aβ_{25-35} at 20 μM for 24 h resulted in some cell death and the

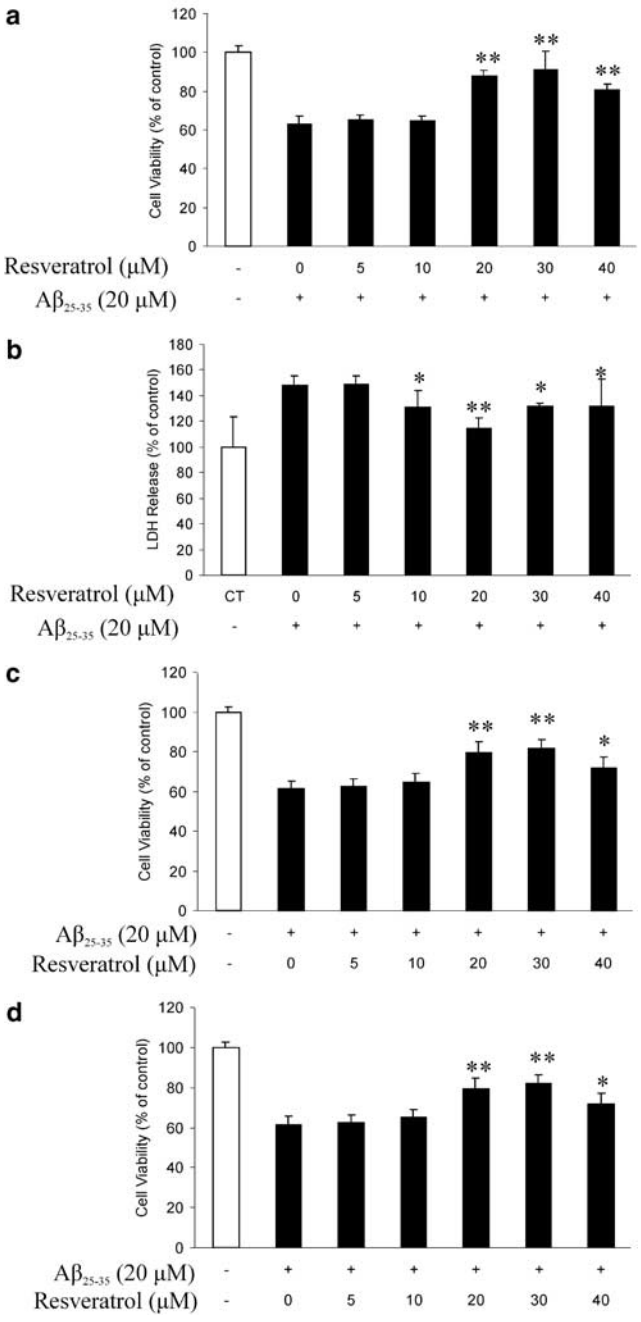

Figure 1 Protective effects of resveratrol against Aβ_{25-35}-induced cell death in hippocampal neurons. Cells were pretreated with resveratrol at various concentrations, 2 h before the addition of Aβ_{25-35} (20 μM) for 24 h. Cell viability was assayed with MTT (a) and LDH (b). Cells were also cotreated (c) or post (2 h)-treated (d) with resveratrol. Percentage of cell viability was relative to vehicle-treated controls (white bars). Values represent mean$\pm$s.e.m of three independent experiments, each performed in triplicates. $*P<0.05$, $**P<0.01$ by Student's *t*-test, compared to group that was treated with Aβ alone.

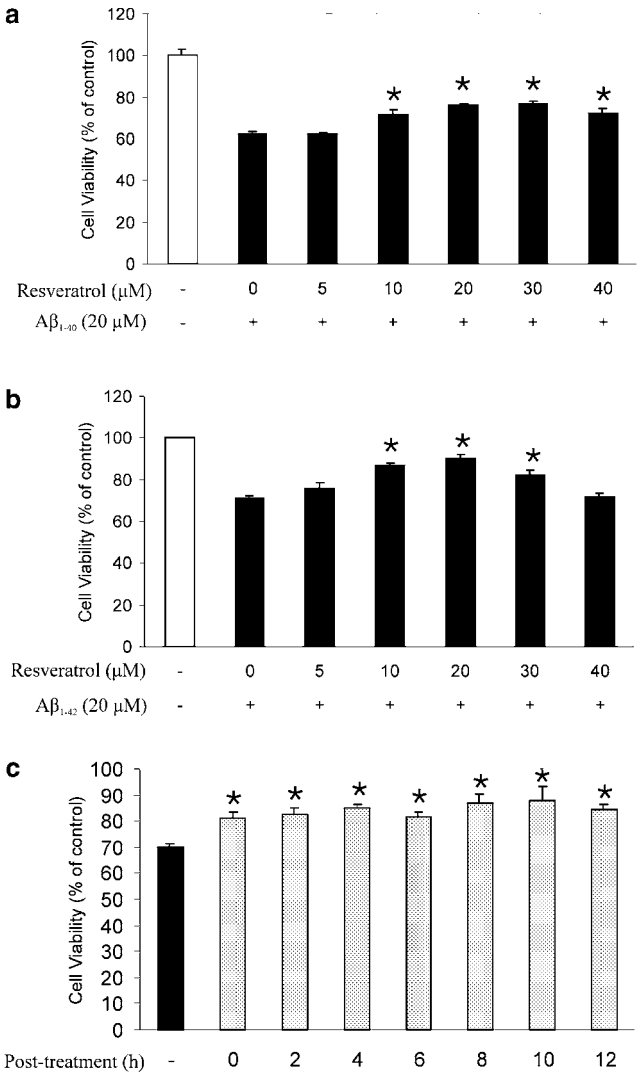

Figure 2 Protective effects of resveratrol against Aβ_{1-40}- and Aβ_{1-42}-induced toxicity and its rescuing effect in hippocampal neurons. Cells were cotreated with resveratrol at various concentrations in the presence of Aβ_{1-40} (20 μM) (a) or Aβ_{1-42} (20 μM) (b) for 24 h. (c) Cells were also post-treated with resveratrol at the indicated time after addition of Aβ_{25-35} (20 μM). Cell viability was assayed with MTT. Percentage of cell viability was relative to vehicle-treated controls (white bars). Values represent mean$\pm$s.e.m of three independent experiments, each performed in triplicates. $*P<0.05$, $**P<0.01$ by Student's *t*-test, compared to group that was treated with Aβ alone.

less neurites (Figure 3b). These morphological changes were counteracted by resveratrol at 20 μM (Figure 3c, d).

PKC activation is neuroprotective

To determine whether the activation of PKC pathways protects neurons against Aβ, we treated cell cultures with increasing concentrations of PMA 1 h before administration of Aβ_{25-35} and cells were then exposed to Aβ_{25-35} (20 μM) for 24 h. As shown in Figure 4, treatment with PMA resulted in a dose-dependent reduction in Aβ toxicity. However, 4α-phorbol, an inactive moiety, had no effect on Aβ toxicity.

PKC inhibitor blocks the neuroprotective effects of resveratrol

To determine whether PKC could be involved in the neuroprotective effects of resveratrol, a broad-spectrum PKC inhibitor, GF 109203X, was used. Treatment of GF 109203X (1 μM) 30 min prior to the addition of resveratrol significantly

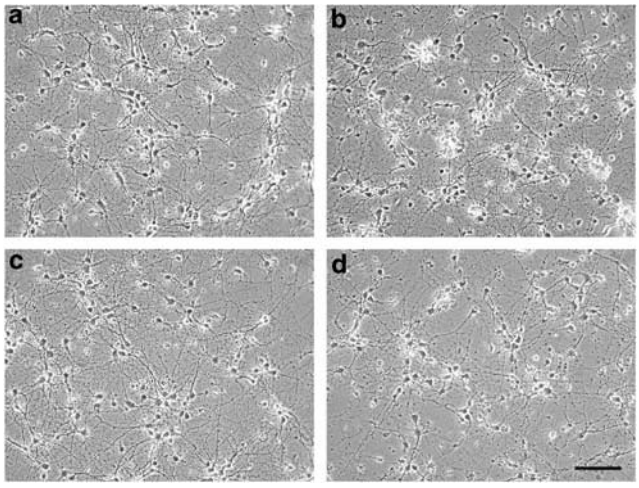

Figure 3 Microscopic analysis of resveratrol against Aβ_{25-35}-induced cell death. Representative phase-contrast photomicrographs of primary cultured rat hippocampal neurons exposure for 24 h to (a) vehicle (0.1% DMSO); (b) Aβ_{25-35} (20 μM); (c) resveratrol (20 μM); and (d) cells pretreated with 20 μM resveratrol for 30 min prior to exposure to Aβ_{25-35} (20 μM). Scale bar = 500 μm.

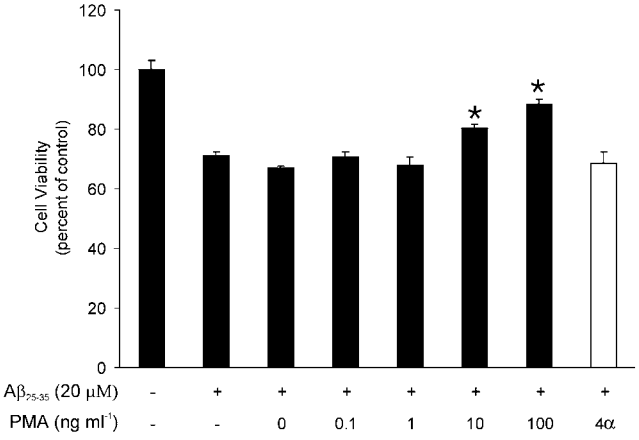

Figure 4 PMA reduces Aβ_{25-35}-induced toxicity in dose-dependent manner. Cultures were treated with PMA (solid bars) at the indicated concentrations and 100 ng ml^{-1} 4α-phorbol (open bar) 1 h before the addition of Aβ_{25-35}. Cell viability was assessed 24 h later using the MTT assay. Data represent the mean ($\pm$s.e.m.) from a representative experiment ($n = 3$). *$P < 0.05$ relative to Aβ_{25-35} alone.

blocked the neuroprotective effects of resveratrol against Aβ_{25-35}-induced cytotoxicity (Figure 4a). By itself, GF 109203X (1 μM) did not affect cell viability (Figure 5a). Inhibitors of other intracellular signaling pathways such as PD98059 (25 μM, MAP kinase) (Figure 5b) and LY294002 (5 μM; PI3 kinase) (Figure 5c) failed to modulate the neuroprotective effects of resveratrol against Aβ_{25-35}-induced toxicity.

Resveratrol activates PKC in primary hippocampal neurons

Since it has been previously shown that the activation by phosphorylation of PKC is closely associated with cell survival (Levites *et al*., 2002), we investigated next if resveratrol was

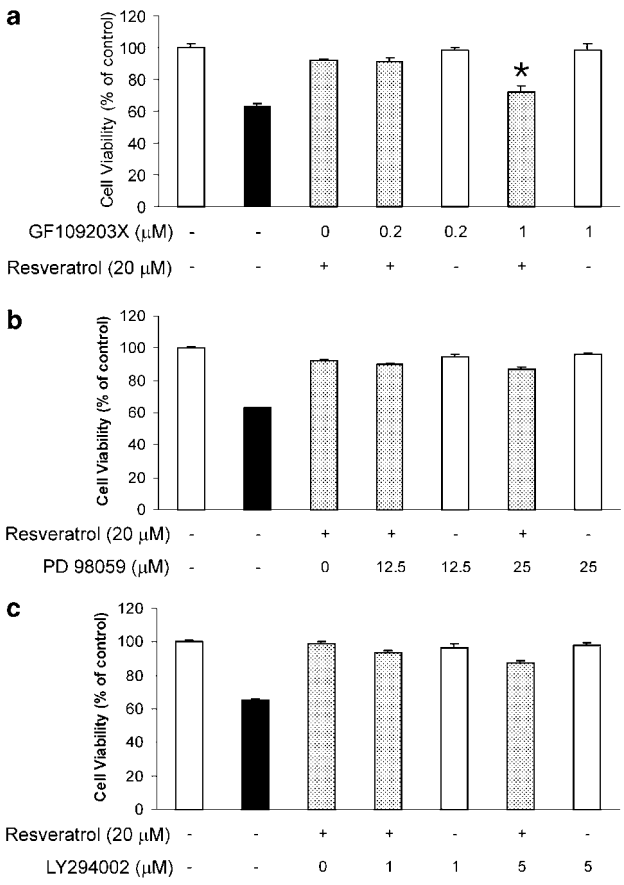

Figure 5 Blockade by a PKC inhibitor of the protective effects of resveratrol against Aβ_{25-35}-induced cell death in hippocampal neurons. Hippocampal neurons were pretreated with GF 109203X (a), PD98059 (b) and LY294002 (c) 30 min before adding resveratrol (20 μM) in the presence (shadow bars) or absence (white bars) of Aβ_{25-35} (20 μM). Treatment with Aβ_{25-35} (20 μM) alone (black bar). After 24 h, the viability of cells was measured using the MTT assay. Results are expressed as mean (%) $\pm$ s.e.m. *$P < 0.01$ by Student's *t*-test, compared to group that was treated with resveratrol (20 μM) in the presence of Aβ_{25-35} (20 μM).

able to promote phosphorylation, as assessed using an anti-phospho-PKC (pan) antibody. First, we determined the time course of PKC-induced phosphorylation by resveratrol. Phosphorylation of PKC showed a maximal increase 30 min after treatment with resveratrol and maintained till 60 min, and then returned to basal level at 120 min (Figure 6a). The phosphorylation of PKC was also induced by resveratrol in a dose-dependent manner, with maximal effects seen at 20–30 μM (Figure 6b), correlating to the optimal concentrations.

Interestingly, the phosphorylation of PKC was decreased 60 min after addition of Aβ_{25-35} (20 μM) (Figure 6c). Moreover, the coadministration of resveratrol at its effective concentration of 20 μM and Aβ_{25-35} (20 μM) abolished the inhibitory effect of Aβ_{25-35} on the phosphorylation of PKC (Figure 6d).

The possible preferential role of a given PKC isozyme in the neuroprotective effect of resveratrol was investigated next using a PKC sampler kit containing PKC isozyme-specific antibodies. The PKC family is composed of at least 12 isozymes, which are classically divided into conventional (α, β_I, β_{II}, γ), novel (δ, ε, η/Δ, θ, μ) and atypical (ζ, λ/ι) ones on the

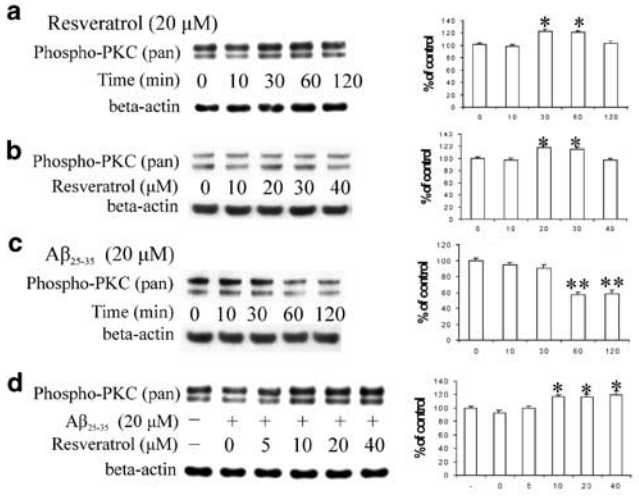

Figure 6 Effects of resveratrol and Aβ_{25-35} on the phosphorylation of PKC in hippocampal cells. The phosphorylation of PKC was detected in cell lysates by Western blot using an anti-phospho-PKC (pan) antibody. (a) Hippocampal cells treated with resveratrol (20 μM) for the indicated times. (b) Hippocampal cells treated with resveratrol at various concentrations for 30 min. (c) Hippocampal cells treated with Aβ_{25-35} (20 μM) for the indicated times. (d) Hippocampal cells treated without or with Aβ_{25-35} (20 μM) in combinations with resveratrol at various concentrations for 30 min. Western blots (left) were probed with anti-phospho-PKC (pan), and bands from phospho-PKC immunoblots were quantified using an MCID program, normalized to β-actin, and represented graphically (right). Results are the mean ± s.e. of three independent experiments. Student *t*-test: $^*P<0.05$, $^{**}P<0.01$ *versus* control.

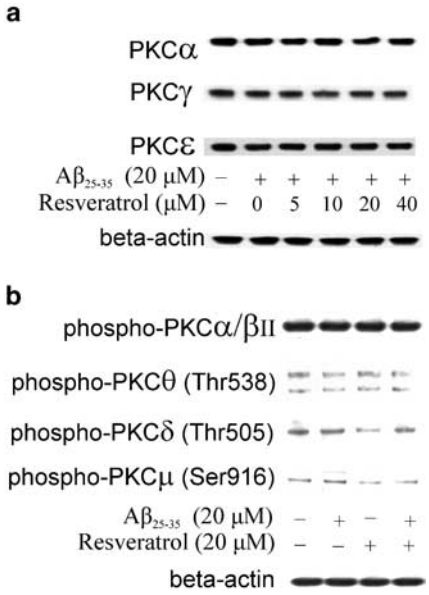

Figure 7 Effects of resveratrol and Aβ_{25-35} on the PKC isoforms and the phosphorylation of PKC isoforms in hippocampal cells. PKC isoforms and the phosphorylation of PKC isoforms were detected in cell lysates by Western blot using a PKC sampler kit and an anti-phospho-PKC antibody sampler kit, respectively. (a) Western blots of PKC isoforms in hippocampal cells treated with Aβ_{25-35} (20 μM) and/or resveratrol at various concentrations for 30 min. (b) Western blots of phospho-PKC isoforms in hippocampal cells treated with Aβ_{25-35} (20 μM) and/or resveratrol (20 μM) for 30 min. Control was treated only with vehicle DMSO at a final concentration of 0.1%. Results are the representative of three independent experiments.

basis of their structure and cofactor requirements (Newton, 1997). It was shown that HT-22 cells, a subclone of a mouse hippocampal cell line, express multiple PKC isozymes, including the cPKC, PKCα, the *n*PKC, PKCδ and PKCε, and the aPKC, PKCζ and PKCλ, but not PKCβ_I, PKCβ_{II}, PKCγ or PKCθ (Maher, 2001). Our results revealed that cultured hippocampal cells expressed all PKCα, PKCβ, PKCγ, PKCδ, PKCε, PKCλ and PKCι, but not PKCθ. Resveratrol (5–40 μM) failed to significantly modulate the expression of PKCα, PKCγ and PKCε (Figure 7a), suggesting the unlikely involvement of these PKC isozymes in the neuroprotective action of resveratrol against Aβ-induced toxicity. Moreover, using a phospho-PKC antibody sampler kit, we also detected a few phospho-PKC isozymes, including phospho-PKC-α/β_{II}, -μ, -δ and -θ in control cultured neurons or following exposure to resveratrol at 20 μM in the presence or absence of Aβ_{25-35} (20 μM). As shown in Figure 7b, resveratrol did not affect the phosphorylation of PKC-α/β_{II}, PKC-μ (Ser916) and PKC-θ (Thr538), but slightly decreased the phosphorylation of PKC-δ, suggesting the likely involvement of PKC-δ (Thr505) in the neuroprotective effects of resveratrol. In addition, resveratrol (up to 40 μM) was unable to modulate the phosphorylation of either Akt kinase (Figure 8a) or ERK1 and ERK2 kinases (Figure 8b), suggesting specificity for its effects on PKC.

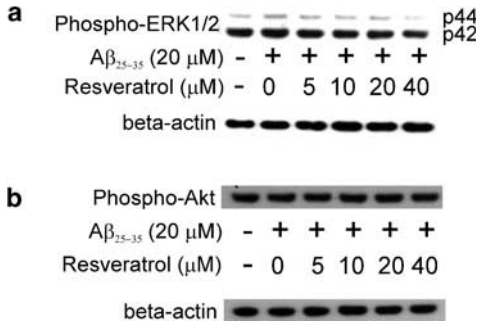

Figure 8 Effects of resveratrol and Aβ_{25-35} on the phosphorylation of Akt and ERK1/2 in hippocampal cells. (a) Western blots of phospho-ERK1/2 in hippocampal cells treated with Aβ_{25-35} (20 μM) and/or resveratrol at various concentrations for 30 min. (b) Western blots of phospho-Akt in hippocampal cells treated with Aβ_{25-35} (20 μM) and/or resveratrol at various concentrations for 30 min. Control was treated only with vehicle DMSO at a final concentration of 0.1%. Results are the representative of three independent experiments.

Discussion

In this study, resveratrol, an active component from grapes, was shown to concentration-dependently protect against Aβ-induced toxicity in cultured hippocampal neurons. Resveratrol was active against various amyloid-related peptides including Aβ_{1-42}, the most neurotoxic amyloid derivative present in the AD brain. Interestingly, resveratrol was able to block Aβ-induced toxicity not only following a pre- or co-treatment with the toxic peptide, but even to rescue neurons post-Aβ exposure. The mechanism(s) involved in the neuroprotective–

neurorescuing effects of resveratrol likely include PKC as revealed by (a) the inhibitory action of GF 109203X, a potent, broadly acting PKC antagonist, on the protective effect of resveratrol against Aβ-induced toxicity; (b) the activation of PKC by PMA which is neuroprotective and (c) the dose-dependent stimulation of PKC phosphorylation by resveratrol, and its reversal of Aβ-induced decrease in PKC phosphorylation. Moreover, resveratrol failed to modulate the phophorylation of Akt, ERK1 and ERK2 kinases demonstrating some specificity for its action on the PKC pathway.

Resveratrol was found to be neuroprotective against all three major neurotoxic amyloid peptides, namely Aβ_{25-35}, Aβ_{1-40}, and most importantly Aβ_{1-42}. Interestingly, the neuroprotective effect of resveratrol was not only observed upon its pre- or co-treatment with the Aβs, but even if added up to 12 h postexposure to the toxic Aβ peptides. These results confirm and extend previous studies that mostly focused on the neuroprotective–neurorescuing properties of resveratrol against the neurotoxic effects of the 25–35 derivative (Jang & Surh, 2002). The neuroprotective–neurorescuing action of resveratrol especially against Aβ_{1-42}-induced neurotoxicity is of particular interest as recent reports have suggested that changes in the Aβ_{1-40}/Aβ_{1-42} ratio is critical for the onset and progression of the symptoms of AD (Hardy & Selkoe, 2002).

Several mechanisms may underlie resveratrol-induced neuroprotection against Aβ neurotoxicity (for a recent review, see Bastianetto & Quirion, 2001). For example, resveratrol has been shown to possess significant free-radical scavenging properties in a variety of cellular types (Chanvitayapongs *et al*., 1997; Subbaramaiah *et al*., 1998; Bastianetto *et al*., 2000; Karlsson *et al*., 2000; Sinha *et al*., 2002) and we have recently reported that it can act as an antioxidant against nitric oxide-induced toxicity (Bastianetto *et al*., 2000). Zhuang *et al*. (2003) also suggested that resveratrol was a potent activator of the heme oxydase system. Besides its antioxidant properties, various intracellular signaling mechanisms have been suggested to be involved, at least partly, in the neuroprotective effects of resveratrol. For example, Miloso *et al*. (1999) have shown that resveratrol can induce the activation of the MAP kinases, ERK1 and ERK2 in SH-SY5Y neuroblastoma cells. However, we failed to observe a significant effect of resveratrol on the phosphorylation of MAP kinases as well as on that of Akt kinases in our model. In contrast, resveratrol potently stimulated the phosphorylation (leading to activation) of PKC, and reversed the inhibitory effect of Aβ peptides on PKC phosphorylation. Moreover, PMA, a potent and chronic activator of PKC, showed a dose-dependent reduction in Aβ-induced toxicity. In addition, GF 109203X, a well-known inhibitor of PKC, blocked the neuroprotective effect of resveratrol against Aβ-induced toxicity as well as its action on the phosphorylation of PKC. Taken together, our results suggest that the PKC pathway, but not MAP and Akt kinases, plays a major role in the neuroprotective–neurorescuing properties of resveratrol against Aβ-induced toxicity in hippocampal neurons. It is of interest to add that PKC has been shown to be implicated in cell survival and programmed cell death (Deacon *et al*., 1997; Maher, 2001; Levites *et al*., 2002).

In an attempt to determine the possible PKC isozymes involved in the neuroprotective action of resveratrol in our model, we examined its effects on the expression as well as phosphorylation of various isozymes. Several studies have suggested roles for PKCα, PKCε, PKCξ and PKCλ/ι in the suppression of programmed cell death (PCD) (Murray & Fields, 1997; Gubina *et al*., 1998; Whelan & Parker, 1998). However, our results showed that resveratrol failed to modulate the expression of PKCα, PKCγ and PKCε in cultured hippocampal neurons, suggesting that these isozymes are unlikely to be involved in the neuroprotective effects of resveratrol against Aβ-induced neurotoxicity. Other isozymes expressed in hippocampal neurons including PKCβI, βII, δ, η, ι, λ should hence be investigated when proper tools such as highly selective antibodies will be available to establish their possible role in resveratrol-induced neuroprotection. It has been shown that PKCδ is associated with the promotion of PCD (Konishi *et al*., 1999). Furthermore, overexpression of PKCδ can induce (Ghayur *et al*., 1996) or potentiate (Konishi *et al*., 1999) PCD. Consistent with these results, our result that resveratrol resulted in decrease in the phosphorylation of PKCδ (Figure 7b) suggests that PKCδ is likely involved in the resveratrol-mediated protection.

Besides a role for PKC in the neuroprotective effects of resveratrol, the possible involvement of novel signaling pathways and transcription factors should be considered especially as some very recent studies have shown, for example, that resveratrol can increase the expression of the transcription factor egr1 (Della Ragione *et al*., 2002) as well as protect against paclitaxel-induced apoptosis in SH-SY5Y cells (Nicolini *et al*., 2003). We are thus in the process of using global genomic and proteomic approaches (Marcotte *et al*., 2003) to potentially uncover new pathways and systems that could be involved in the neuroprotective effects of resveratrol against various toxic insults including the Aβ peptides.

The relevance of our findings to *in vivo* clinical situations remains to be demonstrated. However, some recent studies have revealed the potential neuroprotective beneficial action of moderate red wine intake against various neurological disorders including AD (Orgogozo *et al*., 1997; Leibovici *et al*., 1999) and stroke (Sinha *et al*., 2002) as well as *in vivo* animal models of these diseases (Russo *et al*., 2003; Sinha *et al*., 2002). Future studies aiming at precisely understanding the cellular mechanisms involved in the neuroprotective effects of resveratrol could thus open new avenues for the treatment of these disorders.

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