

## RESEARCH PAPER

Cell signaling pathways in the mechanisms of neuroprotection afforded by bergamot essential oil against NMDA-induced cell death *in vitro*MT Corasaniti<sup>1,2</sup>, J Maiuolo<sup>1</sup>, S Maida<sup>1</sup>, V Fratto<sup>1</sup>, M Navarra<sup>1</sup>, R Russo<sup>3</sup>, D Amantea<sup>3</sup>, LA Morrone<sup>3</sup> and G Bagetta<sup>3</sup>

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**Background and purpose:** The effects of bergamot essential oil (BEO; *Citrus bergamia*, Risso) on excitotoxic neuronal damage was investigated *in vitro*.

**Experimental approach:** The study was performed in human SH-SY5Y neuroblastoma cells exposed to *N*-methyl-D-aspartate (NMDA). Cell viability was measured by dye exclusion. Reactive oxygen species (ROS) and caspase-3 activity were measured fluorimetrically. Calpain I activity and the activation (phosphorylation) of Akt and glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ) were assayed by Western blotting.

**Key results:** NMDA induced concentration-dependent, receptor-mediated, death of SH-SY5Y cells, ranging from 11 to 25% (0.25–5 mM). Cell death induced by 1 mM NMDA (21%) was preceded by a significant accumulation of intracellular ROS and by a rapid activation of the calcium-activated protease calpain I. In addition, NMDA caused a rapid deactivation of Akt kinase and this preceded the detrimental activation of the downstream kinase, GSK-3 $\beta$ . BEO (0.0005–0.01%) concentration dependently reduced death of SH-SY5Y cells caused by 1 mM NMDA. In addition to preventing ROS accumulation and activation of calpain, BEO (0.01%) counteracted the deactivation of Akt and the consequent activation of GSK-3 $\beta$ , induced by NMDA. Results obtained by using specific fractions of BEO, suggested that monoterpene hydrocarbons were responsible for neuroprotection afforded by BEO against NMDA-induced cell death.

**Conclusions and Implications:** Our data demonstrate that BEO reduces neuronal damage caused *in vitro* by excitotoxic stimuli and that this neuroprotection was associated with prevention of injury-induced engagement of critical death pathways. *British Journal of Pharmacology* (2007) **151**, 518–529; doi:10.1038/sj.bjp.0707237; published online 2 April 2007

**Keywords:** bergamot essential oil; neuroprotection; NMDA receptors; excitotoxicity; cell death; SH-SY5Y neuroblastoma cells; Akt; GSK-3 $\beta$ ; calpain I

**Abbreviations:** BEO, bergamot essential oil; GSK-3 $\beta$ , glycogen synthase kinase-3 $\beta$ ; PI3K, phosphatidylinositol 3 kinase; ROS, reactive oxygen species

## Introduction

Excessive activation of *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptors, and hence abnormal Ca<sup>2+</sup> influx through the receptor-associated cation channel, largely contribute to glutamate-mediated neuronal death (Lynch and Guttman, 2002). The increase in intracellular calcium triggers robust activation of calcium-dependent

enzymes, including nitric oxide (NO) synthase (NOS), and mitochondrial calcium overload and dysfunction leading to generation of NO, reactive oxygen species (ROS) and peroxynitrite which initiate neuronal cell demise (Dawson *et al.*, 1991; Corasaniti *et al.*, 1992; Lafon-Cazal *et al.*, 1993; Dugan *et al.*, 1995; Stout *et al.*, 1998; Moncada and Bolanos, 2006). In addition to NOS, Ca<sup>2+</sup> entry through the NMDA receptor-gated cation channel activates the neutral cysteine protease calpain I (Croall and DeMartino, 1991) and activation of calpain I has been implicated in excitotoxic neuronal death both *in vitro* and *in vivo* (Siman *et al.*, 1989; Roberts-Lewis *et al.*, 1994; Corasaniti *et al.*, 1996; Lankiewicz *et al.*, 2000; Bano *et al.*, 2005).

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Survival signaling pathways engaged by activation of Akt kinase (also known as protein kinase B (PKB)), mediate endogenous prosurvival responses to neuronal stress induced by an excitotoxic, that is ischemic, insult (see Fukunaga and Kawano, 2003). The serine/threonine kinase Akt functions as a major downstream target of phosphatidylinositol 3 kinase (PI3K) and, after being phosphorylated, it promotes cell survival by phosphorylating and inhibiting several proteins including Bad, caspase-9 and glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ) (see Brazil and Hemmings, 2001). Several *in vivo* studies have indicated that activation of the PI3K/Akt pathway plays an important role in rescuing neurones of the ischemic penumbra from delayed cell death (see Fukunaga and Kawano, 2003). In animal models of experimental brain ischemia, enhanced Akt phosphorylation has been detected in the ischemic penumbra but not in the ischemic core (Noshita *et al.*, 2001). Double staining experiments demonstrate that phospho-Akt (p-Akt)-positive neurones do not show DNA fragmentation (Noshita *et al.*, 2001), suggesting that phosphorylation-mediated activation of Akt kinase contributes to neuroprotection. However, enhancement of p-Akt expression is transient and it is followed by a dramatic decrease of p-Akt within 24 h or more after ischemia, depending on the experimental model of brain ischemia (focal or global, transient or permanent) (Zhao *et al.*, 2005). Under these experimental conditions, pretreatment with neurotrophic factors able to activate the PI3K/Akt pathway, confers neuroprotection by maintaining the phosphorylation of Akt and thus its activity (see Fukunaga and Kawano, 2003; Maiese *et al.*, 2004; Amantea *et al.*, 2005). However, it should be stressed that a restriction to clinical application of neurotrophic factors is represented by their peptidic moiety which limits their penetration into the brain (Wu, 2005). As a consequence, the availability of compounds endowed with neuroprotective properties and able to cross the blood-brain barrier would be extremely valuable in the development of neuroprotective therapies.

Recently, interest in natural products regarded as potential source of neuroprotective agents has been renewed. In the frame of a research project aimed at investigating the neuropharmacological profile of the essential oil of bergamot (*Citrus bergamia*, Risso), a citrus growing almost exclusively in the south of Italy, in a restricted area of the Calabrian coast, we have assessed the neuroprotective properties of the oil.

According to the Farmacopea Ufficiale Italiana (1991), the bergamot essential oil (BEO), is obtained by cold pressing of the epicarp and, partly, of the mesocarp of the fresh fruit of bergamot. The citrus is cultivated for its essential oil, a product in great demand by perfumery and cosmetic industries but also employed by pharmaceutical, food and confectionery industries. BEO comprises a volatile fraction (93–96% of total) containing monoterpene and sesquiterpene hydrocarbons (such as limonene,  $\alpha$ - and  $\beta$ -pinene,  $\beta$ -myrcene,  $\gamma$ -terpinene, terpinolene, sabinene and  $\beta$ -bisabolene) and oxygenated derivatives (such as linalool, neral, geranial, linalyl acetate, neryl acetate and geranyl acetate) and a nonvolatile fraction (4–7% of total) containing waxes, polymethoxylated flavones, coumarins and psoralens such as bergapten (5-methoxypsoralen) and bergamottine

(5-geranyloxypsoralen) (Mondello *et al.*, 1993; Dugo *et al.*, 2000). The most abundant compounds found in the volatile fraction are the monoterpene hydrocarbons limonene,  $\gamma$ -terpinene, and  $\beta$ -pinene, the monoterpene alcohol, linalool, and the monoterpene ester, linalyl acetate, which altogether constitute more than 90% of the whole oil (Mondello *et al.*, 1995; Verzera *et al.*, 1996, 2003). Oxygenated compounds, namely linalool and linalyl acetate, mark the flavor notes of BEO, whereas the hydrocarbon fraction does not have a fundamental role in determining the olfactory character of BEO. The nonvolatile residue is a natural odor fixative which influences the olfactory properties of the oil; however, it contains about 0.2% bergapten which is responsible for the phototoxicity of BEO (Zaynoun *et al.*, 1977; Ashwood-Smith *et al.*, 1980); therefore, a bergapten-free extract of the essence (BEO-BF) together with a natural essence deprived of the hydrocarbon fraction and of bergapten (BEO-HF/BF) are prepared by extractive industries for perfumery and cosmetic uses.

In the present study, we examined whether BEO could reduce excitotoxic neuronal damage caused by exposure of human SH-SY5Y neuroblastoma cells to NMDA. The finding that BEO protects from NMDA-induced cell death prompted us to investigate the intracellular pathways through which BEO affords neuroprotection together with the identification of the fraction of the oil responsible for neuroprotection. Collectively, our data demonstrate that BEO reduces excitotoxic damage *in vitro* and that neuroprotection is associated with prevention of ROS accumulation, inhibition of calpain I activation and prevention of injury-induced decrease of p-Akt and phospho-GSK-3 $\beta$  (p-GSK-3 $\beta$ ) levels.

## Methods

### *Cell culture, treatments and cytotoxicity study*

Adherent SH-SY5Y human neuroblastoma cells, obtained from ICLC-IST (Genoa, Italy), were cultured in RPMI 1640 (containing 0.407 mM MgSO<sub>4</sub>·7H<sub>2</sub>O) supplemented with heat-inactivated fetal bovine serum (10% v/v), 1 mM sodium pyruvate and 2 mM glutamine at 37°C in a 5% CO<sub>2</sub> atmosphere. The cells, cultured in 75 cm<sup>2</sup> flasks, were seeded in 35 mm six-well plates. Twenty-four hours after plating, the growth medium was replaced with fresh normal medium (control cultures) or with medium supplemented with NMDA (0.25–5 mM) for 24 h. Addition of 1 or 5 mM NMDA brought the pH of the culture medium from 7.60 to 7.55 and 7.08, respectively. For antagonism studies, MK801, CGP40116 and 7-nitroindazole were applied to SH-SY5Y cells 10 min before the addition of NMDA (1 mM) and they were present during the 24 h exposure time. In experiments involving BEO and its fractions, BEO-BF and BEO-BF/HF, these were added to cell cultures 60 min before NMDA application. Preliminary experiments were carried out in to characterize the effects of BEO on cell viability; SH-SY5Y cultures were exposed for 24 h to graded dilutions of BEO before evaluating cell viability. Cell viability was assessed by cell exclusion of trypan blue (0.4% w/v) and cell death was reported as the percentage of stained (non viable) vs total

cells counted (Corasaniti *et al.*, 2001). Data were expressed as mean  $\pm$  s.e.m. percentage cell death.

#### Fluorimetric caspase-3 activity assay

NMDA-treated SH-SY5Y cells were harvested at the indicated times in ice-cold phosphate-buffered saline (PBS) and lysed in ice-cold lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM MgCl<sub>2</sub>, 5 mM EDTA, 0.1% 3[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 5 mM dithiothreitol, 10  $\mu$ g ml<sup>-1</sup> pepstatin A, 10  $\mu$ g ml<sup>-1</sup> leupeptin and 10  $\mu$ g ml<sup>-1</sup> aprotinin). Cell suspensions were briefly sonicated, centrifuged at 12000g for 10 min at 4°C and protein concentration in supernatants was determined by the DC protein assay (Bio-Rad Laboratories, Milan, Italy). The fluorimetric assay for caspase-3 activity was performed as follows. Cell supernatants were diluted in assay buffer (100 mM HEPES, pH 7.4, 5 mM EDTA, 0.1% CHAPS, 5 mM dithiothreitol and 10% glycerol) to a final concentration of 0.6  $\mu$ g protein per  $\mu$ l and incubated in triplicate in a 96-well clear-bottom plate with the fluorogenic substrate acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC; 50  $\mu$ M; Bachem, Bubendorf, Switzerland). Production of fluorescent-free AMC, released by caspase-3 activity, was monitored over 60 min at 37°C using a microplate fluorometer (Victor<sup>2</sup> multilabel counter, Perkin-Elmer Life Sciences; excitation, 355 nm; emission, 460 nm). The specific contribution of caspase-3 activity in each cell extract was determined by preincubating parallel sample aliquots with the caspase-3 preferring inhibitor acetyl-Asp-Glu-Val-Asp-aldehyde (Ac-DEVD-CHO; 50  $\mu$ M; Bachem) for 10 min at 37°C before the addition of the caspase substrate; the difference between the substrate cleavage activity in the absence and presence of Ac-DEVD-CHO was regarded as specific caspase-3 activity. The increase in fluorescence was linear for 40 min after addition of the fluorogenic substrate. Data were analyzed by linear regression within the linear range of the enzymatic reaction and the results expressed as relative fluorescence units (RFU) per min per mg of protein.

#### Determination of ROS production

Accumulation of intracellular ROS, a term that includes both free radicals, such as peroxy, alkoxy and hydroxyl radicals, and nonradicals, such as peroxynitrite and hydrogen peroxide that are oxidizing agent and/or are easily converted into radicals (see Halliwell and Whiteman, 2004), was estimated by fluorescence assay as described previously (Russo *et al.*, 2005), using 2',7'-dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCF-DA; Molecular Probes, Eugene, OR, USA) as a probe (Mattson *et al.*, 1995). H<sub>2</sub>DCF-DA readily diffuses into the cells where intracellular esterases cleave the acetate group of H<sub>2</sub>DCF-DA from the molecule to yield H<sub>2</sub>DCF, which is trapped within the cells. Intracellular ROS (see Halliwell and Whiteman, 2004) oxidize H<sub>2</sub>DCF to form the highly fluorescent compound DCF. SH-SY5Y cells were subcultured from confluent 75 cm<sup>2</sup> flasks and seeded in 96-well plates. Twenty-four hours after plating, the growth medium was replaced with fresh medium containing H<sub>2</sub>DCF-DA (20  $\mu$ M final concentration) and 2.5 mM probene-

cid; after 30 min at 37°C, the cells were washed twice to remove the extracellular H<sub>2</sub>DCF-DA and incubated in a pre-warmed buffer (phosphate-buffered saline (PBS) supplemented with 1.2 mM CaCl<sub>2</sub> and 10 mM glucose, pH 7.4) in the absence or presence of NMDA. In experiments involving BEO, this was applied to SH-SY5Y cells before the loading with H<sub>2</sub>DCF-DA at the same preincubation time used for the cytotoxicity study. Fluorescence was monitored every 5 min for 60 min following the addition of NMDA by using a microplate fluorometer (Victor<sup>2</sup> multilabel counter, Perkin-Elmer Life Sciences; excitation, 495 nm; emission, 530 nm). Data were analyzed according to the following formula:  $(T_x - T_0)/T_0 \times 100$ , where  $T_x$  is the DCF fluorescence measured at the indicated time and  $T_0$  is the DCF fluorescence measured at the beginning of the analysis, and reported as the mean  $\pm$  s.e.m. of eight wells per experimental group.

#### Preparation of cell lysates

Cell monolayers in 100-mm plates were washed with ice-cold PBS and lysed with lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1% Triton, 1 nM okadaic acid, a cocktail of protease inhibitors (code P8340, Sigma, Milan, Italy) and a cocktail of phosphatase inhibitors (code 524625, Calbiochem, La Jolla, CA, USA). Following 5 min incubation on ice, the lysates were collected in microcentrifuge tubes, briefly sonicated and centrifuged at 20800g for 15 min at 4°C. Protein concentration in the supernatants of cell lysates was determined by the DC protein assay (Bio-Rad Laboratories, Milan, Italy).

#### Western-blot analysis

Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 8% for Akt and GSK-3 $\beta$  and 6% for  $\alpha$ -spectrin) and electrotransferred to nitrocellulose membranes (Optitran BA-S 83, Schleicher & Schuell Bioscience, Dassel, Germany). Primary antibodies were incubated overnight at 4°C followed by a horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Immunoreactivity was visualized by enhanced chemiluminescent detection (Amersham Biosciences, GE Healthcare, Milan, Italy) and exposure to X-ray films (Hyperfilm ECL, Amersham Biosciences). Autoradiographic films were scanned and densitometric analysis was carried out using Quantiscan software (Biosoft, Cambridge, UK). The following primary antibodies were used: a rabbit polyclonal antibody for Akt at 1:2000 dilution (Cell Signaling Technology, Beverly, MA, USA), a rabbit polyclonal antibody for p-Akt (Ser473) at 1:2000 dilution (Cell Signaling Technology), a rabbit polyclonal antibody against GSK-3 $\beta$  at 1:2000 dilution (Cell Signaling Technology), a rabbit polyclonal antibody for p-GSK-3 $\beta$  (Ser9) at 1:1000 dilution (Cell Signaling Technology), a mouse anti-spectrin (nonerythroid) monoclonal antibody at 1:4000 dilution (MAB1622; Chemicon International Inc., Temecula, CA, USA), a mouse monoclonal anti-actin antibody at 1:2000 dilution (clone AC-40; Sigma), a mouse monoclonal anti- $\alpha$ -tubulin antibody at 1:40000 dilution (clone B-5-1-2; Sigma). Horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG

(Pierce Biotechnology, Rockford, IL, USA) were used as secondary antibodies.

#### Statistical analysis

Data are expressed as the mean  $\pm$  s.e.m. of the indicated number of independent experiments and evaluated statistically for difference by one-way analysis of variance (ANOVA) followed by Tukey–Kramer or by Dunnett tests for multiple comparisons. A value of  $P < 0.05$  was considered to be significant.

#### Drugs

The essential oil of bergamot (BEO) and its fractions were kindly provided by the company 'Simone Gatto' (San Pier Niceto, Messina, Italy) together with the certificate of analysis carried out by the 'Stazione Sperimentale per le Industrie delle Essenze e dei Derivati dagli Agrumi' (SSEA, Reggio Calabria, Italy). The fractions employed were bergapten-free extract of the essence (BEO-BF) and the natural essence deprived of hydrocarbon fraction and of bergapten (BEO-BF/HF). According to the percentages reported in the literature (Mondello *et al.*, 1995; Verzera *et al.*, 1996, 2003), among other substances present in lower percentages, BEO contained 37.98% D-limonene, 30.02% linalyl acetate, 9.83% linalool, 7.17%  $\gamma$ -terpinene and 6.15%  $\beta$ -pinene. In BEO-BF/HF, the percentages of the above-mentioned compounds were 0.38% limonene, 70.26% linalyl acetate, 18.95% linalool, 0.62%  $\gamma$ -terpinene and 0.03%  $\beta$ -pinene. BEO and its fractions were diluted 1:10 in a 1:9 water/ethanol solution and then further diluted in culture medium to obtain final concentrations of 0.0005, 0.005 and 0.01%; identical volumes of ethanol were added to culture medium to investigate potential effects on NMDA-induced cell death.

MK801 (dizocilpine) and 7-nitroindazole were from Sigma; D-(E)-2-aminomethyl-5-phosphono-3-pentenoic acid (CGP40116) was kindly provided by Ciba-Geigy (Basel, Switzerland). 2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002) and 6-bromoindirubin-3'-oxime (GSK-3 Inhibitor IX) were from Calbiochem and they were supplied as a 10 mM solution in dimethyl sulfoxide (DMSO) and further diluted in culture medium; the maximal concentrations of DMSO in culture medium were 0.2% for LY294002 and 0.01% for GSK-3 Inhibitor IX, which did not affect the percentage cell death caused by NMDA.

## Results

#### Characterization of NMDA-induced cytotoxicity in SH-SY5Y cultures

Human SH-SY5Y neuroblastoma cells express NMDA subtype of glutamate receptors (Sun and Murali, 1998) and exposure to the excitotoxin induces concentration-dependent cell death (Figure 1a). Death caused by NMDA in SH-SY5Y cultures occurs via stimulation of NMDA subtypes of glutamate receptors because CGP40116, a selective competitive NMDA receptor antagonist (Sauer *et al.*, 1993), and the use-dependent channel blocker, MK801, prevented in a

concentration-dependent manner cell death elicited by 1 mM NMDA (Figure 1b and c).

Preincubation with 7-nitroindazole (7-NI), a neuronal NOS preferring inhibitor (Babbedge *et al.*, 1993), concentration dependently reduced NMDA-elicited cell death (Figure 1d), suggesting that exposure of SH-SY5Y cells to NMDA triggers NO accumulation and this contributes to cell demise.

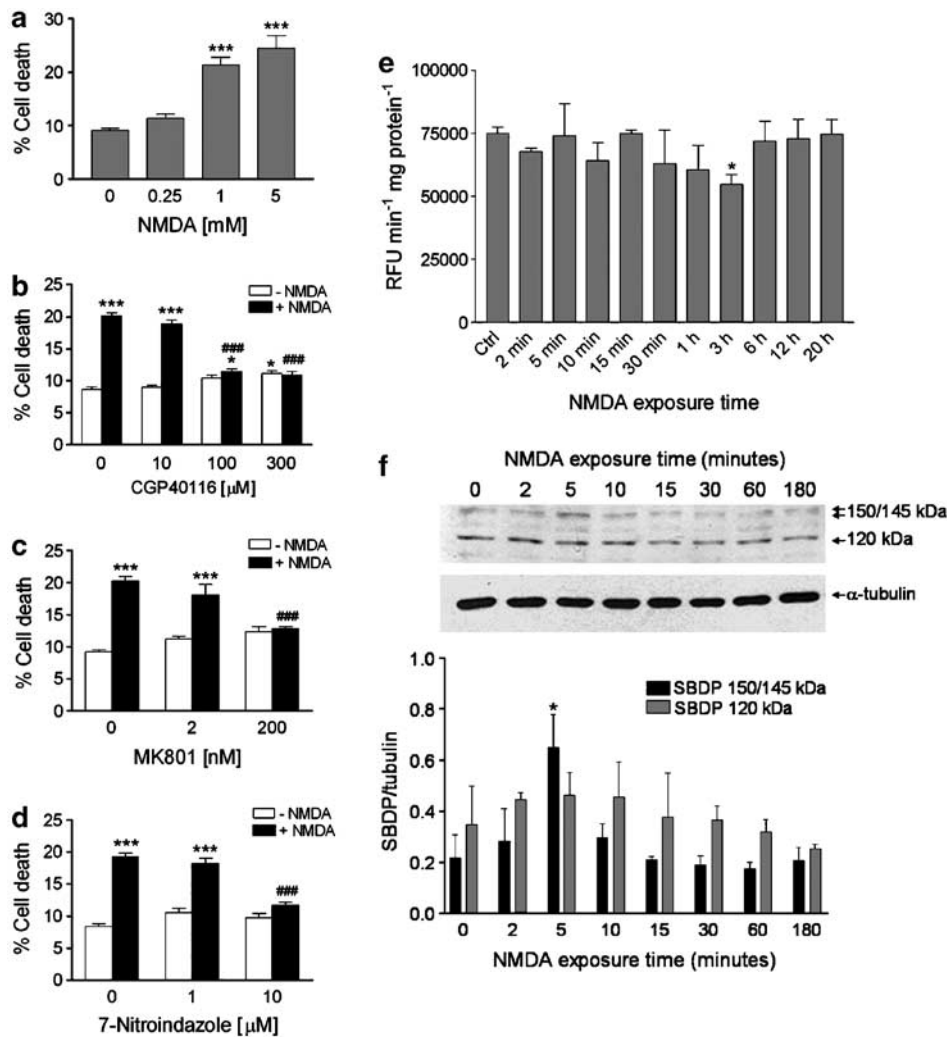
Under our experimental conditions, cell death triggered by NMDA was caspase-independent. In fact, fluorimetric caspase-3 activity assay revealed no activation of caspase-3, the main executioner caspase in neurones (see Yuan and Yankner, 2000) following exposure (2 min to 20 h) of SH-SY5Y cells to the excitotoxin (1 mM; Figure 1e). The latter effect is specific because, exposure of SH-SY5Y cells to a different stimulus, 50  $\mu$ M etoposide for 6 h, increased caspase-3 activity by  $837 \pm 84\%$  ( $P < 0.0001$  vs control =  $74902 \pm 2636$  RFU per min per mg of protein;  $n = 6$ ). Interestingly, NMDA exposure caused a significant reduction of caspase-3 activity after 3 h of treatment, compared to control (Figure 1e).

Quite importantly, activation of the  $\text{Ca}^{2+}$ -activated neutral protease calpain I was detectable early after exposure to NMDA. Activation of calpain was studied by Western-blot analysis of generation of  $\alpha$ -spectrin cleavage fragments (150–145 kDa), characteristic of calpain-mediated proteolysis. The accumulation of the calpain-cleaved 150–145 kDa  $\alpha$ -spectrin breakdown products (SBDP) was evident early after exposure to NMDA and it peaked at 5 min after NMDA addition (Figure 1f). In contrast, no accumulation of the  $\alpha$ -spectrin fragment derived from caspase-mediated proteolysis (120 kDa) was detectable (Figure 1f) and this is in accordance with the lack of caspase-3 activation evaluated by fluorimetric assay (Figure 1e).

As shown in Figure 2, treatment of SH-SY5Y cultures with 1 mM NMDA triggers a significant increase of intracellular ROS generation.

The kinase, Akt, is activated following phosphorylation at Thr308 and Ser473 (Alessi *et al.*, 1996). Interestingly, exposure of SH-SY5Y cells to NMDA induced a fast and transient deactivation of Akt kinase as determined by Western-blot analysis of p-Akt levels, at Ser473 (Figure 3a). Reduced phosphorylation of Akt was evident at 2 and 5 min after NMDA exposure. It was then followed by a trend towards an increase of phosphorylation that peaks at 15–30 min and returned to control levels at 1–3 h after treatment (Figure 3a and b). Such a transient increase in p-Akt at Ser473 following 15 min exposure to NMDA is consistent with previous reports obtained in primary cortical and hippocampal neuronal cultures (Luo *et al.*, 2003), except that the early decline has not been studied previously. At variance with modification of p-Akt levels, NMDA did not affect the expression of total Akt (Figure 3a and b). MK801 (200 nM) enhanced phosphorylation of Akt, typically reduced by 5 min exposure to NMDA (Figure 1e).

Phosphorylation of GSK-3 $\beta$  at Ser9 by Akt inhibits GSK-3 $\beta$  kinase activity (Cross *et al.*, 1995; van Weeren *et al.*, 1998); therefore, we next investigated whether NMDA exposure would reduce levels of GSK-3 $\beta$  phosphorylation at Ser9. In these experiments, NMDA led to a significant reduction of p-GSK-3 $\beta$  levels at Ser9 at 5 but not 2 min after exposure



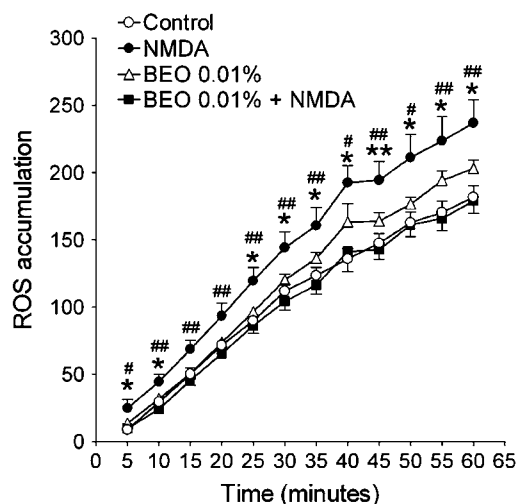
**Figure 1** Characterization of NMDA-induced cytotoxicity in SH-SY5Y cultures. (a) Treatment of SH-SY5Y cells with 1.0 mM NMDA for 24 h induces significant cytotoxic effects as assessed by trypan blue staining; no greater cytotoxicity was observed by incubating the cells with a higher concentration (5.0 mM) of the excitotoxin. A lower concentration (0.25 mM) of NMDA did not significantly affect cell viability. Cell death induced by NMDA (1 mM) was prevented by selective, (b) competitive (CGP40116; 100 and 300  $\mu$ M) and (c) noncompetitive (MK801; 200 nM), antagonists of NMDA subtype of glutamate receptors. (d) 7-Nitroindazole (10  $\mu$ M), a neuronal NOS preferring inhibitor, prevented death of SH-SY5Y cells induced by NMDA (1.0 mM). Each value in a, b, c and d is the mean  $\pm$  s.e.m. of 3–6 experiments. \*\*\* $P$  < 0.001 vs control; ### $P$  < 0.001 vs NMDA given alone (ANOVA followed by Tukey–Kramer multiple comparisons test). (e) NMDA does not increase caspase-3 activity in SH-SY5Y cell cultures. SH-SY5Y cells were exposed to 1 mM NMDA for the indicated periods of time, and then caspase-3 activity was determined in the supernatants of lysed cell suspensions by measuring cleavage of the fluorogenic substrate Ac-DEVD-AMC (see the Methods section). Results are expressed as relative fluorescence units (RFU) per min per mg of protein. Each value is the mean  $\pm$  s.e.m. of three experiments. \* $P$  < 0.05 vs control (ANOVA followed by Dunnett multiple comparisons test). (f) Activation of calpain I after exposure of SH-SY5Y cells to NMDA. Representative Western blot showing the increase of the calpain-specific 150/145 kDa SBDP early after NMDA addition (1 mM). Note the lack of accumulation of 120 kDa  $\alpha$ -spectrin fragment derived from caspase-mediated proteolysis. Histograms in lower panel show results of densitometric analysis of autoradiographic bands corresponding to 150/145 and 120 kDa SBDP. Data were normalized to the values for  $\alpha$ -tubulin. Each value is the mean  $\pm$  s.e.m. of three experiments. \* $P$  < 0.05 vs control (ANOVA followed by Dunnett multiple comparisons test).

(Figure 3c and d), indicating that in NMDA-stimulated SH-SY5Y cells, deactivation of Akt precedes reduction of p-GSK-3 $\beta$  levels.

To test whether or not NMDA-triggered decrease of phosphorylation at Ser9 was accompanied by an elevation of GSK-3 $\beta$  activity that underlies cell death, cells were incubated with NMDA in the presence of GSK-3 inhibitor IX, a selective inhibitor of GSK-3 (Meijer *et al.*, 2003). As shown in Figure 3f, GSK-3 inhibitor IX concentration dependently reduced cell death triggered by NMDA.

*BEO protects against NMDA-induced neuronal death in vitro*  
Preincubation of SH-SY5Y cultures with BEO for 60 min concentration dependently reduced cell death triggered by NMDA (Figure 4a). As shown in Figure 4b, the protective effects cannot be attributed to the vehicle used to dissolve the essential oil because ethanol, applied at the same concentrations present in 0.005 and 0.01% BEO, did not affect NMDA-induced cell death.

Interestingly, BEO (0.01%) prevented the accumulation of intracellular ROS caused by 1 mM NMDA (Figure 2). In



**Figure 2** NMDA (1 mM) induced accumulation of intracellular ROS and this was prevented by preincubating SH-SY5Y cells with BEO (0.01%). ROS were measured by fluorescence assay and the data expressed as  $(T_x - T_0/T_0) \times 100$  as described in the Methods section. Each value is the mean  $\pm$  s.e.m. of eight wells per experimental group. Results were confirmed by three independent experiments. \* $P < 0.05$  and \*\* $P < 0.01$  vs control, respectively; # $P < 0.05$  and ## $P < 0.01$  vs BEO + NMDA, respectively (ANOVA followed by Tukey–Kramer multiple comparisons test).

addition, pretreatment with BEO (0.01%) prevented accumulation of calpain-specific 150–145 kDa SBDP caused by 5 min exposure to 1 mM NMDA (Figure 5).

Exposure of SH-SY5Y cells to NMDA induces a fast and transient deactivation of Akt, which is associated with activation of GSK-3 $\beta$ . Therefore, we next evaluated whether or not BEO counteracts deactivation of Akt induced by NMDA. Interestingly, preincubation with a neuroprotective concentration of BEO (0.01%) reduced deactivation of Akt induced by 2 and 5 min exposure to NMDA (Figure 6a and b). Deactivation of Akt by NMDA is associated with a detrimental activation of GSK-3 $\beta$  (Figure 3); therefore, we next examined the effects of BEO on GSK-3 $\beta$  activation following exposure to NMDA. As shown in Figure 6c and d, BEO enhanced phosphorylation of GSK-3 $\beta$ , typically reduced by 5 min exposure to NMDA.

To investigate the intracellular pathways through which BEO reduces Akt deactivation, cells were preincubated with LY294002, a specific inhibitor of PI3K (Vlahos *et al.*, 1994), for 30 min before addition of BEO and NMDA. Pretreatment with LY294002 concentration dependently reversed the neuroprotection (Figure 7) and prevented enhancement of p-Akt levels induced by BEO (data not shown), whereas NMDA-induced cell death was not affected by any concentration of the enzyme inhibitor (Figure 7 and data not shown).

#### BEO activates Akt via PI3K in serum-starved cells

Serum-starved SH-SY5Y cells have reduced levels of p(Ser473)-Akt and p(Ser9)-GSK-3 $\beta$  (De Sarno *et al.*, 2002). To examine whether or not BEO affects Akt and GSK-3 $\beta$

phosphorylation reduced by serum starvation, SH-SY5Y cells were cultured in serum-free medium for 1 h and then exposed to 0.01% BEO for 5, 10, 20, 60 and 120 min. Western-blot analysis showed that stimulation with BEO restored Akt and GSK-3 $\beta$  phosphorylation reduced by serum deprivation (Figure 8). To investigate the intracellular pathways involved, cells were pretreated with the PI3K inhibitor, LY294002, for 30 min before BEO stimulation. Inhibition of PI3K, which is upstream of Akt (see Brazil and Hemmings, 2001), prevented phosphorylation of Akt and GSK-3 $\beta$  otherwise enhanced by 10 min stimulation with BEO (Figure 8).

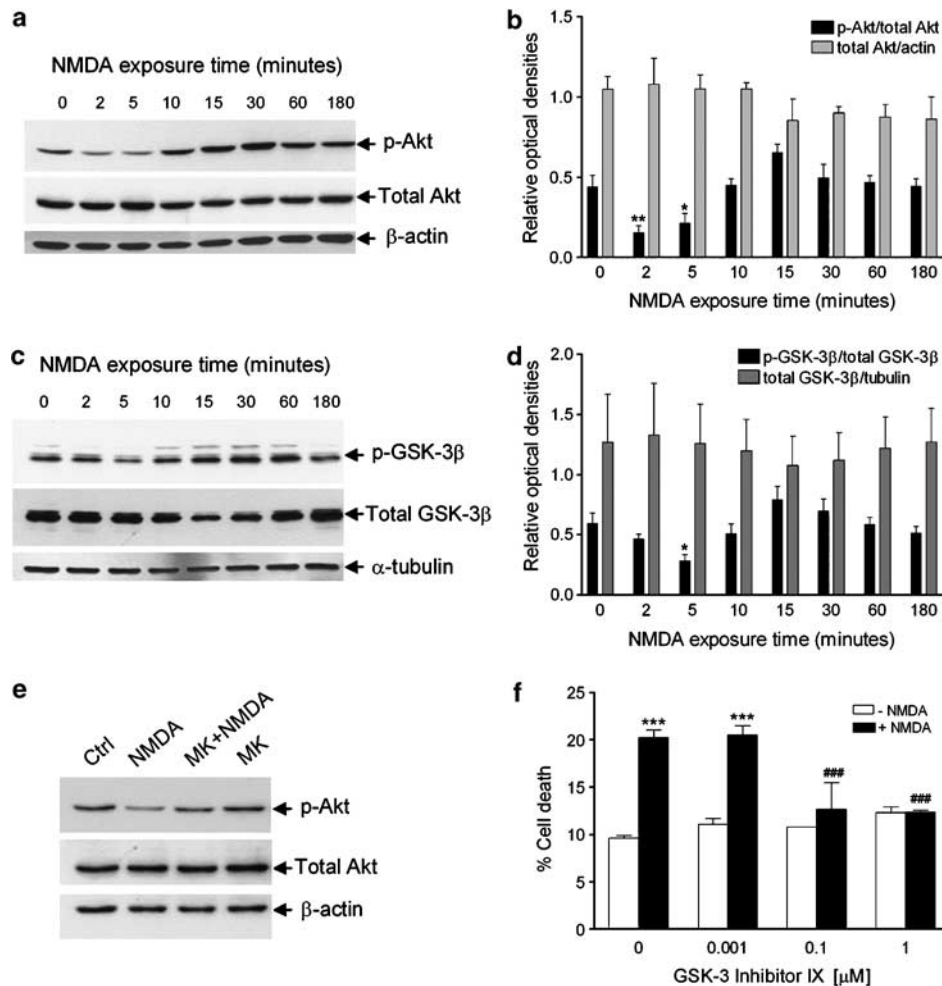
#### Characterization of the fractions of BEO responsible for neuroprotection in vitro

Preincubation of SH-SY5Y cultures with a bergapten-free extract of the essence (BEO-BF) also concentration dependently reduced cell death triggered by NMDA (Figure 9a). Quite importantly, a fraction of the oil deprived of bergapten and of monoterpene hydrocarbons (BEO-BF/HF) failed to rescue SH-SY5Y neuroblastoma cultures from NMDA-induced cell death (Figure 9b) thus suggesting that monoterpene hydrocarbons found in the volatile fraction of BEO may be responsible for neuroprotection.

## Discussion

In the present study, we report the original observation that BEO protects against NMDA-induced cell death by affecting diverse death pathways triggered by the excitotoxin.

Human SH-SY5Y neuroblastoma cells have been shown to express both ionotropic and metabotropic glutamate receptors (Sun and Murali, 1998); however, no study has been until now carried out to characterize the effects on cell viability caused by abnormal stimulation of the NMDA subtype of glutamate receptors. The present experiments show that NMDA produces cell death when applied to human SH-SY5Y neuroblastoma cells in culture. Cytotoxicity elicited by NMDA is concentration-dependent and sensitive to blockade by selective, competitive (CGP40116) and noncompetitive (MK801), antagonists of the NMDA receptor complex. Calcium entry through NMDA receptor-associated cation channel activates calcium-dependent enzymes including NOS (Garthwaite *et al.*, 1988; Kiedrowski *et al.*, 1992). Sustained activation of NOS and the consequent generation of abnormal amounts of NO have been implicated in the mechanisms of excitotoxic cell death triggered *in vitro* by overactivation of glutamate receptors (Dawson *et al.*, 1991; Corasaniti *et al.*, 1992). Interestingly, 7-nitroindazole, a neuronal NOS preferring inhibitor (Babbedge *et al.*, 1993), prevented cell death caused by the excitotoxin, thus suggesting that NO production may take part in the mechanisms of NMDA-evoked death of SH-SY5Y cells. These results are consistent with our previous observations obtained in other neuroectodermal cells, cultured human CHP100 cells, exposed to NMDA (Corasaniti *et al.*, 1992). Collectively, these findings indicate that SH-SY5Y cells in culture represent a useful experimental model to



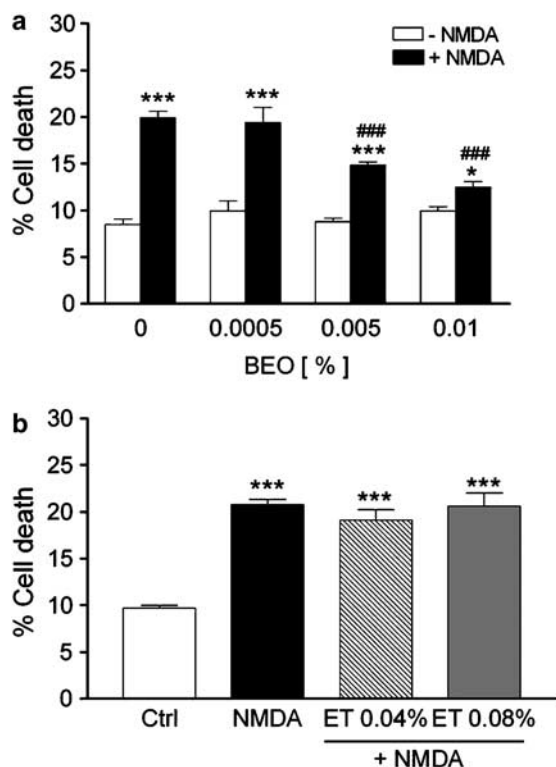
**Figure 3** Time course of NMDA effects on Akt and GSK-3 $\beta$  phosphorylation in SH-SY5Y cells and protection from NMDA-induced cell death by an inhibitor of GSK-3 $\beta$  kinase. SH-SY5Y cells were exposed to 1 mM NMDA for the indicated periods of time, then cellular proteins were extracted for subsequent analysis by Western blotting of Akt and GSK-3 $\beta$  phosphorylation by using a polyclonal antibody specific for (a) Akt phosphorylated at Ser473 and a polyclonal antibody specific for (c) GSK-3 $\beta$  phosphorylated at Ser9. To establish that changes in phospho-Akt (p-Akt) and phospho-GSK-3 $\beta$  (p-GSK-3 $\beta$ ) levels are not due to changes in Akt and GSK-3 $\beta$  protein expression, nitrocellulose membranes were subsequently immunoblotted with an antibody specific for (a) total Akt and (c) total GSK-3 $\beta$ . Equal protein loading in each lane was confirmed by hybridization with an anti- $\beta$ -actin (a) or anti- $\alpha$ -tubulin antibody (c), for Akt and GSK-3 $\beta$  blots, respectively. Histograms in (b) and (d) show the results of the densitometric analysis of autoradiographic bands; the data are reported as the ratio of (b) p-Akt/total Akt and (d) p-GSK-3 $\beta$ /total GSK-3 $\beta$ , whereas total Akt and total GSK-3 $\beta$  levels were normalized to the values yielded for  $\beta$ -actin and  $\alpha$ -tubulin, respectively. Each value is the mean  $\pm$  s.e.m. of 3–6 experiments; \* $P$  < 0.05 and \*\* $P$  < 0.01 vs control, respectively (ANOVA followed by Dunnett multiple comparisons test). (e) MK801 (200 nM; MK), applied to neuroblastoma cultures 10 min before 1 mM NMDA, attenuated reduction of phospho(Ser473)-Akt (p-Akt) levels caused by 5 min exposure to the excitotoxin; a typical example, representative of three independent experiments, is shown. (f) GSK-3 inhibitor IX (0.1 and 1  $\mu$ M) prevented death of SH-SY5Y cells induced by NMDA. GSK-3 inhibitor IX was added to SH-SY5Y cultures 30 min before NMDA application (1.0 mM) and cell death was evaluated 24 h later by trypan blue staining. No protection was afforded by the enzyme inhibitor applied at a lower concentration (0.001  $\mu$ M). Each value is the mean  $\pm$  s.e.m. of 3–6 experiments. \*\*\* $P$  < 0.001 vs control; ### $P$  < 0.001 vs NMDA given alone (ANOVA followed by Tukey–Kramer multiple comparisons test).

begin evaluating the neuroprotective properties of selected compounds against excitotoxicity *in vitro*.

Akt is activated by phosphorylation of Ser473 and Thr308 by a signaling cascade involving PI3K and 3-phosphoinositide-dependent kinase-1 (PDK-1) (see Brazil and Hemmings, 2001), whereas de-phosphorylation is regulated by protein phosphatase 2A (Andjelkovic *et al.*, 1996). Activated Akt promotes cell survival by phosphorylating and, thus, inactivating proteins implicated in promoting cell death such as Bad, caspase-9 and GSK-3 $\beta$  (Cardone *et al.*, 1998; see

Brazil and Hemmings, 2001). In particular, Akt inactivates GSK-3 $\beta$  by phosphorylating the enzyme at Ser9 (Cross *et al.*, 1995; van Weeren *et al.*, 1998).

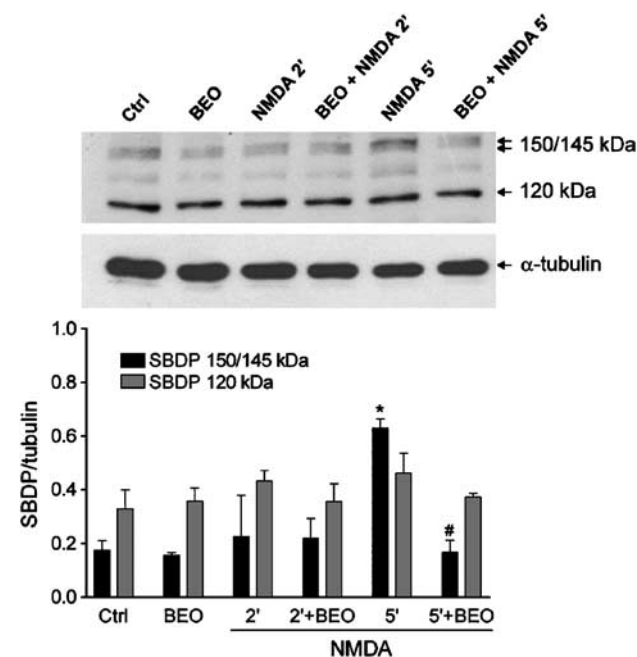
Exposure of SH-SY5Y cells to NMDA resulted in a rapid deactivation of Akt kinase and this preceded activation of GSK-3 $\beta$  as monitored by Western-blot analysis of p-GSK-3 $\beta$  at Ser9. Quite importantly, a selective inhibitor of GSK-3 (Meijer *et al.*, 2003) was able to rescue SH-SY5Y cells from death caused by NMDA, thus suggesting that reduction of p-GSK-3 $\beta$  levels was associated with an increase of kinase



**Figure 4** BEO reduces cell death induced by NMDA in SH-SY5Y cultures. (a) BEO (final concentrations in medium: 0.0005, 0.005 and 0.01%) was added to SH-SY5Y cultures 60 min before application of 1 mM NMDA and cell death was assessed by trypan blue exclusion assay 24 h later; no protection against NMDA-induced cell death was produced by BEO at the lowest concentration (0.0005%). (b) Ethanol (ET, 0.04 and 0.08%), the vehicle used to dissolve BEO, does not affect 1.0 mM NMDA-induced cell death; these concentrations of ethanol correspond to those found in 0.005 and 0.01% BEO. Each value is the mean  $\pm$  s.e.m. of 4–12 experiments. \* $P$  < 0.05 and \*\*\* $P$  < 0.001 vs control, respectively; ### $P$  < 0.001 vs NMDA given alone (ANOVA followed by Tukey–Kramer multiple comparisons test).

activity which underlies cell death. Consequently, the ability of BEO to maintain GSK-3 $\beta$  phosphorylation in NMDA-treated cells may well correlate with neuroprotection. The finding that BEO is able to prevent deactivation of Akt kinase and the downstream activation of GSK-3 $\beta$  induced by NMDA suggests an intriguing mechanism of neuroprotection. Indeed, pharmacological inhibition of GSK-3 $\beta$ , a critical activator of cell death in different models of neuronal insults (see Grimes and Jope, 2001; Bhat *et al.*, 2004), has been recently reported to reduce neuronal death resulting from excitotoxicity *in vitro* (Facci *et al.*, 2003; Kelly *et al.*, 2004) and cerebral ischemia *in vivo* (Kelly *et al.*, 2004), indicating that GSK-3 $\beta$  may represent a useful target for neuroprotective strategies.

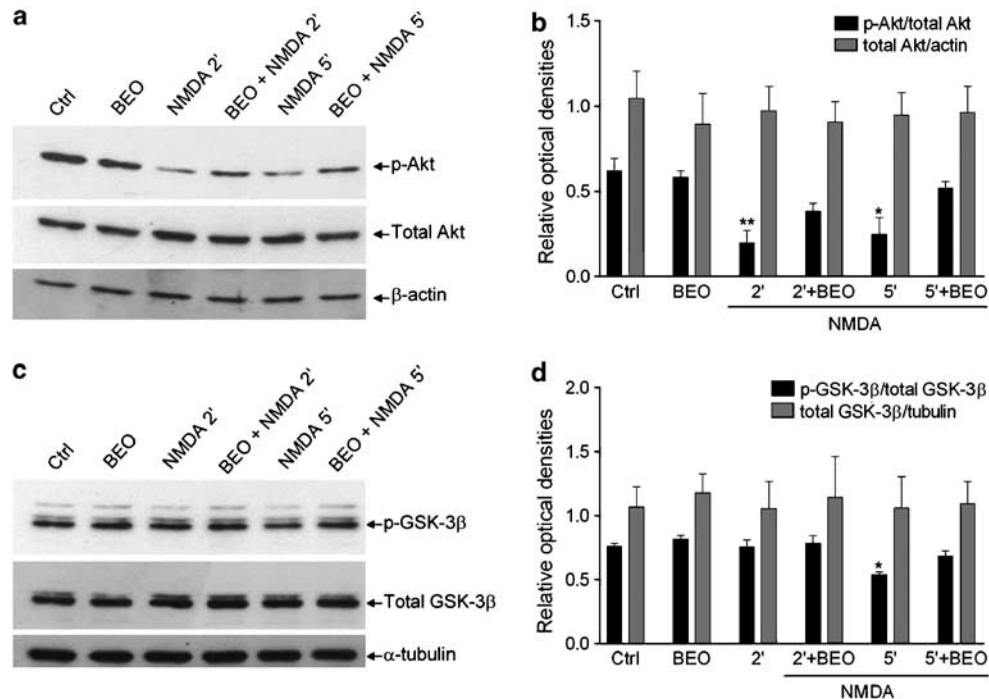
The mechanism through which NMDA receptor activation deactivates Akt in SH-SY5Y cells remains to be elucidated; however, the finding that this effect is prevented by the use-dependent NMDA receptor channel blocker, MK801, suggests that calcium influx may be implicated. Activation of NMDA receptors affects the activity of protein phosphatases 1 and 2 (Mulkey *et al.*, 1994). Of interest, in cerebellar



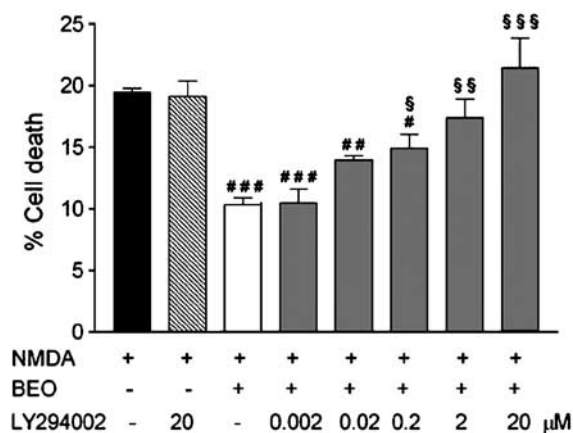
**Figure 5** BEO prevented NMDA-induced activation of calpain I. Exposure of SH-SY5Y cells to 1 mM NMDA induced activation of calpain as assessed by Western-blotting analysis of generation of  $\alpha$ -spectrin cleavage fragments (150/145 kDa) characteristic of calpain-mediated proteolysis. A significant accumulation of calpain-specific 150/145 kDa SBDP was reached at 5 min after NMDA exposure and this was prevented by a pretreatment (60 min beforehand) with BEO (0.01%). Exposure to NMDA and BEO, given alone or in combination, did not affect generation of 120 kDa  $\alpha$ -spectrin fragment derived from caspase-mediated proteolysis. Histograms in lower panel show results of densitometric analysis of autoradiographic bands corresponding to 150/145 and 120 kDa SBDP. Data were normalized to the values yielded for  $\alpha$ -tubulin. Each value is the mean  $\pm$  s.e.m. of three experiments. \* $P$  < 0.05 vs control and # $P$  < 0.05 vs NMDA given alone (ANOVA followed by Tukey–Kramer multiple comparisons test).

granule cells, protein phosphatase inhibitors such as okadaic acid and calyculin A prevent glutamate-induced reduction of Akt phosphorylation and activity (Chalecka-Franaszek and Chuang, 1999). Similarly, okadaic acid prevented the dephosphorylation of Akt induced in retinal ganglion and amacrine cells by injection of NMDA into the vitreous cavity of rats (Nakazawa *et al.*, 2005). Altogether, these observations suggest that NMDA-induced reduction of Akt phosphorylation in SH-SY5Y cells might be caused by activation of protein phosphatase 2A. Under our experimental conditions, cell death induced by NMDA was not affected by LY294002, a specific PI3K inhibitor (Vlahos *et al.*, 1994), suggesting that inhibition of Akt phosphorylation does not facilitate death caused by NMDA. Interestingly, the protection afforded by BEO against NMDA-induced cell death is concentration dependently antagonized by LY294002, indicating that neuroprotection is mediated by PI3K. The latter deduction is further supported by the finding that LY294002 inhibited the ability of BEO to restore Akt phosphorylation reduced by serum deprivation. Therefore, increased Akt phosphorylation by BEO does not seem to be the consequence of inhibition of a phosphatase dephosphorylating Akt, as the





**Figure 6** BEO reduced NMDA-induced decrease of phospho-Akt and phospho-GSK-3 $\beta$  levels in SH-SY5Y cells. (a) Exposure of SH-SY5Y cells to 1 mM NMDA for 2 and 5 min induced deactivation of Akt kinase as determined by Western-blot analysis of phospho(Ser473)-Akt (p-Akt) levels and this was attenuated by BEO (0.01%) applied to neuroblastoma cultures 60 min before NMDA exposure. (b) Densitometric analysis of immunoreactive bands shows that the significant changes in phospho(Ser473)-Akt levels induced by BEO in NMDA-treated cells were not associated with changes in total Akt immunoreactivity. (c) Exposure of SH-SY5Y cells to 1 mM NMDA for 5 but not for 2 min induced activation of GSK-3 $\beta$  kinase as determined by Western-blot analysis of phospho(Ser9)-GSK-3 $\beta$  (p-GSK-3 $\beta$ ) levels and this is attenuated by BEO (0.01%) applied to neuroblastoma cultures 60 min before NMDA addition. As confirmed by densitometric analysis of immunoreactive bands (d), BEO did not affect the expression of total GSK-3 $\beta$  in NMDA-treated cells. Each value in b and d is the mean  $\pm$  s.e.m. of 3–6 experiments; \* $P$  < 0.05 and \*\* $P$  < 0.01 vs control, respectively (ANOVA followed by Tukey–Kramer multiple comparisons test).

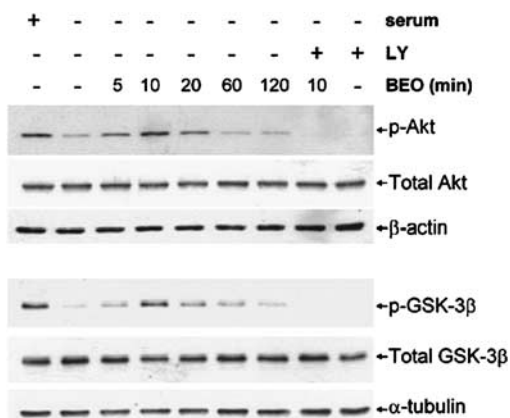


**Figure 7** LY294002 concentration dependently reversed the neuroprotection afforded by BEO against NMDA-induced cell death. SH-SY5Y cells were treated with LY294002 (0.002–20  $\mu$ M), a specific inhibitor of PI3K, for 30 min before the addition of BEO (0.01%, added 1 h before NMDA) and of 1 mM NMDA for 24 h and, then, cell death assessed by means of trypan blue staining. SH-SY5Y cells were treated with LY294002 (20  $\mu$ M) for 30 min before exposure to 1 mM NMDA for 24 h. LY294002, given alone, did not affect NMDA-induced cell death. Each value is the mean  $\pm$  s.e.m. of 4–6 experiments. # $P$  < 0.05, ## $P$  < 0.01 and ### $P$  < 0.001 vs NMDA, respectively; \$ $P$  < 0.05, \$\$ $P$  < 0.01 and \$\$\$ $P$  < 0.001 vs BEO + NMDA, respectively (ANOVA followed by Tukey–Kramer multiple comparisons test).

level of p-Akt in the presence of an inhibitor of PI3K is unaltered by BEO. It has been previously shown that withdrawal of serum from cultured rat cortical neurons reduces phosphorylation of Akt on Ser473 and enhances GSK-3 $\beta$  activity and that addition of brain-derived neurotrophic factor (BDNF) restores Akt phosphorylation and suppresses GSK-3 $\beta$  activity (Hetman *et al.*, 2000). Here, we have provided evidence that BEO restores Akt and GSK-3 $\beta$  phosphorylation decreased by serum deprivation. In addition, treatment with LY294002 inhibited BEO-induced phosphorylation of GSK-3 $\beta$  on Ser9, indicating that BEO negatively regulates GSK-3 $\beta$  activity in SH-SY5Y cells via a PI3K-dependent mechanism that involves Akt.

Treatment of SH-SY5Y cells with NMDA causes a significant accumulation of intracellular ROS and a rapid activation of the calcium-activated neutral protease calpain I. The latter observation is in line with our previous finding that calpain I is implicated in death of human CHP100 neuroblastoma cells caused by NMDA (Corasaniti *et al.*, 1996).

Under the present experimental conditions, death of SH-SY5Y cells by NMDA was not preceded nor it is accompanied by activation of caspase-3. Despite reports of caspase-dependent excitotoxic neuronal death, lack of caspase-3 activation has been previously observed in primary rat hippocampal neurones treated with NMDA (Lankiewicz *et al.*, 2000; Luo *et al.*, 2003), suggesting that activation of

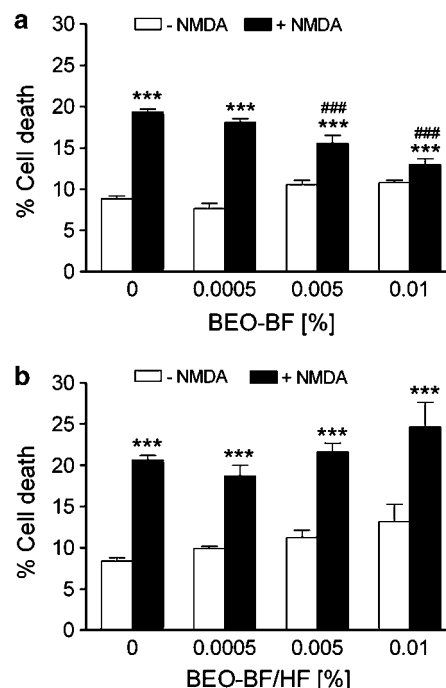


**Figure 8** BEO restored Akt and GSK-3β phosphorylation, reduced by serum withdrawal, via a PI3K-dependent mechanism. SH-SY5Y cells were maintained in serum-containing medium (+serum) or cultured in serum-free medium for 1 h (-serum); serum-starved cells were exposed to 0.01% BEO for 5, 10, 20, 60 and 120 min (min), then cellular proteins were extracted for subsequent analysis by Western blotting of Akt and GSK-3β phosphorylation by using polyclonal antibodies specific for Akt phosphorylated at Ser473 or for GSK-3β phosphorylated at Ser9. Serum deprivation caused a dramatic decline in phospho-Akt (p-Akt) and phospho-GSK-3β (p-GSK-3β) immunoreactivity. Application of BEO for 5–20 min enhanced phosphorylation of Akt and GSK-3β reduced by serum deprivation; this effect peaked at 10 min, then it progressively declined. A preincubation for 30 min with the specific PI3K inhibitor, LY294002 (LY; 20 μM), before exposure to BEO for 10 min, prevented the effects of the essential oil on Akt and GSK-3β phosphorylation. Representative immunoblots from three independent experiments are shown.

caspace-3 activation following exposure to NMDA was owing early and robust activation of calpain I induced by the excitotoxin.

BEO prevents accumulation of calpain-specific 150–145 kDa SBDP caused by 5 min exposure of SH-SY5Y cells to NMDA, indicating that inhibition of calpain activation may be implicated in neuroprotection. In addition to activation of Ca<sup>2+</sup>-dependent enzymes, such as NOS and calpain I, Ca<sup>2+</sup> entry through the NMDA receptor-gated cation channel triggers mitochondrial Ca<sup>2+</sup> overload and dysfunction leading to generation of ROS which contribute to cell demise (Lafon-Cazal *et al.*, 1993; Dugan *et al.*, 1995; Stout *et al.*, 1998). Interestingly, pretreatment of SH-SY5Y cultures with BEO prevented accumulation of intracellular ROS induced by NMDA. Collectively, these observations suggest that BEO affects diverse death pathways triggered by alteration of Ca<sup>2+</sup> homeostasis induced by the excitotoxin.

Protection afforded by BEO against NMDA-induced cell death is reproduced by BEO-BF supporting the suggestion that bergapten was not involved in the protective actions. Quite importantly, BEO-BF/HF failed to rescue SH-SY5Y neuroblastoma cultures from cell death induced by NMDA,



**Figure 9** Monoterpene hydrocarbons could be involved in the neuroprotection afforded by BEO against NMDA-induced cell death. SH-SY5Y cultures were incubated with a bergapten-free extract of the essence (BEO-BF (a)) or with a fraction of the oil deprived of bergapten and of monoterpene hydrocarbons (BEO-BF/HF (b)) for 60 min before application of 1 mM NMDA and cell death was assessed by trypan blue exclusion assay 24 h later. (a) BEO-BF, but not (b) BEO-BF/HF, concentration dependently reduced NMDA-induced cell death. Each value is the mean ± s.e.m. of 4–8 experiments. \*\*\**P* < 0.001 vs control; ###*P* < 0.001 vs NMDA given alone (ANOVA followed by Tukey–Kramer multiple comparisons test).

thus suggesting that monoterpene hydrocarbons found in the volatile fraction of BEO may be responsible for neuroprotection. The most abundant monoterpene hydrocarbons found in the volatile fraction of BEO are limonene, γ-terpinene, and β-pinene (Mondello *et al.*, 1995; Verzera *et al.*, 1996, 2003). Several reports on limonene show it to interfere with mevalonate metabolism (Elson, 1995) and protein isoprenylation (Crowell *et al.*, 1994; Kawata *et al.*, 1994), although no study has addressed its neuroprotective potential. Further investigation is needed to identify the monoterpene hydrocarbon/s responsible for neuroprotection afforded by BEO.

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## Conflict of interest

The authors state no conflict of interest.

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