British Journal of Pharmacology (2009), 157, 1072–1084
 0007-1188/09
www.brjpharmacol.org

RESEARCH PAPER

Clovamide and rosmarinic acid induce neuroprotective effects in *in vitro* models of neuronal death

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Background and purpose: Phenolic compounds exert cytoprotective effects; our purpose was to investigate whether the isosteric polyphenolic compounds clovamide and rosmarinic acid are neuroprotective.
Experimental approach: Three *in vitro* models of neuronal death were selected: (i) differentiated SH-SY5Y human neuroblastoma cells exposed to *tert*-butylhydroperoxide (*t*-BOOH), for oxidative stress; (ii) differentiated SK-N-BE(2) human neuroblastoma cells treated with L-glutamate, for excitotoxicity; and (iii) differentiated SH-SY5Y human neuroblastoma cells exposed to oxygen-glucose deprivation/reoxygenation, for ischaemia–reperfusion. Cell death was evaluated by lactate dehydrogenase measurements in the cell media, while the mechanisms underlying the effects by measuring: (i) *t*-BOOH-induced glutathione depletion and increase in lipoperoxidation; and (ii) L-glutamate-induced intracellular Ca^{2+} overload (fura-2 method) and inducible gene expression (*c-fos, c-jun*), by reverse transcriptase-PCR. The ability of compounds to modulate nuclear factor-κB and peroxisome proliferator-activated receptor-γ activation was evaluated by Western blot in SH-SY5Y cells not exposed to harmful stimuli.
Key results: Both clovamide and rosmarinic acid (10–100 μmol·L^{-1}) significantly protected neurons against insults with similar potencies and efficacies. The EC_{50} values were in the low micromolar range (0.9–3.7 μmol·L^{-1}), while the maximal effects ranged from 40% to –60% protection from cell death over untreated control at 100 μmol·L^{-1}. These effects are mediated by the prevention of oxidative stress, intracellular Ca^{2+} overload and *c-fos* expression. In addition, rosmarinic acids inhibited nuclear factor-κB translocation and increased peroxisome proliferator-activated receptor-γ expression in SH-SY5Y cells not exposed to harmful stimuli.
Conclusion and implications: Clovamide and rosmarinic acid are neuroprotective compounds of potential use at the nutritional/pharmaceutical interface.
British Journal of Pharmacology (2009) **157,** 1072–1084; doi:10.1111/j.1476-5381.2009.00213.x; published online 21 May 2009

Keywords: polyphenolics; neuroprotection; oxidative stress; excitotoxicity; ischaemia–reperfusion; PPARγ; NF-κB

Abbreviations: 15-deoxy-$\Delta^{12,14}$-PGJ_2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2; $[Ca^{2+}]_i$, intracellular calcium concentration; DMEM, Dulbecco modified Eagle's medium; DMSO, dimethylsulphoxide; F12, Nutrient Mixture Ham's F12; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; GSH, glutathione; LDH, lactate dehydrogenase; MFI, mean fluorescence ratio; MPP^+, 1-methyl-4-phenylpyridinium; NF-κB, nuclear factor-κB; OGD, oxygen-glucose deprivation; PBS, phosphate-buffered saline; PPARγ, peroxisome proliferator-activated receptor-γ; RA, retinoic acid; ROS, reactive oxygen species; RT-PCR, reverse transcriptase-PCR; *t*-BOOH, *tert*-butylhydroperoxide; TBARS, thiobarbituric acid-reacting substances; TBS-T, Tris-buffered saline Tween-20

Introduction

The nervous system is extremely vulnerable to a wide range of harmful stimuli, such as oxidative stress, excitotoxicity and ischaemia–reperfusion injury, and is particularly susceptible to irreversible damages because it has a reduced capacity for regeneration (Choi and Rothman, 1990; Lipton and Rosenberg, 1994; Barnham *et al.*, 2004; Gould, 2007).

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Received 11 November 2008; revised 16 January 2009; accepted 19 January 2009

Substantial pieces of evidence have mounted over the years showing the biochemical and cellular events associated with neuronal death (Gassen and Youdim, 1999; Lipton, 1999; Vanlangenakker *et al*., 2008). Common players in necrotic cell death, irrespective of the specific stimulus, are oxidative stress and Ca^{2+} overload. Oxidative stress, in which the extracellular and intracellular production of reactive oxygen species (ROS) and reactive nitrogen species overwhelms physiological antioxidant defences, such as the intracellular glutathione (GSH) system, initiates damage to lipids (lipoperoxidation), proteins and DNA (Miyamoto *et al*., 2003; Sayre *et al*., 2008). This results in mitochondrial dysfunction, deregulation of ion balance and loss of membrane integrity (Beal *et al*., 1993; Trushina and McMurray, 2007). During necrosis, cellular Ca^{2+} regulating systems are compromised, and intracellular calcium concentrations ($[Ca^{2+}]_i$) become elevated. Intracellular Ca^{2+} overload leads to bioenergetic effects and activation of proteases and phospholipases, resulting in synaptic dysfunction, impaired neuronal plasticity and cell death (Bano and Nicotera, 2007; Mattson, 2007). Furthermore, injured neurons become depolarized and increase the release of neurotransmitters by Ca^{2+}-dependent exocytosis and reversal transport (Kim *et al*., 1995). The resultant build-up of extracellular neurotransmitters compromises the physiological functions of neurons. In particular, the accumulation of L-glutamate causes an overload of intracellular Ca^{2+} (Choi, 1992), leading to neurotoxicity (excitotoxicity) (Olney, 1986) and free radical production (Pellegrini-Giampietro *et al*., 1988), creating a dramatic vicious cycle (Hoyt *et al*., 1997).

Ischaemia–reperfusion is another complex type of cell injury engaging many of these independently fatal terminal pathways, involving loss of membrane integrity and ATP, progressive proteolysis, generalized depolarization, excitotoxicity, cytosolic Ca^{2+} overload, oxidative stress and lipoperoxidation, all contributing to neuronal death (Lipton, 1999; White *et al*., 2000).

The complex series of biochemical and molecular events triggered by harmful stimuli activate a number of protein signalling mechanisms, involving Ca^{2+}/calmodulin-dependent kinases and mitogen-activated protein kinases, such as extracellular signal-regulated kinase and c-Jun N-terminal kinase. These signals act as modulators of nuclear transcriptional responses, such as c-Jun N-terminal kinase-mediated early gene (e.g. *c-jun* and *c-fos*) expression (Shan *et al*., 1997; Pláteník *et al*., 2000; Tormos *et al*., 2004) and nuclear transcription factor [e.g. nuclear factor-κB (NF-κB)] activation, responsible for the survival or damage of cells (Mémet, 2006; Pizzi and Spano, 2006; Mehta *et al*., 2007).

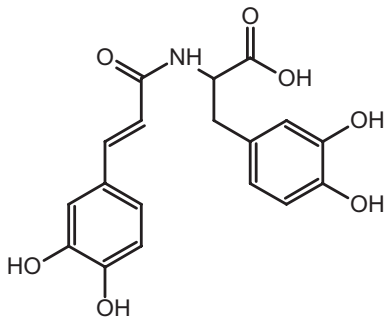
Furthermore, necrotic cells release pro-inflammatory factors, which activate phagocytes, causing the induction of inflammatory gene expression and the amplification of cellular insults (Zipp and Aktas, 2006). The cascade of inflammatory events can be modulated by the activation of several nuclear receptors, such as peroxisome proliferator-activated receptor (PPAR)γ, which inhibit the expression of pro-inflammatory genes at the transcriptional level by antagonizing the actions of nuclear transcription factors, such as NF-κB, activator protein 1, and signal transducer and activator of transcription 1 (Daynes and Jones, 2002).

In the CNS, PPARγ stimulation exerts significant neuroprotective effects in both *in vitro* and *in vivo* models (Dehmer *et al*., 2004; Schütz *et al*., 2005; Culman *et al*., 2007). Rosiglitazone, a potent PPARγ agonist, protects SH-SY5Y cells against the neurotoxin 1-methyl-4-phenylpyridinium (MPP^+)-induced cytotoxicity (Jung *et al*., 2007), and PPARγ ligands inhibit both NF-κB activation and cell growth in SH-SY5Y human neuroblastoma cell lines (Kim *et al*., 2003).

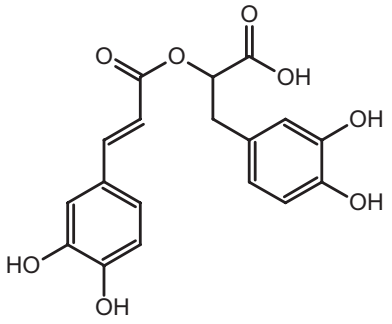
In spite of the overall weight of evidence, neuroprotective strategies have not yet been fully defined and the systematic search for new neuroprotective compounds is still matter of intense research. Recently, the scientific interest in this field has increasingly focused on natural products.

Phytochemicals, including vitamin C, vitamin E, β-carotene and phenolic compounds, are endowed with cytoprotective effects, (Fang *et al*., 2002; Soobrattee *et al*., 2005), and the consumption of food containing these compounds has been proposed for the prevention of pathological (Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis, stroke, retinal degeneration) or physiological (aging) neuronal death (Engelhart *et al*., 2002; Zandi *et al*., 2004; Steele *et al*., 2007).

Among phytochemicals, phenolic compounds are an important class of quasi-vitamins of potential use at the nutritional/pharmaceutical interface (Williams *et al*., 2004). Rosmarinic acid (α-*O*-caffeoyl-3,4-dihydroxyphenyl lactic acid; Figure 1) is widely distributed in *Labiatae* herbs, like rosemary, sweet basil and perilla (Petersen and Simmonds, 2003), and it exerts antimicrobial, antiviral, antioxidant, anti-



clovamide



rosmarinic acid

Figure 1 Chemical structure of the compounds used in this study.

inflammatory and immunosuppressive effects (van Kessel *et al*., 1986; al-Sereiti *et al*., 1999; Zheng and Wang, 2001; Kang *et al*., 2003). Clovamide (*N*-caffeoyl-L-dihydroxyphenylalanine; Figure 1), the amide isostere of rosmarinic acid, belongs to a family of structurally related polyphenolic-amino acid conjugates, first described in red clover (*Trifolium pratense*) (Yoshihara *et al*., 1974; 1977), and later in fractions of African blackwood (*Dalbergia melanoxylon*) (van Heerden *et al*., 1980), cocoa (*Theobroma cacao*) (Sanbongi *et al*., 1998), *Capsicum* spp. and coffee beans (*Coffea arabica*) (Clifford *et al*., 1989). Because clovamides are difficult to obtain by isolation, they have been less investigated than rosmarinic acid, but the efficient synthesis of clovamides (Park, 2005; Arlorio *et al*., 2008), together with the detection of clovamides in common foodstuffs, such as cocoa and coffee, have recently sparked research on the biological properties of these compounds (Park and Schoene, 2003; Park and Schoene, 2006).

The aim of our study was to evaluate if clovamide or rosmarinic acid exert neuroprotective effects against oxidative stress, excitotoxicity and ischaemia–reperfusion injury. For this purpose, three different *in vitro* models were selected: (i) differentiated SH-SY5Y human neuroblastoma cell lines, exposed to *tert*-butylhydroperoxide (*t*-BOOH; 100 μmol·L^{-1}, 3 h), as a model of oxidative stress (Lombardi *et al*., 2002); (ii) differentiated SK-N-BE(2) human neuroblastoma cell lines, treated with to L-glutamate (1 mmol·L^{-1}, 24 h), as a model of excitotoxicity (Lombardi *et al*., 2007); and (iii) differentiated SH-SY5Y human neuroblastoma cell lines, exposed to oxygen-glucose deprivation (OGD; 5 h)/reoxygenation, as a model of ischaemia–reperfusion injury (Miglio *et al*., 2004).

The data we have obtained demonstrate that both clovamide and rosmarinic acid protect neurons against different harmful stimuli by mechanisms presumably related to their ability to prevent *t*-BOOH-induced oxidative stress and L-glutamate-induced intracellular Ca^{2+} overload/*c-fos* expression. We also present evidence that rosmarinic acid inhibits NF-κB nuclear translocation and increase PPARγ expression, in SH-SY5Y cells not exposed to harmful stimuli.

Methods

Cell cultures

Human neuroblastoma cells, SH-SY5Y and SK-N-BE(2) cell lines, were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Dulbecco modified Eagle's medium (DMEM) or DMEM–Nutrient Mixture Ham's F12 (DMEM/F12) (1:1), supplemented with 10% foetal calf serum, penicillin (100 IU·mL^{-1}), streptomycin (100 μg·mL^{-1}) and L-glutamine (2 mmol·L^{-1}) respectively. Cell culture medium was replaced every 2 days, and the cultures were maintained at 37°C in 95% air/5% CO_2 in a humidified incubator. Cells were differentiated into the neuron-like type by treatment with 10 μmol·L^{-1} all-*trans* retinoic acid (RA), added to the cell culture medium every 2 days for either 7 days (oxidative stress model) (Lombardi *et al*., 2002) or 14 days (excitotoxicity and OGD/reoxygenation) (Miglio *et al*., 2004; Lombardi *et al*., 2007). The day before each experiment, differentiated cells were plated in a six-well culture plate (0.5–1 × 10^6 cells·well^{-1}).

In vitro *models of neuronal death*

The *in vitro* model of oxidative stress used human SH-SY5Y cells differentiated by treatment with 10 μmol·L^{-1} RA for 7 days, as previously described (Lombardi *et al*., 2002). Briefly, the cells were washed twice with phosphate-buffered saline (PBS) and were exposed to *t*-BOOH (100 μmol·L^{-1}) in the experimental buffer (in mmol·L^{-1}: NaCl 138, KCl 2.7, $CaCl_2$ 1.2, $MgCl_2$ 1.2, PBS 10, glucose 10; pH 7.4) for 3 h at 37°C. Afterwards, the cells were returned to standard culture conditions, and cell death was measured.

The *in vitro* model of excitotoxicity used human SK-N-BE(2) cells differentiated by treatment with 10 μmol·L^{-1} RA for 14 days, as previously described (Lombardi *et al*., 2007). The cells were washed twice with PBS, incubated in Neurobasal medium supplemented with B27 (24 h at 37°C) to increase their sensitivity to the excitotoxic insult and exposed to L-glutamate (1 mmol·L^{-1} in PBS) added to the cell culture medium. Cell death was then measured after 24 h of cell culture incubation at 37°C.

The *in vitro* model of OGD/reoxygenation used human SH-SY5Y cells differentiated by treatment with 10 μmol·L^{-1} RA for 14 days, as previously described (Miglio *et al*., 2004). The cells were exposed to OGD for 5 h, followed by 20 h of reoxygenation at 37°C. Briefly, the standard culture medium was replaced with a glucose-free buffer (OGD buffer) (in mmol·L^{-1}: 154 NaCl, 5.6 KCl, 5.0 HEPES, 3.6 $NaHCO_3$, 2.3 $CaCl_2$; pH 7.4), bubbled with an anaerobic gas mixture (95% N_2–5% CO_2) for at least 2 h before using, with or without increasing concentrations (0.1–100 μmol·L^{-1}) of clovamide or rosmarinic acid, 1 μmol·L^{-1} (+)-MK-801, or 50 μmol·L^{-1} vitamin E. The cell cultures were then placed into an anaerobic chamber (Oxoid, Basingstoke, Hampshire, UK), flushed with the anaerobic gas mixture (95% N_2–5% CO_2) and maintained at 37°C for 5 h. Then, the culture plates were removed from the anaerobic chamber, returned to the standard culture medium and placed in a humidified incubator at 37°C for 20 h of recovery (reoxygenation). Afterwards, cell death was measured.

Cell death was quantified by using the Cytotoxicity Detection Kit (Roche Diagnostic, Monza, Italy), based on the measurement of lactate dehydrogenase (LDH) activity in the experimental media. Cell death was expressed as percentage of LDH released from damaged cells over that released from cells treated with vehicle alone (controls), according to the manufacturer's instructions.

Determination of intracellular GSH and thiobarbituric acid-reacting substances levels

Intracellular GSH content was measured on protein-free extracts according to Lombardi *et al*. (2002) with minor modifications. To assay GSH, 100 μL of 5,5′-dithio-bis(2-nitrobenzoic acid) (6 mmol·L^{-1}), 25 μL of protein-free extracts, 875 μL of NADPH (0.3 mmol·L^{-1}) and 10 μL of GSH reductase (10 U·mL^{-1}) were mixed together, and the absorbance changes monitored at 412 nm with a spectrophoto-

meter (Beckman DU-68, Beckman Instruments, Milan, Italy). Intracellular GSH contents were quantified from a standard curve obtained with known amounts of GSH and expressed as percentage of GSH (%GSH) calculated as $(x \times 100)/y$ where x and y are the rates of absorbance increase in drug-treated and drug-untreated cells, respectively, normalized on the sample protein concentration.

Lipoperoxidation was evaluated at the end of the experiments in cell lysates by measuring the thiobarbituric acid-reacting substances (TBARS) (Miglio *et al.*, 2004). TBARS content was determined by measuring the fluorescence intensity at 550 nm (excitation) and 532 nm (emission) in the butanol phase by a spectrofluorometer (FP-777, Jasco, Tokio, Japan). TBARS contents were expressed as percentage of TBARS (%TBARS) calculated as $(x \times 100)/y$ where x and y are the rates of absorbance increase in drug-treated and drug-untreated cells, respectively, normalized on the sample protein concentration.

Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured at single cell level in fura-2-loaded cells by using a digital calcium image system according to Lombardi *et al.* (2007). All measurements were taken at 40-fold magnification. Emitted fluorescence was taken with a grey-scale CCD camera (SensiCam; PCO, Kelheim, Germany) and was analysed with Axon Imaging Workbench 4.0 software (Axon Instruments, Union City, CA, USA). Data were expressed as the mean of the ratio of fluorescence emitted by excitation at 340 and 380 nm of *n*-monitored cells.

mRNA isolation and reverse transcriptase-PCR (RT-PCR)

RNA extraction and RT-PCR analyses were performed as previously described (Miglio *et al.*, 2005). PCR was performed in a 25 μL reaction mixture containing 2 μg of cDNA, 2.5 μL of ×10 buffer, 1.5 μL of $MgCl_2$ (50 mmol·L^{-1}), 0.5 μL of a dNTPs mix (10 mmol·L^{-1}) (Invitrogen), 2.5 U of Taq DNA polymerase (Invitrogen) and 2.5 μL of each primer (Table 1). RT-PCR amplicons were resolved in a 2% agarose gel by electrophoresis, and signals were quantified with densitometric analysis software (NIH Image 1.32; National Institutes of Health, Bethesda, MD, USA). Data are expressed as the ratio of the signal obtained for each gene (*c-fos*, *c-jun*) in one sample divided by that obtained for the reference gene [glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*)] in the same sample.

NF-κB activation

Differentiated SH-SY5Y cells were evaluated either without any treatment, to assess constitutive, basal NF-κB activation, or in the presence of clovamide or rosmarinic acid (10 μmol·L^{-1}; 2 h). The nuclear and cytosolic extracts (20 μg protein for each sample) were obtained by the Nuclear Extraction Kit (Active Motifs, Rixensart, Belgium), according to the manufacturer's instructions, and were used to evaluate NF-κB p50 and p65 subunits by Western blot, as previous described (Bardelli *et al.*, 2005). For these assays, commercial antibodies were used. The rabbit polyclonal anti-NF-κB p50 (ab 7971) and anti-NF-κB p65 (ab 7970) antibodies were obtained from Abcam (Cambridge, UK); the mouse monoclonal anti-lamin A/C (sc-7293) antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and the mouse monoclonal anti-β-actin (A-5441) antibody was obtained from Sigma (St. Louis, MO, USA). Extracts were challenged for 2 h at room temperature with the specific antibody at final concentration of 1 μg·mL^{-1}, and the band was semi-quantified by densitometry. Results are expressed as the nuclear/cytosolic ratio for each NF-κB subunit.

PPARγ protein expression and quantification

Differentiated SH-SY5Y cells were untreated or treated with clovamide, rosmarinic acid, or 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15-deoxy-$\Delta^{12,14}$-PGJ_2) (an endogenous PPARγ agonist) for 6 h to evaluate possible effects on PPARγ expression. Differentiated cells were washed twice with ice-cold PBS and harvested in the lysis buffer containing: sodium dodecyl sulphate (3%), Tris (0.25 mol·L^{-1}) and phenyl-methyl-sulphonyl fluoride (1 mmol·L^{-1}). Cells were lysed by sonication, and, when necessary, cell lysates were stored at –80°C. Protein concentration was obtained with the Bradford-based assay. Protein samples (20 μg) were analysed by SDS-PAGE (10%) polyacrylamide gel and electro-blotted on nitrocellulose membrane (Protran, Perkin Elmer Life Sciences, Boston, MA, USA). Immunoblots were performed by using the following antibodies: monoclonal mouse anti-human PPARγ [E-8; Santa Cruz, CA, USA; 1:1000 in Tris-Buffered Saline Tween-20 (TBS-T) 5% milk] and monoclonal mouse anti-human β-actin

Table 1 PCR primers and protocols used in this study

PCR primers		*Amplicon size (bp)*	*Denaturation*	*Annealing*	*Extension*	*Cycles*
c-fos	F: 5′GGAATAAGATGGCTGCAGCCAAATGCC-3′	388	95°C, 30 s	70°C, 90 s	72°C, 60 s	35
NM_OO5252[a]	R: 5′-GGGAACAGGAAGTCATCAAAGGGCTC-3′					
c-jun	F: 5′-CTGCAAAGATGGAAACGACCT-3′	240	95°C, 30 s	61°C, 90 s	72°C, 60 s	35
NM_OO2228[a]	R: 5′-GGATTATCAGGCGCTCCAG-3′					
GAPDH	F: 5′-GGTCGGAGTCAACGGATTTGG-3′	1000	96°C, 30 s	60°C, 30 s	72°C, 45 s	25
NM_OO2046[a]	R: 5′-ACCACCCTGTTGCTGTAGCCA-3′					

GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
[a]Accession number NCBI sequence database (GenBank).

(A-5441; Sigma, St. Louis, MO, USA; 1:5000 in TBS-T, 3% bovine serum albumin). Proteins were visualized with an enzyme-linked chemiluminescence detection kit according to the manufacturer's instructions (Perkin Elmer). Chemiluminescence signals were analysed under non-saturating conditions with an image densitometer (Versadoc, Bio-Rad, Hercules, CA, USA). Semi-quantification of PPARγ protein was performed as previously described (Amoruso *et al.*, 2007), by calculating the ratio between PPARγ and β-actin protein expression; the latter was selected as a reference housekeeping protein.

Data analysis

Results are expressed as means ± SEM of at least four experiments. Statistical significance was evaluated by either the Student's *t*-test or the ANOVA-test for paired varieties. Differences were considered statistically significant when $P < 0.05$. Data were fitted as sigmoidal concentration–response curves and analysed with a four-parameter logistic equation by using the software Origin version 6.0 (Microcal Software, Northampton, MA, USA).

Materials

(+)–MK 801 was obtained from Tocris Bioscience (Bristol, UK). 15-deoxy-$\Delta^{12,14}$-PGJ_2 was from Biomol (Plymouth Meeting, PA, USA). DMEM, DMEM/F12 (1:1), Neurobasal medium, B27 supplement and foetal calf serum were from Gibco (Invitrogen, Milan, Italy). Rosmarinic acid, L-glutamine, L-glutamate, penicillin, streptomycin, all-*trans*-RA, *t*-BOOH and vitamin E (α-tocopherol) and all other reagents were from Sigma-Aldrich (Milan, Italy). Clovamide was kindly provided by Professor Giovanni Appendino (DISCAFF, University of 'Piemonte Orientale', Novara, Italy).

Clovamide, rosmarinic acid and Vitamin E were dissolved in dimethylsulphoxide (DMSO); L-glutamate and (+)–MK 801 were dissolved in PBS. Final drug concentrations were obtained by dilution of stock solutions in the experimental buffers. Final concentration of DMSO was always less than 0.1%; equivalent amount of this solvent was always added to either clovamide- or rosmarinic acid-untreated cells, and it has no effects on cell viability. Clovamide, rosmarinic acid or vitamin E were added to the experimental buffers 1 h before either *t*-BOOH, L-glutamate, or OGD and were maintained for the entire experiment.

Results

Neuroprotective effects of clovamide and rosmarinic acid

To determine whether clovamide or rosmarinic acid may exert neuroprotective effects we evaluated their ability to protect neurons against three different harmful stimuli: oxidative stress, excitotoxicity and ischaemia–reperfusion injury.

tert-butylhydroperoxide induces a rapid, progressive and dose-dependent increase of free radical production in the differentiated SH-SY5Y cells (Amoroso *et al.*, 1999). Three hours cell exposure to 100 μmol·L^{-1} *t*-BOOH in experimental buffer at 37°C significantly ($P < 0.01$; $n = 6$) induced cell death, as indicated by the increase in the LDH activity measured in the experimental media of *t*-BOOH-treated cells (Figure 2A). Clovamide or rosmarinic acid concentrations between 10 and 100 μmol·L^{-1} significantly ($P < 0.01$; $n = 6$) prevented cell death (Figure 2A). Clovamide and rosmarinic acid showed similar potencies and efficacies: the calculated EC_{50} values were 3.6 μmol·L^{-1} and 3.7 μmol·L^{-1}, whereas the maximal cytoprotective effects for clovamide and rosmarinic acid were obtained at 100 μmol·L^{-1}. Vitamin E (50 μmol·L^{-1}), a lipid-soluble radical scavenger (van Acker *et al.*, 1993), used as internal positive control, significantly ($P < 0.01$; $n = 6$) reduced cell death (Figure 2A).

L-glutamate induces excitotoxicity in differentiated SK-N-BE(2) cells expressing NMDA-type of ionotropic glutamate receptors (Lombardi *et al.*, 2007). Twenty-four hours cell exposure to L-glutamate (1 mmol·L^{-1}) significantly ($P < 0.01$; $n = 6$) increased cell death, which was prevented by clovamide or rosmarinic acid (10–100 μmol·L^{-1}; $P < 0.01$; $n = 6$; Figure 2B). The calculated EC_{50} values were 3.7 μmol·L^{-1} and 2.5 μmol·L^{-1}, whereas the maximal cytoprotective effects for clovamide and rosmarinic acid were obtained at 100 μmol·L^{-1}. (+)-MK-801 (1 μmol·L^{-1}), a selective and non-competitive NMDA receptor antagonist (Wong *et al.*, 1986) or vitamin E (50 μmol·L^{-1}), used as internal positive controls, both produced a significant ($P < 0.01$; $n = 6$) reduction in cell death induced by L-glutamate (Figure 2B).

As ischaemia–reperfusion injury engages many of the terminal pathways involved in the oxidative stress- and excitotoxicity-induced cell death (Lipton, 1999), we then aimed to confirm the neuroprotective effects of clovamide or rosmarinic acid in an *in vitro* model of ischaemia–reperfusion. Exposure of differentiated SH-SY5Y cells to 5 h OGD leads to ischaemia-induced cell death (Miglio *et al.*, 2004). To study if clovamide or rosmarinic acid are able to protect neurons against this insult, increasing concentrations (0.1–100 μmol·L^{-1}) of these compounds were added to the ischaemic buffer, and cell death was measured either at the end of the OGD period (5 h) (Figure 2C) or after 20 h of reoxygenation (Figure 2D). Clovamide or rosmarinic acid concentrations between 10 and 100 μmol·L^{-1} significantly ($P < 0.05$; $n = 6$) prevented cell death (Figure 2C and D). The EC_{50} values were 0.9 μmol·L^{-1} and 1.5 μmol·L^{-1} (at the end of OGD), and 1.6 μmol·L^{-1} and 1.8 μmol·L^{-1} (at the end of OGD/reoxygenation) for clovamide and rosmarinic acid respectively. The maximum cytoprotective effects at the end of the OGD and at 20 h for clovamide and rosmarinic acid were obtained at 100 μmol·L^{-1}. Vitamin E (50 μmol·L^{-1}), used as positive control, was similarly cytoprotective ($P < 0.05$; $n = 6$) (Figure 2C and D).

Taken together these results clearly demonstrate that both clovamide and rosmarinic acid exert significant neuroprotective effects against different harmful stimuli.

Antioxidant effects of clovamide or rosmarinic acid

To investigate the mechanisms underlying the neuroprotective effects of clovamide and rosmarinic acid, we first studied the ability of these compounds to reduce damages induced by oxidative stress, such as depletion of intracellular GSH and increase in lipoperoxidation. We measured the levels of intra-

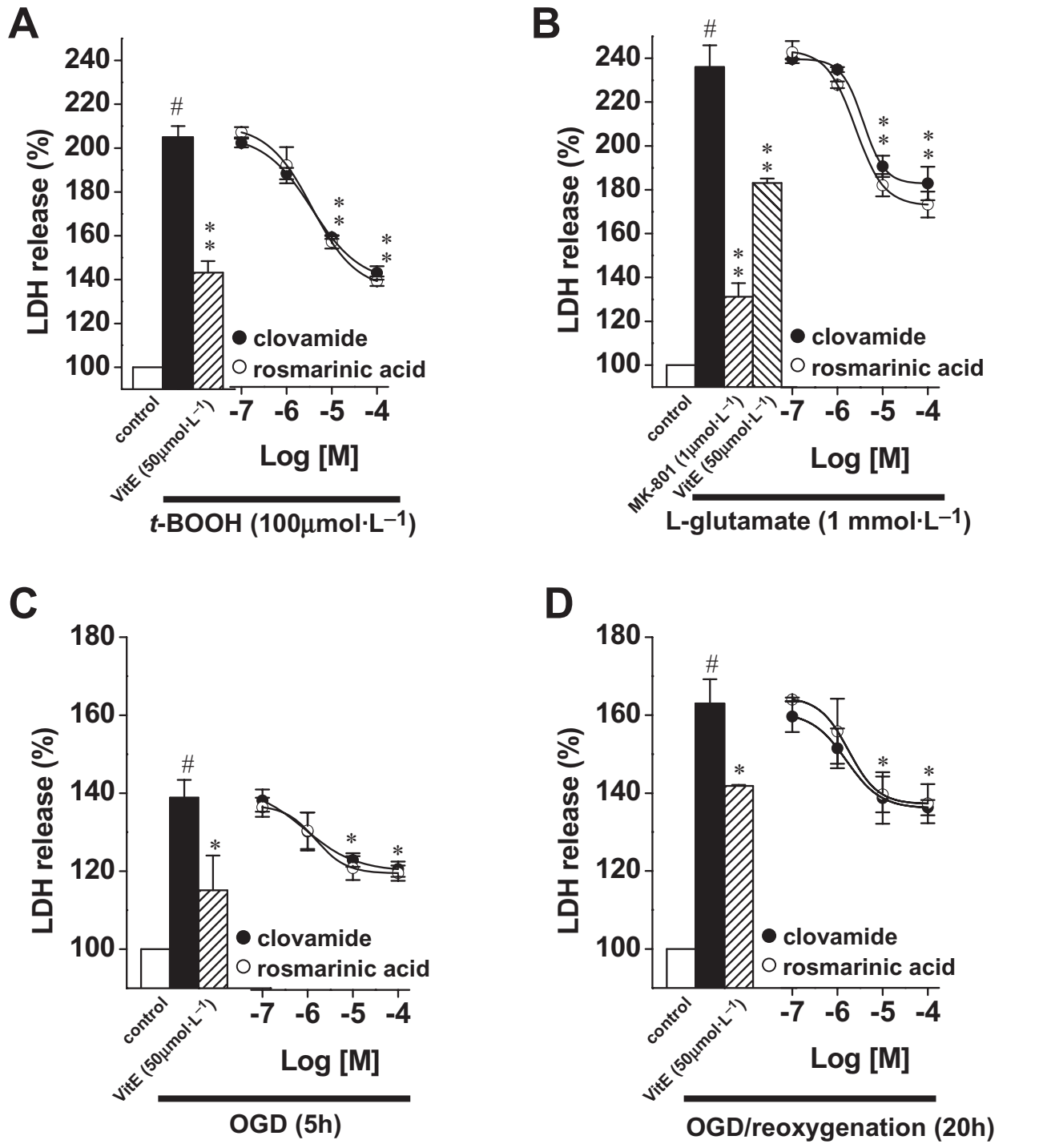


Figure 2 Concentration–response curves of the neuroprotective effects of clovamide or rosmarinic acid. (A) Effects of increasing concentrations of clovamide or rosmarinic acid (0.1–100 μmol·L^{-1}) on *t*-BOOH (100 μmol·L^{-1}; 3 h)-induced cell death, expressed as percentage of LDH released from damaged cells over that released from cells treated with DMSO alone (control). (B) Effects of increasing concentrations of clovamide or rosmarinic acid (0.1–100 μmol·L^{-1}) on L-glutamate (1 mmol·L^{-1}; 24 h)-induced cell death, expressed as percentage of LDH released from damaged cells over that released from cells treated with DMSO alone (control). (C and D) Effects of increasing concentrations of clovamide or rosmarinic acid (0.1–100 μmol·L^{-1}) on OGD- (5 h) (C) or OGD (5 h)/reoxygenation (20 h) (D)-induced cell death, expressed as percentage of LDH released from damaged cells over that released from cells treated with DMSO alone (control). Vitamin E (VitE; 50 μmol·L^{-1}) and (+)–MK 801 (MK-801; 1 μmol·L^{-1}) were used as internal positive controls. The data represent mean ± SEM of at least six experiments run in triplicate. #$P < 0.01$ versus cells treated with DMSO alone (control); **$P < 0.01$, *$P < 0.05$ versus *t*-BOOH (A)-, L-glutamate (B)-, or OGD (C and D)-treated cells. DMSO, dimethylsulphoxide; LDH, lactate dehydrogenase; OGD, oxygen-glucose deprivation; *t*-BOOH, *tert*-butylhydroperoxide.

cellular GSH and the contents of TBARS, as index of lipoperoxidation, in differentiated SH-SY5Y cells exposed to *t*-BOOH (100 μmol·L^{-1}; 3 h) in the presence/absence of clovamide or rosmarinic acid. *t*-BOOH significantly ($P < 0.01$; $n = 5$) decreased the intracellular GSH levels (Figure 3A) and increased the contents of TBARS (Figure 3B), relative to those in untreated cells. Clovamide or rosmarinic acid (10–100 μmol·L^{-1}) abolished these effects ($P < 0.01$; $n = 5$). The EC_{50} values were 4.2 μmol·L^{-1} and 8.8 μmol·L^{-1} for the effects on GSH levels, and 2.0 μmol·L^{-1} and 3.4 μmol·L^{-1} for the effects on TBARS levels respectively. Vitamin E (50 μmol·L^{-1}), used as internal positive control, also produced significant ($P < 0.01$; $n = 5$) antioxidant effects for both GSH and TBARS (Figure 3A and B).

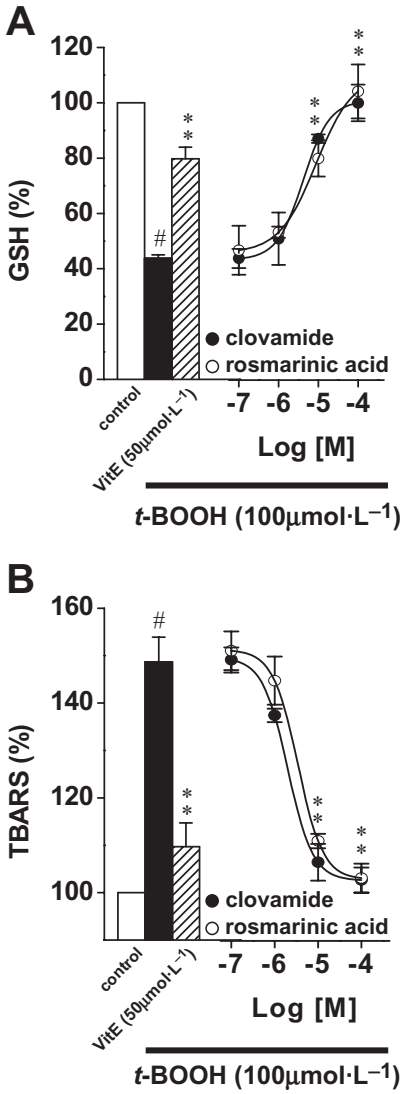


Figure 3 Antioxidant effects of clovamide or rosmarinic acid. (A) Ability of increasing concentrations (0.1–100 μmol·L^{-1}) of either clovamide or rosmarinic acid in reducing *t*-BOOH-induced GSH decrease, expressed as percentage of GSH over cells treated with DMSO alone (control). (B) Ability of increasing concentrations (0.1–100 μmol·L^{-1}) of either clovamide or rosmarinic acid in reducing lipoperoxidation (TBARS increase), expressed as percentage of the TBARS level in cells treated with DMSO alone (control). Vitamin E (VitE; 50 μmol·L^{-1}) was used as internal positive control. The data represent mean ± SEM of at least five experiments run in triplicate. #$P < 0.01$ versus cells treated with DMSO alone (control); **$P < 0.01$ versus *t*-BOOH-treated cells. DMSO, dimethylsulphoxide; GSH, glutathione; *t*-BOOH, *tert*-butylhydroperoxide; TBARS, thiobarbituric acid-reacting substances.

Effects of clovamide and rosmarinic acid on L-glutamate-induced $[Ca^{2+}]_i$ increase

As protracted entry of Ca^{2+} into neurons is one of the main mechanisms leading to neuronal death (Choi, 1988) induced by excitotoxicity, we then evaluated if clovamide and rosmarinic acid may prevent L-glutamate-induced $[Ca^{2+}]_i$ increase. The mean fluorescence ratio (MFI) was used as the measure of $[Ca^{2+}]_i$, and this ratio in fura-2-loaded cells exposed to DMSO alone (control) was significantly increased by exposure to L-glutamate (1 mmol·L^{-1}; 24 h; $P < 0.01$; $n = 6$; Figure 4). When clovamide or rosmarinic acid (10 μmol·L^{-1}) were added to the experimental buffer, the L-glutamate-induced $[Ca^{2+}]_i$ increase was significantly ($P < 0.05$; $n = 6$) prevented (Figure 4).

Effects of clovamide and rosmarinic acid on c-jun *or* c-fos *expression*

As the abnormal transcriptional induction of nuclear responses amplifies the progression of cascade induced by

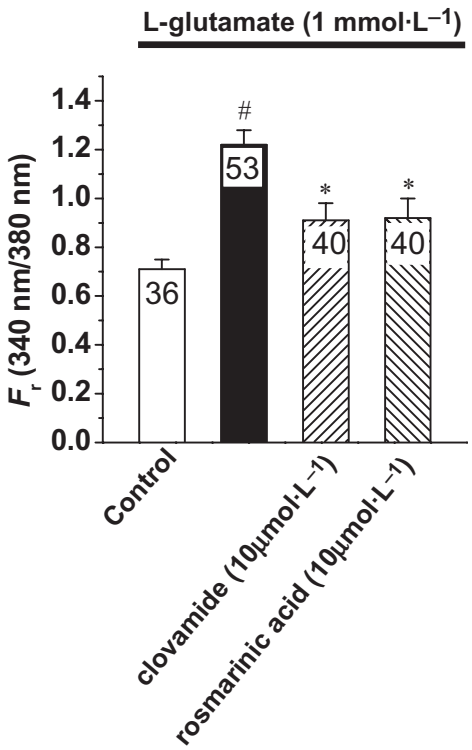


Figure 4 Effects of clovamide or rosmarinic acid on the $[Ca^{2+}]_i$ increase induced by L-glutamate treatment. $[Ca^{2+}]_i$ was measured at single cell level in fura-2/acetoxymethyl ester-loaded cells exposed to L-glutamate (1 mmol·L^{-1}; 24 h) in the absence or presence of 10 μmol·L^{-1} clovamide or rosmarinic acid. The values are expressed as F_r (MFI) mean of 36–53 cells monitored. The data represent mean ± SEM of at least six experiments run in triplicate. #$P < 0.01$ versus cells treated with DMSO alone (control); *$P < 0.05$ versus L-glutamate-treated cells. $[Ca^{2+}]_i$, intracellular calcium concentration; DMSO, dimethylsulphoxide; MFI, mean fluorescence ratio.

excitotoxicity (Hughes and Dragunow, 1995), we evaluated the ability of clovamide and rosmarinic acid to modulate L-glutamate-induced expression of immediate early genes, such as *c-jun* and *c-fos* (Lidwell and Griffiths, 2000; Raivich and Behrens, 2006). SH-SY5Y cells were treated with 1 mmol·L^{-1} L-glutamate for 24 h, and *c-fos* and *c-jun* expressions were measured by semi-quantitative RT-PCR analysis. Low constitutive levels of gene expression were detected in cells treated with DMSO alone (control); cell exposure to L-glutamate significantly ($P < 0.01$; $n = 4$) induced an up-regulation of the expression of *c-fos* and *c-jun* (Figure 5). Clovamide or rosmarinic acid (10 μmol·L^{-1}) abolished ($P < 0.01$; $n = 4$) L-glutamate-induced *c-fos* gene expression, whereas they did not significantly modify L-glutamate-induced *c-jun* gene expression at all concentrations tested (0.1–100 μmol·L^{-1}) (Figure 5A and B).

Effects of clovamide or rosmarinic acid on NF-κB activation

As NF-κB in the CNS is responsible for the activation of cellular signals mediating cell survival or damage (Mémet, 2006), we then investigated whether clovamide or rosmarinic acid are able to modulate NF-κB activation in SH-SY5Y cells not exposed to harmful stimuli. Activated NF-κB is usually present in the cytoplasm as a p50/p65 heterodimer and after activation translocates into the nucleus (Kim *et al*., 2008). Therefore we evaluated the ability of clovamide or rosmarinic acid to affect the nuclear translocation of the NF-κB subunits p50 and p65. Figure 6A and B show the nuclear/cytoplasmic ratio of NF-κB subunits, and this ratio was significantly ($P < 0.05$; $n = 4$) inhibited by rosmarinic acid (10 μmol·L^{-1}) for both p50 and p65 subunits, whereas clovamide at the same concentration was not effective (reduction not statistically significant).

Effects of clovamide or rosmarinic acid on PPARγ expression

To clarify whether clovamide or rosmarinic acid inhibits NF-κB nuclear translocation through the modulation of the cellular expression of PPARγ, a nuclear receptor able to down-regulate NF-κB activation (Kim *et al*., 2003), we measured PPARγ expression in SH-SY5Y cells, not exposed to harmful stimuli, untreated or treated with these compounds.

A low basal constitutive protein expression (PPARγ/β-actin ratio = 0.100 ± 0.002; $n = 5$) was detected in untreated control

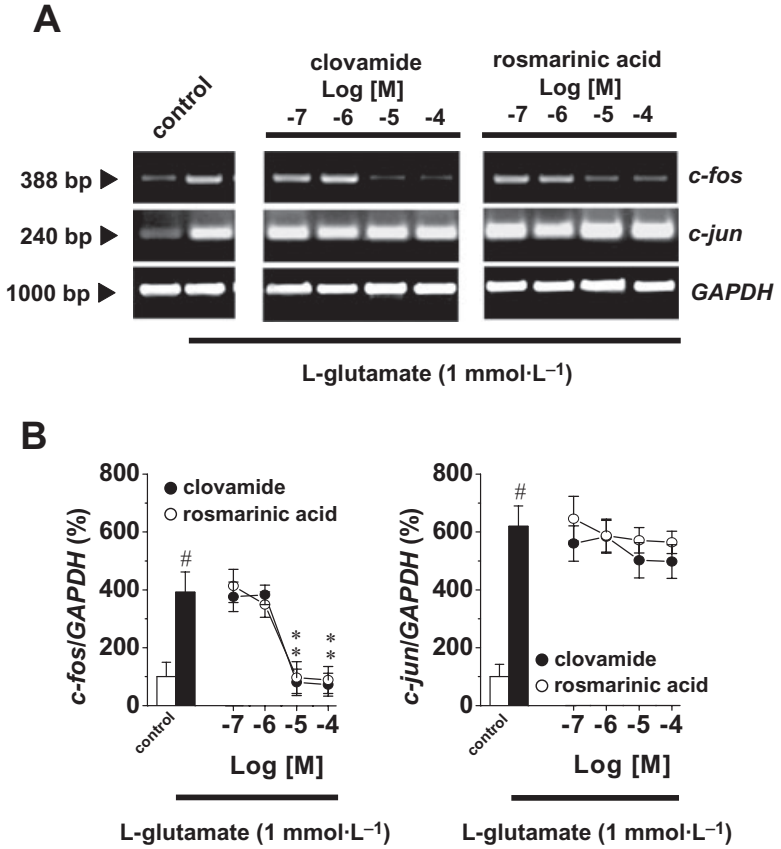


Figure 5 Effects of clovamide or rosmarinic acid on L-glutamate-induced *c-fos* and *c-jun* gene expression. (A) *c-fos* and *c-jun* gene expression was quantified by semi-quantitative RT-PCR in SK-N-BE(2) cells untreated or treated with L-glutamate (1 mmol·L^{-1}; 24 h) in the absence or presence of increasing concentration (0.1–100 μmol·L^{-1}) of clovamide or rosmarinic acid. The extracted total mRNA was reverse transcribed into its related cDNA, and PCR was carried out to amplify *c-fos* and *c-jun* cDNA by using specific primers (see Table 1). Expression of *GAPDH* was used as a loading control. PCR products were visualized with ethidium bromide on a 1% agarose gel. (B) The signals are densitometrically analysed; data, calculated as mean ± SEM of at least four determinations, are expressed as the ratio of the signal obtained for each sample divided by that obtained for *GAPDH* in the same sample to permit between-sample comparisons. #$P < 0.01$ versus cells treated with DMSO alone (control); **$P < 0.01$ versus L-glutamate-treated cells. DMSO, dimethylsulphoxide; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; RT-PCR, reverse transcriptase-PCR.

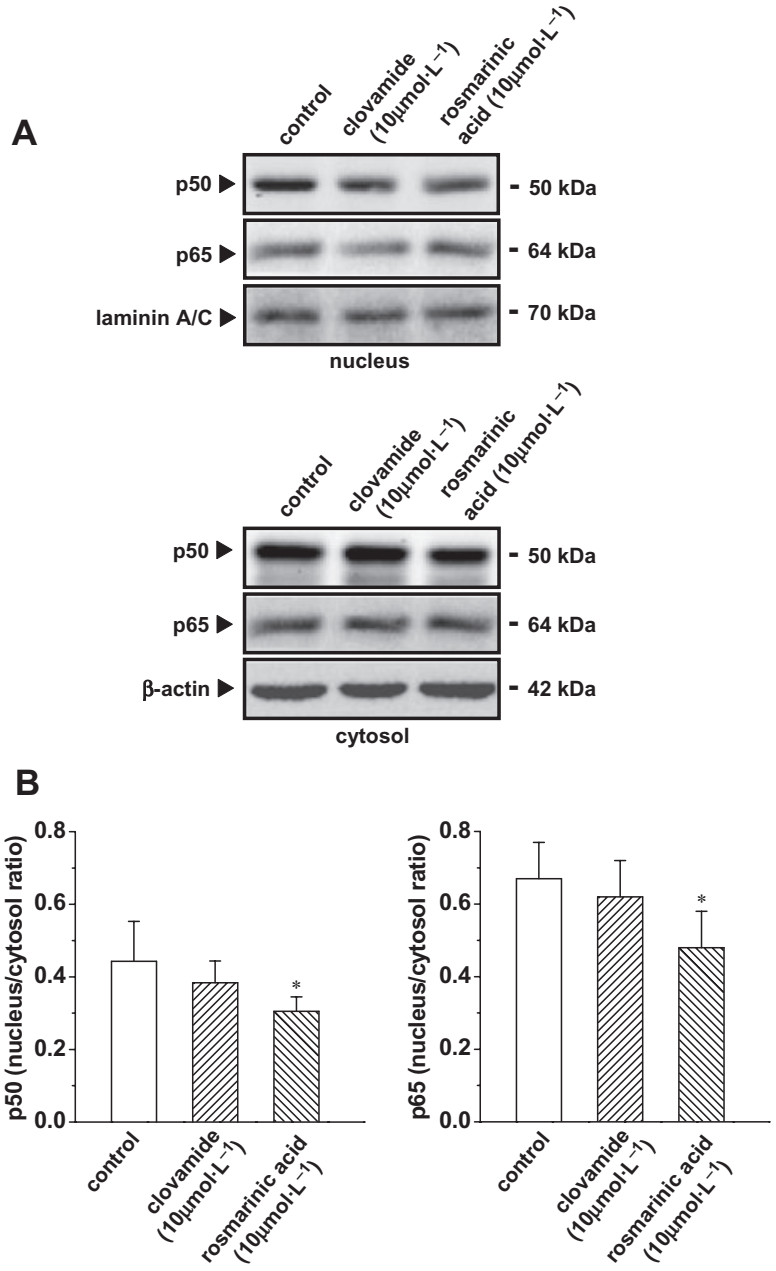


Figure 6 Effects of clovamide or rosmarinic acid on NF-κB activation in SH-SY5Y cells not exposed to harmful stimuli. Both compounds were used at 10 μmol·L^{-1}; results are presented as the nuclear/cytoplasmic ratio of NF-κB p50 and p65 subunits (see *Methods*) of at least four determinations. *$P < 0.05$ versus cells treated with DMSO alone (control). DMSO, dimethylsulphoxide; NF-κB, nuclear factor-κB.

cells. When cells were treated (6 h) with 15-deoxy-$\Delta^{12,14}$-PGJ_2, an endogenous PPARγ ligand (Kim *et al.*, 2003), used as internal positive control, a concentration-dependent (2–20 μmol·L^{-1}) enhancement ($P < 0.01$; $n = 5$) of PPARγ protein expression was measured, with maximal effects (ratio 0.41 ± 0.03) at 20 μmol·L^{-1} (Figure 7A). Similar results were obtained after treatment of cells for 6 h with clovamide or rosmarinic acid (10 μmol·L^{-1}). As shown in Figure 7B, clovamide or rosmarinic acid significantly enhanced PPARγ expression by about 1.5-fold ($P < 0.05$; $n = 5$) and fourfold ($P < 0.01$; $n = 5$) over untreated control cells respectively (Figure 7B). In these conditions, rosmarinic acid was more potent than 15-deoxy-$\Delta^{12,14}$-PGJ_2, the endogenous PPARγ ligand used as a positive control, in increasing PPARγ expression (fourfold increase at 10 and 20 μmol·L^{-1} respectively) (Figure 7B).

Discussion

Results reported here clearly show that both clovamide and rosmarinic acid are able to protect neurons from injury in three *in vitro* models of neuronal death: oxidative stress, excitotoxicity and OGD/reoxygenation. The neuroprotective effects are linked to their ability to modulate the progression of events leading to neuronal death.

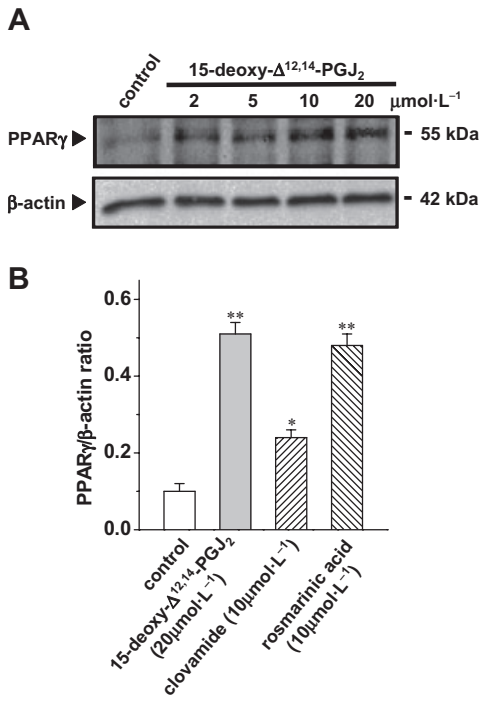


Figure 7 Effects of clovamide or rosmarinic acid on PPARγ expression in SH-SY5Y cells not exposed to harmful stimuli. (A) The endogenous PPARγ agonist 15-deoxy-$\Delta^{12,14}$-PGJ_2, used as positive internal control, increases PPARγ expression in a concentration-dependent manner (2–20 μmol·L^{-1}) in comparison with control. (B) Effects of 10 μmol·L^{-1} clovamide or rosmarinic acid and 20 μmol·L^{-1} 15-deoxy-$\Delta^{12,14}$-PGJ_2 on PPARγ expression. Results are presented as PPARγ/β-actin ratio (see *Methods*) of at least five determinations. *$P < 0.05$; **$P < 0.01$ versus cells treated with DMSO alone (control). 15-deoxy-$\Delta^{12,14}$-PGJ_2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2; PPARγ, peroxisome proliferator-activated receptor-γ.

Rosmarinic acid, a widely distributed natural compound, is present in several plants and is commercially available in a highly (>97%) purified form, while clovamide is present only in few natural sources and is not commercially available. To overcome this problem Arlorio *et al.* (2008), from our Department, reported the synthesis of clovamide, and we have taken advantage of this opportunity to study its biological activities further. In all experiments we have performed, the activity of clovamide was always compared with that of rosmarinic acid, because we aimed to clarify the role of the ester/amide isostery on the activity of caffeic conjugates. Furthermore, as polyphenolics have complex pharmacokinetic properties and are poorly adsorbed after oral administration (Baba *et al.*, 2004; Konishi *et al.*, 2005), we have hypothesized that specific structural modifications, such as the substitution of an ester function with an amide in the clovamide molecule, could confer an higher metabolic stability to the compound and increase its bioavailability. From our results, clovamide and rosmarinic acid exhibit *in vitro* similar potencies and efficacies: the calculated EC_{50} values were in the low micromolar range (0.9–3.7 μmol·L^{-1}), while the maximal protective effects were around 50% inhibition of cell death at 100 μmol·L^{-1}. The overall results demonstrate that the substitution of an ester with an amide is not sufficient to significantly change the activities of these compounds in evoking neuroprotective effects. On the other hand, this result is not surprising, due to the close structural similarity of clovamide and rosmarinic acid molecules.

To study possible neuroprotective effects of clovamide and rosmarinic acid, we have used three different *in vitro* models of cell death (oxidative stress, excitotoxicity and OGD/reoxygenation), extensively used in our laboratories for testing compounds (Lombardi *et al.*, 2002; 2007; Miglio *et al.*, 2004). Here we have decided to use all three experimental models to increase the predictive power for the *in vivo* effects, which are currently under evaluation in our laboratories.

The models of oxidative stress and excitotoxicity strictly reproduce toxic damages induced by free radicals and excitatory amino acid accumulations (Choi, 1988; Beal *et al.*, 1993), while OGD/reoxygenation is a complex model that reproduces *in vitro* the multiple fatal cellular events contributing *in vivo* to ischaemia–reperfusion damages (Lipton, 1999). For our experiments, two human neuroblastoma cell lines [SH-SY5Y and SK-N-BE(2)] were used, and these cells were differentiated into the neuronal type by treatment with 10 μmol·L^{-1} RA before experiments. Undifferentiated tumour cells are less vulnerable to chemical changes (Benedetti *et al.*, 1984), and neuroblastoma cell lines acquire morphological, neurochemical and electrophysiological properties of neurons (Biedler *et al.*, 1978) only after adequate RA treatments. In our experience, SH-SY5Y and SK-N-BE(2) human cell lines, which derive from different cell clones, need to be treated with the same concentration (10 μmol·L^{-1}) of RA for different periods of time (7 days for oxidative stress and 14 days for excitotoxicity or ischaemia) for assuring easily measurable and reproducible responses to the different insults (Lombardi *et al.*, 2002; 2007; Miglio *et al.*, 2004). This can probably be ascribed to the particular sensitivity of SH-SY5Y cells, treated with RA for 7 days, to ROS (Amoroso *et al.*, 1999) and to the necessity of 14 days of RA treatments for both cell lines to express functional glutamate receptors (Nair *et al.*, 1996; Lombardi *et al.*, 2007), which play a key role in excitotoxicity and ischaemia–reperfusion insults.

In all three models, a cell pretreatment (1 h) with the compounds was necessary to achieve significant neuroprotective effects. Both clovamide and rosmarinic acid were, indeed, ineffective when added after or simultaneously to harmful stimuli (data not shown). This property, undoubtedly, could represent an unfavourable pharmacological characteristic for acute drug treatments, but we are confident that chemical modifications on the molecules will be helpful to overcome this problem (SAR studies are currently in progress in our laboratories).

The neuroprotective effects of both clovamide and rosmarinic acid seem to be linked to the antioxidant/radical scavenging properties of these compounds and to their ability to modulate some of the intracellular cascade events leading to neuronal death. The ability of clovamide and rosmarinic acid to scavenge free radical species has been already reported in a cell-free system (Arlorio *et al.*, 2008), and our data confirm and complete these previous observations in cell-based systems. Furthermore, we add new insights showing that both clovamide and rosmarinic acid are able to modulate some of the intracellular events (e.g. Ca^{2+} overload, *c-fos* expression) involved in neuronal death (Lipton and Rosen-

berg, 1994; Barnham *et al*., 2004; Mémet, 2006). From our data, both compounds are able to prevent *c-fos*, but not *c-jun* expression. This might be ascribed to differences in the levels of gene responses of cells to a same stimulus (see Figure 5), or to intrinsic experimental problems. Additional experiments aimed to reproduce the effects of clovamide or rosmarinic acid on the modulation of other immediate early gene products, such as *jun-B* and *zif/268*, might help to clarify this point.

As previously reported by Kim *et al*. (2003), SK-N-SH and SK-N-MC human neuroblastoma cell lines express a constitutively high level of NF-κB activity, which is inhibited, in a time-dependent manner, by the PPARγ agonist, 15-deoxy-$\Delta^{12,14}$-PGJ_2. We have expanded these observations to SH-SY5Y cells, showing that these cells also exhibited constitutive NF-κB activity and providing evidence that rosmarinic acid (10 μmol·L^{-1}) inhibited the nuclear translocation of NF-κB p50 and p65 subunits, while clovamide was ineffective at the same concentration. This activity of rosmarinic acid could be potentially relevant to explain not only the neuroprotective effects herein described, but also the wide range of biological effects, such as antimicrobial, antiviral and immunosuppressive, which have been demonstrated for this compound by other groups (see Introduction).

Finally, we showed that SH-SY5Y cells express the nuclear receptor PPARγ, and that clovamide or rosmarinic acid treatment can enhance the expression of this protein. This result is consistent with published data, showing that PPARγ is expressed at low levels in SH-SY5Y cells (Valentiner *et al*., 2005), while it is expressed at high levels in two other human neuroblastoma cell lines (SK-N-SH and SK-N-MC), which are precursors of SH-SY5Y cells (Kim *et al*., 2003). Of note, PPARγ stimulation produces neuroprotection both *in vitro* and *in vivo* models (Dehmer *et al*., 2004; Schütz *et al*., 2005; Culman *et al*., 2007), and rosiglitazone, a potent PPARγ agonist, protects SH-SY5Y cells against the neurotoxin MPP^+-induced cytotoxicity by inhibiting mitochondrial dysfunction and ROS overproduction (Jung *et al*., 2007). Rosiglitazone induces the activities of scavenger enzymes (superoxide dismutase and catalase), while it does not inhibit ROS production per se. Similar mechanisms might contribute to the protective effects we have measured in the excitotoxicity and OGD/reoxygenation models that are characterized by longer kinetics for cell death. Further experiments will be necessary to clarify this point.

On the other hand, PPARγ activation is able to down-regulate the nuclear translocation of NF-κB subunits in SK-N-SH and SK-N-MC cells (Kim *et al*., 2003). Our results, demonstrating that rosmarinic acid is able to activate PPARγ and to inhibit NF-κB, suggest that the effects evoked by this compound are likely due, at least in part, to its ability to modulate nuclear events.

It is noteworthy that rosmarinic acid, but not clovamide, was effective in inhibiting NF-κB activation, and that rosmarinic acid was more effective than clovamide in inducing PPARγ expression at the same concentration (10 μmol·L^{-1}). This result allows us to speculate that the substitution of ester function with amide into the clovamide molecule can significantly reduce the activity of the compound at the nuclear level. The determination of the effects evoked by compounds bearing other functional groups (e.g. a tertiary amide lacking the ability to form hydrogen bonds) at the same position will clarify this point.

Taken together, our data shed new light on the promising pharmacological properties of clovamide and rosmarinic acid, disclosing that these isosteric polyphenolics are neuroprotective in three different *in vitro* models of neuronal death, and identifying new molecular targets for their actions.

Acknowledgement

The study was supported by grants from the University of 'Piemonte Orientale Amedeo Avogadro' (Italy).

Conflict of interest

None.

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