

# Flavonoids protect neurons from oxidized low-density-lipoprotein-induced apoptosis involving c-Jun N-terminal kinase (JNK), c-Jun and caspase-3

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Oxidative stress has been associated with neuronal loss in neurodegenerative diseases and during age-associated cognitive decline. Flavonoids have been proposed to play a useful role in protecting the central nervous system against oxidative and excitotoxic stress, although the mechanism of action is unknown. Using oxidized low-density lipoprotein (oxLDL) as the oxidative insult we investigated the mechanism of neurotoxicity and attempted to identify possible sites of action of two of the most potent protective flavonoids, epicatechin and kaempferol, in cultured primary neurons. Using cultured striatal neurons and selective phosphospecific antibodies we addressed the potential role of extracellular signal-regulated kinases 1/2 (ERK1/2) and c-Jun N-terminal kinase (JNK). OxLDL stimulated a Ca<sup>2+</sup>-dependent activation of both ERK1/2 and JNK that was strongly inhibited by pre-treatment with low micromolar concentrations of epicatechin. Neurotoxicity induced by oxLDL, however, was neither reduced nor enhanced by inhibiting ERK1/2 activation with mitogen-activated protein kinase kinase (MEK) inhibitors, suggesting that this cascade is unlikely to be involved in either

oxLDL toxicity or the protective effects of flavonoids. oxLDL caused a sustained activation of JNK that resulted in the phosphorylation of the transcription factor c-Jun, which was abolished in neurons pre-treated with flavonoids. Furthermore, oxLDL induced the cleavage of procaspase-3 and increased caspase-3-like protease activity in neurons, an effect which was strongly inhibited by pre-exposure to either epicatechin or kaempferol. In addition, a caspase-3 inhibitor reduced oxLDL-induced neuronal death, implicating an apoptotic mechanism. A major *in vivo* metabolite of epicatechin, 3'-O-methyl-epicatechin was as effective as epicatechin in protecting neurons. Thus dietary flavonoids might have potential as protective agents against neuronal apoptosis through selective actions within stress-activated cellular responses, including protein kinase signalling cascades.

**Key words:** epicatechin, ERK, MAP kinase, oxidative stress, striatal neuron.

## INTRODUCTION

There is increasing evidence that oxidative stress contributes to the neuropathology of a number of neurodegenerative disorders [1], including Alzheimer's [2], Parkinson's [3] and Huntington's [4] diseases. Oxidative stress has also been implicated in neuronal loss associated with cognitive decline, cerebral ischaemia and seizures [5]. Consequently, there is growing interest in establishing therapeutic and dietary strategies to combat oxidative-stress-induced damage to the central nervous system, and attention is turning towards the potential neuroprotective effects of dietary antioxidants, especially flavonoids. Indeed, studies in humans and animals suggest that flavonoids, a large group of polyphenolic compounds found in fruit and vegetables, may play a useful role in preventing age-related cognitive, motoric and mood decline [6–9] and protect against oxidative stress [10] as well as cerebral ischaemia/reperfusion injuries [11]. The precise mechanisms by which flavonoids exert their neuroprotective actions *in vivo* are presently unknown and it is currently unclear whether or not these compounds function as hydrogen-donating antioxidants or exert their neuroprotective actions independently of such properties.

Accumulating evidence suggests that flavonoids interact selectively within mitogen-activated protein kinase (MAPK) sig-

nalling cascades [12,13]. This could have important implications with regard to their possible sites of action in neurons, since members of the MAPK family are believed to be involved in signalling to neuronal survival, regeneration and death [14–16].

The molecular mechanisms underlying oxidative-stress-induced neuronal damage are emerging and appear to involve an apoptotic mode of death in which extracellular signal-regulated kinases 1 and 2 (ERK1/2) [17–19] and c-Jun N-terminal kinase (JNK) [20] have been strongly implicated. Furthermore, it is becoming clear that products of lipid peroxidation such as 4-hydroxy-2,3-nonenal (4-HNE), lipid hydroperoxides (LOOHs) and oxysterols are important mediators of oxidative-stress-mediated apoptosis in the central nervous system [21,22], possibly through activation of the transcription factor activator protein-1 (AP-1) complex [20].

Oxidized low-density lipoprotein (oxLDL), which contains typical products of lipid peroxidation, such as LOOHs, 4-HNE or oxysterols, has been shown to be neurotoxic [21,23,24]. Furthermore, recent studies have demonstrated that oxLDL enters primary cultured striatal neurons and induces cell death [25]. Pre-treatment of neurons with flavonoids, in particular epicatechin, protected against oxLDL-induced neuronal death [25].

Abbreviations used: oxLDL, oxidized low-density lipoprotein; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; ERK1/2, extracellular signal-regulated kinases 1 and 2; JNK, c-Jun N-terminal kinase; 4-HNE, 4-hydroxy-2,3-nonenal; LOOH, lipid hydroperoxide; DCDHF-DA, 2',7'-dichlorodihydrofluorescein diacetate; DCF, 2',7'-dichlorofluorescein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; LDH, lactate dehydrogenase; HBTM, HEPES-buffered toxicity medium; HBM, HEPES-buffered incubation medium; AP-1, activator protein-1; pAb, polyclonal antibody; Dil, 1,1'-dioctadecyl-3,3',3'-tetra-methyl-indocarbocyanine perchlorate; DEVD-CHO, Asp-Glu-Val-Asp-aldehyde.

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In this study, the potential roles of ERK1/2, JNK, c-Jun and caspases in the mechanisms underlying neuronal cell damage resulting from oxidative stress mediated by oxLDL have been investigated. In addition, we have addressed the question concerning the mechanism of the neuroprotective effects of flavonoids in relation to inhibitory actions involving downstream signalling components that have been linked to neuronal apoptosis.

## MATERIALS AND METHODS

### Materials

Epicatechin and kaempferol were purchased from Extrasynthese (Genay, France). 3'-O-Methyl-epicatechin was a gift from the laboratory of Dr J. P. E. Spencer (King's College, London, U.K.). Swiss mice were obtained from Harlan (Bicester, Oxon, U.K.). PD98059 and U0126 were from Alexis (San Diego, CA, U.S.A.). 2',7'-Dichlorodihydrofluorescein diacetate (DCDHF-DA) was obtained from Molecular Probes (Eugene, OR, U.S.A.) and the protein assay reagent was purchased from Bio-Rad (Hemel Hempstead, Herts., U.K.). Anti-ACTIVE MAPK polyclonal antibody (pAb) and anti-ACTIVE JNK pAb were obtained from Promega (Madison, MI, U.S.A.). Anti-phospho-Jun (Ser-73) pAb was from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Anti-pro-caspase-3 (H-277) pAb, anti-ERK1/ERK2 pAb and anti-JNK1 pAb were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Biotinylated goat anti-rabbit IgG was obtained from Vector Laboratories (Burlingame, CA, U.S.A.) and the enhanced chemiluminescence (ECL) reagent and Hyperfilm-ECL<sup>®</sup> were purchased from Amersham (Little Chalfont, Bucks., U.K.). The fluorescent probe 1,1'-dioctadecyl-3,3,3',3'-tetra-methyl-indocarbocyanine perchlorate (DiI) and the colorimetric caspase-3 assay were from Sigma (Poole, Dorset, U.K.). Cell-permeable caspase-3 inhibitor Asp-Glu-Val-Asp-aldehyde (DEVD-CHO) was from Calbiochem (Nottingham, U.K.). All other reagents were obtained from Sigma or Merck (Poole, Dorset, U.K.).

### Cell culture

Primary cultures of mouse striatal neurons were prepared essentially as described previously [26]. In brief, striata were dissected from 14–16 day-old Swiss mouse embryos and mechanically dissociated, using a fire-polished Pasteur pipette in PBS supplemented with glucose (33 mM). Cells were plated into 24- or 6-well Nunc multi-well plates that had been coated previously overnight with 15 µg/ml poly-L-ornithine and then with culture medium supplemented with 10% bovine calf serum for 2 h.

After removal of the final coating solution, cells were seeded ( $1 \times 10^6$  cells/ml; 6- and 24-well plates containing 2.0 ml or 0.5 ml of medium, respectively) in a serum-free medium composed of a mixture of Dulbecco's modified Eagle's medium and F-12 nutrient (1:1) supplemented with glucose (33 mM), glutamine (2 mM), sodium bicarbonate (6.5 mM), Hepes (pH 7.4, 5 mM), streptomycin (100 µg/ml) and penicillin (60 µg/ml). A mixture of hormones and salts composed of insulin (25 µg/ml), transferrin (100 µg/ml), putrescine (60 µg/ml), progesterone (20 nM) and sodium selenate (30 nM) was also added to the cell-culture medium. The cells were cultured at 37 °C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>. After 5–7 days in serum-free culture medium, the majority of cells were of a neuronal phenotype (> 98%) and there were < 2% detectable glial elements, as determined by a lack of glial fibrillary acidic protein (GFAP) immunoreactivity (results not shown).

### LDL isolation and oxidation

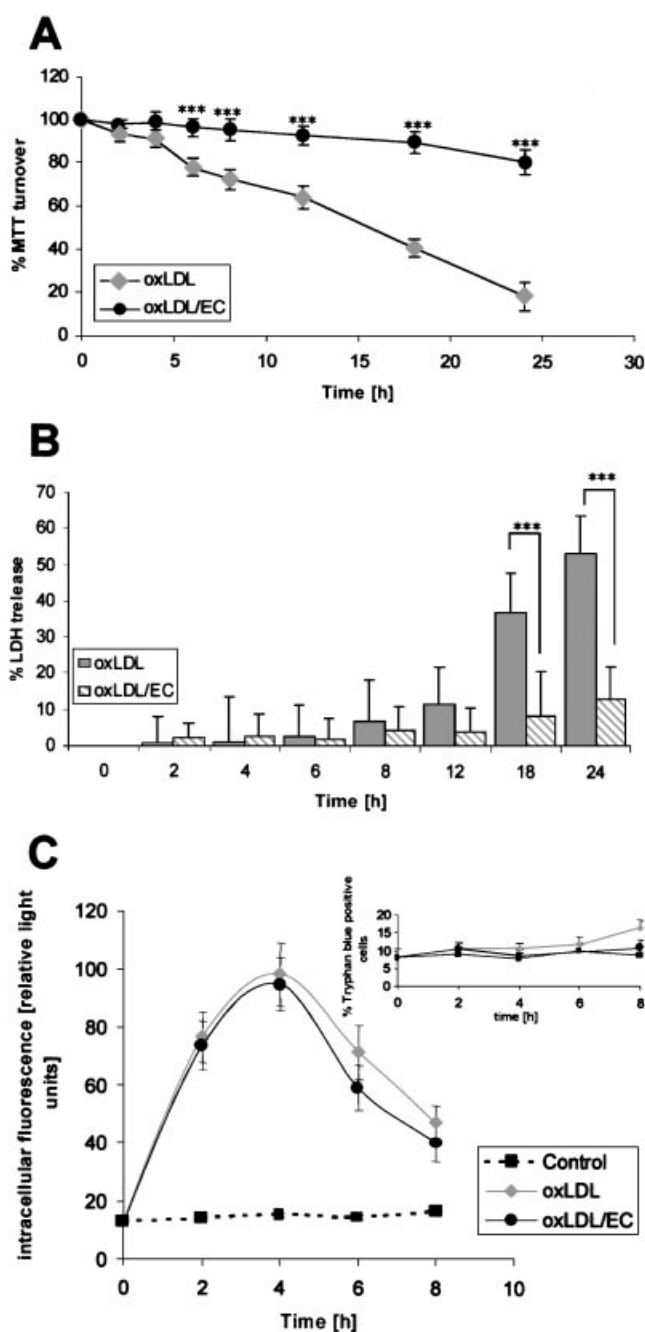
LDL was isolated as described previously [25,27] and stored under nitrogen at 4 °C until use within 1 week of isolation. The concentration of EDTA in LDL preparations was reduced prior to oxidation by dialysis against PBS (10 mM) containing 10 µM EDTA for 6 h. Lipoprotein concentration was expressed in terms of protein content [28]. Conditions for LDL oxidation were: 62.5 µg/ml LDL in PBS (10 mM) containing a final concentration of 5 µM CuSO<sub>4</sub> incubated at 37 °C for 45 min. Oxidation was followed by monitoring the increased formation of conjugated dienes at 234 nm and concentrations of conjugated dienes were calculated using an absorbance coefficient of  $2.8 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$  [29]. The concentration of LOOHs formed during LDL oxidation was also measured using the FOX assay (where FOX is ferrous-oxidation Xylenol Orange) as described previously [30].

To assess the extent of oxidation of LDL in terms of the modified surface charge on the apolipoprotein B100, lipoprotein electrophoresis was performed using a Beckman Paragon<sup>®</sup> electrophoresis system as described previously [25]. The concentration of LOOHs following oxidation was  $467 \pm 41$  nmol/mg of LDL protein ( $n = 5$ ) as determined using the FOX assay, and the concentration of conjugated dienes was  $227 \pm 36$  nmol/mg of LDL protein ( $n = 5$ ) as measured spectrophotometrically. The alteration of the surface charge of apolipoprotein B100, as assessed by measuring the electrophoretic mobility relative to BSA, was  $0.3 \pm 0.04$  ( $n = 3$ ) and  $0.47 \pm 0.03$  ( $n = 3$ ) for native LDL and oxLDL, respectively.

### Cell treatment

Flavonoid stock solutions were obtained by dissolving the compounds in 50% methanol. Neurons were cultured for 5 days, then the culture medium was replaced by Hepes-buffered toxicity medium (HBTM, pH 7.4: 5 mM Hepes, 154 mM NaCl, 4.6 mM KCl, 2.3 mM CaCl<sub>2</sub>, 33 mM glucose, 5 mM NaHCO<sub>3</sub>, 1.1 mM MgCl<sub>2</sub> and 1.2 mM Na<sub>2</sub>HPO<sub>4</sub>), and oxLDL (12.5 µg of protein/ml) was applied to the neurons for up to 24 h to investigate the neurotoxic effect of oxLDL. The potential neuroprotective effects of flavonoids against oxLDL-mediated cell death were investigated after pre-treating the neurons for 18 h with flavonoids by applying a flavonoid stock solution (50% methanol) directly into the cell medium to a final concentration of 30 µM (final concentration of methanol in the medium was less than 1%). The medium was then removed, and the cells were washed twice to remove exogenous flavonoids and replaced by HBTM. OxLDL (6.25–12.5 µg of protein/ml) was administered to the neuronal cultures and incubated for up to 24 h. Neuronal cell injury was evaluated after treatments by visual observation using a microscope (Leica, Fluovert) and by colorimetric assay (MTT assay) for mitochondrial function using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as described previously [26]. The data obtained were transformed into the percentage of MTT reduction, and since native LDL showed no effect compared with untreated controls it was taken to be 100%. To parallel and verify the MTT assay, lactate dehydrogenase (LDH)-release assays (LDH assays) were performed as described previously [25]. The absorbance data were transformed into the percentage of LDH release taking Triton X-100-treated control cultures as 100% enzyme release.

To assess the involvement of caspase-3 in oxLDL-induced neurotoxicity, neurons were pre-treated for 15 min with the cell-permeable caspase-3 inhibitor DEVD-CHO (100 µM), exposed to oxLDL (12.5 µg of protein/ml) for a further 18 h and cell damage was measured by the MTT assay as described above.



**Figure 1** Epicatechin protects neurons against oxLDL-mediated damage without significantly altering the increase in intracellular oxidative stress caused by oxLDL

Striatal neurons were exposed to oxLDL (12.5  $\mu\text{g}$  of protein/ml) for up to 24 h following pre-treatment with epicatechin (EC, 30  $\mu\text{M}$ ). Neuronal damage was assessed by MTT assay (A) and by LDH release (B) as described in the Materials and methods section. For (A) results are expressed as the percentage of MTT reduction relative to a native LDL control, which was defined as 100% (variations in MTT reduction for the native LDL control were less than 15% as judged from the absorbance data). Data given are means  $\pm$  S.D. from four independent cultures, each performed in triplicate. OxLDL induced a time-dependent decline in MTT turnover that was significantly attenuated by epicatechin ( $n = 6$ ,  $***P < 0.001$ , unpaired two-tailed Student's  $t$  test). For (B) results are expressed as the percentage of LDH release relative to a Triton X-100-treated control (100%). Epicatechin pre-treatment significantly reduced LDH release at 18 and 24 h ( $n = 4$ ,  $***P < 0.001$ ). (C) Striatal neurons were pre-treated with epicatechin (30  $\mu\text{M}$ ) prior to administration of oxLDL (12.5  $\mu\text{g}$  of protein/ml) for 2, 4, 6 and 8 h. Following incubation, cells were loaded with 100  $\mu\text{M}$  DCDHF-DA in HBTM for 30 min in 5%  $\text{CO}_2/95\%$  air at 37  $^\circ\text{C}$ . DCF fluorescence was measured as described in the Materials and

The possible effects of flavonoid pre-treatment on oxLDL uptake were investigated by labelling the oxLDL with the fluorescent label Dil as described previously [25]. The labelled oxLDL (12.5  $\mu\text{g}$  of protein/ml) was then exposed to neuronal cultures (3 h of incubation) that had been pre-treated with epicatechin (30  $\mu\text{M}$ ) for 18 h as detailed above.

### Measurements of intracellular oxidative stress

The effects of oxLDL on intracellular redox status were measured using DCDHF-DA, which is hydrolysed inside cells to form DCDHF and emits fluorescence following oxidation by various oxidants to form 2',7'-dichlorofluorescein (DCF). Thus the fluorescence emitted by DCF directly reflects the overall oxidative status of a cell [31]. In this work two different approaches have been taken to investigate the increase in intracellular oxidative stress in neurons following an oxidative insult. This was necessary to counteract the leakage of the probe from the intracellular space into the medium, which has been observed to occur in this system to a significant extent increasingly after 60 min of loading (results not shown).

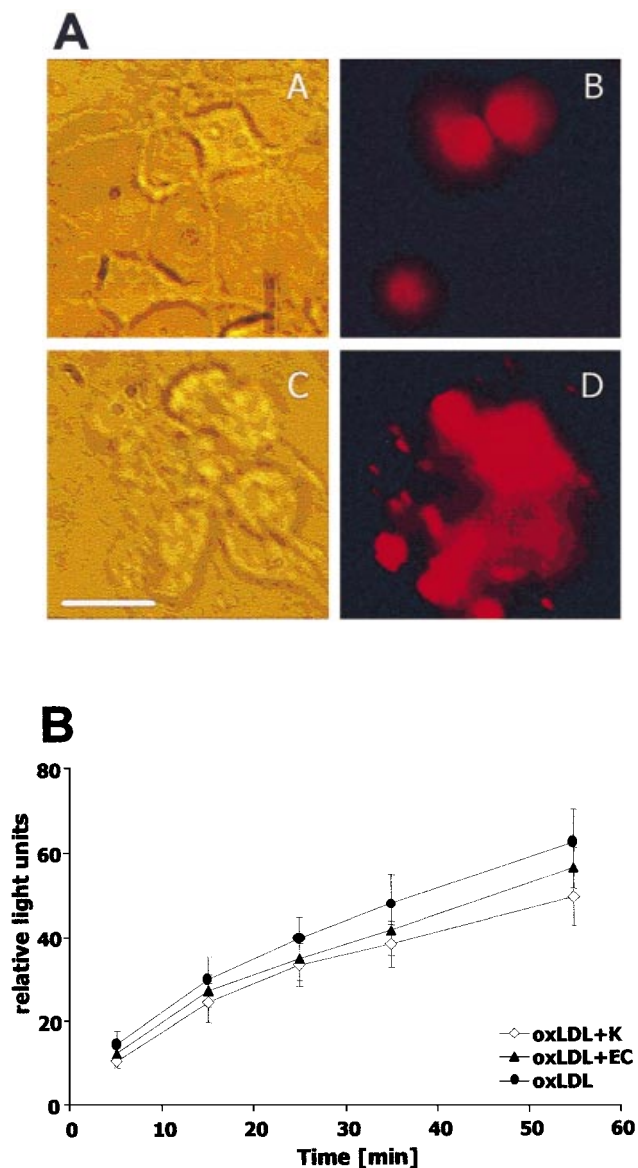
First, the acute effects of an oxidative insult were investigated on-line for 55 min by pre-loading the neurons with DCDHF-DA prior to the administration of oxLDL. Briefly, neurons pre-treated with flavonoids as described above were washed twice, and the medium was replaced by HBTM containing 100  $\mu\text{M}$  DCDHF-DA and incubated for 30 min in 5%  $\text{CO}_2/95\%$  air at 37  $^\circ\text{C}$ . Following incubation, the neuronal cultures were washed three times and incubated with oxLDL (12.5  $\mu\text{g}$  of protein/ml) in HBTM. The fluorescence was followed for up to 55 min using a SPECTRAmax<sup>®</sup> Gemini microplate spectrofluorometer (Molecular Devices). The temperature was maintained at 37  $^\circ\text{C}$  and the emission was recorded at 530 nm after exciting at 500 nm. Each well was scanned in the instrument's well-scan mode, accumulating data from 21 independent points/well, which were then transformed into an average signal expressed in relative light units. All data were normalized with respect to the change in fluorescence of untreated controls (no oxLDL).

Secondly, chronic effects on the intracellular redox status following an oxidative insult were investigated by applying oxLDL (12.5  $\mu\text{g}$  of protein/ml) to neuronal cultures for up to 8 h and subsequently loading the neurons with the probe (100  $\mu\text{M}$  DCDHF-DA for 30 min) at different time points. The fluorescence was then measured immediately using the instrument settings detailed above and results were expressed in relative light units.

### Immunoblotting

Immunoblotting analysis was performed essentially as described previously [26] with minor modifications. Neurons cultured in 6-well Nunc plates were pre-treated for 18 h with flavonoids (30  $\mu\text{M}$ ) or vehicle as detailed in the toxicity studies above. Neurons were then washed twice in HEPES-buffered incubation medium (HBM): 140 mM NaCl, 5 mM KCl, 5 mM  $\text{NaHCO}_3$ , 1.2 mM  $\text{Na}_2\text{HPO}_4$ , 1.2 mM  $\text{CaCl}_2$ , 5.5 mM glucose and 20 mM HEPES, pH 7.4, and then pre-incubated for 5 min at 37  $^\circ\text{C}$  in the presence or absence of  $\text{CaCl}_2$  or U0126 (10  $\mu\text{M}$ ) as appro-

methods section. Data are expressed as relative light units. No significant differences ( $n = 4$ ) were observed between the epicatechin and non-pre-treated neurons at any of the time points tested. The insert demonstrates Trypan Blue exclusion data for parallel-treated neurons. These results are expressed the percentage of Trypan Blue-positive neurons compared with total cell number (300 neurons counted). No significant changes ( $n = 4$ ) were observed with up to 8 h of incubation.



**Figure 2** Pre-treatment with epicatechin does not alter oxLDL uptake into cultured neurons

(A) Striatal neurons pre-treated with epicatechin (panels C and D) or vehicle control (panels A and B), then exposed to Dil-labelled oxLDL (12.5  $\mu\text{g}$  of protein/ml) for 3 h and subsequently observed using light (panels A and C) or fluorescence (panels B and D) microscopy. Scale bar, 15  $\mu\text{m}$ . (B) Neurons were pre-treated with epicatechin (EC, 30  $\mu\text{M}$ ) or kaempferol (K, 30  $\mu\text{M}$ ) and then loaded with 100  $\mu\text{M}$  DCDHF-DA in HBTM for 30 min in 5%  $\text{CO}_2$ /95% air at 37  $^\circ\text{C}$ . Following a washing step, oxLDL (12.5  $\mu\text{g}$  of protein/ml) was applied and the fluorescence was measured for up to 55 min as described in the Materials and methods section. There was no significant difference ( $n = 3$ ) in the increase of oxLDL-mediated intracellular fluorescence between flavonoid-pretreated and non-pretreated striatal cultures.

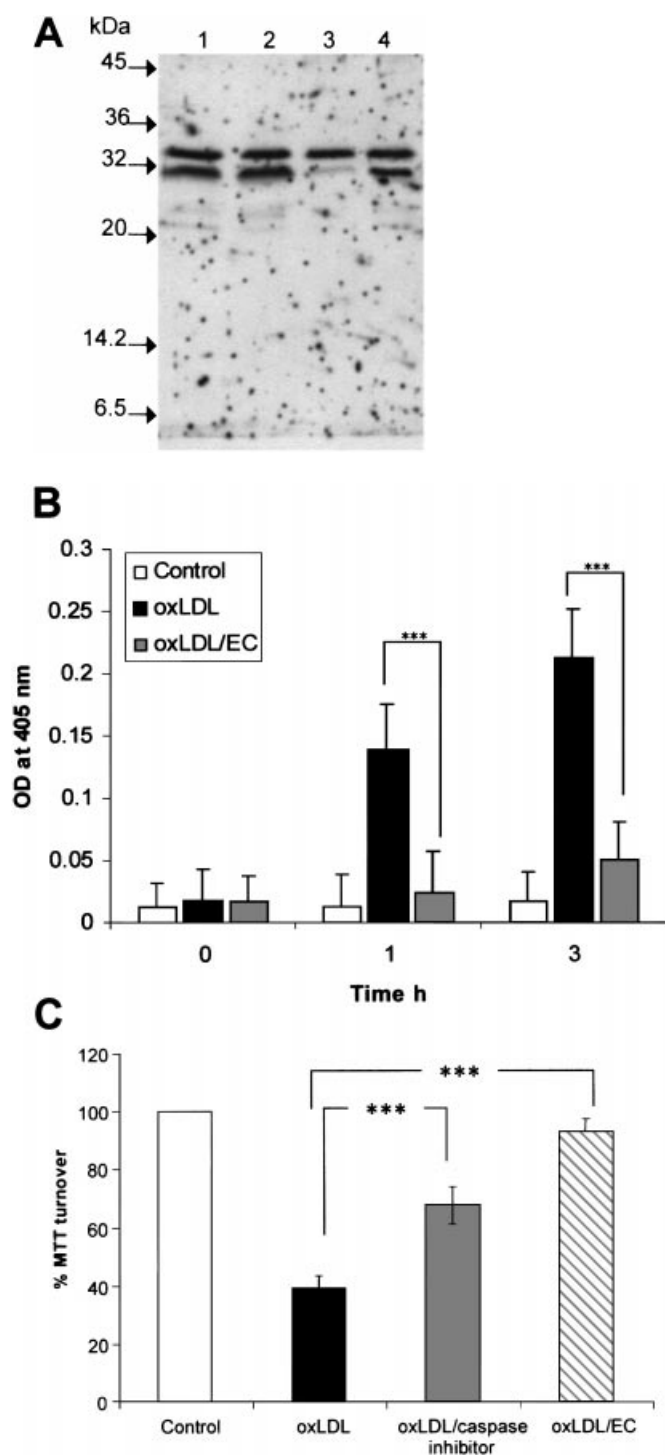
appropriate. Cells were then stimulated with oxLDL (6.25–25  $\mu\text{g}$  of protein/ml) in HBM from 15 to 120 min at 37  $^\circ\text{C}$  in the presence or absence of  $\text{CaCl}_2$ . The HBM was removed and the plates were quickly washed with ice-cold PBS, pH 7.4, containing EGTA (200  $\mu\text{M}$ ) and placed immediately on ice. The cell monolayer was rapidly scraped from the plate into ice-cold lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 2 mM EDTA, 2 mM EGTA, 0.5 mM PMSF, 10  $\mu\text{g}/\text{ml}$  leupeptin, 10  $\mu\text{g}/\text{ml}$  antipain, 1  $\mu\text{g}/\text{ml}$  pepstatin A, 1 mM  $\text{Na}_3\text{VO}_4$  and 50 mM NaF)

and left on ice to solubilize for 45 min. Lysates were centrifuged at 1000  $g$  for 5 min at 4  $^\circ\text{C}$  to remove unbroken cell debris and nuclei. Protein concentration in the supernatants was determined by the Bio-Rad Bradford protein assay<sup>®</sup> (Bio-Rad). Samples were incubated for 2 min at 95  $^\circ\text{C}$  in boiling buffer, giving 62.5 mM Tris (pH 6.8) 2% SDS, 5% 2-mercaptoethanol, 10% glycerol and 0.0025% Bromophenol Blue in the final sample. Boiled samples (20–30  $\mu\text{g}/\text{lane}$ ) were run on 9–12% SDS/polyacrylamide gels and proteins were transferred on to nitrocellulose membranes (Hybond-ECL<sup>®</sup>, Amersham) by semi-dry electroblotting (1.5 mA/cm<sup>2</sup>). The nitrocellulose membrane was then incubated in a blocking buffer (20 mM Tris, pH 7.5, 150 mM NaCl; TBS) containing 4% (w/v) skimmed milk powder for 30 min at room temperature followed by 2  $\times$  5 min washes in TBS supplemented with 0.05% (v/v) Tween 20 (TTBS). Blots were then incubated with anti-ACTIVE MAPK pAb (1:5000 dilution), anti-ACTIVE JNK pAb (1:5000 dilution), anti-phospho-Jun (Ser-73) pAb, anti-pro-caspase-3 (H-277) pAb (1:500 dilution), anti-ERK1/ERK2 pAb (1:1000 dilution) or anti-JNK1 pAb (1:1000 dilution) in TTBS containing 1% (w/v) skimmed milk powder (antibody buffer) overnight at room temperature on a three-dimensional rocking table. The blots were washed for 2  $\times$  5 min in TTBS and then incubated with goat anti-rabbit IgG conjugated with horseradish peroxidase (1:1000 dilution, Sigma) in antibody buffer for 1 h. Finally the blots were washed for 2  $\times$  5 min in TTBS, rinsed in TBS and exposed to ECL reagent<sup>®</sup> for 1–2 min as described in the manufacturer's protocol (Amersham). Blots were exposed to Hyperfilm-ECL<sup>®</sup> (Amersham) for 2–5 min in an autoradiographic cassette and developed. Bands were analysed using BioImage<sup>®</sup> Intelligent Quantifier software (Ann Arbor, MI, U.S.A.). Molecular masses of the bands were calculated from comparison with pre-stained molecular-mass markers (27000–180000 Da and 6500–45000 Da, Sigma) that were run in parallel with the samples. The equal loading and efficient transfer of proteins was confirmed by staining the nitrocellulose with Ponceau Red (Sigma).

#### Measurement of caspase-3 activity

After 5 days in culture neurons were pre-treated with flavonoids as described above, washed twice and the medium replaced by HBTM containing oxLDL (12.5  $\mu\text{g}$  of protein/ml). After incubation in 5%  $\text{CO}_2$ /95% air at 37  $^\circ\text{C}$  for 60 min the neurons were washed and a cell lysate was prepared as described in the previous section.

The activity of caspase-3-like proteases in the lysate was determined using a colorimetric caspase-3 assay kit (Sigma) according to the manufacturer's protocol with minor modifications. Briefly, the reaction mixture (total volume, 100  $\mu\text{l}$ ) contained 30  $\mu\text{l}$  of cell lysate and 10  $\mu\text{l}$  of the caspase-3 substrate acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide (final concentration, 200  $\mu\text{M}$ ) in assay buffer, and the assay was carried out in a 96-well plate. To account for non-specific hydrolysis of the substrate, a control reaction mixture contained 30  $\mu\text{l}$  of cell lysate, 10  $\mu\text{l}$  of substrate and 10  $\mu\text{l}$  (final concentration, 20  $\mu\text{M}$ ) of the specific caspase-3 inhibitor acetyl-DEVD-CHO in assay buffer. Both mixtures were incubated for 90 min at 37  $^\circ\text{C}$  and the absorbance was read at 405 nm using a SPECTRAMax<sup>®</sup> 190 microplate photometer (Molecular Devices). Absorbance data obtained using the caspase-3 inhibitor were subtracted from that without caspase-3 inhibitor to correct for any non-specific hydrolysis. Vehicle controls and blanks were incorporated and caspase-3 protein was used as a positive control.



**Figure 3** Epicatechin inhibits oxLDL-induced activation of caspase-3, which is involved in neuronal death

Neurons were treated with oxLDL (12.5  $\mu$ g of protein/ml) for up to 3 h following pre-treatment with epicatechin (EC, 30  $\mu$ M). Neuronal lysates were immunoblotted using anti-pro-caspase-3 (H-277) pAb to demonstrate the cleavage of pro-caspase-3 and in parallel experiments the activity of caspase-3-like proteases in the neuronal lysates was determined using a spectrophotometric caspase-3 assay. (A) The blot represents a 60 min incubation of striatal neurons with oxLDL. Lane 1, control (0 h); lane 2, control (60 min); lane 3, oxLDL (60 min); lane 4, neurons treated with oxLDL (60 min) following pre-treatment with epicatechin. OxLDL caused the disappearance of a band at 32 kDa, which corresponds to pro-caspase-3. This loss of band intensity at 32 kDa is attenuated by pre-treatment with epicatechin. (B) The activity of caspase-3-like proteases in the lysate was determined by a colorimetric caspase-3 assay using

### Statistical analysis

Data are expressed as means  $\pm$  S.D. Statistical comparisons were made using an unpaired two-tailed Student's *t* test with a confidence level of 95%. The significance level was set at  $P < 0.05$ .

### RESULTS

Neuronal cultures were exposed to oxidative stress in the form of oxLDL with or without pre-treatment with flavonoids. In all experiments the flavonoids were removed from the media and the neurons washed prior to the addition of oxLDL to prevent any extracellular interactions between the flavonoid and the oxidative insult. The effects of oxLDL on neuronal viability, intracellular oxidative stress, caspase-3 activation and signalling through MAPK pathways were investigated.

#### OxLDL induces neuronal death

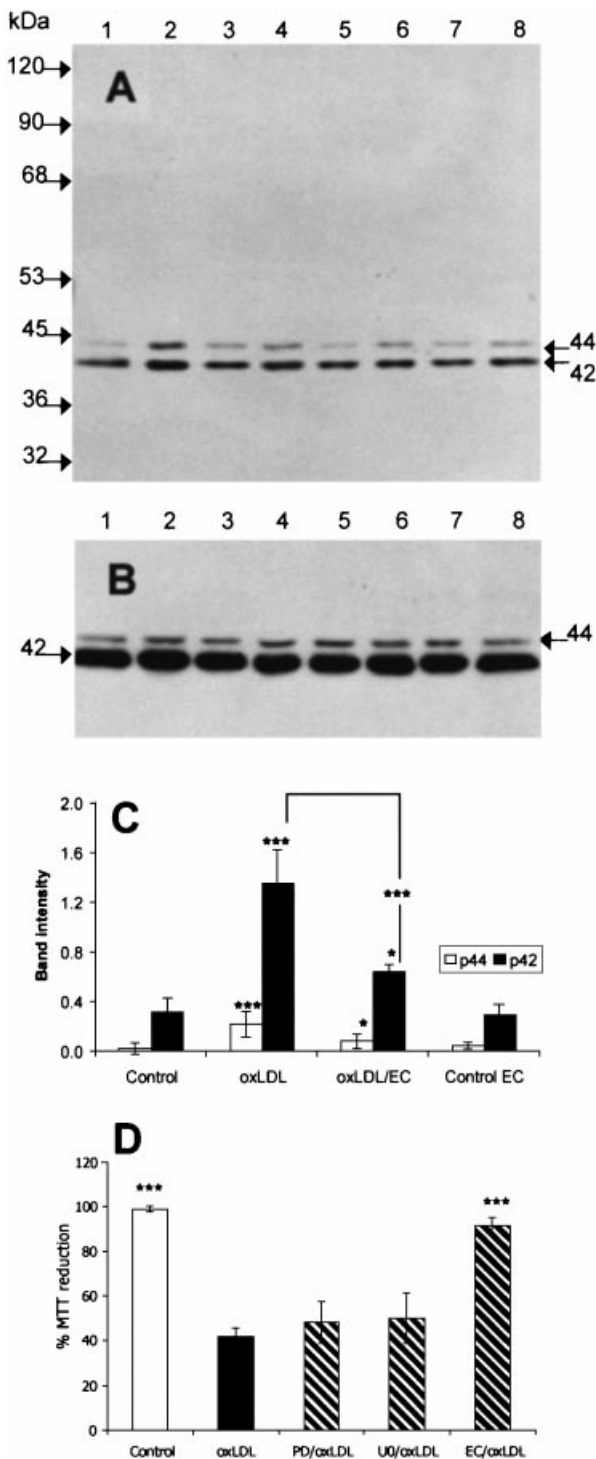
Neuronal cultures were incubated with oxLDL (12.5  $\mu$ g of protein/ml) for up to 24 h and then mitochondrial function was measured using the MTT assay and membrane integrity was assessed by LDH release. A significant time-dependent decline in MTT reduction and increase in LDH release were observed (Figures 1A and 1B). Native LDL caused no significant loss of MTT reduction or increase in LDH release under the same conditions compared with untreated controls (results not shown) [25]. No significant changes in mitochondrial function were observed when cultured striatal neurons were treated with either CuSO<sub>4</sub> (10  $\mu$ M) or EDTA (10  $\mu$ M) for 18 h.

To investigate the neuroprotective effects of flavonoids, the neurons were pre-treated with epicatechin and kaempferol (final concentration 30  $\mu$ M) in medium for 18 h. The neurons were then washed and exposed to oxLDL for up to 24 h. Pre-treatment with epicatechin (Figure 1A) or kaempferol (results not shown) strongly attenuated the oxLDL-mediated loss of mitochondrial function and membrane integrity (Figures 1A and 1B). Vehicle controls had no effect on either mitochondrial function or the oxLDL-mediated LDH release. Epicatechin and kaempferol at a concentration of 30  $\mu$ M displayed no neurotoxic effects on control cultures as evaluated by MTT reduction and morphological assessment [25].

#### OxLDL and intracellular oxidative stress

The effects of chronic oxLDL exposure on the intracellular redox status of neurons was assessed using DCDHF-DA as described in the Materials and methods section. OxLDL provoked a time-dependent increase in intracellular fluorescence (Figure 1C), with the maximum reached 4 h after the administration of oxLDL. There was no significant difference in the increase in intracellular fluorescence between untreated neurons or those pre-treated with epicatechin (30  $\mu$ M) following oxLDL administration. The intracellular fluorescence declined between 4 and 8 h in the absence of any measurable loss of cellular integrity, as determined using Trypan Blue exclusion in parallel experiments (Figure 1C and inset). After 8 h, the onset of oxLDL neurotoxicity (LDH

the caspase-3 substrate acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide as detailed in the Materials and methods section. Data are expressed as the increase in absorbance (OD) at 405 nm due to the release of *p*-nitroanilide. Neurons incubated with oxLDL (12.5  $\mu$ g of protein/ml) for up to 3 h showed a strong increase in caspase-3-like proteases activity, which was significantly ( $n = 3$ ) attenuated by pre-treatment with epicatechin. (C) Pre-treatment of striatal neurons with epicatechin (30  $\mu$ M, 18 h) and with the caspase-3-specific inhibitor DEVD-CHO (100  $\mu$ M, 15 min) protected significantly ( $n = 3$ ) against oxLDL-induced neuronal damage as determined by MTT turnover. For (B) and (C), \*\*\* $P < 0.001$ .



**Figure 4** OxLDL-induced activation of ERK1/2 is attenuated by epicatechin but blocking the signalling to ERK1/2 with MEK inhibitors is not neuroprotective

Neuronal lysates were immunoblotted using anti-ACTIVE MAPK pAb (1:5000 dilution; **A**) or anti-ERK1/2 pAb (1:1000 dilution; **B**) following a 15 min (lanes 1–4) or 60 min (lanes 5–8) exposure to oxLDL (12.5  $\mu$ g of protein/ml). Lanes 1 and 5, untreated control cultures; lanes 2 and 6, cultures treated with oxLDL; lanes 3 and 7, cultures pre-treated with epicatechin (30  $\mu$ M); lanes 4 and 8, cultures treated with oxLDL following pre-treatment with epicatechin (30  $\mu$ M). (**C**) Data obtained from immunoblot experiments representing a 15 min incubation of striatal neurons with oxLDL (from **A**, lanes 1–4) were analysed using BioImage Intelligent Quantifier software. Data were obtained from four independent experiments and are presented as means  $\pm$  S.D. OxLDL led to a significant increase in active ERK1/2 compared with control cells ( $***P < 0.001$ ). Pre-treatment with epicatechin (EC) attenuated the activation of

release, Figure 1B; Trypan Blue exclusion, results not shown) prevented further meaningful measurements of intracellular fluorescence.

#### Does flavonoid pre-treatment alter oxLDL uptake into neurons?

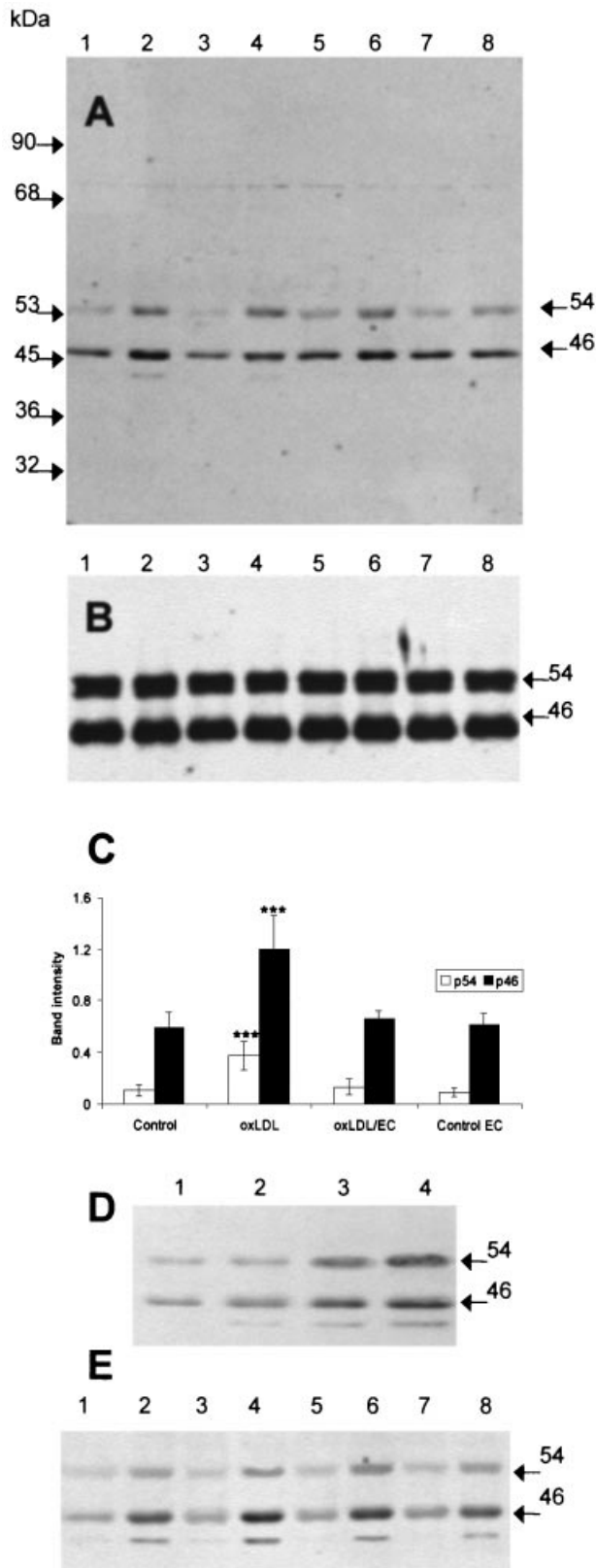
Neurons were pre-treated with epicatechin (30  $\mu$ M) for 18 h and then exposed to Dil-labelled oxLDL (12.5  $\mu$ g/protein/ml) for 3 h as described above. Incubation of labelled oxLDL with non-pretreated (Figure 2A, panels A and B) or epicatechin-pretreated neurons (Figure 2A, panels C and D) resulted in uptake of oxLDL into the cells, as reflected by the strongly fluorescent staining of the neuronal cell bodies. To demonstrate that the fluorescence was co-localized with individual striatal neurons, fluorescence-positive cells were also examined by light microscopy (Figure 2A, panels A and C).

An alternative approach was employed to address the same question by investigating whether flavonoid pre-treatment altered the intracellular redox status in response to acute oxLDL exposure measured for up to 55 min. No significant differences in DCF fluorescence could be detected between control and flavonoid (epicatechin and kaempferol)-pretreated cells following oxLDL administration (Figure 2B), suggesting that pre-treatment with flavonoids does not affect the ability of oxLDL to generate intracellular oxidative stress. Collectively, these data suggest that the neuroprotective actions of flavonoids are not due to their ability to inhibit oxLDL uptake in primary neurons. In support of an action unrelated to receptor function, flavonoids also protected neurons against non-receptor-mediated oxidative stress in the form of exogenously applied hydrogen peroxide (results not shown).

#### Involvement of caspase-3 in oxLDL-induced neuronal toxicity

In previous experiments we demonstrated that oxLDL-induced neuronal cell death involves DNA fragmentation, suggesting a possible apoptotic process [25]. We further investigated the possible involvement of apoptosis in oxLDL-mediated neurotoxicity by measuring the cleavage of pro-caspase-3 and the activity of caspase-3-like proteases. Striatal neurons were challenged with oxLDL for 60 min and the neuronal lysates were then analysed for caspase-3 activity by a spectrophotometric assay and for pro-caspase-3 cleavage by immunoblotting. OxLDL stimulated a 9-fold increase in caspase-3-like activities at 1 h and a 13-fold increase at 3 h (Figure 3B). Pre-treatment with epicatechin prior to treatment with oxLDL resulted in significant attenuation of the oxLDL-induced increase in caspase-3-like activities at both 1 and 3 h (Figure 3B). Parallel immunoblots for anti-pro-caspase-3 (H-277) pAb were used to investigate the activation of caspase-3 in the same lysates used for caspase-3 activity measurements by following the cleavage of the precursor protein pro-caspase-3 (also designated CPP32). Lysates from untreated controls exhibited one band at the expected molecular mass for pro-caspase-3 (32 kDa) and another band at 34 kDa

ERK1/2 by oxLDL ( $***P < 0.001$ ), although there was a less significant difference compared with the control ( $*P < 0.05$ ). (**D**) The effects of the MEK inhibitors U0126 and PD98059 on oxLDL-mediated neurotoxicity were investigated. Neurons were pre-treated with PD98059 (PD, 50  $\mu$ M) or U0126 (UO, 10  $\mu$ M) for 15 min prior to an 18 h exposure to oxLDL in the continued presence of the MEK inhibitors and were then assessed for neuronal injury using the MTT assay as described above. Results are expressed as the percentage of MTT reduction relative to an untreated control, which was defined as 100% (variations in MTT reduction for the controls were less than 10% as judged from the absorbance data). Data given are means  $\pm$  S.D. from three independent cultures, each performed in triplicate. Blocking the activation of ERK1/2 was neither neuroprotective nor neurotoxic. Pre-treatment with epicatechin strongly increased MTT reduction compared with non-pretreated cultures ( $***P < 0.001$ ).



**Figure 5** Epicatechin inhibited oxLDL-mediated activation of JNK

Neuronal lysates were immunoblotted using anti-ACTIVE JNK pAb (A) or anti-JNK1 pAb (B) following a 15 min (lanes 1–4) or 60 min (lanes 5–8) exposure to oxLDL (12.5  $\mu$ g of protein/ml). Lanes 1 and 5, untreated control cultures; lanes 2 and 6, cultures treated with oxLDL; lanes 3 and 7, cultures pre-treated with epicatechin (30  $\mu$ M); lanes 4 and 8, cultures

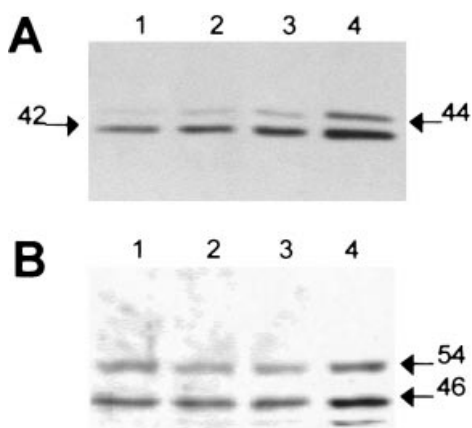
(Figure 3A). There was a significant reduction in the levels of p32 in lysates derived from oxLDL-treated (60 min) neurons (Figure 3A). Pre-treatment with epicatechin prior to administration of oxLDL strongly reversed the loss of the band at 32 kDa, demonstrating significant attenuation of the effect of oxLDL. In contrast, the band at 34 kDa was not influenced by either oxLDL or epicatechin (Figure 3A). No subunits derived from the cleavage of pro-caspase-3 (p11, p20) were detected. In support of a direct role for caspase-3 in oxLDL-induced neuronal death, pre-incubation with the selective caspase-3 inhibitor DEVD-CHO significantly protected the neurons, as measured by MTT turnover (Figure 3C).

#### The effect of oxLDL on MAPK signalling

MAPK signalling cascades (ERK1/2 and JNK) have been associated with oxidative-stress-induced neuronal death signalling [17,19,32,33]. Therefore we investigated the possible involvement of MAPK signalling in oxLDL-mediated neurotoxicity by focusing on ERK1/2 and JNK. To test whether the stimulation of striatal neurons with oxLDL led to activation of the ERK1/ERK2 forms of MAPK, immunoblotting of neuronal homogenates with anti-ACTIVE MAPK pAb, which recognizes the dually phosphorylated Thr/Glu/Tyr region within the catalytic core of the active form of ERK1 (44 kDa) and ERK2 (42 kDa), was performed. Under non-stimulated control conditions the levels of active ERK1/2 were low. Treatment with oxLDL (12.5  $\mu$ g of protein/ml) for 15 min resulted in activation of ERK1 that was 10-fold over basal levels and of ERK2 that was 4-fold over basal (Figures 4A and 4C). The levels of ERK1/2 declined following 60 min of incubation with oxLDL, demonstrating that the activation of ERK1/2 by oxLDL is time-dependent (Figure 4A). To investigate the effects of flavonoids, striatal neurons were pre-incubated with epicatechin (30  $\mu$ M) for 18 h prior to washing and application of oxLDL (12.5  $\mu$ g of protein/ml). Pre-treatment with epicatechin significantly reduced the levels of active ERK1/2 mediated by a 15 min exposure to oxLDL (Figures 4A and 4C) without changing the total level of ERK1/2 (Figure 4B).

To establish whether the activation of ERK1/2 plays a significant role in the process of oxidative-stress-induced neuronal death, the effects of the MAPK kinase (MEK) inhibitors U0126 and PD98059 on oxLDL-mediated neurotoxicity were investigated. PD98059 (50  $\mu$ M) and U0126 (10  $\mu$ M) abolish oxidative activation of ERK1/2 in striatal neurons ([17], and A. Crossthwaite and R. J. Williams, unpublished work). Neurons were pre-treated with U0126 and PD98059 for 15 min prior to an 18 h exposure to oxLDL in the continued presence of the MEK inhibitors and were then assessed for neuronal injury by the MTT assay. Blocking the activation of ERK1/2 had no significant effect on oxLDL-induced neurotoxicity (Figure 4D). Control experiments using PD98059 (50  $\mu$ M) and U0126 (10  $\mu$ M)

treated with oxLDL following a pre-treatment with epicatechin (30  $\mu$ M). (C) Data obtained from immunoblot experiments representing a 60 min incubation of striatal neurons with oxLDL (from A, lanes 5–8) were analysed using BiImage Intelligent Quantifier software. Data were obtained from four independent experiments and are presented as means  $\pm$  S.D. OxLDL led to a significant increase in the levels of active JNK (p54/p46) compared with control cells ( $***P < 0.001$ ). Pre-treatment with epicatechin (EC) strongly attenuated the activation of JNK by oxLDL (no significant difference compared with control). (D) Exposure of oxLDL to striatal neurons (60 min) caused a concentration-dependent increase in JNK activation. Lanes 1–4 correspond to oxLDL at concentrations of 0, 6.25, 12.5 and 25  $\mu$ g of protein/ml, respectively. (E) OxLDL (12.5  $\mu$ g of protein/ml) mediated a sustained activation of JNK for up to 120 min following administration. Lanes 1, 3, 5 and 7 correspond to control cultures at 15, 60, 90 and 120 min of incubation, respectively. Lanes 2, 4, 6 and 8 reflect neuronal cultures incubated with oxLDL for 15, 60, 90 and 120 min, respectively.



**Figure 6** Activation of ERK1/2 and JNK by oxLDL is calcium-dependent

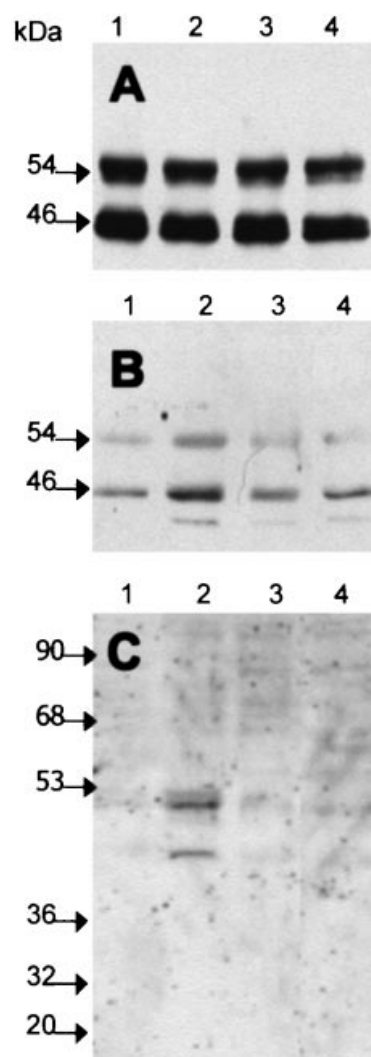
Neurons were treated with (lanes 2 and 4) and without (lanes 1 and 3) oxLDL (12.5  $\mu$ g of protein/ml) for 15 min (A) or 60 min (B) in the presence (lanes 3 and 4) or absence (lanes 1 and 2) of  $\text{CaCl}_2$  and then immunoblotted for anti-ACTIVE MAPK pAb (A) or anti-ACTIVE JNK pAb (B).

in the absence of oxLDL showed no significant effects on mitochondrial function or cell morphology (results not shown).

Since the activation of ERK1/2 by oxLDL did not appear to be directly involved in oxLDL-mediated neurotoxicity, the possible involvement of JNK was investigated. Immunoblotting of neuronal lysates was carried out using an anti-ACTIVE JNK pAb, which preferentially recognizes the dually phosphorylated Thr/Pro/Tyr region within the catalytic core of the active form of JNK, corresponding to Thr-183 and Tyr-185 of mammalian JNK2. Stimulation of striatal neurons with oxLDL (12.5  $\mu$ g of protein/ml) for 15 min resulted in an activation of JNK (p46 and p54) above basal levels (Figure 5A) and this activation was sustained for up to 120 min, as demonstrated by high levels of p46/p54 (Figures 5A, 5C and 5E), without altering the total levels of JNK (Figure 5B). Incubation with oxLDL for 60 min resulted in an increase in active JNK of 3.5-fold and 2-fold above the basal level for p54 and p46, respectively (Figure 5C). These effects were concentration-dependent (Figure 5D). The effect of epicatechin on oxLDL-mediated JNK activation in striatal neurons was investigated following the incubation of neurons with epicatechin (30  $\mu$ M) for 18 h prior to washing and application of oxLDL (12.5  $\mu$ g of protein/ml) for 60 min. Pre-treatment with epicatechin strongly inhibited the oxLDL-induced activation of p46/p54 (Figures 5A and 5C). The stimulating effect of oxLDL at 60 min was reduced by epicatechin from 3.5-fold activation above basal (no pre-treatment) to 0.3-fold for p54 and from 2-fold activation above basal (no pre-treatment) to 0.1-fold for p46 (Figure 5C) without altering the total levels of JNK (Figure 5B).

#### Calcium and the activation of ERK1/2 and JNK

Since calcium has been proposed to play a role in oxidative-stress-mediated neuronal signalling [17], we investigated the calcium-dependency of oxLDL-induced activation of ERK1/2 and JNK. Neurons were treated with oxLDL (12.5  $\mu$ g of protein/ml) for 15 min (ERK1/2 activation) or 60 min (JNK activation) in the presence or absence of  $\text{CaCl}_2$ . The removal of  $\text{CaCl}_2$  prevented the oxLDL-induced activation of both ERK1/2 (Figure 6A) and JNK (Figure 6B).



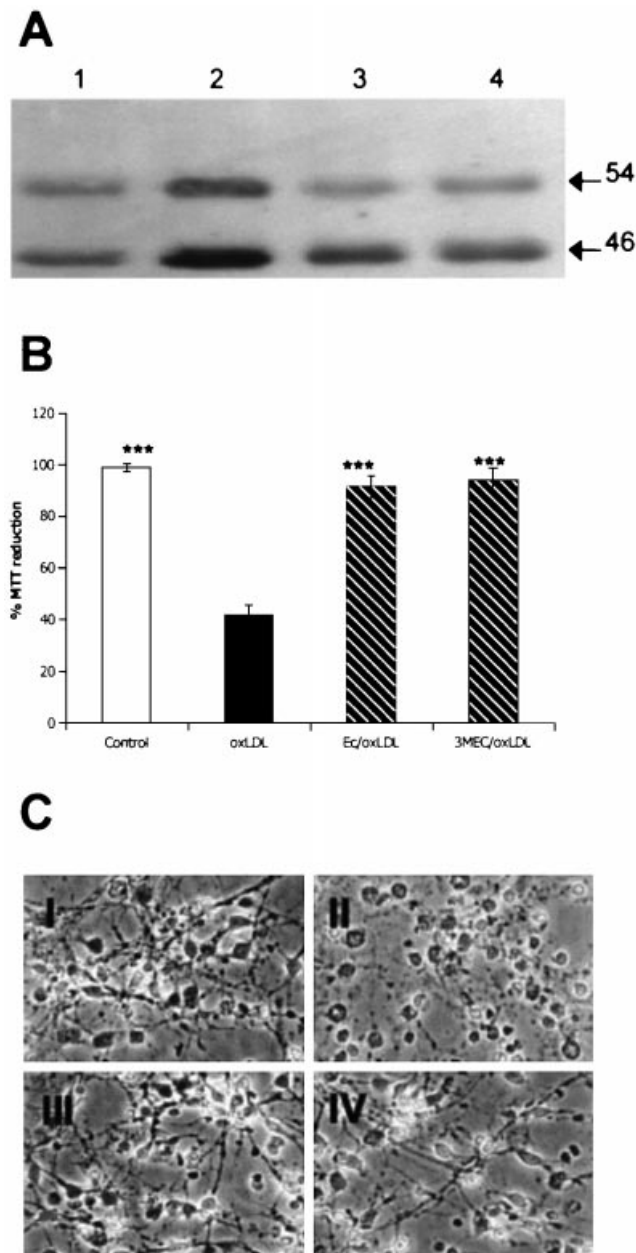
**Figure 7** Epicatechin and kaempferol abolish oxLDL-mediated phosphorylation of c-Jun

Neurons were incubated for 60 min with oxLDL and the neuronal lysates were immunoblotted using (A) anti-JNK1 pAb, (B) anti-ACTIVE JNK pAb and (C) anti-phospho-Jun (Ser-73) pAb. Lanes 1, control; lanes 2, oxLDL (12.5  $\mu$ g of protein/ml); lane 3 and 4, cultures treated with oxLDL (12.5  $\mu$ g of protein/ml) following pre-treatment with either epicatechin (30  $\mu$ M) or kaempferol (30  $\mu$ M), respectively. A representative blot of two independent experiments is shown.

#### OxLDL-induced phosphorylation of c-Jun

Recent findings suggest that activated JNK may induce neuronal apoptosis through the phosphorylation and stimulation of c-Jun [15,16]. Therefore, we investigated whether or not oxLDL-mediated activation of JNK culminated in the phosphorylation of c-Jun in striatal neurons. Neuronal lysates derived from striatal neurons treated with oxLDL for 60 min were immunoblotted and then probed with anti-ACTIVE JNK pAb and in parallel with anti-phospho-Jun pAb recognizing pSer-73 of the c-Jun sequence (identical sequence in JunD). The stimulation of striatal neurons with oxLDL (60 min) led to an activation of JNK (p46/p54; Figure 7B), in parallel with the appearance of two bands recognized by the anti-phospho-Jun antibody corresponding to pSer73-c-Jun (higher band) and pSer73-JunD (lower band), which is also known to be detected by this antibody





**Figure 8** The *in vivo* metabolite 3'-O-methyl-epicatechin parallels the neuroprotective actions of unmetabolized native epicatechin

(A) Neuronal lysates were immunoblotted using anti-ACTIVE JNK pAb following a 60 min exposure to oxLDL. Lane 1, control cultures; lane 2, oxLDL (12.5  $\mu\text{g}$  of protein/ml); lanes 3 and 4, neuronal cultures treated with oxLDL following pre-treatment with epicatechin (30  $\mu\text{M}$ ) or 3'-O-methyl-epicatechin (30  $\mu\text{M}$ ), respectively. (B) 3'-O-Methyl-epicatechin protects against oxLDL-induced neuronal injury as determined by MTT assay. Striatal neurons were exposed to native LDL (control) or oxLDL (12.5  $\mu\text{g}$  of protein/ml) for 18 h following pre-treatment with epicatechin (Ec, 30  $\mu\text{M}$ ) or 3'-O-methyl-epicatechin (3MEC, 30  $\mu\text{M}$ ). Neuronal damage was assessed by MTT assay as described in the Materials and methods section. Results are expressed as the percentage of MTT reduction relative to a native LDL control, which was defined as 100% (variations in MTT reduction for the native LDL control were less than 15% as judged from the absorbance data). Data are means  $\pm$  S.D. from four independent cultures, each performed in triplicate. Pre-treatment with epicatechin and 3'-O-methyl-epicatechin significantly increased MTT reduction compared with non-pre-treated cultures ( $***P < 0.001$ ). (C) Epicatechin and 3'-O-methyl-epicatechin protect against oxLDL-mediated morphological changes in striatal neurons. The morphological appearance of the neurons was examined after exposure to native and oxLDL using light microscopy. (I) Control cultures of striatal neurons exhibit a characteristic shape and intact cell body as well as a finely developed dendritic network. (II) OxLDL (12.5  $\mu\text{g}$  of protein/ml; 18 h exposure) caused a loss of dendrites and

(Figure 7C). Neurons were pre-treated with epicatechin and kaempferol (both 30  $\mu\text{M}$ ) for 18 h, washed and challenged with oxLDL for 60 min to investigate the effect of flavonoid pre-treatment on oxLDL-induced activation of c-Jun. Pre-treatment with epicatechin and kaempferol strongly inhibited the activation of JNK by oxLDL (60 min; Figure 7B) and abolished the phosphorylation of c-Jun (Figure 7C). Parallel controls using an antibody against total JNK showed that neither the pre-treatment with the flavonoids nor the time period of the experiment altered total levels of JNK (Figure 7A).

#### Effects of 3'-O-methyl-epicatechin

It has been demonstrated recently that epicatechin is metabolized extensively *in vivo* [34] and that one of the major metabolites that results is the methylated product 3'-O-methyl-epicatechin. This suggests that in animal and human studies 3'-O-methyl-epicatechin might be a more physiologically relevant form of this flavonoid. Therefore, the ability of 3'-O-methyl-epicatechin to protect neurons and to influence oxidative-stress-stimulated signalling events was investigated. Pre-treatment with epicatechin or 3'-O-methyl-epicatechin prior to oxLDL administration (12.5  $\mu\text{g}$  of protein/ml) led to MTT reductions of 90% and 93% for epicatechin and 3'-O-methyl-epicatechin respectively, as compared with 43% MTT reduction following treatment with oxLDL alone (Figure 8B). In addition, morphological assessment of the neurons demonstrated that epicatechin and 3'-O-methyl-epicatechin exerted similar degrees of neuroprotection (Figure 8C). Immunoblots of neuronal lysates for active ERK1/2 (results not shown) and active JNK (Figure 8A) demonstrated a clear inhibition of the oxLDL-mediated activation of ERK1/2 and JNK by 3'-O-methyl-epicatechin.

#### DISCUSSION

This study demonstrates that oxLDL is a rapid activator of ERK1/2 and JNK in striatal neurons and thereby highlights the potential importance of MAPK cascades as pivotal mediators of oxidative stress signalling in neurons. Furthermore, the results of this study show for the first time that flavonoids such as epicatechin, kaempferol and the *in vivo* metabolite 3'-O-methyl-epicatechin are potent intracellular inhibitors of oxLDL-induced neuronal cell death, possibly by counteracting JNK, c-Jun and caspase-3 activation. Thus dietary flavonoids might be considered as being potential neuroprotective agents against neurodegenerative diseases.

Previous work in animal models has shown that dietary supplementation with flavonoid-rich extracts can retard the onset of age-related loss of neuronal function [6,35]. Furthermore, the oral administration of catechin protects against ischaemia/reperfusion-induced neuronal death in gerbils [11], and grape polyphenols block neurodegenerative changes caused by chronic ethanol administration [36]. In the work described here, direct evidence is provided to show that flavonoids are highly effective in protecting neurons from oxidative insults.

Epicatechin is extensively metabolized *in vivo* and one of the major metabolites is the methylated product 3'-O-methyl-epicatechin [34], suggesting that this could be a physiologically important form of the flavonoid. Importantly, 3'-O-methyl-epicatechin was found to have the same ability to attenuate oxLDL-mediated neuronal death as the unmetabolized native

shrinkage of the cell body. (III and IV) Pre-treatment with either epicatechin (III, 30  $\mu\text{M}$ ) or 3'-O-methyl-epicatechin (IV, 30  $\mu\text{M}$ ) protected cultured neurons from oxLDL-induced morphological changes.

epicatechin, showing that this modification to the antioxidant moiety of the flavonoid does not have an unfavourable effect on its biological activity. Although clear beneficial effects of flavonoids have been shown both *in vivo* and *in vitro*, the mechanism by which these compounds exert their neuroprotective action is unknown. Many studies have confirmed the powerful antioxidant actions of flavonoids *in vitro* and their protection against oxidative stress through their hydrogen-donating properties [37]. However, our findings suggest that pre-treatment with flavonoids does not strongly reduce the overall intracellular oxidative stress in neurons generated by exposure to oxLDL and does not alter oxLDL uptake, suggesting that other mechanisms of protection against cell death as a consequence of oxidative insults are involved.

There is evidence from non-neuronal models that flavonoids can act selectively within MAPK signalling cascades [12,13], which might be significant, as members of the MAPK family such as ERK1/2 and JNK are involved in signalling to neuronal survival, regeneration, development and death [14–16,38]. ERK1/2 and JNK are generally considered as having opposing actions on neuronal apoptosis [39]. ERK1/2 is usually associated with pro-survival signalling [40,41] through mechanisms that may involve activation of cAMP-response-element-binding protein (CREB), the up-regulation of the anti-apoptotic protein Bcl-2 and non-transcriptional inhibition of BAD (Bcl-2/Bcl-X<sub>L</sub>-antagonist causing cell death) [41]. JNK on the other hand has been strongly linked to transcription-dependent apoptotic signalling [15,16], possibly through the activation of c-Jun [42] and other AP-1 proteins including JunB, JunD and activating transcription factor (ATF)-2 [43].

Oxidative stress stimulates the phosphorylation and activation of ERK1/2 in cultured neurons [17], although whether or not this plays a role in the pathology of neurodegenerative disorders such as Alzheimer's disease is controversial [44,45]. A number of reports have suggested that direct inhibition of MEK, the upstream activator of ERK1/2, blocks excitotoxic [46] and oxidative-stress-induced cell death [18,19,33]. These are somewhat unexpected findings given the proposed pro-survival characteristics of the ERK1/2 cascade in neurons. Indeed, neurotrophin signalling through ERK1/2 rescues cerebellar granule cells from oxidative-stress-mediated apoptosis [47]. In this study, oxLDL rapidly stimulated ERK1/2 phosphorylation through MEK in a strictly Ca<sup>2+</sup>-dependent manner. This activation of ERK1/2 was strongly inhibited when striatal neurons were pre-treated with the flavonoid epicatechin, or one of its major *in vivo* metabolites 3'-O-methyl-epicatechin, at a concentration that blocked oxLDL-induced neuronal death. However, preventing ERK1/2 activation with the MEK inhibitors PD98059 or U0126 had no effect on neuronal loss caused by oxLDL, suggesting strongly that the neuroprotective effects of these flavonoids were not linked to their potent inhibitory action within the ERK1/2 cascade. Furthermore, the inability of MEK inhibitors to protect neurons was consistent with previous work that also failed to establish a clear role for ERK1/2 in neuronal dysfunction [26]. Thus the physiological relevance of oxLDL-induced activation of ERK1/2 and its inhibition by flavonoids in striatal neurons remains to be established.

There is very strong evidence linking the activation of JNK to neuronal loss in response to a wide array of pro-apoptotic stimuli in both developmental and degenerative death signalling [16,43]. In the context of oxidative insults in neurons, JNK has been found to be activated by dopamine [32], by 4-HNE [20,22] and through reduced expression of superoxide dismutase (SOD) 1 [48]. In the present study, oxLDL stimulated a rapid concentration- and Ca<sup>2+</sup>-dependent phosphorylation of JNK (p54 and

p46) that was sustained well above basal levels for at least 2 h. This sustained activation of JNK was severely inhibited in neurons that had been pre-exposed to epicatechin or 3'-O-methyl-epicatechin, suggesting that flavonoids may be exerting neuroprotective actions through an attenuation of a pro-apoptotic signalling cascade lying downstream of JNK.

Phosphorylation of the AP-1 protein c-Jun on Ser-63 and Ser-73 by JNK causes increased transcriptional activity [49], which has been linked to stress-induced apoptosis. OxLDL caused a pronounced phosphorylation of c-Jun and another protein probably corresponding to JunD, which mirrored the activation of JNK. These findings support the recently reported increase in AP-1 binding in neurons exposed to the lipid-peroxidation product 4-HNE [20]. Significantly, the oxLDL-induced activation of c-Jun was abolished in neurons pre-exposed to flavonoids despite the presence of residual JNK activity. This implies either that a threshold level of phosphorylated JNK is required to activate c-Jun or that there is a subcellular population of JNK in striatal neurons that is not involved in the transduction of signals through to c-Jun. The complete inhibition of phosphorylation of c-Jun by flavonoids suggests that these polyphenolic compounds may have powerful anti-apoptotic actions in neurons.

Activated c-Jun has been proposed to regulate the expression of pro-apoptotic genes that may activate Bax to induce the release of cytochrome *c* from mitochondria, culminating in a stimulation of caspases 9 and 3 [15]. There is some evidence linking oxidative stress to caspase activation in neurons. Caspase inhibitors have been shown to block 4-HNE-induced activation of JNK [20] and to reduce oxidative-stress-mediated neuronal death [24]. In this study, oxLDL strongly activated pro-caspase 3 and increased caspase-3 activity in striatal neurons. Blocking caspase-3 activity with a selective inhibitor protected neurons against oxLDL-induced neurotoxicity. The activation of caspase-3 was completely blocked in flavonoid-pretreated neurons, providing compelling evidence in support of a potent anti-apoptotic action of flavonoids in these cells. Thus our findings suggest that flavonoids may function by acting selectively within neuronal death signalling cascades. This could be at the level of preserving Ca<sup>2+</sup> homeostasis, and thereby preventing Ca<sup>2+</sup>-dependent activation of JNK, or may involve direct interactions with upstream signalling components required for the recruitment of MAPKs in neurons. It has been proposed that caspase-3 activates JNK [20] although this is unlikely in our model as oxLDL-induced JNK activation is detectable in advance of any measurable caspase-3 activity. This correlates with recent reports that indicate an important role for JNK upstream of caspases [43,50]. The challenge now is to determine the precise site(s) of action of flavonoids in the sequence of events that regulate oxidative-stress-induced cell death in the central nervous system and to further evaluate the potential of dietary flavonoids as neuroprotective agents.

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