

# Neuroprotective actions of eicosapentaenoic acid on lipopolysaccharide-induced dysfunction in rat hippocampus

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## Abstract

Eicosapentaenoic acid (EPA) protects hippocampus from age-related and irradiation-induced changes that lead to impairment in synaptic function; the evidence suggests that this is due to its anti-inflammatory effects, specifically preventing changes induced by the proinflammatory cytokine, interleukin-1 $\beta$  (IL-1 $\beta$ ). In this study, we have investigated the possibility that EPA may prevent the effects of lipopolysaccharide (LPS) administration, which have been shown to lead to deterioration of synaptic function in rat hippocampus. The data indicate that treatment of hippocampal neurones with EPA abrogated the LPS-induced increases in phosphorylation of the mitogen-activated protein kinase, c-Jun N-terminal kinase (JNK), the transcription factor, c-Jun and the

mitochondrial protein, Bcl-2. In parallel, we report that intraperitoneal administration of LPS to adult rats increases phosphorylation of JNK, c-Jun and Bcl-2 in hippocampal tissue and that these changes are coupled with increased IL-1 $\beta$  concentration. Treatment of rats with EPA abrogates these effects and also blocks the LPS-induced impairment in long-term potentiation in perforant path-granule cell synapses that accompanies these changes. We propose that the neuroprotective effect of EPA may be dependent on its ability to inhibit the downstream consequences of JNK activation.

**Keywords:** c-Jun N-terminal kinase, eicosapentaenoic acid, hippocampus, interleukin-1 $\beta$ , lipopolysaccharide, long-term potentiation.

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There is increasing awareness that inflammatory changes may be a significant contributory factor in the aetiology of a number of neurodegenerative disorders highlighting the importance of understanding the changes that occur as a consequence of inflammation. Systemic administration of lipopolysaccharide (LPS), a component of the cell wall of Gram-negative bacteria, has been reported to consistently induce an increase in concentration of the proinflammatory cytokine, interleukin-1 $\beta$  (IL-1 $\beta$ ) in hippocampus (Loscher *et al.* 2000; Vereker *et al.* 2000a; Nolan *et al.* 2002; Kelly *et al.* 2003) and is recognized as a useful model for investigation of changes that accompany inflammation in brain.

Among the several changes that occur as a consequence of LPS administration, is impairment in cognitive function; thus LPS inhibits hippocampal-dependent, but not hippocampal-independent forms of learning (Pugh *et al.* 1998; Hauss-Wegrzyniak *et al.* 1999; Shaw *et al.* 2001). A deficit in long-term potentiation (LTP) in perforant path-granule cell synapses has also been described in LPS-treated rats and this deficit is accompanied by evidence of cell stress; thus stimulation of the mitogen-activated protein kinase, c-Jun

N-terminal kinase (JNK), in hippocampus has been coupled with apoptotic changes and with impairment in LTP (Vereker *et al.* 2000b), and there is convincing evidence that these changes are mediated by IL-1 $\beta$  (Vereker *et al.* 2000b; Nolan *et al.* 2002).

A number of recent studies have identified that the *n*-3 polyunsaturated fatty acid, eicosapentaenoic acid (EPA) possesses anti-inflammatory properties (Hayashi *et al.* 1999; Babcock *et al.* 2000), reducing production of proinflammatory cytokines in several cell types (McCarty 1999; Curtis

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**Abbreviations used:** EPA, eicosapentaenoic acid; EPSP, excitatory postsynaptic potential; IL-1 $\beta$ , interleukin-1 $\beta$ ; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; LTP, long-term potentiation; NBM, neurobasal medium; PARP, poly(ADP-ribose) polymerase; p-Bcl-2, phosphorylated Bcl-2; p-c-Jun, phosphorylated c-Jun; p-JNK, phosphorylated JNK.

*et al.* 2000; Wallace *et al.* 2000; Calder and Zurier 2001). Consistently, EPA suppresses the LPS-induced increase in circulating IL-1 $\beta$  (Sadeghi *et al.* 1999), and EPA treatment has been shown to prevent the age-related increases in IL-1 $\beta$  concentration in hippocampus and cortex and the accompanying increases in translocation of cytochrome *c* from mitochondria to the cytosol and activation of caspase-3 (Martin *et al.* 2002). Several proteins play a role in maintaining the integrity of the mitochondrial membrane; one of these is Bcl-2, which has been identified as anti-apoptotic because (a) it forms heterodimers with pro-apoptotic mitochondrial membrane proteins inactivating their pro-apoptotic effect, and (b) it influences the permeability transition pore, preventing membrane rupture and non-specific release of apoptotic mediators such as cytochrome *c* (Kluck *et al.* 1997; Yang *et al.* 1997). A decrease in Bcl-2 expression and increased expression of the pro-apoptotic protein, Bax have been associated with activation of JNK (Miyashita *et al.* 1994; Fuchs *et al.* 1998) and, in addition, phosphorylation of Bcl-2 by JNK appears to antagonize its anti-apoptotic effect (Park *et al.* 1996; Maundrell *et al.* 1997). Thus JNK exerts a significant influence on mitochondrial function.

We proposed that the neuroprotective effect of EPA, which was observed in hippocampus of aged rats, may result from its ability to prevent mitochondrial membrane disruption, which occurs downstream of JNK activation, and to address this proposal we investigated the effects of LPS on JNK activation and mitochondrial function *in vitro* and *in vivo*. We report that LPS induced phosphorylation of both JNK and Bcl-2, accompanied by cytochrome *c* translocation, and that EPA treatment prevented these changes. The evidence suggests that the neuroprotective effect of EPA may derive from its ability to prevent the LPS-induced increase in the permeability of mitochondrial membrane.

## Experimental procedures

### Animals

Primary hippocampal neurones were isolated from Wistar rats 1 day postpartum (Bio Resources Unit, Trinity College, Dublin 2, Ireland). Twenty-four male Wistar rats (250–350 g; Bio Resources Unit, Trinity College) were also used in this study. Animals were housed in groups of six in a controlled environment (20–22°C ambient temperature; 12:12 h light : dark cycle) under veterinary supervision in accordance with national ethical guidelines.

### Preparation and treatment of primary hippocampal neurones

Rats (1 day postpartum) were decapitated, the hippocampi were removed, cross-chopped and incubated in phosphate-buffered saline containing trypsin (0.25  $\mu$ g/mL) for 25 min at 37°C. The tissue was triturated in phosphate-buffered saline containing soyabean trypsin inhibitor (0.2  $\mu$ g/mL) and DNase (0.2 mg/mL) and filtered through a sterile mesh filter (40  $\mu$ m). Following centrifugation (2000 *g* for 3 min at 20°C), the pellet was resuspended in neurobasal medium

(NBM; Gibco, Paisley, UK), supplemented with heat-inactivated horse serum (10%), penicillin (100 U/mL), streptomycin (100 U/mL), glutamax (2 mM) and B-27 (1%). Suspended cells were plated out on 24-well plates at a density of  $1 \times 10^5$  cells on circular coverslips (10 mm diameter) coated with poly-L-lysine (60  $\mu$ g/mL) and incubated in a humidified atmosphere containing 5% CO<sub>2</sub> : 95% air at 37°C. After 48 h, cytosine-arabinofuranoside (5 ng/mL) was included in the NBM for 24 h to prevent proliferation of non-neuronal cells. Media were exchanged every 3 days and neurones were grown in culture for up to 5 days prior to treatment. To assess the possibility that these cultures were contaminated with glia, cells were stained with glial fibrillary acidic protein; no perceptible staining was observed, suggesting that the contribution of glia to the observations reported here is minimal.

Hippocampal neurones were treated with either ethyl-EPA containing 0.2% DL- $\alpha$ -tocopherol (600  $\mu$ M final concentration in NBM; Laxdale Ltd, Stirling, UK) or oleic acid (600  $\mu$ M final concentration in NBM; Sigma, Dorset, UK). Ethyl-EPA and oleic acid were made as 600 mM stock solutions in dimethyl sulfoxide and stored under nitrogen at –20°C until needed. At 24 h, neurones were incubated in the presence/absence of LPS from *Escherichia coli* serotype 0111:B4 (100 ng/mL in NBM; Sigma, Dorset, UK) with either ethyl-EPA or oleic acid. At 48 h, the supernatant was removed; the neurones were rinsed in phosphate-buffered saline and harvested as described below.

### Eicosapentaenoic acid treatment *in vivo*

Twenty-four male Wistar rats were used. Food intake was measured daily for 2 weeks and at the end of this period, one group of 12 rats was randomly assigned to a control treatment regime and the other group of 12 rats was assigned to the experimental treatment regime; the latter group were fed on normal laboratory chow supplemented with 500 mg ethyl-EPA per rat per day containing 0.2% DL- $\alpha$ -tocopherol (Laxdale Ltd, Stirling, UK) for 4 weeks. The rats assigned to the control treatment regime received standard laboratory chow supplemented with monounsaturated fatty acids to ensure isocaloric intake. Rats were offered 100% of their average daily food intake so that the full daily allowance of fatty acids would be ingested. Food and water intake did not vary between groups and there was no significant difference in daily food intake before and after dietary modifications were made.

### Induction of long-term potentiation in perforant path-granule cell synapses *in vivo*

At the end of the 4 week control or EPA treatment, rats were anaesthetized by intraperitoneal administration of urethane (1.5 g/kg) and injected intraperitoneally with either LPS from *Escherichia coli* serotype 0111:B4 (100  $\mu$ g/kg in saline; Sigma, Dorset, UK) or saline. After 3 h, recording and stimulating electrodes were placed in the molecular layer of the dentate gyrus (2.5 mm lateral and 3.9 mm posterior to bregma) and perforant path, respectively (angular bundle, 4.4 mm lateral to lambda). Stable baseline recordings were made before electrophysiological recording commenced at test shock frequency (1/30 s), 10 min before and 40 min after tetanic stimulation (three trains of stimuli; 250 Hz for 200 ms; intertrain interval, 30 s; for details see Lonergan *et al.* 2002). Rats were killed by decapitation; the hippocampi were removed, cross-chopped into slices (350  $\times$  350  $\mu$ m) and frozen separately in 1 mL of Krebs solution

(136 mM NaCl, 2.54 mM KCl, 1.18 mM  $\text{KH}_2\text{PO}_4$ , 1.18 mM  $\text{MgSO}_4$ , 16 mM  $\text{NaHCO}_3$ , 10 mM glucose, 1.13 mM  $\text{CaCl}_2$ ) containing 10% dimethyl sulfoxide. For analysis, thawed slices were rinsed three times in Krebs solution and used as described below.

#### **Analysis of JNK phosphorylation, c-Jun phosphorylation, Bcl-2 phosphorylation, Bax expression, cytochrome *c* translocation and poly(ADP-ribose) polymerase cleavage**

JNK phosphorylation, c-Jun phosphorylation, cytochrome *c* translocation, Bcl-2 phosphorylation and Bax expression were assessed in samples prepared from primary hippocampal neurones. In the case of JNK phosphorylation and c-Jun phosphorylation, hippocampal neurones were harvested in lysis buffer (20 mM HEPES, pH 7.4, 10 mM KCl, 1.5 mM  $\text{MgCl}_2$ , 1 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulphonyl fluoride, 2 mg/mL leupeptin, 2 mg/mL aprotinin, 200 mM sucrose), incubated on ice for 15 min and equalized for protein concentration (Bradford 1976). Aliquots were added to sample buffer [0.5 mM Tris-HCl, pH 6.8, 20% glycerol, 2% sodium dodecyl sulfate, 5%  $\beta$ -mercaptoethanol, 0.05% bromophenol blue (w/v)] to a final concentration of 500  $\mu\text{g/mL}$  and boiled for 3 min. Cytochrome *c* translocation was assessed in cytosolic fractions and Bcl-2 phosphorylation and Bax expression were assessed in mitochondrial fractions. Hippocampal neurones were digitonin-permeabilized for 5 min on ice in cytosolic extraction buffer (250 mM sucrose, 70 mM KCl, 137 mM NaCl, 4.3 mM  $\text{Na}_2\text{HPO}_4$ , 1.4 mM  $\text{KH}_2\text{PO}_4$ , pH 7.2, 100  $\mu\text{M}$  phenylmethylsulphonyl fluoride, 10  $\mu\text{g/mL}$  leupeptin, 2  $\mu\text{g/mL}$  aprotinin, containing 200  $\mu\text{g/mL}$  digitonin) and the supernatant (cytosolic fraction) was equalized for protein concentration and used for analysis of cytochrome *c* translocation. Aliquots were added to sample buffer to a final concentration of 100  $\mu\text{g/mL}$  and boiled for 3 min. The remaining cells were harvested in mitochondrial lysis buffer (50 mM Tris-Base, pH 7.4, 150 mM NaCl, 2 mM EGTA, 2 mM EDTA, 0.1 mM phenylmethylsulphonyl fluoride, 0.2% Triton X-100, 0.3% igeal CA-630, 10 mg/mL leupeptin, 2 mg/mL aprotinin), centrifuged (10 000 *g* for 15 min at 4°C) and the supernatant (mitochondrial fraction) was equalized for protein concentration and used for analysis of Bcl-2 phosphorylation and Bax expression. Aliquots were added to sample buffer to a final concentration of 400  $\mu\text{g/mL}$  and boiled for 3 min.

JNK phosphorylation, c-Jun phosphorylation, cytochrome *c* translocation, Bcl-2 phosphorylation, Bax expression and poly (ADP-ribose) polymerase (PARP) cleavage were also analysed in samples prepared from hippocampal tissue using a method described previously (Lonergan *et al.* 2002). In the case of JNK phosphorylation, c-Jun phosphorylation and PARP cleavage, tissue was homogenized in lysis buffer and equalized for protein concentration. Aliquots were added to sample buffer to a final concentration of 1 mg/mL and boiled for 5 min. In the case of cytochrome *c* translocation, hippocampal homogenate was digitonin-permeabilized for 20 min on ice in cytosolic extraction buffer and centrifuged (15 000 *g* for 10 min at 4°C). The supernatant (cytosolic fraction) was equalized for protein concentration and aliquots were added to sample buffer to a final concentration of 300  $\mu\text{g/mL}$  and boiled for 3 min. Bcl-2 phosphorylation and Bax expression were assessed in mitochondrial fractions; pellets were resuspended in mitochondrial lysis buffer and equalized for protein concentration. Aliquots were added to sample buffer to a final concentration of 800  $\mu\text{g/mL}$  and boiled for 3 min.

In all cases, proteins were loaded on to sodium dodecyl sulfate–polyacrylamide gels and separated by application of a constant current (32 mA) for 25–30 min, transferred onto nitrocellulose membranes (225 mA for 90 min) and probed with the appropriate antibody. The primary antibodies for phospho-JNK, phospho-c-Jun, cytochrome *c*, Bax and PARP were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The primary antibody for phospho-Bcl-2 was obtained from Oncogene Research Products (San Diego, CA, USA). Immunoreactive bands were detected with horseradish peroxidase-conjugated secondary antisera (Sigma, Dorset, UK) using the SuperSignal West Dura chemiluminescence reagents (Pierce, Chester, UK). Bands were quantified using densitometry (ZERO-Dscan Image Analysis System) and values expressed as arbitrary units. All blots were stripped (ReBlot Plus, Chemicon, Temecula, CA, USA) and in some cases reprobed for analysis of total (rather than phosphorylated) JNK and in other cases reprobed with an anti-actin antibody to confirm equal loading of proteins.

#### **Analysis of hippocampal concentrations of interleukin-1 $\beta$**

IL-1 $\beta$  concentration was analysed in hippocampal homogenate by enzyme-linked immunosorbent assay (R & D Systems, Minneapolis, MN, USA) as described previously (Martin *et al.* 2002). Antibody-coated (1.0  $\mu\text{g/mL}$  goat anti-rat IL-1 $\beta$  antibody diluted in phosphate-buffered saline, pH 7.3) 96-well plates were incubated overnight at room temperature (20–22°C), washed several times with phosphate-buffered saline containing 0.05% Tween 20, blocked for 1 h at room temperature with blocking buffer (phosphate-buffered saline, pH 7.3; 5% sucrose, 1% bovine serum albumin, 0.05%  $\text{NaN}_3$ ) and incubated with IL-1 $\beta$  standards (0–1000 pg/mL) or samples for 2 h at room temperature. The plate was washed with phosphate-buffered saline, incubated with secondary antibody (350 ng/mL biotinylated goat anti-rat antibody diluted in phosphate-buffered saline containing 1% bovine serum albumin and 2% normal goat serum) for 2 h at room temperature, washed again and incubated with horseradish peroxidase-conjugated streptavidin (diluted in phosphate-buffered saline containing 1% bovine serum albumin) for 20 min at room temperature. Substrate solution (1 : 1 mixture of  $\text{H}_2\text{O}_2$  and tetramethylbenzidine) was added, incubation continued at room temperature in the dark for 30 min and the reaction stopped using 1 M  $\text{H}_2\text{SO}_4$ . Absorbance was read at 450 nm, values were corrected for protein (Bradford 1976) and expressed as pg/mg protein.

#### **Statistical analysis**

Data are expressed as means  $\pm$  SEM. A one-way analysis of variance (ANOVA) was performed to determine whether there were significant differences between conditions. When this analysis indicated significance (at the 0.05 level), *post hoc* Student Newman–Keuls test analysis was used to determine which conditions were significantly different from each other (Prism, GraphPad Software, San Diego, CA, USA).

## **Results**

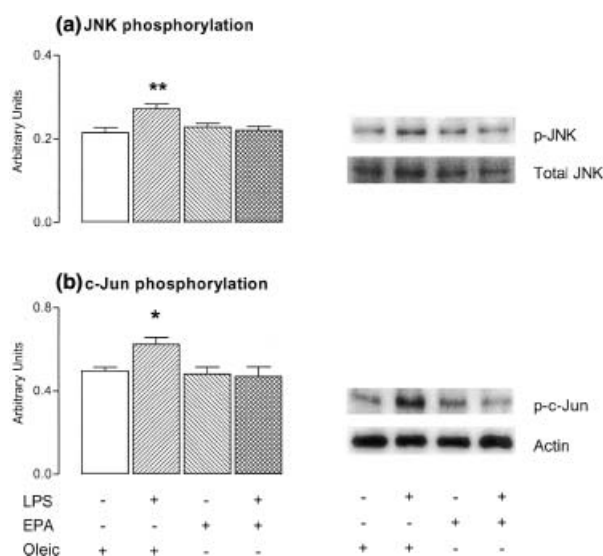
### **JNK and c-Jun phosphorylation in hippocampal neurones**

Incubation of hippocampal neurones in the presence of LPS leads to a significant increase in expression of phosphorylated

JNK (p-JNK). Figure 1 shows one immunoblot indicating that p-JNK, but not total JNK, expression was increased in LPS-treated cells. The mean data obtained from densitometric analysis indicate a statistically significant LPS-induced enhancement in p-JNK ( $p < 0.01$ ; ANOVA; Fig. 1a) but not in total JNK. EPA treatment completely abrogated the LPS-induced change so that mean p-JNK expression was similar in LPS-treated samples as in controls and significantly reduced compared with the value in the LPS-treated sample ( $p < 0.01$ ; ANOVA). In parallel with this observation, we found that phosphorylation of c-Jun was significantly increased in LPS-treated neurones compared with control-treated neurones ( $p < 0.05$ ; ANOVA) and that EPA prevented the LPS-induced change (Fig. 1b).

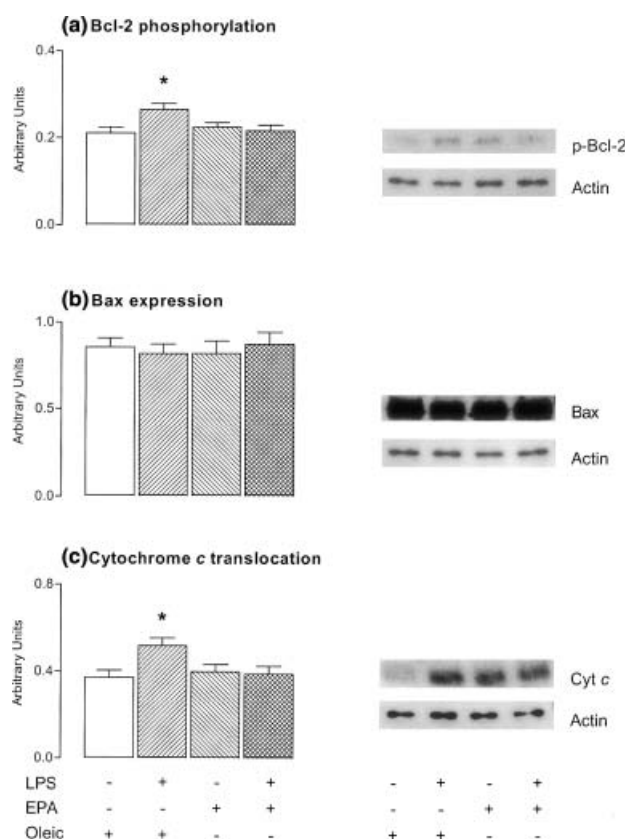
### Bcl-2 phosphorylation, Bax expression and cytochrome *c* translocation in hippocampal neurones

Because the mitochondrial protein, Bcl-2 is a substrate for JNK, we analysed expression of phosphorylated Bcl-2 (p-Bcl-2) in a mitochondrial fraction prepared from cells in all treatment groups. The immunoblot and mean data obtained from densitometric analysis, shown in Fig. 2(a),



**Fig. 1** The lipopolysaccharide (LPS)-induced increases in phosphorylation of c-Jun N-terminal kinase (JNK) and c-Jun in hippocampal neurones are abrogated by treatment with eicosapentaenoic acid (EPA). Hippocampal neurones were treated with EPA (600  $\mu$ M) or oleic acid (600  $\mu$ M) for 48 h with the addition of LPS (100 ng/mL) or control medium at 24 h. Cells were harvested and samples prepared (see text) for analysis of expression of phosphorylated JNK (p-JNK) and c-Jun (p-c-Jun). LPS significantly increased expression of p-JNK and p-c-Jun ( $*p < 0.05$ ;  $**p < 0.01$ ; ANOVA) and LPS-induced changes were abrogated by EPA. Mean arbitrary values ( $\pm$  SEM;  $n = 6$ ), obtained from densitometric analysis are presented and sample blots are shown. In all cases, blots were stripped and reprobed with total JNK (a) or actin (b) to confirm equal loading of proteins.

demonstrate that phosphorylation of Bcl-2 (p-Bcl-2) was enhanced in LPS-treated neurones. This increase was statistically significant ( $p < 0.05$ ; ANOVA), but expression of p-Bcl-2 was similar in samples prepared from the other treatment groups and protein loading was similar in all treatment groups as indicated by the expression of actin. In contrast to Bcl-2, Bax has been identified as a pro-apoptotic mitochondrial protein and an increase in its expression has been associated with loss of mitochondrial membrane integrity and apoptosis (Pastorino *et al.* 1998). In this study, we observed no change in Bax expression in mitochondrial fractions following any of the treatments (Fig. 2b).



**Fig. 2** The lipopolysaccharide (LPS)-induced increases in phosphorylation of Bcl-2 and cytochrome *c* translocation in hippocampal neurones are abrogated by treatment with eicosapentaenoic acid (EPA). Hippocampal neurones were treated with EPA (600  $\mu$ M) or oleic acid (600  $\mu$ M) for 48 h with the addition of LPS (100 ng/mL) or control medium at 24 h. Cells were harvested and samples prepared for analysis of expression of phosphorylated mitochondrial Bcl-2 (p-Bcl-2), mitochondrial Bax and cytosolic cytochrome *c* (Cyt *c*). LPS significantly increased expression of p-Bcl-2 and Cyt *c* ( $*p < 0.05$ ; ANOVA;  $n = 6$  in both cases) and LPS-induced changes were abrogated by EPA. Mitochondrial Bax expression was unaffected by treatment. Mean arbitrary values obtained from densitometric analysis are presented and sample blots are shown. In all cases, blots were stripped and reprobed with actin to confirm equal protein loading.

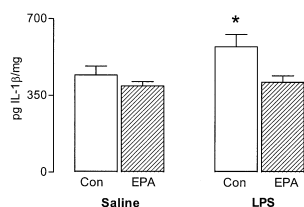
The interaction between Bcl-2 and pro-apoptotic proteins dissolves when Bcl-2 is phosphorylated and therefore its protective effect on mitochondrial membrane integrity is lost. Consistent with this, we observed that LPS treatment of cells resulted in translocation of cytochrome *c* to the cytosol, paralleling the LPS-induced increase in phosphorylation of Bcl-2 (Fig. 2c). Thus, expression of cytochrome *c* in the cytosol was significantly increased in LPS-treated preparations ( $p < 0.05$ ; ANOVA), whereas this effect was abrogated by EPA so that mean values were similar in the other three treatment groups.

### Interleukin-1 $\beta$ concentration in hippocampal tissue

We considered that if EPA had the ability to reverse the increase in mitochondrial membrane permeability in cultured hippocampal cells, it might exert a similar effect *in vivo* and therefore groups of rats were treated with/without EPA for 4 weeks and then treated with LPS or saline. Hippocampal tissue prepared from these rats was first analysed for concentration of IL-1 $\beta$  because we have previously observed that IL-1 $\beta$  triggers an increase in JNK activation in hippocampus (O'Donnell *et al.* 2000; Vereker *et al.* 2000b; Kelly *et al.* 2001). Figure 3 indicates that intraperitoneal injection of LPS significantly increased hippocampal IL-1 $\beta$  concentration ( $p < 0.05$ ; ANOVA), but there was no evidence of a similar increase in IL-1 $\beta$  concentration in hippocampal samples prepared from LPS-treated rats that were treated with EPA, in which mean values were similar to those in saline-treated controls.

### JNK and c-Jun phosphorylation in hippocampal tissue

Expression of p-JNK in hippocampal tissue precisely mirrored the changes observed *in vitro*; thus phosphorylation of JNK was significantly increased in hippocampal tissue prepared from LPS-treated rats ( $p < 0.05$ ; ANOVA; Fig. 4a) but there was no evidence of similar changes in tissue prepared from rats treated with both LPS and EPA. In parallel with this observation, we report that phosphorylation of c-Jun was significantly increased in hippocampal preparations obtained from LPS-treated, compared with saline-treated, rats



**Fig. 3** The lipopolysaccharide (LPS)-induced increase in interleukin-1 $\beta$  (IL-1 $\beta$ ) is inhibited by eicosapentaenoic acid (EPA). IL-1 $\beta$  concentration was significantly increased in hippocampal tissue obtained from rats that were injected intraperitoneally with LPS (100  $\mu$ g/kg;  $*p < 0.05$ ; ANOVA;  $n = 6$ ) but this change was not observed in LPS-treated rats that received EPA.

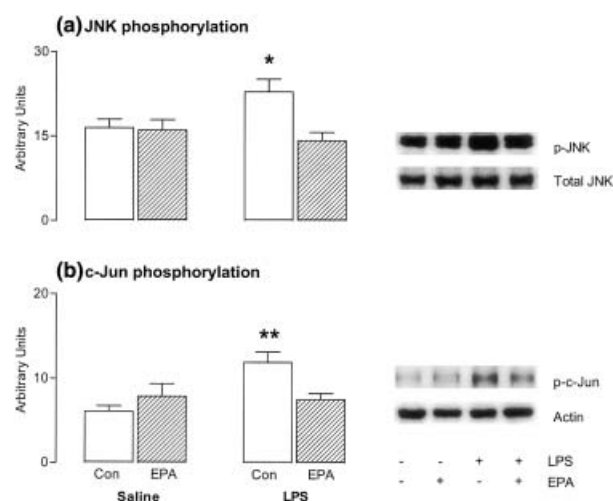
( $p < 0.01$ ; ANOVA) and that EPA prevented this LPS-induced change (Fig. 4b).

### Bcl-2 phosphorylation and Bax expression in hippocampal tissue

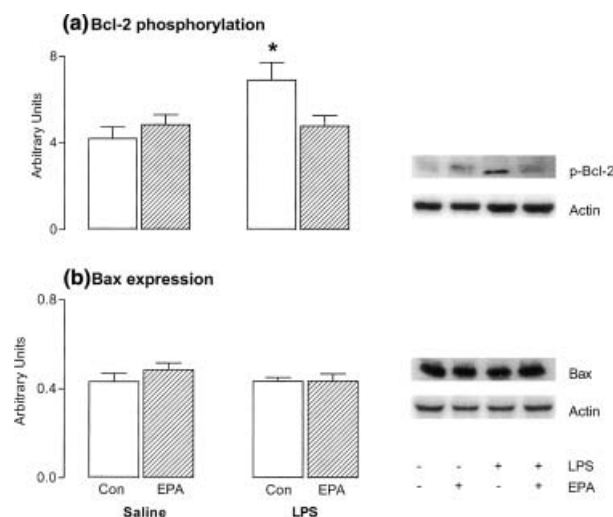
In a striking parallel with the results obtained *in vitro*, the data indicate that the increase in JNK phosphorylation was accompanied by an increase in phosphorylation of Bcl-2 in mitochondrial preparations obtained from hippocampus of LPS-injected rats; Fig. 5(a) demonstrates that mean phosphorylation was significantly increased in LPS-treated, compared with saline-treated rats ( $p < 0.05$ ; ANOVA), but no similar effect was observed in preparations obtained from LPS-treated rats that received EPA. In contrast to the LPS-induced increase in Bcl-2 phosphorylation, there was no evidence of any change in Bax expression (Fig. 5b).

### Cytochrome *c* translocation and poly(ADP-ribose) polymerase cleavage in hippocampal tissue

Consistent with the hypothesis that phosphorylation of Bcl-2 leads to increased mitochondrial membrane permeability, is the finding that cytochrome *c* translocation was significantly increased in cytosolic preparations obtained from hippocampus of LPS-treated rats ( $p < 0.01$ ; ANOVA; Fig. 6a). Pre-treatment of rats with EPA prevented this LPS-induced change so that cytosolic cytochrome *c* expression was similar in this group and in the control group. In an effort to



**Fig. 4** The lipopolysaccharide (LPS)-induced increases in phosphorylation of c-Jun N-terminal kinase (JNK) and c-Jun are inhibited by eicosapentaenoic acid (EPA). Expression of phosphorylated JNK (p-JNK) and c-Jun (p-c-Jun) was significantly increased in hippocampal tissue obtained from rats that were injected intraperitoneally with LPS (100  $\mu$ g/kg;  $*p < 0.05$ ;  $**p < 0.01$ ; ANOVA;  $n = 6$ ) but this change was not observed in LPS-treated rats that received EPA. These trends are evident in the sample immunoblots, which also show, in stripped and reprobed blots, that neither total JNK nor actin expression was altered by treatment.

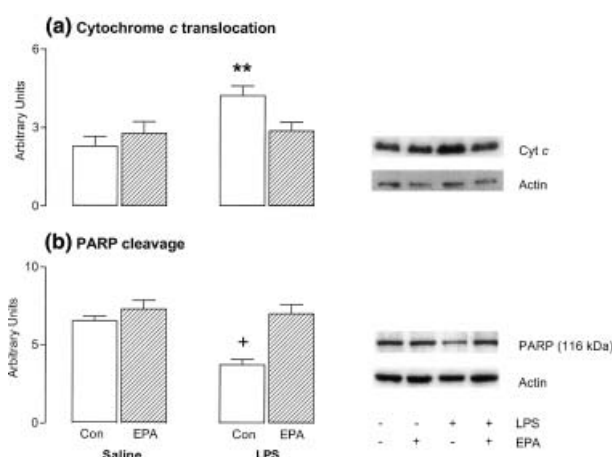


**Fig. 5** The lipopolysaccharide (LPS)-induced increase in phosphorylation of Bcl-2 is inhibited by eicosapentaenoic acid (EPA). Expression of phosphorylated Bcl-2 (p-Bcl-2) was significantly increased in a mitochondrial preparation obtained from hippocampus of rats that were injected intraperitoneally with LPS (100  $\mu$ g/kg; \* $p$  < 0.05; ANOVA;  $n$  = 6) but this change was not observed in LPS-treated rats that received EPA. Bax expression was unaffected by treatment. Sample immunoblots for p-Bcl-2 and Bax are shown and accompanied by sample blots indicating that actin expression did not change with treatment.

consolidate this finding, we assessed one of the downstream cellular consequences of cytochrome *c* translocation, i.e. cleavage of the DNA repair enzyme, PARP. This was examined by analysis of expression of the 116 kDa holoenzyme in tissue prepared from hippocampus. Figure 6(b) shows that PARP expression was significantly decreased in preparations obtained from LPS-treated rats compared with expression in the other treatment groups ( $p$  < 0.001; ANOVA); specifically and importantly, the LPS-induced change was not evident in hippocampal samples prepared from LPS-treated rats that received EPA.

#### Long-term potentiation *in vivo*

These data pointed to a neuroprotective effect of EPA, preventing the threatening effects of LPS on cell function. We predicted that synaptic function might also be protected by EPA and in an effort to test this prediction, we analysed LTP in perforant path-granule cell synapses in the four treatment groups of rats, as we have previously observed a robust and reproducible inhibitory effect of LPS on LTP (Vereker *et al.* 2000a; Nolan *et al.* 2002; Kelly *et al.* 2003). Figure 7(a) shows that delivery of three trains of tetanic stimulation to the perforant path led to a marked and immediate increase in the mean population excitatory postsynaptic potential (EPSP) slope in saline-treated control rats. This change was sustained for the duration of the experiment. LPS significantly inhibited expression of LTP;



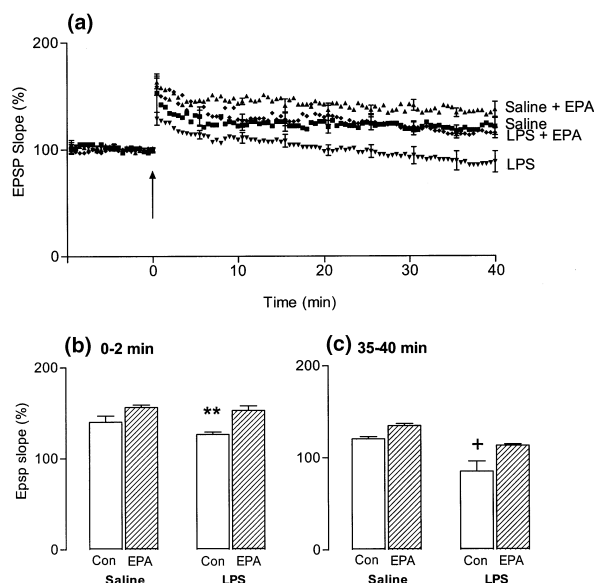
**Fig. 6** The lipopolysaccharide (LPS)-induced increases in cytochrome *c* translocation and poly(ADP-ribose) polymerase (PARP) cleavage are inhibited by eicosapentaenoic acid (EPA). Expression of cytochrome *c* (Cyt *c*) was significantly increased in a cytosolic fraction prepared from hippocampus of rats that were injected intraperitoneally with LPS (100  $\mu$ g/kg; \*\* $p$  < 0.01; ANOVA;  $n$  = 6) but this change was not observed in LPS-treated rats that received EPA. Expression of 116 kDa PARP was significantly decreased in hippocampal tissue prepared from LPS-treated rats (\* $p$  < 0.05; ANOVA;  $n$  = 6) but this change was not observed in LPS-treated rats that received EPA. Immunoblots reprobed with anti-actin revealed that actin expression was unchanged by treatment.

thus the mean percentage increase in EPSP slope in the last 5 min of the experiment (compared with the mean value in the 5-min period immediately prior to stimulation) in saline-treated rats was  $120.62 \pm 2.46$  and the corresponding value in LPS-treated rats was  $85.20 \pm 11.01$  ( $p$  < 0.01; ANOVA; Fig. 7c). EPA treatment significantly attenuated the effect of LPS ( $p$  < 0.01; ANOVA) but did not completely reverse it, although there was no significant difference between the value in the saline-treated controls and that in the group treated with LPS and EPA. Figure 7(b) shows that LPS also attenuated the immediate change in EPSP slope; thus the mean percentage changes in the 2-min period immediately following tetanic stimulation in saline-treated and LPS-treated rats were  $140.27 \pm 6.78$  and  $126.54 \pm 2.62$ , respectively ( $p$  < 0.01; ANOVA). The corresponding value in LPS-treated rats that received EPA was  $152.85 \pm 5.27$ .

#### Discussion

The findings of this study indicate that treatment with EPA provided protection from the damaging effects of LPS on hippocampal neurones and hippocampal function. The data presented indicate that EPA prevents the LPS-induced increase in IL-1 $\beta$  concentration in hippocampus and the accompanying IL-1 $\beta$ -induced signalling.

We first investigated the effect of EPA *in vitro* and found that several LPS-induced changes in hippocampal neurones



**Fig. 7** The lipopolysaccharide (LPS)-induced inhibition of long-term potentiation (LTP) is blocked by eicosapentaenoic acid (EPA). (a) Intraperitoneal injection of LPS blocked tetanus-induced LTP in perforant path-granule cell synapses but this effect was suppressed by pretreatment of rats with EPA. The data are expressed as the mean percentage change in population excitatory postsynaptic potential (EPSP) slope (compared with the EPSP slope in the 5 min immediately prior to tetanic stimulation). Values are means  $\pm$  SEM of six rats in each treatment group. (b) and (c) The mean percentage changes in EPSP slopes in the 2 min (b) and 35–40 min (c) following tetanic stimulation indicate the significant inhibitory effect of LPS (\*\* $p < 0.01$ ; + $p < 0.01$ ; ANOVA) on LTP. Pre-treatment with EPA reversed the effect of LPS, so that there was a significant difference in mean percentage EPSP slope in rats treated with LPS and those treated with LPS + EPA ( $p < 0.01$ ; ANOVA; at both time intervals).

were attenuated by co-incubation of cultured cells with EPA. LPS stimulated JNK activation, as previously shown in neutrophils (Nick *et al.* 1996), macrophages (Nick *et al.* 1996; MacKichan and DeFranco 1999), microglial cultures (Pyo *et al.* 1998) and in hippocampus following LPS injection (Vereker *et al.* 2000a). Several reports have implicated the JNK pathway in apoptosis in a variety of cultured cells, including some of neuronal origin (Xia *et al.* 1995; Choi *et al.* 1999; Kumagai *et al.* 1999; Mielke *et al.* 2000). It is considered that JNK-stimulated phosphorylation of the transcription factor, c-Jun, represents an important step in the cascade of events leading to apoptosis (Neary 1997; Ip and Davis 1998; Harper and LoGrasso 2001). In addition, there is convincing evidence that Bcl-2 phosphorylation by JNK (Maundrell *et al.* 1997) antagonizes its anti-apoptotic action (Haldar *et al.* 1995; Park *et al.* 1996; Yamamoto *et al.* 1999) and one consequence of this is increased permeability of the mitochondrial membrane. Therefore, in an effort to consolidate the finding that LPS induces a potentially damaging sequence of changes in hippocampus, stimulated by JNK

activation, we analysed phosphorylation of c-Jun, Bcl-2 and cytochrome *c* translocation. The data indicate that LPS induced parallel increases in phosphorylation of JNK, c-Jun, Bcl-2 and also in translocation of cytochrome *c*, suggesting that JNK phosphorylates Bcl-2, which consequently compromises its anti-apoptotic effect, leads to leakage of the mitochondrial membrane and translocation of cytochrome *c* to the cytosol. Disruption of the mitochondrial transmembrane potential and cytochrome *c* translocation from the mitochondria to the cytosol are relatively early events in apoptotic cell death (Cortopassi and Wong 1999) and lead to sequential activation of caspase-9 and caspase-3 in several tissues (Liu *et al.* 1996; Slee *et al.* 1999; Kannan and Jain 2000), including hippocampus (Lonergan *et al.* 2002). Here we found that cytochrome *c* translocation was associated with cleavage of the DNA repair enzyme and caspase-3 substrate, PARP; cleavage of PARP results in its inability to repair DNA, and therefore is considered to be a reliable indicator of apoptosis (Martinou and Sadoul 1996; O'Brien *et al.* 2001). This series of changes have been identified as factors that herald the demise of the cell; thus the present observations point to LPS as an inducer of apoptosis in hippocampal cells *in vitro*, an effect that has been reported in fibroblasts (Alikhani *et al.* 2003), neurones and glia (Picot *et al.* 2003), and macrophages (Comalada *et al.* 2003). Of particular significance is the observation that EPA acted as a neuroprotective agent, preventing each of these LPS-induced changes.

Each of the LPS-induced effects, which we observed *in vitro*, were also observed in hippocampal tissue prepared from rats injected intraperitoneally with LPS. Thus the LPS-induced increases in phosphorylation of JNK and c-Jun were accompanied by increased phosphorylation of Bcl-2, increased concentration of cytochrome *c* in the cytosol and increased cleavage of PARP. IL-1 $\beta$  concentration was also enhanced in hippocampus prepared from LPS-treated rats; it is possible that IL-1 $\beta$  triggers the increase in JNK phosphorylation and the consequent downstream changes, as we have previously reported that several LPS-induced changes, including the increase in JNK phosphorylation, are blocked by treatment of rats with an inhibitor of caspase-1 (Vereker *et al.* 2000a). Moreover the increase in JNK activation and at least some of the downstream effects of this, which are induced by LPS treatment, are also induced by IL-1 $\beta$  (Martin *et al.* 2002).

We report that these changes in hippocampus are accompanied by a deficit in LTP in perforant path-granule cell synapses and this confirms our previous findings (Vereker *et al.* 2000a). It is possible that IL-1 $\beta$  mediates this effect of LPS because impaired LTP is coupled with increased IL-1 $\beta$  concentration in hippocampus (Vereker *et al.* 2000a; Martin *et al.* 2002; Minogue *et al.* 2003) and data from several groups have reported that IL-1 $\beta$  leads to attenuation of LTP (Katsuki *et al.* 1990; Bellinger *et al.* 1993; Cunningham

*et al.* 1996). Similarly, activation of JNK may be a key component in the inhibition of LTP because both IL-1 $\beta$ -induced (Curran *et al.* 2003) and LPS-induced (Barry and Lynch 2003) inhibition of LTP are abrogated by inhibitors of JNK.

One of the most significant findings presented here is that EPA acted as a neuroprotective agent, preventing the damaging effects triggered by LPS both *in vitro* and *in vivo*. Thus EPA blocked the LPS-associated increases in phosphorylation of JNK, c-Jun and Bcl-2 *in vitro* and the subsequent translocation of cytochrome *c* and cleavage of PARP. Consistent with the present findings is the report that docosahexaenoic acid protects Neuro-2A and PC12 cells from apoptosis induced by serum starvation (Kim *et al.* 2000; Calder 2001). This effect was attributed to docosahexaenoic acid-induced accumulation of phosphatidylserine, which enhanced membrane translocation of raf-1 (Kim *et al.* 2001). Interestingly we have shown that treatment of cortical neurones with phosphatidylserine attenuates the LPS-induced increase in TUNEL staining (Nolan *et al.* 2004).

Although this neuroprotective effect of EPA *in vitro* is significant, the finding that a similar neuroprotective effect was observed following treatment of rats with EPA is of particular importance. *In vivo*, EPA prevented the LPS-induced increase in IL-1 $\beta$  concentration in hippocampus, the activation of JNK and the downstream consequences of JNK activation, i.e. phosphorylation of Bcl-2, cytochrome *c* translocation and PARP cleavage. Coupled with these changes, we report that EPA treatment attenuated the deficit in LTP triggered by LPS injection. These LPS-induced changes are broadly similar to those observed in hippocampal tissue prepared from aged rats (Martin *et al.* 2002), rats exposed to  $\gamma$ -irradiation (Lonergan *et al.* 2002) and rats treated with amyloid- $\beta$  peptide (Minogue *et al.* 2003). Consistent with a neuroprotective role for EPA, we have previously reported that treatment with EPA abrogated the changes associated with age (Martin *et al.* 2002) and irradiation (Lonergan *et al.* 2002).

A number of studies have suggested that EPA and other fish oils possess anti-inflammatory actions (Hayashi *et al.* 1999; Babcock *et al.* 2000), reducing production of proinflammatory cytokines in circulating cells (McCarty 1999; Sadeghi *et al.* 1999; Wallace *et al.* 2000; Calder and Zurier 2001), chondrocytes (Curtis *et al.* 2000) and macrophages (Calder 2001; Babcock *et al.* 2002). Similarly, dietary supplementation with *n*-3 polyunsaturated fatty acids decreases monocyte and neutrophil chemotaxis in human subjects and ameliorates symptoms of autoimmune disease in some animal models (Calder 2001) and prevents the age-related increase in IL-1 $\beta$  concentration in hippocampus and cortex (Martin *et al.* 2002). The present findings add further support to the body of evidence that indicates an anti-inflammatory role for *n*-3 polyunsaturated fatty acids; specifically EPA prevents LPS-induced stress-related changes in hippocampal

neurones and EPA also protects against several of these changes that are also observed *in vivo*.

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