Analysis of Interleukin-1β-induced Cell Signaling Activation in Rat Hippocampus following Exposure to Gamma Irradiation

PROTECTIVE EFFECT OF EICOSAPENTAENOIC ACID†

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Among the many reported effects of irradiation in cells is activation of the stress-activated protein kinase, c-Jun N-terminal kinase (JNK), which has been shown to result in apoptotic cell death. The trigger that leads to JNK activation has not been identified, although, in rat hippocampus at least, irradiation-induced apoptosis has been coupled with increased accumulation of reactive oxygen species (ROS). Significantly, irradiation-induced changes in hippocampus are abrogated by treatment of rats with the polyunsaturated fatty acid, eicosapentaenoic acid (EPA). A close coupling between ROS accumulation and concentration of the pro-inflammatory cytokine, interleukin-1β (IL-1β) in hippocampus has been reported, and the evidence suggests that IL-1β may be responsible for the enhanced ROS production. Here we set out to assess the possibility that whole body γ-irradiation increases IL-1β concentration in hippocampus and to investigate the consequences of such a change. We present evidence that reveals that the irradiation-induced increase in IL-1β concentration in hippocampus is accompanied by increased expression of IL-1 type I receptor and IL-1 accessory protein and increased activation of IL-1 receptor-activated kinase. These changes, which were coupled with increased activation of JNK and evidence of apoptotic cell death, were absent in hippocampus of rats that received EPA treatment. Significantly, EPA treatment enhanced hippocampal IL-10 concentration that was inversely correlated with IL-1β concentration. The data are consistent with the idea that EPA exerts anti-inflammatory and neuroprotective effects in the central nervous system.

Interleukin-1β (IL-1β) exerts a wide range of effects in the CNS, most of which have been attributed to its interaction with IL-1 type I receptor (IL-1RI (1)), although recent evidence has revealed actions of IL-1β that are independent of interaction with IL-1RI (2). IL-1RI activation by IL-1β initiates formation of a complex that requires recruitment of IL-1 accessory protein (IL-1aP), an adaptor protein, MyD88, and a kinase, IL-1 receptor-associated kinase (IRAK) (1). Phosphorylation of IRAK is a pivotal step in activating the cascade of events that lead to the IL-1β-induced response; events in this cascade include activation of the stress-activated protein kinase, c-Jun N-terminal kinase (JNK). Increased IL-1β concentration in hippocampus is associated with impairment in synaptic function, and activation of JNK significantly contributes to this; for example, deficits in synaptic transmission, and consequently in long-term potentiation (LTP), have been coupled with increased IL-1β concentration and increased JNK activation in aged rats (3, 4) and lipopolysaccharide-treated rats (5). Hippocampal and cortical cell death has also been reported to accompany these changes (4–6).

Radiotherapy treatment for brain tumors has been shown to result in deficits in hippocampal-dependent cognitive function (7, 8), and in animal studies, exposure to irradiation exerts profound effects on hippocampal function that have been coupled with apoptosis. For example, irradiation inhibits LTP in CA1 in vitro (9) and in dentate gyrus in vivo (10) and impairs memory/learning (11, 12). These changes have been shown to be accompanied by apoptotic cell death (10, 13), decreased neurogenesis (14, 15), and apoptosis of proliferating stem cells in dentate gyrus (16), the area of the hippocampus that has been shown to be particularly susceptible to irradiation (17). Although several changes have been observed following irradiation, the cell signaling events leading to irradiation-induced cell loss have not been explained. In a recent study, we have coupled irradiation-induced impairment in synaptic function with accumulation of reactive oxygen species (ROS) in hippocampus and with apoptotic changes (10). Significantly, treatment of rats with eicosapentaenoic acid (EPA) prevented these changes. Others have reported that inflammatory changes occur in brain following irradiation and increases in IL-1β and tumor necrosis factor α mRNA expression have been described previously (18). Interestingly, a close correlation between inflammation, as assessed by IL-1β concentration, and accumulation of ROS has been identified in hippocampus. Specifically, IL-1β increases activity of superoxide dismutase (but not catalase and glutathione peroxidase) resulting in increased ROS accumulation (3, 19).

Here we have investigated the possibility that exposure of rats to whole body irradiation induces an increase in hippocampal IL-1β concentration and IL-1β-induced signaling. The data suggest that the irradiation-induced apoptotic changes in hippocampus are a consequence of increased IL-1β concentration and the subsequent up-regulation of JNK activation. We report...
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that these changes are prevented by treatment with EPA, which increases hippocampal concentrations of the anti-inflammatory cytokine, IL-10.

EXPERIMENTAL PROCEDURES

Animals—Thirty-six adult (3 months) male Wistar rats were housed in groups of six in the BioResources Unit, Trinity College, Dublin and maintained under a 12-h light schedule (ambient temperature 22–23 °C) with veterinary supervision for the duration of the study. Rats were randomly assigned to one of six treatment groups. Food and water intake was measured daily for 2 weeks, and at the end of this period, 12 rats were fed on normal laboratory chow supplemented with ethyl cellulose (EPA) 250 mg (1%; dissolved in corn oil; Sigma, UK), 12 rats were fed on laboratory chow supplemented with 500 mg (2%) of EPA, and the remaining 12 rats received control diet (laboratory chow to which only corn oil was added). Sufficient diet was prepared for 2 or 3 days at a time, and rats were offered 100% of their average daily food intake so that the full daily allowance of EPA would be ingested; dietary supplementation continued for 4 weeks. Food and water intake, and weight gained did not vary between groups, and there was no significant difference in daily food and water intake before and after dietary modifications were made.

At the end of this 4-week period, six rats from each treatment groups were exposed to whole body irradiation (10 Gy at a rate of 10 Gy/min; NordLab Gammacell cesium137 irradiator). The remaining rats were sham-irradiated. Rats were monitored for 4 days following irradiation and were then killed by cervical dislocation and decapitation. Hippocampal tissue was dissected free, sliced (350 μm) using a McIlwain tissue chopper, and stored at −80 °C in Krebs solution containing 10% dimethyl sulfoxide as previously described (3) until required for analysis.

The experimental conditions were chosen on the basis of findings from preliminary experiments in which irradiation dose and changes in hippocampal IL-1β concentration at 1, 2, and 4 days following irradiation were assessed. The data from these experiments indicated that 10 Gy at 10 Gy/min was not associated with weight loss or gastrointestinal disturbance, which occurred at higher doses. The data also indicated that the increase in hippocampal IL-1β concentration was maximal 4 days after irradiation.

Analysis of Hippocampal Concentrations of IL-1β and IL-10—Hippocampal slices were thawed by agitation in a water bath at 37 °C for 1.5–2 min. Tissue was homogenized in Krebs solution containing 2 mM CaCl2, and samples were analyzed for concentrations of IL-1β and IL-10 by ELISA (R&D Systems) as previously described (4). Briefly, in the case of IL-1β, antibody-coated (100 μl; 1.0 μg/ml) final concentration, diluted in phosphate-buffered saline (PBS), pH 7.3; goat anti-rat IL-1β antibody) 96-well plates were incubated overnight at room temperature, washed several times with PBS containing 0.05% Tween 20, blocked for 1 h at room temperature with 5% milk, rabbit polyclonal IgG, Santa Cruz Biotechnology) in 0.1% BSA in TBS-T, whereas cytochrome c (1:600; rabbit anti-rat IL-1RI antibody) was loaded onto either 10% IL-1RI or 12% (cytochrome c) gels. Proteins were separated by application of 30-mA constant current for 25–30 min, transferred onto nitrocellulose strips (225 mA for 75 min), blocked for 2 h at room temperature in 2% bovine serum albumin (BSA) in Tris-buffered saline (TBS), and immunoblotted overnight at 4 °C with the appropriate antibody diluted in 0.1% BSA in TBS-T (0.1% Tween 20).

IL-1β and IL-10 expression was determined by immunoblotting with a rabbit anti-rat IL-1RaP antibody (1:500; QED Biosciences Inc., San Diego, CA). PARP cleavage was detected by incubating nitrocellulose strips with an antibody to both the cleaved and uncleaved forms of PARP (1:1000; BIOSOURCE). To estimate IRAK activity, proteins were immunoblotted with a rabbit polyclonal anti-IRAK-1 antibody (1:500; Stressgen Biotechnologies Corp., Canada). To assess ERK, JNK, or p38 activity, proteins were immunoblotted with antibodies that specifically targeted the respective kinases (1:500; mouse monoclonal anti-p38, Santa Cruz Biotechnology, Santa Cruz, CA), phosphorylated JNK (G-7, 1:300, Santa Cruz Biotechnology), or phosphorylated p38 (1:1000; mouse monoclonal IgG, Cell Signaling Technology). These nitrocellulose blots were stripped and reprobed for ERK (1:500; mouse monoclonal IgG, Santa Cruz Biotechnology), JNK (F-3, 1:200, Santa Cruz Biotechnology), and p38 (A-12, 1:100; Santa Cruz Biotechnology) to assess expression of total ERK, JNK, and p38.

In all cases, nitrocellulose strips were washed in TBS-T and incubated for 2 h at room temperature with secondary antibody diluted in TBS-T containing 0.1% BSA (horseradish peroxidase-linked anti-rabbit antibody (Amersham Biosciences, UK) at 1:1000, and 1:2000 dilution for IRAK, IL-1RaP, and PARP, respectively). Nitrocellulose strips were incubated with a phosphate-buffered anti-mouse IgG (Sigma, UK) in the case of anti-active p-ERK (1:1000), the unphosphorylated form of ERK (1:1000), anti-active p-JNK (1:300), the unphosphorylated form of JNK (1:400), phosphorylated p38 (1:2000), and p38 (1:800). Protein complexes were visualized with SuperSignal (Pierce), and nitrocellulose strips were exposed to film for 5 to 10 min depending on the antibody and processed using a Fuji x-ray processor. Quantitation of protein bands was achieved by densitometric analysis using two software packages, Grab It (Grab It Annotating Grabber 2.04.7, Synotics, UK) and GelWorks (GelWorks ID, Version 2.51, UVP Ltd.) for densitometry and densitometry, respectively. GelWorks provides a single file containing all the results (block units) for each gel. The values presented here are means of data generated from six separate experiments.

Analysis of IL-1RI Expression and Cytochrome c Translocation—IL-1RI expression was assessed in the membrane fraction, whereas cytochrome c analysis was performed on the cytosolic fraction. Samples were prepared by homogenizing hippocampal slices in lysis buffer, followed by centrifugation (15,000 × g for 10 min at 4 °C). The resulting pellet (membrane fraction) was resuspended in lysis buffer, equalized for protein, and added to half the volume of sample buffer (final concentration, 50 μg/ml). Both preparations were boiled for 5 min and loaded onto either 10% (IL-1RI) or 12% (cytochrome c) gels. Proteins were separated, transferred onto nitrocellulose strips, and immunoblotted.

IL-1RI membrane expression was assessed by incubating the nitrocellulose strips in the primary antibody (1:800; rabbit anti-rat IL-1RI IgG, Santa Cruz Biotechnology) for 1 h, washed in PBS containing 0.1% BSA in Tris-buffered saline (0.1% BSA in Tris-buffered saline), and incubated in the primary antibody for 1 h at 0–4 °C. Cytochrome c translocation was determined by incubating the nitrocellulose strips in cytochrome c (0.1250 mg in PBS-T containing 2% nonfat dried milk, rabbit polyclonal IgG, Santa Cruz Biotechnology). Immunoreactive bands were detected using peroxidase-conjugated anti-rabbit antibody (1:1000, Sigma) and SuperSignal (Pierce) and quantified by densitometry as measured from the film as before.
Irradiation up-regulates IL-1β-induced cell signaling; this was prevented by EPA. Mean IL-1β concentration (pg/mg ± S.E.) was significantly increased in the hippocampus of irradiated rats that had received control diet (A; p < 0.05; ANOVA). This effect was prevented in irradiated rats that had received either 1% or 2% EPA diet, where the IL-1β concentration was similar to the sham-irradiated groups. There was a corresponding significant increase in IL-1RI (B; p < 0.05; ANOVA) and IL-1RAcP (C; p < 0.001; ANOVA) expression. A similar trend was evident when the ratio of phosphorylated (100 kDa) to unphosphorylated (80 kDa) IRAK was assessed, although significance was not reached (D). In B–D the bar graphs are represented by sample immunoblots. In the case of D, the sample immunoblots represent the 80-kDa unphosphorylated and the 100-kDa phosphorylated forms of IRAK.

**Preparation of Primary Hippocampal Neuronal Cultures**—Primary hippocampal neurons were established from 1-day postpartum Wistar rats and maintained in neurobasal medium (Invitrogen). Four experimental groups (n = 6/group) were assessed, which comprised sham-irradiated or γ-irradiated cells, with/without inhibitor (specific details below). Rats were decapitated, and the hippocampus was dissected free and incubated in sterile phosphate-buffered saline (PBS, Sigma) containing trypsin (0.25%; Sigma) and DNase (0.2 mg/ml; Sigma) and gently filtered through a sterile mesh filter (40 μm). Following centrifugation (2000 × g for 3 min) at 20 °C, the pellet was resuspended in neurobasal medium supplemented with heat-inactivated horse serum (10%; Sigma), penicillin (100 units/ml; Invitrogen), and glutamax (2 mM; Invitrogen). Suspended cells were plated out at a density of 1 × 10⁴ cells on circular 10-mm diameter coverslips, coated with poly-L-lysine (40 μg/ml; Sigma), and incubated in a humidified atmosphere containing 5% CO₂:95% O₂ at 37 °C. After 48 h, cytosinearabinofuranoside (5 ng/ml; Sigma) was incubated in the culture medium to prevent proliferation of non-neuronal cells. Twenty-four hours later, preparations were randomly divided into one of four treatment groups: sham-irradiated or γ-irradiated hippocampal neurons incubated in the presence or absence of the caspase-1 inhibitor, Ac-YVAD-CMK. In the case of those to be treated with caspase-1 inhibitor, neurons were pretreated for 1 h with Ac-YVAD-CMK (100 μM; Calbiochem). Cells that were assigned to the irradiated group underwent γ-irradiation of 20 Gy at a rate of 10 Gy/min in a Nordion cesium¹³⁷ irradiator. The following day, cells were fixed with 4% paraformaldehyde in TBS and stored at 4 °C in TBS until required for immunocytochemistry. In a separate set of experiments, neurons were preincubated with the specific JNK inhibitor DJNK1 (1 μM; Alexis) for 1 h prior to γ-irradiation and processed as above.

**Immunocytochemistry and TUNEL Staining of Hippocampal Neurons**—Sham- and γ-irradiated hippocampal neuronal cultures, which were pretreated with either Ac-YVAD-CMK (100 nm) or DJNK1 (1 μM), were immunostained for either p-JNK or anti-active caspase-3. Cells were permeabilized in 0.1% Triton X-100 and 20 μg/ml proteinase K in TBS for 10 min and then washed in TBS. Non-reactive sites were blocked with TBS containing 10% normal goat serum (Vector, UK) and incubated for 2 h at room temperature. Cells were washed and incubated overnight at 4 °C in primary antibody; p-JNK (1:150; Santa Cruz Biotechnology) or anti-active caspase-3 antibody (1:150; Promega) di-
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**RESULTS**

Fig. 1A shows that IL-1β concentration was similar in hippocampal tissue prepared from the three groups of sham-irradiated rats. Exposure to irradiation significantly enhanced IL-1β concentration in rats that received control diet by over 50% (p < 0.05; ANOVA, compared with sham-irradiated rats that received control diet). A preliminary time-course study demonstrated that, when the concentration of IL-1β was assessed 1, 2, and 4 days after γ-irradiation, the levels increased significantly with time. Treatment with EPA prevented the irradiation-induced increase in IL-1β concentration. These findings were paralleled by changes in expression of membrane-associated IL-1RI (Fig. 1B). Thus receptor expression was similar in hippocampal tissue prepared from the three groups of sham-irradiated rats, and although exposure to irradiation significantly increased IL-1RI in hippocampus of rats that received control diet (p < 0.05; ANOVA), there was no significant change in irradiated rats that were treated with EPA. Consistent with the developing theme that irradiation up-regulated IL-1β-associated signaling is the finding that IL-1RaP expression, which was similar in hippocampus of all sham-irradiated rats, was significantly enhanced in tissue prepared from irradiated rats that were maintained on the control diet (Fig. 1C; p < 0.001; ANOVA). Analysis of IRAK (80 and 100 kDa) revealed that irradiation enhanced the expression of the phosphorylated (100 kDa) form but did not alter expression of the unphosphorylated (80 kDa) form. Fig. 1D presents the data obtained from analysis of the ratio of 100- to 80-kDa IRAK. The data demonstrate that the mean ratio was enhanced in hippocampal tissue prepared from rats that were exposed to irradiation (although this did not reach statistical significance; p < 0.11) but that treatment with EPA attenuated this change.

That increased IL-1β concentration is positively correlated with components of the IL-1β signaling pathway is illustrated in Fig. 2. A significant correlation exists between IL-1β concentration (pg/mg) and IL-1RI expression (A; p < 0.04; r² = 0.18). IL-1RaP expression (B; p < 0.0001; r² = 0.71), and IRAK activity (C; p < 0.01; r² = 0.21). The γ-irradiation enhancement of IL-1RI and IL-1RaP expression was also significantly correlated (D; p < 0.001; r² = 0.81, open triangles), although this was not the case when the sham-irradiated groups were assessed (filled squares).

Statistical Analysis—Data are expressed as the mean ± S.E. 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).

**Fig. 2.** IL-1β concentration and elements of the signaling pathway are positively correlated. There was a significant positive correlation between IL-1β concentration (pg/mg) and IL-1RI expression (A; p < 0.04; r² = 0.18). IL-1RaP expression (B; p < 0.0001; r² = 0.71), and IRAK activity (C; p < 0.01; r² = 0.21). The γ-irradiation enhancement of IL-1RI and IL-1RaP expression was also significantly correlated (D; p < 0.001; r² = 0.81, open triangles), although this was not the case when the sham-irradiated groups were assessed (filled squares).
Exposure to irradiation resulted in a significant decrease in ERK phosphorylation; thus the mean value in hippocampus prepared from irradiated rats that received the control diet was significantly reduced compared with the corresponding value in the sham-irradiated group (Fig. 4A; p < 0.05; ANOVA). Although EPA treatment failed to affect ERK phosphorylation in tissue prepared from sham-irradiated rats, it prevented the irradiation-induced effect. Although protein loading was similar in all experiments, the data indicate that total ERK expression was also decreased in tissue prepared from irradiated rats maintained on the control diet (Fig. 4B; p < 0.05; ANOVA), and in parallel with its effect on ERK phosphorylation, EPA treatment reversed the irradiation-associated decrease in total ERK expression.

That activation of JNK leads to mitochondria membrane perturbation has been previously demonstrated (10, 21), and the data presented here is consistent with this. Fig. 5A shows that cytochrome c translocation was significantly increased in tissue prepared from rats that were exposed to irradiation (p < 0.05; ANOVA). Cytochrome c has been shown to activate caspase-3 (12), which in turn leads to cleavage of PARP. Fig. 5B shows that in parallel with the irradiation-induced increase in cytochrome c translocation, PARP cleavage was significantly enhanced (p < 0.001; ANOVA). Treatment with EPA did not affect either measure in sham-irradiated rats, but it prevented the effects of irradiation. In parallel with upstream markers of cell death, there was also evidence of increased DNA fragmentation in the irradiated group as assessed by TUNEL labeling (Fig. 5C; p < 0.01; ANOVA), and this was prevented by the EPA diet.

Fig. 3. EPA abolishes the irradiation-induced increase in JNK activity. p-JNK expression was significantly increased in hippocampus of irradiated rats on control diet (A; p < 0.001; ANOVA), but this activity was decreased to that of sham-irradiated levels when irradiated rats received EPA. However, there was no difference in total JNK levels across all groups (B). When p-JNK activity was assessed at different γ-irradiation doses, its expression was significantly increased in a dose-dependent manner (C; p < 0.001; ANOVA). In contrast, p38 activation was not altered by irradiation (D), and a similar pattern was evident for total p38 levels (E). The histograms represent the means ± S.E. of six observations; the data were calculated by densitometric analysis and are expressed as arbitrary values. In A–E, sample immunoblots reflect the results obtained from statistical analyses.
Previous data have indicated that EPA may act as an anti-inflammatory agent and therefore we considered that it might act by increasing IL-10 production. Fig. 6A shows that IL-10 concentration was decreased in hippocampal tissue prepared from irradiated rats, but this did not reach statistical significance. However, the data show that irradiated rats that were treated with 1% EPA exhibited a significant increase in hippocampal IL-10 concentration (p < 0.01; ANOVA). Fig. 6B demonstrates that irradiation significantly increases IL-1β release from cultured primary hippocampal neurons (p < 0.001; ANOVA), and this effect was prevented by pretreatment with IL-10 (p < 0.01). A significant inverse relationship is evident between the concentrations of IL-10 and IL-1β in vivo (Fig. 6C; r² = 0.40; p < 0.01).

To establish a sequential role for IL-1β and JNK in irradiation-induced apoptosis, γ-irradiated hippocampal neurons were assessed in vitro following pretreatment with either Ac-YVAD-CMK or DJNKI1. Gamma-irradiation significantly increased...
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![IL-10, IL-1β concentrations](image)

**Fig. 6.** EPA induced a significant increase in IL-10 concentration. Although IL-10 levels were decreased in the control-treated irradiated group, results did not reach statistical significance. However, in the 1% EPA-treated irradiated group, the concentration of IL-10 was significantly increased when compared with the sham-irradiated group (A; p < 0.05; ANOVA). The concentration of IL-1β released from primary hippocampal neurons was significantly increased following irradiation, when compared with the sham-irradiated group (B; ***p < 0.001; ANOVA). Pretreatment with 100 ng/ml IL-10 (1% EPA) directly decreased the irradiation-induced increase in IL-1β (B; +++ p < 0.01; ANOVA). When in vivo concentrations of IL-1β and IL-10 were compared, a significant inverse correlation was revealed (C; r² = 0.40; p < 0.01).

The total number of JNK-positive cells (Fig. 7A; p < 0.05; ANOVA), and this increase was reversed by preincubation with the caspase-1 inhibitor Ac-YVAD-CMK indicating that IL-1β mediated the irradiation-induced increase in JNK immunostaining. Cells that were positive for p-JNK appeared to fall into two categories: those that were immunostained globally in a punctate manner and those that were smaller in morphology and more densely stained. The subpopulation of densely immunostained JNK-positive cells were also significantly increased in the irradiated group (Fig. 7B; p < 0.01; ANOVA), and the effect of irradiation was not observed in cells that were preincubated in the presence of Ac-YVAD-CMK. A micrograph represents both JNK-positive subtypes (Fig. 7C).

Two methods were used to evaluate apoptosis; neurons were assessed for anti-active caspase-3 immunostaining and by directly labeling fragmented DNA using TUNEL staining. Significantly, irradiation increased the numbers of caspase-3- and TUNEL-positive hippocampal cells (Figs. 8, A–D). Both indicators of apoptosis were significantly decreased in neurons that were incubated in the presence of Ac-YVAD-CMK, the caspase-1 inhibitor (Fig. 8A; p < 0.001, ANOVA and Fig. 8C; p < 0.01; ANOVA). Similar results were obtained in the irradiated neurons that were preincubated with DJNKI1 (Fig. 8B; p < 0.01, ANOVA and Fig. 8D; p < 0.05; ANOVA). Anti-active caspase-3 immunostaining is represented in Fig. 8E and TUNEL labeling in Fig. 8F.

**DISCUSSION**

Here we demonstrate that exposure of rats to γ-irradiation resulted in an increase in IL-1β concentration and IL-1β-associated signaling events in hippocampus and that these changes were coupled with evidence of cell death. Significantly, the data show that treatment of rats with EPA completely abrogated the irradiation-induced increase in IL-1β and prevented cell death. EPA increased IL-10 concentration in hippocampus, and, because we have shown that the irradiation-induced increase in IL-1β is prevented by IL-10, we propose that this may be the key to the protective effect of EPA.

IL-1β concentration was significantly increased in hippocampal tissue prepared from irradiated, compared with sham-irradiated, rats; this is consistent with an earlier observation that indicated that irradiation increased expression of tumor necrosis factor α and IL-1β mRNA in brain tissue (18). A similar irradiation-induced increase in IL-1β concentration has been observed in a number of cells and tissues (22, 23), and increased circulating IL-1β concentration has been reported in patients following radiotherapy for brain tumors (24). In this study, EPA treatment prevented the irradiation-associated increase in IL-1β concentration and in other studies, EPA has been shown to have anti-inflammatory properties (25, 26). Specifically, it has been reported that EPA inhibits production of IL-1β by monocytes (27) and IL-6 by macrophages (28). In a recent study from this laboratory, we found that EPA treatment for 8 weeks attenuated the age-related increase in IL-1β concentration in hippocampus (6), whereas the lipopolysaccharide-induced increase in IL-1β concentration in hippocampus was inhibited by 4 weeks treatment with EPA (10). The mechanism by which this action occurs requires further elucidation, but it has been suggested that substitution of EPA for arachidonic acid, which would prevent cyclooxygenase-dependent production of prostaglandin E₂, may be a key element in blocking IL-1β formation (25).

There is a clear-cut correlation between IL-1β and elements of the IL-1β-induced signaling pathway as well as the fact that the interaction between IL-1RI and IL-1RAcP was potentiated by γ-irradiation. Data from three separate laboratories highlight an interesting parallel in keratinocytes: exposure of these...
cells to UV irradiation has been reported to increase IL-1β concentration (22), IL-1RI expression (29), and IRAK phosphorylation (30). The likely outcome of these changes is up-regulation of IL-1β-induced signaling predicting increases in phosphorylation of JNK and p38 as previously described (3, 5). We found that activation of JNK, but not p38, was increased in tissue prepared from irradiated rats, which is consistent with the in vitro evidence presented here, as well as previous findings indicating that JNK phosphorylation in cultured cells was stimulated by exposure to irradiation (31). Significantly, the present data indicate that treatment with EPA attenuated these irradiation-induced increases.

Here we report that in addition to preventing the irradiation-induced increases in IL-1β signaling, EPA increases hippocampal IL-10 concentration; indeed there is an inverse correlation between hippocampal concentrations of IL-10 and IL-1β.

The finding that EPA increases IL-10 has been confirmed in a recent study in which we observed that EPA abrogated the inhibitory effect of lipopolysaccharide on LTP and that this was accompanied by an EPA-induced increase in IL-10.3 Significantly, we also found that IL-10 mimicked the effect of EPA by blocking the lipopolysaccharide-induced deficit in LTP (32). These data support previous observations indicating that supplementation of infant formula with docosahexaneoic acid, of which EPA is precursor, resulted in increased the proportion of CD4-positive T cells and, concomitantly increased IL-10 production (33). Similarly it has been shown that the beneficial effects of EPA in experimental acute pancreatitis are coupled with enhanced production of IL-10 (34).

The question of the mechanism of action of IL-10 arises, and here we show that it prevents the irradiation-induced increase in IL-1β in hippocampal neurons. Interestingly, it has also been shown that IL-10 down-regulates IL-1 mRNA in splenocytes (35) and IL-1 production in synovial tissue cells (36). That IL-10 prevents IL-1β-induced cell signaling events is probably as a consequence of its ability to decrease membrane expression of IL-1RI, as previously reported (37).

JNK activation triggers apoptosis in a variety of cultured cells (38); it has been proposed that phosphorylation of the mitochondrial membrane protein bcl-2 by JNK may be a significant event in induction of apoptosis. Bcl-2 phosphorylation results in its inactivation (39, 40), which leads to loss of mitochondrial membrane integrity and translocation of mitochondrial enzymes like cytochrome c to the cytosol (41). Here, we show both in vivo and in vitro that JNK activity was increased by irradiation. This was associated with increased cytochrome c translocation in vivo and increased activation of caspase-3 here, as well as in several studies (42, 43). The irradiation-induced increase in cytochrome c translocation, together with increased cleavage of the caspase-3 substrate, PARP, and increased TUNEL-labeling in vivo and in vitro are considered to be reliable indicators of apoptosis (44, 45). Taken together the data suggest that exposure of rats to γ-irradiation leads to apoptosis in hippocampus, providing an explanation for the marked cell loss observed in hippocampus following irradiation (46). Among the important findings of this study is the observation that the irradiation-induced increase in JNK activation is dependent on IL-1RI, because it is blocked by inhibition of stained population (B). At least 400 cells were counted at 100× magnification in each experimental group and there were n = 6 per group. A micrograph (C) represents the various cell populations analyzed and comprise negatively stained cells (narrow arrows), a globally immunostained JNK-positive cell (filled arrow), and a small densely stained JNK-positive cell (open arrow).

3 T. Kavanagh and M. A. Lynch, unpublished observation.

**Fig. 7. Inhibition of irradiation-induced IL-1β production decreased JNK activity in vitro.** Irradiation increased the total number of JNK-positive cells (A; p < 0.05; ANOVA) and when subdivided into two populations, those that were densely immunostained and apoptotic in morphology were also significantly increased in number (B; p < 0.01; ANOVA). When irradiated hippocampal cells were pretreated with Ac-YVAD-CMK (the caspase-1 inhibitor), the total number of JNK-positive cells was decreased (A) as well as the more densely stained population (B). At least 400 cells were counted at 100× magnification in each experimental group and there were n = 6 per group. A micrograph (C) represents the various cell populations analyzed and comprise negatively stained cells (narrow arrows), a globally immunostained JNK-positive cell (filled arrow), and a small densely stained JNK-positive cell (open arrow).
and that irradiation-induced apoptosis is mediated by IL-1β and JNK activation. EPA treatment inhibited these irradiation-induced changes, and the significance of this finding is 2-fold: first it suggests an interdependence of these factors and, second, it identifies a neuroprotective effect of EPA.

Irradiation decreased activation of ERK, which is generally considered to be a survival factor (47), and it is therefore possible that this contributed to the irradiation-induced apoptosis. It is of interest that EPA treatment reversed the irradiation-induced decrease in ERK activation. These observations in hippocampus therefore mirror the changes observed in Jurkat T cells in which apoptotic changes were linked with activation of JNK, but not activation of either p42 mitogen-activated protein kinase or p38 (48).

The question of the mechanism underlying the protective effect of EPA remains to be addressed. The present data indicate that EPA enhanced IL-10 concentration in hippocampus of rats that were exposed to irradiation. IL-10 is a potent anti-inflammatory cytokine, which we have shown abrogates irradiation-induced IL-1β release in hippocampal neurons. Additionally, IL-10 has been shown to inhibit the IL-1β-induced increase in activation of IRAK and JNK (37), and it suppresses the effects of IL-1β on LTP (37), behavior (49), and fever (50). We propose that the ability of EPA to caspase-1, and that irradiation-induced apoptosis is mediated by IL-1β and JNK activation. EPA treatment inhibited these irradiation-induced changes, and the significance of this finding is 2-fold: first it suggests an interdependence of these factors and, second, it identifies a neuroprotective effect of EPA.

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increase IL-10 expression may significantly contribute to the protective effect of EPA.

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