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RESEARCH PAPER

Eicosapentaenoic acid suppression of systemic inflammatory responses and inverse up-regulation of 15-deoxy$\Delta^{12,14}$ Prostaglandin J_2 production

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BACKGROUND AND PURPOSE

Eicosapentaenoic acid (EPA) has been shown to suppress immune cell responses, such as cytokine production and downstream PG production *in vitro*. Studies *in vivo*, however, have used EPA as a minor constituent of fish oil with variable results. We investigated the effects of EPA on systemic inflammatory responses as pure EPA has not been evaluated on immune/inflammatory responses *in vivo*.

EXPERIMENTAL APPROACH

Rabbits were administered polyinosinic: polycytidylic acid (poly I:C) i.v. before and after oral treatment with EPA for 42 days (given daily). The responses to IL-1β and TNF-α were also studied. Immediately following administration of poly I:C, body temperature was continuously monitored and blood samples were taken. Plasma levels of IL-1β, PGE_2 (PGE_2), and 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2) were measured by enzyme immunoassay.

KEY RESULTS

Following EPA treatment, the fever response to poly I:C was markedly suppressed compared with pretreatment responses. This was accompanied by a parallel reduction in the poly I:C-stimulated elevation in plasma levels of IL-1β and PGE_2. Paradoxically, the levels of 15d-PGJ_2 were higher following EPA treatment. EPA treatment did not significantly alter the fever response or plasma levels of PGE_2 in response to either IL-1β or TNF-α.

CONCLUSION AND IMPLICATIONS

Oral treatment with EPA can suppress immune/inflammatory responses *in vivo* via a suppression of upstream cytokine production resulting in a decreased fever response and indirectly reducing circulating levels of PGE_2. EPA also enhances the production of the cytoprotective prostanoid 15d-PGJ_2 indicating the therapeutic benefit of EPA.

Abbreviations

EPA, eicosapentaenoic acid; NSAIDs, non-steroidal anti-inflammatory drugs; poly I:C, polyinosinic:polycytidylic acid

Introduction

The C20:5 fatty acid, eicosapentaenoic acid (EPA), has been shown to have direct modulatory functions in cells and tissues. EPA can directly result in vasorelaxation of either noradrenaline or high potassium pre-contracted aortae in an endothelium-independent manner (Engler *et al.*, 2000). This vasoactive action appears to be mediated via the activation of

K^+/ATP channels. It has also been shown that EPA can also have an opposite effect on other ion channels. EPA can directly inhibit Na^+ channels in cell lines (Nakajima *et al.*, 2009). A direct modulatory effect of EPA *in vitro* has also been demonstrated in immune cells. Human alveolar macrophages incubated with EPA released much lower levels of the obligatory pro-inflammatory cytokines IL-1β and TNF-α in response to LPS stimulation (Mickleborough *et al.*, 2009). This would indicate that EPA should have an anti-inflammatory action *in vivo*; however, although the direct actions of pure EPA have been clearly demonstrated *in vitro*, very few studies have been able to confirm this *in vivo*. There are many studies that have implied the modulatory actions of EPA but have used a heterogenous mixture of 'fish oils', which have a very wide variety of different fatty acids with EPA comprising as little as 4% from some sources and a maximum of 14% in others (Ackman *et al.*, 1988). The present study used pure EPA.

Studies using heterogenous fish oils have shown that markers of inflammation are down-regulated, indicating that fish oils do contain components that are anti-inflammatory (De Caterina *et al.*, 2000). The mechanism by which EPA-containing agents suppress inflammation remains unclear. Potential interference could occur at several points in the sequence of steps involved in the development of systemic inflammatory responses (Romanovsky *et al.*, 2005). These include production of cytokines (upstream mediators), suppression of prostanoids (downstream mediators of symptoms) or the synthesis of novel anti-inflammatory eicosanoids. Recent studies indicate that 15-deoxy-$\Delta^{12,14}$-PG J_2 (15d-PGJ_2), a cyclopentenone PG and dehydration product of PGD_2 (a major product of immune/inflammatory cells, including monocytes, macrophages and dendritic cells) possesses anti-inflammatory activity (Herlong and Scott, 2006). Elevated plasma levels of 15d-PGJ_2 appear to play a role in abrogating the inflammatory response that contributes to cell death in neurological disorders following acute ischaemic stroke (Blanco *et al.*, 2005) indicating that it is an important cytoprotective mediator. However, the main critical downstream mediator of systemic inflammatory responses (the fever response) is PGE_2 and is the primary therapeutic target for non-steroidal anti-inflammatory drugs (NSAIDs) (Mackowiak, 2000).

We have previously characterized in depth the responses to the TLR3 ligand, polyinosinic : polycytidylic acid (poly I:C), which induces a systemic inflammatory response (Abul *et al.*, 1987; Rotondo *et al.*, 1987; 1988) manifest as a fever when administered intravenously to rabbits. The changes in body temperature (fever) are a direct result of the sequential production of cytokines including IL-1β (see Fortier *et al.*, 2004) and TNF-α as a first wave and the consequent production of PGs, particularly PGE_2. The prostanoids act at a variety of specific receptors, both in the peripheral circulation (to regulate cytokine production) and in the CNS at the hypothalamic thermoregulatory centre (see Alexander *et al.*, 2011 for prostanoid receptor classification). The levels of PGE_2increase in both the peripheral circulation and in the cerebrospinal fluid (Rotondo *et al.*, 1988; Davidson *et al.*, 2001) in response to Poly I:C (and also to LPS in a qualitatively identical manner). The aim of the present study was, therefore, to determine whether pure EPA could modulate a systemic inflammatory challenge and have any effect on blood levels of either IL-1β, PGE_2 or 15d-PGJ_2. Since pro-inflammatory cytokines are induced in response to challenge with poly I:C, modulation of the response to IL-1β and TNF-α following EPA supplementation was also determined as this would be a strong indicator of what level in the sequence of events may be modulated preferentially by EPA. We report that EPA suppresses the systemic response in rabbits to challenge with poly I:C. This occurs simultaneously with a decrease in plasma levels of IL-1β and PGE_2 and an increase in plasma levels of 15d-PGJ_2. Our results suggest that EPA attenuates systemic inflammatory responses by suppressing pro-inflammatory cytokine release and promoting the production of 'anti-inflammatory/cytoprotective' PGs.

Materials and methods

Measurement of body temperature

Dutch rabbits (1.9–2.6 kg) were used throughout the study, and while not being used for experiments, rabbits were individually caged and maintained at 21–23°C. Lights were on 10–12 h per day and food and water were available *ad lib*. All procedures were carried out in compliance with the ethical guidelines laid down by the Home Office. In order to minimize any error in body temperature measurements due to restraint stress, rabbits were accustomed to conventional stocks over a period of 5 days before being used in any experiments. All experiments were performed at an ambient temperature of 22–24°C. Body temperature was measured using Yellow Springs rectal thermistor probes (401 series). Probes were connected to a Biopac Systems model MP100 data acquisition unit and Acqknowledge software (supplied by D. Med Systems Ltd, Oxford, UK) controlled by an Apple Macintosh computer.

Administration of EPA

Rabbits were given 40 $mg \cdot kg^{-1}$ of purified EPA orally every day for a period of 42 days. Purified EPA, comprised 94% EPA ethyl ester, 4.6% free fatty acids (EPA) and 0.17% α-tocopherol (National Institutes of Health, Bethesda, MD, USA – Biomaterials Test Program).

Immunostimulation challenge of animals with Poly I:C, TNF-α and IL-1β

Immediately prior to EPA supplementation (control/pretreatment) and at various intervals thereafter, animals were challenged with different immunomodulatory stimuli (see EPA tretament overview figure). A group of animals were administered 2.5 $\mu g \cdot kg^{-1}$ of polyinosinic: polycytidylic acid (poly I:C). On each occasion, the animal's body temperature was monitored continuously for 5 h, and blood samples were taken at various intervals (as shown in the results section). In a separate series of experiments groups of rabbits were also challenged with either (i) rabbit recombinant rabbit IL-1β (2000 $U \cdot kg^{-1}$ – see Davidson *et al.*, 1990) or (ii) TNF-α (10 $\mu g \cdot kg^{-1}$ – see Davidson *et al.*, 1992) and body temperature measured for 3 or 4 h respectively (the duration of fever responses to each stimuli). Blood samples were taken immediately before administration of either of the stimuli and during the peak increase in fever response. Poly I:C (Sigma,

Dorset, UK), TNF-α and IL-1β (Glaxo, Geneva, Switzerland) were dissolved in sterile saline and administered i.v. via the marginal ear vein.

Blood sampling

Blood samples were taken immediately prior to injection of any of the stimuli and at 90 and 210 min after poly I:C or after 45 min for IL-1β and 60 min for TNF-α (peak fever responses for each of these cytokines). Plasma levels of PGE_2, 15d-PGJ_2 and rabbit IL-1β were determined using commercially available assay kits. Briefly, samples were taken from the marginal ear vein at various times as described in the results section by making a small longitudinal incision with a sterile scalpel blade. The first few drops of blood were discarded and 1 mL collected into either ice-cold Eppendorf tubes containing 100 μL of 4.5 mM-EDTA and 50 μL of 0.5 mg·mL^{-1} ketoprofen (PG analysis) or empty Eppendorf tubes (IL-1β analysis). Immediately after collection, blood samples were centrifuged at 13 000 g for 1 min and plasma either processed further as described below for PG measurement or stored at –80°C for subsequent measurement of rabbit IL-1β.

Processing of samples for PG measurements

Aliquots of plasma (0.5 mL) were transferred to Eppendorf tubes containing 50 μL of 1 M HCl to give a final pH of 3.5–4.0. The acidified plasma was then passed through a Sep-Pak C_{18} column (Waters Associates, Milford, MA, USA), previously prepared by successive washing with 2 mL of methanol and 5 mL of distilled water. The column was then washed with 5 mL of distilled water and the eluate discarded. A further 2 mL of methanol was then passed through the column, the first 0.5 mL of eluate was discarded, and the remainder collected into Eppendorf tubes.

Samples were stored at –80°C for subsequent analysis of PGE_2 and 15d-PGJ_2. Recovery of PGs from samples was determined by the addition of different known concentrations of [^{3}H] PGE_2 or authentic 15d-PGJ_2 to acidified plasma, which were then passed through Sep-Pak C_{18} columns as described above and radioactivity in the eluate determined by liquid scintillation counting or amount estimated by ELISA respectively. The efficiency of the Sep-Pak C_{18} columns in extracting PGE_2 was estimated to be greater than 94% and recovery of 15d-PGJ_2 from samples was routinely found to be greater than 86%.

Measurement of PGE_2 and 15d-PGJ_2

For measurement of PG in each sample, aliquots of 100 μL of methanol were placed into Eppendorf tubes and evaporated to dryness under nitrogen. Assay buffer (100 μL), was then added to each tube and PGE_2 and 15d-PGJ_2 determined using the corresponding commercially available assay kit as directed by the manufacturer. PGE_2 was estimated using R & D Systems Parameter PGE_2 EIA kit. The inter-assay and intra-assay coefficient of variance (CV) were 8.4 and 3.8% respectively (100 pg·mL^{-1}). 15d-PGJ_2 was estimated using Assay Designs Correlate-EIA 15d-PGJ_2 EIA kit (Ann Arbor, MI, USA). The inter-assay and intra-assay coefficient of variance CV were 6.6 and 5.4% respectively (250 pg·mL^{-1}). To obviate potential measurement artefacts, this assay was evaluated for its ability to measure the target PG using authentic pure 15d-PGJ_2 purchased from an alternative source (Cayman Chemicals, SPI-Bio, Montigny-le-Bretonneux, France) and found to have 93% reactivity compared with the standard supplied in the kit. The ability of the kit antibodies to bind to EPA and so generate false results was also evaluated. Absorbance values of samples of plasma, spiked with EPA then extracted using Sep-Pak C_{18} columns as described earlier were below the limit of detection indicating the assay did not erroneously measure EPA.

Measurement of IL-1β

IL-1β levels in plasma samples were measured using an ELISA Kit (Endogen, supplied by Bradsure, Loughborough, UK). Briefly, aliquots of plasma (100 μL) or standard authentic IL-1β were placed into the wells of 96-well plates pre-coated with primary anti-rabbit IL-1β antibody. Plates were incubated for 2 h at 22°C after which wells were washed with PBS buffer and HRP-labelled secondary anti-rabbit IL-1β antibody (100 μL) was added and incubated for a further 2 h at 22°C. Plates were finally washed three times with PBS and tetramethylbenzidine was added (100 μL) for 30 min at 22°C after which 0.1N H_2SO_4 was added (50 μL) and the absorbance (at 450 nm) of each well measured in a plate reader.

Presentation of data and statistics

Body temperature measurements are shown either as the change in body temperature from basal (ΔT i.e. Δ°C), or as a thermal response index (TRI). TRI_5, TRI_4 and TRI_3values represent the magnitude of changes (area under the curves – Δ°C.hr) in body temperature over 5, 4 and 3 h respectively (this matched the duration of responsiveness of animals to the different stimuli, Poly I:C, TNF-α and IL-1β respectively). A TRI value of 1 represents an increase of 1°C for 1 h, thus a TRI5 is the magnitude of the response (area under the curve) over 5 h. Plasma levels of PGE_2, 15d-PGJ_2 and rabbit IL-1β are presented as pg·mL^{-1} of plasma. All results are expressed as the mean of *n* experiments ± the SEM. Data were analysed using a paired Student's *t*-test, each animal acting as its own control.

Results

Effect of EPA on poly I:C-induced fever

Poly I:C (2.5 μg·kg^{-1} i.v.) produced a biphasic increase in body temperature, the first peak occurring 90 min and the second peak 210 min after injection. EPA supplementation attenuated both peaks of the fever response. Δ*T*-values following EPA administration for 28 and 42 days in comparison with the pretreatment levels (control) are shown in Figure 1. The magnitude of suppression of the response by EPA increased with increasing duration of administration. TRI_5 values were significantly reduced from 3.69 ± 0.32°Ch before EPA (pretreatment) to 2.54 ± 0.34°Ch ($P < 0.05$) after 28 days and to 1.71 ± 0.22°Ch ($P < 0.001$) after 42 days (Figure 2). Suppression by EPA appeared to be maintained after supplementation was discontinued, TRI_5 values of 1.87 ± 0.21°Ch obtained on day 63 (21 days following the end of EPA administration) were lower than pretreatment controls ($P < 0.001$). In a separate series of treatments animals were administered poly I:C (2.5 μg·kg^{-1} i.v.) then sham-treated (no EPA was administered)

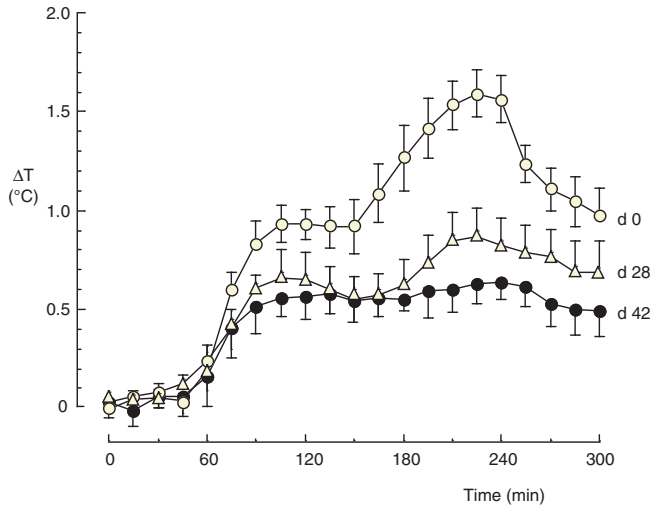

Figure 1

Effect of EPA on the fever response to poly I:C. Rabbits were challenged with poly I:C (2.5 μg·kg^{-1} i.v.) at time 0 and body temperature monitored for 5 h. Body temperature is presented as the change from basal (ΔT), immediately prior to injection of poly I:C. Values shown are for pre-EPA (pretreatment -day 0, open circles), and following daily oral administration of EPA (100 mg·kg^{-1}) for 28 days (open triangles) and 42 days (closed circles). All values represent mean ± SEM for n = 8 animals.

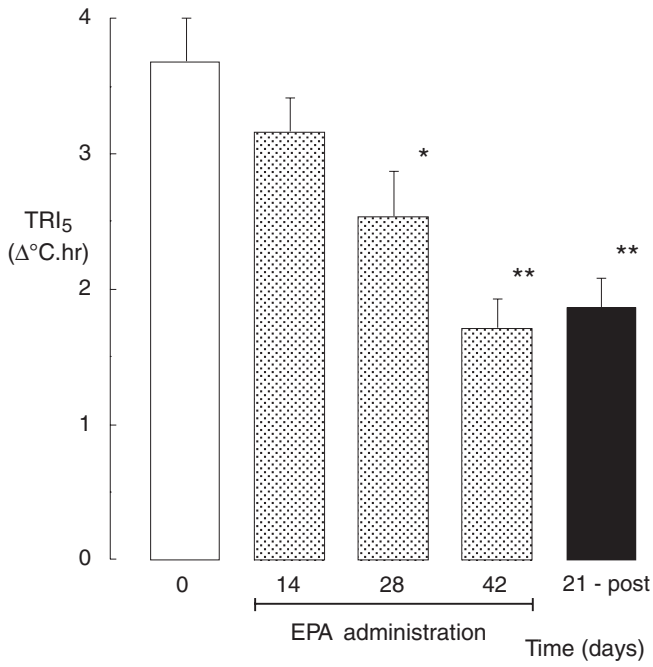

Figure 2

Magnitude of the fever response to poly I:C before, during and post-EPA administration. Rabbits were challenged with poly I:C (2.5 μg·kg^{-1} i.v.), and body temperature measured for 5 h. The magnitude of the fever responses are presented as TRI_5 (Δ°C.hr) values prior to administration of 100 mg·kg^{-1} EPA (pretreatment, open bar), following daily oral administration of EPA for up to 42 and 21 days after EPA administration was stopped (patterned bars). All values represent mean ± SEM, for n = 8 animals. $^{**}P < 0.01$ and $^{*}P < 0.05$ compared with pretreatment (paired t-test).

for 42 days prior to administration of another dose of poly I:C (2.5 μg·kg^{-1} i.v.) on day 42. The fever responses prior to treatment compared with the response on day 42 were not significantly different with a TRI_5 value of 3.61 ± 0.29°Ch on day 0 and 3.78 ± 0.36°Ch on day 42 (n = 5) confirming that in EPA-treated animals, the inhibitory action is due specifically to administration of the EPA.

Effect of EPA on poly I:C-induced changes in plasma PGE_2

Blood samples were therefore taken immediately prior to injection of poly I:C and at 90 and 210 min thereafter for measurement of PGE_2. EPA attenuated the poly I:C-induced increases in plasma levels of PGE_2 (Figure 3). PGE_2 levels were reduced from 190 ± 31 pg·mL^{-1} at 90 min and 200 ± 37 pg·mL^{-1} at 210 min in control pretreatment animals to 105 ± 10 pg·mL^{-1} and 112 ± 10 pg·mL^{-1}, respectively, following 42 days of EPA administration (both $P < 0.05$).

Effect of EPA on poly I:C-induced changes in plasma 15d-PGJ_2

Blood samples were also taken immediately prior to injection of Poly I:C and at 90 and 210 min thereafter for measurement of 15d-PGJ_2. Administration of poly I:C induced an increase in circulating levels of 15d-PGJ_2 (Figure 4). The Poly I:C induced an 11.7-fold and a 7.4-fold increase in blood levels of 15d-PGJ_2 at 90 min and 210 min respectively compared to pretreatment. In contrast to its suppressive effect on PGE_2 levels (Figure 3), 42 days of treatment with EPA appeared to enhance both basal and poly I:C-induced increases in blood levels of 15d-PGJ_2 (Figure 4). Blood levels of 15d-PGJ_2 in response to poly I:C were increased from 75 ± 11 pg·mL^{-1}, 880 ± 49 pg·mL^{-1} and 557 ± 43 pg·mL^{-1} to 184 ± 19 pg·mL^{-1}, 1193 ± 86 pg·mL^{-1} and 958 ± 85 pg·mL^{-1} following 42 days of EPA treatment at 0, 90 and 210 min after poly I:C challenge respectively (Figure 4).

Effect of EPA on poly I:C-induced changes in plasma IL-1β

Poly I:C stimulates the production of IL-1β, therefore, the effect of EPA on plasma levels of endogenous rabbit IL-1β in response to this stimulus were determined (Figure 4). Blood samples were taken from animals both before (pretreatment) and 42 days after EPA supplementation commenced. EPA appeared to reduce basal levels of IL-1β compared with pre-supplementation levels; however, this difference was not statistically significantly different. Poly I:C increased plasma levels of IL-1β by approximately 4.7-fold compared with pre-challenge levels, and this increase was significantly suppressed by EPA. Plasma levels of IL-1β were reduced from 170.1 ± 19.8 pg·mL^{-1} (pretreatment) to 77.7 ± 12.8 pg·mL^{-1} ($P < 0.01$) following administration of EPA.

Effect of EPA on IL-1β- and TNF-α induced fever

As cytokines are intermediate mediators in systemic inflammatory responses, the effect of EPA administration on the

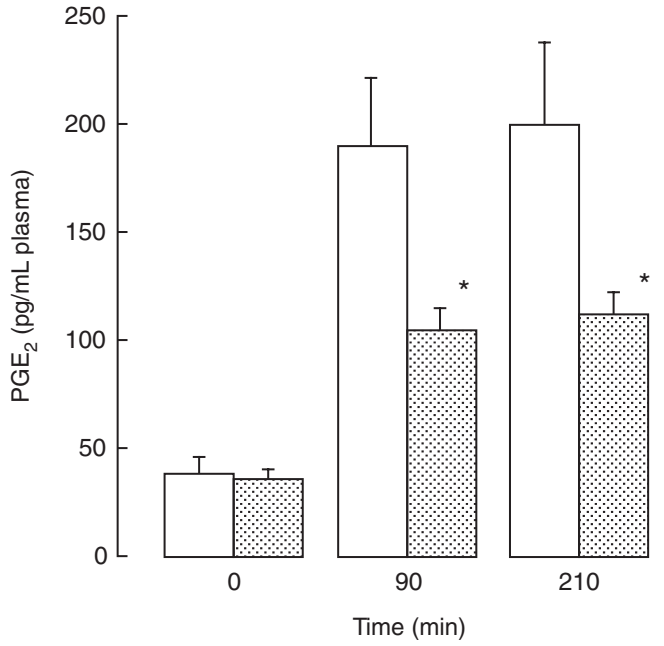

Figure 3

Effect of EPA on plasma levels of PGE_2 following i.v. administration of poly I:C. Blood samples were taken at 0 min, immediately prior to injection and at 90 min and 210 min after injection of poly I:C (2.5 μg·kg^{-1} i.v.). Plasma levels of PGE_2 in response to poly I:C before administration of EPA (open bars, pretreatment) and after administration of EPA for 42 days (patterned bars) were determined by ELISA. All values represent mean ± SEM, for $n = 8$ animals. *$P < 0.05$ versus the respective pretreatment value (paired t-test).

fever response to the cytokines, IL-1β and TNF-α was also determined. IL-1β (2000 U·kg^{-1} i.v.) was administered to animals prior to supplementation (pretreatment – day 0) and 42 days after supplementation with EPA. Similarly, TNF-α (10 μg·kg^{-1} i.v.) was administered to animals prior to supplementation (pretreatment – day 0) and 42 days after treatment with EPA. EPA treatment did not significantly alter the response to either IL-1β or TNF-α. Prior to EPA administration (day 0), the TRI_3 value for i.v. treatment with IL-1β was 0.90 ± 0.10, and following EPA administration (day 42), the TRI_3 value for IL-1β was 0.93 ± 0.09 ($n = 5$). The TRI_4 value for TNF-α given before EPA treatment was 1.48 ± 0.17 and following EPA administration (day 42), the value was 1.36 ± 0.14 ($n = 4$).

Effect of EPA on IL-1β- and TNF-α induced changes in plasma PGE_2 & 15d-PGJ_2 levels

Plasma levels of both PGE_2 and 15d-PGJ_2 were measured during the fever responses to IL-1β and TNF-α as described earlier. Blood samples were taken from animals immediately prior to the injection of either IL-1β or TNF-α (labelled controls – pretreatment values) or at the peak of fever responses for either cytokine, 45 min for IL-1β and 60 min for TNF-α. In animals that were given either IL-1β or TNF-α prior to EPA treatment, the plasma levels of PGE_2 increased by approximately threefold (Figure 5). The ability of IL-1β or TNF-α to increase the plasma levels of PGE_2 were not significantly altered following 42 days of EPA treatment (Figure 5).

The plasma levels of 15d-PGJ_2 in animals given either IL-1β or TNF-α, prior to EPA treatment, also increased compared with basal levels (Figure 5). Following 42 days of EPA treatment, the levels of 15d-PGJ_2 were moderately higher in

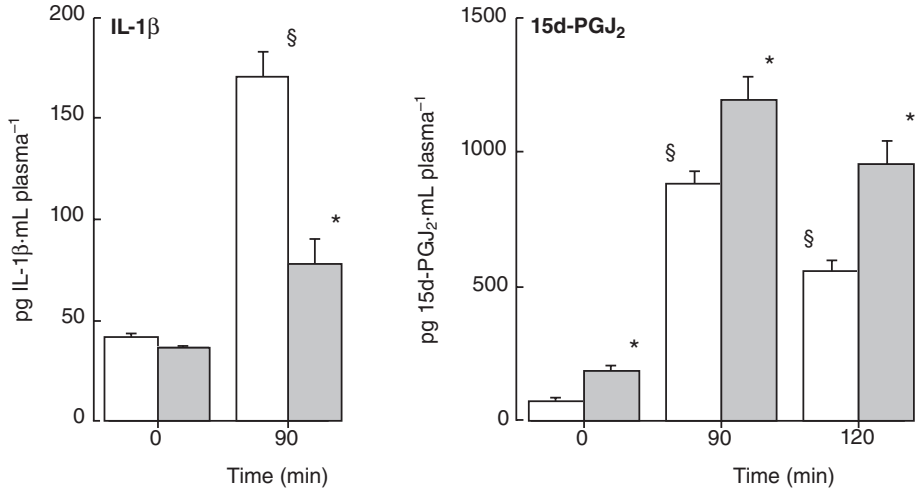

Figure 4

Effect of EPA treatment on plasma levels of IL-1β and 15d-PGJ_2 following i.v. administration of poly I:C. Blood samples were taken at 0 min (immediately prior to injection), 90 and 210 min after injection of poly I:C (2.5 μg·kg^{-1} i.v.). Plasma levels of IL-1β were measured by ELISA in 0 and 90 min samples. 15d-PGJ_2 levels were measured in 0, 90 and 120 min samples by EIA. This was carried out for animals before administration of EPA (open bars, pretreatment) and after administration of EPA for 42 days (patterned bars). All values represent mean ± SEM, for $n = 8$ animals. §$P < 0.01$ versus 0 min and *$P < 0.05$ versus pre-EPA treatment (paired t-test).

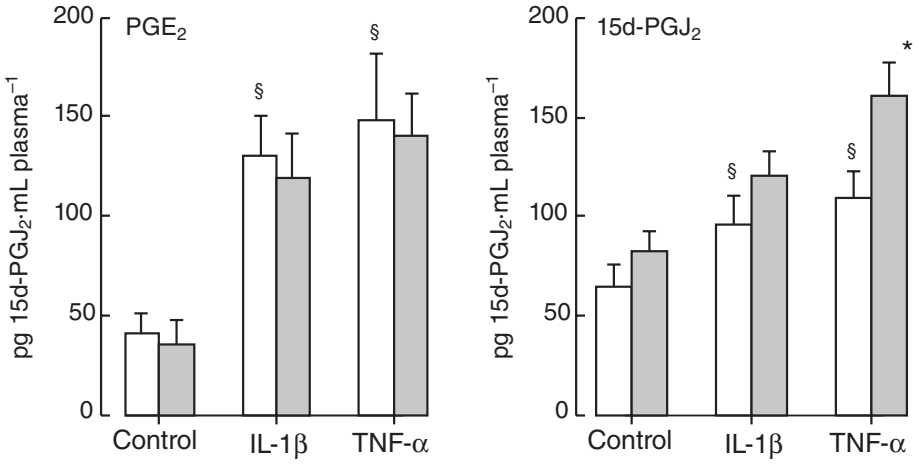

Figure 5

Effect of EPA on plasma levels of PGE_2 and 15d-PGJ_2 following i.v. administration of either IL-1β- or TNF-α. Rabbits were challenged with either IL-1β (2000 U·kg^{-1} i.v.) or TNF-α (10 μg·kg^{-1} i.v.), both before (controls – pretreatment, open bars) and after 42 days (patterned bars) administration of EPA. Blood samples were taken immediately prior to (control) and following injection of either IL-1β (45 min) or TNF-α (60 min). Plasma levels of PGE_2 were determined by ELISA, and 15d-PGJ_2 were determined by EIA. $^{\S}P < 0.01$ versus control (pretreatment) and $^{*}P < 0.05$ versus pre-EPA treatment (paired *t*-test).

EPA-treated animals. The EPA treatment did not significantly alter the plasma level 15d-PGJ_2 in response to IL-1β (Figure 5), but it did increase the 15d-PGJ_2 levels in response to TNF-α ($P < 0.05$).

Discussion

Effect of EPA on poly I:C-induced fever

This study examined the effect of daily administration of EPA on the fever response and circulating levels of inflammatory mediators, systemic manifestations of inflammation. In this study, the immunomodulatory effects of pure EPA (>98%) as evaluated and was administered orally in the manner of a pharmaceutical preparation. This was distinct from other *in vivo* studies, where the EPA is given as a fish-oil emulsion incorporated into dietary chow. The advantage of this approach over previous work using fish oils is that each animal received a directly quantifiable amount of EPA with little influence on overall calorific intake. Fever was induced by i.v. administration of the TLR3 ligand poly I:C, a synthetic double-stranded polyribonucleotide, used experimentally to model viral activation *in vivo*, which we have previously shown to induce reproducible fevers in rabbits qualitatively identical to those of LPS, but without demonstrating the profound tolerance observed with LPS (Rotondo *et al.*, 1987). EPA supplementation attenuated both peaks of the fever response (Figure 1). The magnitude of suppression increased with increasing duration of supplementation, reaching a maximum at 42 days (Figure 2). Our observations clearly demonstrate that EPA can suppress immune/inflammatory responses *in vivo*. The dose of EPA used in the present study was comparable with that used in many human studies. We administered 40 mg·kg^{-1}·day, approximately equivalent to 2.8 g·day^{-1} in human studies (70 kg subjects). Some human studies have used as much as 4 g·day^{-1} (Woodman *et al.*, 2003). In our own human studies, we had previously used 1 g·day^{-1} EPA in subjects (Bonner *et al.*, 1997; Davidson *et al.*, 1997). A recent meta-study of EPA trials has shown that generally, the EPA doses used in human studies is between 1 g·day^{-1} and 4 g·day^{-1} (Xin *et al.*, 2012a,b). Thus, the present study used an EPA dose intermediate to those commonly used in human studies.

Effect of EPA on Poly I:C-induced blood levels of inflammatory mediators

Poly I:C stimulated increases in plasma levels of both primary and secondary inflammatory mediators, i.e. cytokines and prostanoids. Levels of IL-1, PGE_2 and 15d-PGJ_2 increased following administration of poly I:C (Figures 3,4). This is in agreement with other studies which have shown that poly I:C an stimulate IL-1 production (Matsukura *et al.*, 2006; Mignot *et al.*, 2012). Indeed, the fever response to poly I:C is dependent on IL-1 and has been shown to increase circulating levels of IL-1β in rats (Fortier *et al.*, 2004).

EPA attenuated the poly I:C-induced increases in plasma levels of PGE_2 and this occurred simultaneously with changes in body temperature (Figures 1,3). Since PGs of the E series, particularly PGE_2, are thought to be the final mediators of fever (Milton and Wendlandt, 1971; Ivanov and Romanovsky, 2004), this suggests that the antipyretic and anti-inflammatory actions of EPA, result in part from a reduction in the synthesis and/or release of PGE_2. EPA can reduce plasma levels of PGE_2 by a number of potential mechanisms that have been extensively proposed previously. EPA competitively inhibits the incorporation of AA into membrane phospholipids, therefore reducing the amount of substrate for synthesis of two-series prostanoids such as PGE_2 and so on, effectively reducing their synthesis (Rubin and Laposata, 1992). EPA could also competitively reduce the amount two-

series eicosanoids synthesized by COX enzymes (Lands, 1992). Both COX-1 and COX-2 can oxygenate a range of *n*–3 and *n*–6, 18–22 carbon fatty acids, optimal catalytic efficiency for both isoforms occurs with AA. The difference in structure between EPA and AA, an additional double bond at C17/C18, is responsible for the 'strained' conformation with which EPA binds to the cyclo-oxygenase active site in the COX enzyme, which slows its enzymatic conversion rate and effectively causes EPA to act as an inhibitor (Malkowski *et al.*, 2001). EPA itself is also a substrate for COX and has been shown to be converted to three-series prostanoids (e.g. PGE_3 or PGD_3). This has been quoted as the most likely explanation, whereby EPA can alter end function. Ingestion of fish oil has been shown to reduce concentrations of two-series PGs and to increase three-series prostaglandins *in vivo* (Fischer *et al.*, 1988; Knapp, 1990), which is cited as evidence that switching to three-series prostanoids is responsible for a change in function. This would seem the least plausible explanation, as it would assume a difference in potency between two- and three-series prostanoids, especially E-series PGs. With respect to immune cell responses, this is definitively not the case. It has been demonstrated that PGs E_1, E_2 and E_3 exert equipotent actions on mononuclear cells by suppressing inflammatory cytokine production (Haynes *et al.*, 1992; Dooper *et al.*, 2002). This would clearly indicate that diverting PG production from PGE_2 to PGE_3 would not alter the magnitude of a final response, that is a fever in this case. This does not appear to happen in the present study, rather a decrease in all E series PGs following EPA treatment is the most likely explanation. This view is strengthened by our assay measurement methodology, as the anti-PGE_2 antibody used in this study has an 81.5% cross-reactivity with PGE_3, that is it cannot discriminate E_2 from the E_3. It is without doubt that PGE_3 would have been produced in the present study; however, it was not possible to directly quantitate this. To date, it has been difficult to measure PGE_3 specifically as antibodies that selectively bind to PGE_3, have been difficult to produce. Although physical methods can easily discriminate PGE_2 from PGE_3 (see our previous study, Rotondo *et al.*, 1994), unfortunately, these methods are not sufficiently sensitive to measure the relatively low levels circulating *in vivo*. A similar study (conducted over 8 weeks) showed that the relative levels of PGE_3 compared with PGE_2 were very low following EPA treatment *in vivo* (albeit given as heterogenous fish oils) with a resultant ratio of circa 600:1 PGE_2 to PGE_3, which was elicited from monocytes *ex vivo* (Kearns *et al.*, 1999). Despite the relative ratios, our data clearly show an overall reduction in the PGE measured, which would include PGE_3, and are very likely secondary to a reduction in the levels of cytokines upstream.

Effect of EPA on poly I:C-induced 15d-PGJ_2

Circulating blood levels of 15d-PGJ_2 increased during the fever response to poly I:C (Figure 4). This observation is similar to those of other groups who have detected increased levels of 15d-PGJ_2 in the cerebrospinal fluid of rats 1 day and 2 days after intaperitoneal injection of high doses of the TLR4 ligand, LPS (Mouihate *et al.*, 2004). Enhanced biosynthesis of 15d-PGJ_2 following immune challenge may contribute to the self-limiting nature of inflammatory responses in general and specifically to poly I:C. Many clinical and experimental studies have shown that increases in body temperature during fever rarely exceed a set limit, and 15d-PGJ_2 appears to belong to a group of endogenous anti-inflammatory/cytoprotective molecules, of which a number have already been identified (e.g. glucocorticoids, α-melanocyte-stimulating hormone, arginine vasopressin and IL-10). EPA treatment increased basal levels of plasma 15d-PGJ_2 compared with pretreatment levels (Figure 4), but had no effect on basal levels of PGE_2 (Figure 3). This may reflect a selective action of EPA on PGD_2 pathways, for example augmentation of PGD synthase activity. In addition, we found that EPA treatment further enhanced poly I:C-stimulated increases in plasma levels of 15d-PGJ_2 (Figure 4). This suggests that 15d-PGJ_2 may contribute to the anti-inflammatory activity of EPA. Our observations suggest that the anti-inflammatory actions of EPA may result from a combination of reduction in PGE_2 biosynthesis (secondary to a reduction in cytokine production) and possibly an increase in 15d-PGJ_2 synthesis.

There are other possible mechanisms by which EPA could down-regulate IL-1 production. Other novel anti-inflammatory eicosanoids may mediate these actions. These include the lipoxins, resolvins, docosatrienes and neuroprotectins (Serhan, 2005). Resolvin E1 (5S,12R,18R-trihydroxy-EPA; RvE1) is an anti-inflammatory mediator endogenously synthesized from EPA by a novel transcellular mechanism involving the sequential actions of aspirin-acetylated COX-2 and 5-lipoxygenase during the spontaneous resolution phase of acute localized inflammation (Arita *et al.*, 2005; Serhan, 2005) and are potential candidates.

Previous work by our group has demonstrated that both glucocorticoids and the glucocorticoid-derived intermediate, annexin-1, can attenuate poly I:C-induced fever (Milton *et al.*, 1989; Davidson *et al.*, 1991) and that these changes in both fever responses and plasma PGE_2 levels (Rotondo *et al.*, 1988) have similar profiles to those following EPA treatment (Figures 1,3). It is also possible that the actions of EPA are actually mediated via steroids. It was interesting to note that EPA pretreatment appeared to attenuate the second peak of the fever response to Poly I:C greater than the first peak (Figure 1), which is also the case for steroids (Davidson *et al.*, 1991). Unsaturated free fatty acids can increase blood levels of cortisol (Widmaier *et al.*, 1995), whereas saturated fatty acids have no effect. This may be a direct action of unsaturated fatty acids on the adrenal cortex, as it has been shown that fatty acids can induce steroidogenesis directly in adrenocortical cells (Sarel and Widmaier, 1995). It has also been demonstrated that EPA can specifically amplify the release of cortisol levels in humans following activation of serotoninergic pathways with fenfluoramine (Buydens-Branchey *et al.*, 2011). This demonstrates the capability of EPA to modulate steroid pathways. We have previously shown that a novel steroid, 7β OH-epiandrosterone, can selectively enhance the production of 15d-PGJ_2 while down-regulating PGE_2 production (Davidson *et al.*, 2008). This is similar to the actions of EPA in the present study where EPA appears to increase levels of 15d-PGJ_2 but suppresses PGE_2 levels stimulated by poly I:C. This strengthens the possibility that EPA suppresses cytokine levels via the up-regulation of steroid production which in turn selectively enhances 15d-PGJ_2 levels. An EPA effect on steroid levels may also be responsible for the longer-lasting actions of EPA following cessation of administration. It is clear that EPA has a prolonged action following the end of

administration irrespective of the mechanism (Figure 2). This is well acknowledged in *n*–3 research, which makes crossover studies difficult and ambiguous. The 'washout' of EPA and indeed other long-chain fatty acids used *in vivo* is delayed. The study of Metherel *et al*. (2009) showed that levels of EPA in various blood compartments, including cells, were still significantly above the pretreatment levels 8 weeks after cessation of supplementation. The study used a dose of EPA comparable with the dose used in the present study (3.2 $g \cdot day^{-1}$/subject), but EPA was only administered for 28 days (Metherel *et al*., 2009). In an earlier study with a very short 7-day administration of EPA, it was established that the half-life of EPA (in cell phospholipids) following cessation was 2.31 days (Zuijdgeest-van Leeuwen *et al*., 1999). This would equate to circa 33% of the administration period.

Effect of EPA on PPARs

EPA may also act as an anti-inflammatory agent by binding to peroxisome proliferator-activated receptors (PPARs) that exert their regulatory activity at the gene level. EPA can bind to all three subtypes of PPAR but has the greatest affinity for PPAR-γ (Xu *et al*., 1999). In this study, we observed that EPA enhanced the amount of poly I:C-induced 15d-PGJ_2 present in plasma (Figure 4), and since 15d-PGJ_2 is reported to be an endogenous ligand for PPAR-γ (Forman *et al*., 1995), it is possible that EPA may also activate PPARs indirectly through enhanced 15d-PGJ_2 production/activity. However, there is increasing evidence that the activation of PPAR-γ by the levels of 15d-PGJ_2 normally found following activation is highly unlikely (see Davidson *et al*., 2012 for discussion).

Effect of EPA on poly I:C-induced increases in IL-1β

It is not known whether poly I:C itself can directly stimulate the production of PGs; however, it does induce the synthesis and release of endogenous pro-inflammatory intermediates, such as IL-1, TNFα and interferon, which are pro-inflammatory and induce fever in the rabbit. In this study, we demonstrated that poly I:C increased plasma levels of endogenous IL-1β compared with pre-challenge levels, and this increase was suppressed by EPA (Figure 4), implying that EPA can attenuate the inflammatory response by suppressing pro-inflammatory cytokine production. Various *in vitro* studies in cultured cells have shown that EPA can decrease IL-1β and TNF-α mRNA expression by inhibiting activation of the transcription factor, nuclear factor-kappaB (NFκB) (Novak *et al*., 2003; Zhao *et al*., 2004; Li *et al*., 2005), and NF-κB plays a pivotal role in signalling pathways leading to the production of pro-inflammatory cytokines.

Effect of EPA on IL-1-induced systemic inflammatory responses

In this study, we found that purified EPA did not attenuate IL-1β-induced fever. Previous work by other groups have reported that supplementation with fish oils reduces fever in response to IL-1 (Pomposelli *et al*., 1989; Cooper and Rothwell, 1993). Discrepancies between the data presented here and those of the above authors may be a result of using heterogenous preparations (fish oil), different animal models, different fever-inducing agents or alternative sources of IL-1 and routes of administration. In this study, we have attempted to simulate a 'naturally' occurring fever response by using rabbit IL-1β, and in order to gain a clearer understanding of the effect of an individual fatty acid and rather than an undefined mixture, we have administered a defined dose of pure EPA. This definitively indicates that EPA specifically attenuates the production of IL-1 (Figure 4) and not the actions of exogenously administered IL-1 (Figure 5), which bypasses the induction step in response to Poly I:C. Similarly, the actions of TNF-α were also affected by EPA treatment (Figure 5).

The data presented here clearly illustrate that EPA can down-regulate systemic inflammatory responses to the TLR3 ligand, poly I:C, specifically suppressing the endogenous production of IL-1β with the consequential reduction of PGE_2 levels. This is because of the sequential production of IL-1β followed by prostanoid biosynthesis (see Introduction) and that we have demonstrated that the direct actions of the exogenous upstream mediators (IL-1β and TNF-α) are unaffected by EPA. We also demonstrate the novel observation that EPA can enhance production of 15d-PGJ_2 following an immune challenge. This also raises the possibility that EPA acts as an effective anti-inflammatory agent *in vivo* by suppressing PGE_2 synthesis secondary to a decrease in proinflammatory IL-1β and by enhancing production of 15d-PGJ_2. Our observations are consistent with the view that EPA can definitively modulate immune/inflammatory responses *in vivo*.

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Conflict of interest

There are no conflicts of interests in the execution of this study.

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