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Fatty acid-mediated inhibition of IL-12 production by murine macrophages is independent of PPARγ

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Our laboratory has reported that *n*-3 PUFA can reduce host resistance to *Listeria* infection, in part, by impairing *in vivo* IL-12 biosynthesis. Recently, PUFA were shown to be ligands for PPAR, a novel family of nuclear receptors with three isoforms: PPARα, PPARδ/β and PPARγ. PPARγ is expressed in immune cells, such as T cells and macrophages. Two PPARγ agonists, 15-deoxy-$\Delta^{12,14}$-prostaglandin (PG) J_2 and rosiglitazone, have been shown to have immunomodulatory activity *in vitro*, including inhibiting IL-12 biosynthesis. We hypothesized that *n*-3 PUFA inhibit IL-12 production through activating PPARγ. We used thioglycolate-elicited mouse peritoneal macrophages to study the effect of various fatty acids and their oxidized metabolites on *in vitro* IL-12 production. Our present results demonstrate that both *n*-3 and *n*-6 PUFA can reduce *in vitro* IL-12 biosynthesis, though less potently than 15-deoxy-PGJ_2 and rosiglitazone. GW9662, a PPARγ antagonist, reversed the inhibitory effect of rosiglitazone, but not that of PUFA. Our present findings suggest that fatty acid-mediated inhibition of IL-12 production is independent of PPARγ.

Polyunsaturated fatty acids: Peroxisome proliferator-activated receptor-γ: Interleukin 12: Macrophages

Dietary *n*-3 PUFA are capable of affecting immune responses in both animals and human subjects. Studies have shown that *n*-3 PUFA can diminish cell-mediated immune function, including lymphocyte proliferation, cytotoxicity of natural killer cells, as well as antigen-presentation by macrophages (Calder, 1996, 1998; Harbige, 1998; Wu & Meydani, 1998). One mechanism by which *n*-3 PUFA modulate immune function is by alteration of cytokine production. Dietary supplementation with *n*-3 PUFA has been reported to diminish, not change and enhance, the production of various cytokines, including IL-1, IL-2, IL-6 and TNF-α (Endres, 1996; Meydani, 1996; Blok *et al.* 1997; Calder, 1997; Grimble & Tappia, 1998; Wallace *et al.* 2001). Our laboratory was first to demonstrate that *n*-3 PUFA consumption was associated with a significant impairment of *in vivo* IL-12 production in mice (Fritsche *et al.* 1999, 2000).

IL-12 is produced by activated myelomonocytic cells (D'Andrea *et al.* 1992; Hsieh *et al.* 1993). The biologically active form of IL-12 is a heterodimeric molecule (p70) composed of a 40 kDa (p40) and a 35 kDa (p35) subunit (Podlaski *et al.* 1992). IL-12 is a critical factor for the development of T helper 1 cells and initiation of cell-mediated immune responses against a variety of pathogens (Hsieh *et al.* 1993; Trinchieri, 1995). The unique ability of IL-12 to direct T helper 1 cell development and cellular immunity explains its key role in the development of certain inflammatory and autoimmune diseases (Adorini *et al.* 1997). Therefore, we believe it is essential to define the mechanisms by which *n*-3 fatty acids affect IL-12 production in order to maximize the potential therapeutic anti-inflammatory effects without compromising important anti-microbial or anti-tumour functions of the immune system.

Recent studies suggest that fatty acids share many properties with classic steroid hormones in that they can bind nuclear receptors and regulate target gene expression. A variety of fatty acids were shown to be ligands for a novel family of nuclear receptors called PPAR (Krey *et al.* 1997). Three isoforms of PPAR have been identified: PPARα, PPARδ and PPARγ (Kliewer *et al.* 1994). PPARγ has been shown to be an important modulator of inflammation (Gelman *et al.* 1999). It mainly serves as a down-regulator of the activity of many immune cells (Jiang *et al.* 1998; Ricote *et al.* 1998). Many different fatty acids can bind and activate PPARγ, including PUFA from both the *n*-6 and *n*-3 families (Krey *et al.* 1997; Xu *et al.* 1999). To date, the exact contribution of the PPARγ pathway to the immunoregulatory properties of fatty acids has not been defined. Our hypothesis was that *n*-3 PUFA inhibit IL-12 production through activating PPARγ. In the present study we used thioglycolate-elicited mouse macrophages as an *in vitro* cell model system to study the effects of fatty acids and PPARγ ligands on IL-12 biosynthesis.

Abbreviations: AA, arachidonic acid; DHA, docosahexaenoic acid; IFN, interferon; LA, linoleic acid; LPS, lipopolysaccharide; PG, prostaglandin.

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Materials and methods

Reagents

Mouse recombinant interferon (IFN) γ was purchased from R&D (Minneapolis, MN, USA). Lipopolysaccharide (LPS) from *Escherichia coli* was purchased from Sigma Chemical Co. (St Louis, MO, USA). 15-Deoxy-prostaglandin (PG) J_2, all fatty acids and fatty acids metabolites were purchased from Caymen Chemical (Ann Arbor, MI, USA). Rosiglitazone was extracted with ethanol from rosiglitazone maleate tablets purchased from SmithKline Beecham Pharmaceuticals (Philadelphia, PA, USA). GW9662, a PPARγ antagonist, was a gift from GlaxoSmithKline (Research Triangle Park, NC, USA). WY14643, a selective PPARα activator, was purchased from Biomol (Plymouth Meeting, PA, USA).

Animals

Female specific-pathogen-free BALB/cAnNHsd mice (6–10 weeks old) were used in all experiments (Harlan, Indianapolis, IN, USA). Upon arrival mice were placed in polycarbonate micro-isolator cages (three to four animals per cage) in an environmentally controlled room (21–24°C, 50–60 % relative humidity). A 12 h light–dark cycle was maintained throughout the study. Animals had free access to distilled water and food (Mouse Chow 5008; Ralston Purina Co., Richmond, IN, USA). Animal housing, handling and sample collection procedures conformed to policies and recommendations of the University of Missouri's Laboratory Animal Care and Use Committee.

Cell collection and stimulation

Mouse peritoneal macrophages were used for all experiments. To collect these cells, mice were injected intraperitoneally with 2 ml sterile Brewer's thioglycolate broth (Sigma Chemical Co.). After 4 d, peritoneal exudate cells were collected by flushing the peritoneal cavity twice with 8 ml sterile ice-cold PBS. Cells were pelleted in a centrifuge (500 ***g***, 10 min) and then resuspended in Roswell Park Memorial Institute 1640 medium (GibcoBRL, Grand Island, NY, USA) containing fetal bovine serum (20 g/l), 25 mM-HEPES and 2 mM-glutamine. The concentration of cells was determined electronically (Coulter Electronics Inc., Miami, FL, USA) and then adjusted to a final concentration of 2×10^6/ml. Aliquots of each cell preparation were deposited on a glass slide and stained (Wright's stain) for cell differentials. Our cell populations were routinely 85–95 % macrophages, based on cell morphology and staining properties. The remaining cells in our cell preparations were predominately lymphocytes, with a small number of neutrophils, eosinophils, basophils and mast cells. We made no effort to further enrich our cell preparations for macrophages.

Unstained cells were cultured in twenty-four-well or forty-eight-well tissue culture plates (Corning; Corning, NY, USA). To stimulate IL-12 production, IFNγ (10 U/ml) was added to the wells, followed by the addition of LPS (0·1 μg/ml) or *Listeria monocytogenes* (10^6 colony forming units/ml). One hour after addition of Listeria, gentamycin (50 μg/ml) was added to Listeria-treated wells to kill extracellular bacteria. Control wells received the same amount of antibiotic at this time. After 24 h of incubation, the plates were centrifuged (500 ***g*** for 10 min) and then supernatant fractions were collected and stored at −70°C for future cytokine analysis.

Treatment of cells with PPARγ ligands, fatty acids, fatty acid metabolites and PPARγ antagonist

In a pilot study we demonstrated that PPARγ ligands were equally effective if added 4 h before or simultaneously with the addition of stimuli, but did not show any effect if added 4 h after the stimulation of cells (results not shown). Therefore, in all subsequent experiments PPARγ ligands were added to cells shortly before (5–15 min) the addition of stimuli. PPARγ ligands were delivered to cells dissolved in ethanol. To simulate the cell treatment with the PPARγ agonists, fatty acids and the various fatty acid metabolites tested were also delivered in ethanol. The PPARγ antagonist GW9662 was dissolved in dimethylsulfoxide according to the manufacturer's instructions. Cells were incubated for 2 h with GW9662 before stimulating IL-12 biosynthesis. Control cells were cultured with dimethylsulfoxide only. The final concentration of ethanol and dimethylsulfoxide never exceeded 4 and 2 g/l respectively. These concentrations were not toxic to the cells, as determined by cell viability at the end of the incubation period (described later).

Following treatment with PPARγ ligands and immune stimulation as described earlier, cells were incubated for an additional 22 h at 37°C in a 5 % CO_2 atmosphere. Plates were gently spun (200 ***g***, 5 min) to pellet the cells, then the supernatant fractions were collected for IL-12 determination via ELISA.

Cell viability assay

In all experiments the viability of cells following treatment was determined using Cell Titer 96 Aqueous One Solution (Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, to 50 μl supernatant fraction and cells that were left behind in each well, 50 μl fresh media and 20 μl MTS tetrazolium dye (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) were added. Plates were incubated at 37°C for 30 min, and then 100 μl liquid from each well was transferred to a ninety-six-well ELISA plate to determine the absorbance at 492 nm using a plate reader.

IL-12 and TNF-α determinations

Commercially available ELISA kits (R&D) were used for measuring the concentration of IL-12p70 and TNF-α concentrations in the cell culture supernatant fraction samples. Briefly, ninety-six-well ELISA plates (Nunc, Naperville, IL, USA) were coated with capture antibody (e.g. rat anti-mouse IL-12p70 antibody) overnight. The next day, plates were treated with blocking buffer (i.e. bovine serum albumin (10 g/l), 50 g sucrose/l PBS with 0·5 g sodium azide/l)

to block non-specific binding. Various dilutions of samples and standards were incubated in the plates for 2 h before biotinylated goat anti-mouse cytokines antibody was added. After 2 h, plates were washed three times with wash buffer (PBS with 0·5 g Tween 20/l) followed by the addition of streptavidin–horseradish (*Armoracia Rusticana*) peroxide solution. Following a 20 min equilibration period, one-step substrate (DAKO Corp., Carpinteria, CA, USA) was added. After 20 min colour development the reaction was stopped with 2 M-H_2SO_4 acid. All the treatments were conducted at room temperature. The absorbance was measured on an ELISA plate reader at 450 nm with a reference reading at 620 nm. The detection limit for the IL-12p70 and TNF-α ELISA kits were 23 and 16 pg/ml respectively. All samples were assayed in duplicate.

Statistical analyses

Results are expressed as mean values with their standard errors. Cytokine production data were analysed by one-way ANOVA. For most experiments, cell preparations from separate mice were treated as independent experimental units. Data represent values from replicate wells from the same pooled cell population. In all cases, when a significant difference ($P<0·05$) occurred among different treatment groups, treatment mean differences were identified by Fisher's protected least significant difference test. All analyses were conducted on a Macintosh computer with StatView II software (version 1.3.2; Abacus Concepts, Berkeley, CA, USA).

Results

PPARγ ligands inhibit IL-12 and TNF-α production by mouse peritoneal macrophages

Two PPARγ ligands, 15-deoxy-PGJ_2 and rosiglitazone, were tested for their effect on IL-12 production by thioglycolate-elicited murine macrophages following either infection with *L. monocytogenes* or LPS stimulation. PPARγ agonists dose-dependently inhibited IL-12 production, but had little or no effect on TNF-α production. For example, *L. monocytogenes*-induced IL-12 production was reduced by approximately 50 % by 0·1 μM-rosiglitazone, with little additional inhibition noted at 1·0 μM. 10·0 μM-rosiglitazone decreased IL-12p70 biosynthesis further (approximately 30 % of untreated cells) and tumour necrosis α production was significantly diminished (approximately 50 % of controls). We observed similar rosiglitazone-mediated reductions in IL-12 production from mouse macrophages stimulated with LPS rather than with *L. monocytogenes* (results not shown). We determined cell viability at the end of each experiment and found that the inhibitory effects of PPARγ agonist treatment on macrophage IL-12 production were not due to cell toxicity. Rosiglitazone was not toxic to these cells at all of the concentrations used; however, 10 μM-15-deoxy-PGJ_2 did induce a considerable degree of cell death (results not shown). We also tested the impact of WY14643, a selective PPARα activator, on IL-12 biosynthesis and found that it was without effect at concentrations <10 μM. At a concentration of 10 μM, WY14643 modestly reduced mouse macrophage IL-12p70 production by 10 to 15 % of untreated cells.

Fatty acids and their oxidation products reduce IL-12 production

The addition of non-esterified PUFA in their native form (i.e. unoxidized) to mouse peritoneal macrophages decreases IL-12p70 production (Fig. 1(A)). The reduction was dose-dependent and varied depending on the fatty acid used. Of the fatty acids tested, arachidonic acid (AA, 20 : 4*n*-6) and docosahexaenoic acid (DHA, 22 : 6*n*-3) were the most potent at reducing IL-12 production. The impact of linoleic acid (LA, 18 : 2*n*-6) was more variable than that of AA or DHA, and EPA (20 : 5*n*-3) had the least effect on IL-12p70 production. Exposure of cells to NEFA concentrations ≥ 50 μM reduced cell viability (results not shown). Therefore, all subsequent experiments were conducted at a concentration of 25 μM.

Next, we tested oxidized metabolites of LA, AA, EPA (i.e. 9-hydroxyoctadecadienoic acid, 9-hydroxyeicosatetraenoic acid and 9-hydroxyeicosapentaenoic acid respectively). These fatty acid metabolites dose-dependently reduced IL-12p70 production (Fig. 1(B)). The rank order of their potency was 9-hydroxyeicosatetraenoic acid >9-hydroxyoctadecadienoic acid >9-hydroxyeicosapentaenoic acid, which is consistent with the relative potency of the native fatty acids these metabolites were derived from (i.e. AA, LA and EPA). Furthermore, it was clear that the oxidized metabolites were more potent than the native fatty acid, the former having an IC_{50} (median inhibitory concentration) between 1 and 4 μM.

GW9662, a PPARγ antagonist, partially reversed rosiglitazone's effect on IL-12 production, but not that of fatty acids

GW9662 is a putative PPARγ antagonist. In order to determine whether this drug functioned as a PPARγ antagonist in our cell culture system, we pretreated our mouse macrophage preparations with varying concentrations of GW9662. After 2 h we added rosiglitazone (20 μM) to the cells and then stimulated IL-12 production with IFNγ and LPS as described previously. As expected, rosiglitazone inhibited IL-12 production. The pretreatment of cells with GW9662 at 10 μM almost completely abrogated inhibitory effects of rosiglitazone on mouse macrophage IL-12 production (results not shown). If PUFA-mediated inhibition of IL-12 production is mediated by PPARγ, then treatment with this PPARγ antagonist should abrogate the effect of PUFA. Therefore, mouse peritoneal macrophages were pretreated with GW9662 for 2 h before the addition of various PUFA at 25 μM. PUFA treatment reduced IL-12 production, yet GW9662 pretreatment did not prevent the inhibitory actions of these PUFA on IL-12 production (Fig. 2).

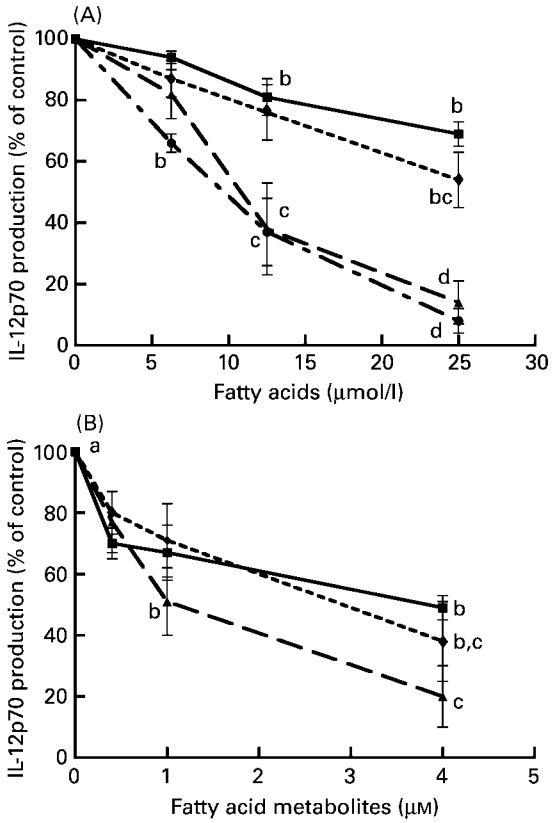

Fig. 1. PUFA and their oxidized metabolites diminish IL-12p70 production by murine macrophages. Thioglycolate-elicited macrophages were isolated from the peritoneums of female specific-pathogen-free BALB/c mice and cultured as described on p. 734. Briefly, cells were resuspended in Roswell Park Memorial Institute 1640 medium with fetal bovine serum (20 g/l) at a final concentration of 2×10^6/ml and cultured in forty-eight- or twenty-four-well tissue culture plates. Fatty acids (Fig. 1(A); –■–, linoleic acid; –▲–, arachidonic acid; – - –•– - –, docosahexaenoic acid; –-◆–-, EPA) or various oxidized metabolites (Fig. 1(B); –■–, 9-hydroxyoctadecadienoic acid; –-▲–-, 9-hydroxyeicosatetraenoic acid; – –◆– –, 9-hydroxyeicosapentaenoic acid) dissolved in ethanol were added to cell suspensions at the indicated concentrations. After 5–10 min, IL-12p70 biosynthesis was stimulated by adding interferon γ (10 U/ml) and lipopolysaccharide (0·1 μg/ml). Cells were cultured at 37°C in a 5% CO_2 environment for 24 h. Supernatant fractions were collected, then analysed for IL-12p70 by ELISA. Values are means from three wells with their standard errors shown by vertical bars (representative of three independent experiments). The actual concentrations of IL-12p70 in the untreated cultures were 1056 (SED 152) and 803 (SED 71) pg/ml for Fig. 1(A) and Fig. 1(B) respectively. [a,b,c,d]Mean values with unlike superscript letters were significantly different ($P<0·05$).

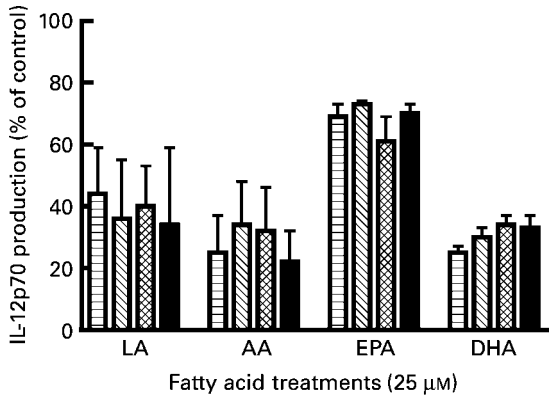

Fig. 2. Pretreatment with a PPARγ antagonist does not reverse the inhibitory effect of PUFA on lipopolysaccharide-induced IL-12p70 production by mouse macrophages. ▤, 0·0 μM-GW9662; ▧, 0·1 μM-GW9662; ▨, 1·0 μM-GW9662; ■, 10·0 μM-GW9662. LA, linoleic acid; AA, arachidonic acid; DHA, docosahexaenoic acid. Thioglycolate-elicited mouse peritoneal macrophages were collected and cultured as described on p. 734. GW9662, a PPARγ antagonist, was added to cells at a final concentration of 10 μM. After 2 h, LA, AA, EPA or DHA was added to the cells at a final concentration of 25 μM, immediately followed by interferon γ and lipopolysaccharide stimulation. Cells were cultured at 37°C for 24 h. Cell-free supernatant fractions were assayed for IL-12p70 by ELISA. Values are means with their standard errors shown by vertical bars (three independent experiments). While PUFA treatment significantly reduced IL-12p70 production ($P<0·005$), GW9662 did not have a significant effect on IL-12p70 production under any of the conditions studied here ($P>0·05$). The actual concentrations of IL-12p70 in the cultures treated with vehicle alone were 1137 (SEM 121) pg/ml for the LA, AA and EPA-treated cells and 1655 (SEM 182) pg/ml for the DHA-treated cells.

Discussion

In the present study we established an *in vitro* cell culture model to establish whether long-chain *n*-3 PUFA were reducing IL-12 production via a PPARγ dependent process. We used thioglycolate-elicited mouse peritoneal macrophages, which are known to express high levels of PPARγ (Huang *et al.* 1999). Next we confirmed what others have previously reported (Chung *et al.* 2000; Alleva *et al.* 2002), that *in vitro* treatment of these cells with two compounds that bind and activate PPARγ (i.e. 15-deoxy-PGJ_2 and rosiglitazone) reduce the secretion of this pro-inflammatory cytokine in a dose-dependent manner. Several research teams, however, have demonstrated that some of the actions of 15-deoxy-PGJ_2 can occur independently of PPARγ (Petrova *et al.* 1999; Thieringer *et al.* 2000; Hinz *et al.* 2003). In contrast to 15-deoxy-PGJ_2, rosiglitazone at low concentrations (≤ 1 μM) is believed to function exclusively through activation of PPARγ (Willson *et al.* 2000). The concentration of rosiglitazone at which we observed 50% inhibition of IL-12 production (i.e. 0·1 μM) is similar to the EC_{50} (half effective concentration) reported in a PPAR-GAL4 transactivation assay (Willson *et al.* 2000).

Researchers have shown that a number of fatty acids can bind and activate PPARγ at μM concentrations (Xu *et al.* 1999). Oxidized PUFA in particular (i.e. 9-hydroxyoctadecadienoic acid) are putative PPARγ agonists (Nagy *et al.* 1998). Thus, after demonstrating that our *in vitro* cell system was sensitive to PPARγ-dependent regulation of IL-12 biosynthesis, we tested the impact of various native and oxidized PUFA. We found that similar to known PPARγ agonists, treatment of murine macrophages with native and oxidized PUFA reduced IL-12 production in a dose-dependent manner. The relative potency we observed was rosiglitazone > oxidized PUFA > native PUFA. Among the PUFA tested the relative potency was DHA = AA > EPA = LA.

Previously our laboratory has reported that *n*-3 PUFA found in fish oils, EPA or DHA were equally effective at reducing *in vivo* IL-12 production in mice (Fritsche *et al.* 1999, 2000). Thus, we were surprised when DHA distinguished itself from EPA in its potency for reducing

IL-12 production *in vitro*. It is possible that DHA might have unique actions over those of EPA. For example, DHA, but not EPA, was recently proposed to be a natural ligand of retinoid X receptor (de Urquiza *et al.* 2000). This receptor heterodimerizes with PPARγ, as well as with other nuclear transcription factors (Willson *et al.* 2000; Chawla *et al.* 2001). The physiological consequences of DHA's interaction with retinoid X receptor are poorly understood at this time. It is unclear if interaction with retinoid X receptor is what distinguishes DHA's greater potency on IL-12 biosynthesis over that of the closely related *n*-3 fatty acid, EPA.

The relatively high potency of the *n*-6 fatty acid, AA, over its precursor *n*-6 fatty acid (i.e. LA) is more easily understood. We have determined that the inhibitory activity of exogenous AA requires endogenous cyclooxygenase (COX)-2 activity. In contrast, the use of COX-2 inhibitors did not significantly alter the inhibition of IL-12 biosynthesis by LA, EPA or DHA (results not shown).

Our original hypothesis was that *n*-3 PUFA down-regulated IL-12 production through a PPARγ-dependent process. However, our *in vitro* results with a PPARγ antagonist, GW9662, suggest that fatty acid-mediated inhibition of IL-12 production is independent of PPARγ. We found that pretreatment of cells with GW9662 was able to abrogate the inhibitory action of rosiglitazone, a PPARγ-specific agonist, on *in vitro* IL-12 biosynthesis. However, GW9662 pretreatment did not alter the inhibitory effect of PUFA treatment on mouse macrophage IL-12 production. At the time of these studies, biphenol A diglycidyl ether was the only other PPARγ antagonist known (Wright *et al.* 2000). We found biphenol A diglycidyl ether to be toxic to primary mouse peritoneal macrophages and were unable to successfully carry out any experiments with it (results not shown).

The concentrations of PUFA that we used in the present study were relatively low ($\leq 25\,\mu M$) compared with those commonly used in transactivation assays that originally defined PUFA as PPAR agonists (Kliewer *et al.* 1997). Clearly, the potency of fatty acids to activate PPARγ is much lower than that of synthetic activators such as rosiglitazone (Krey *et al.* 1997). At this time the question as to whether fatty acids serve as endogenous PPARγ ligands remains controversial. However, it seems feasible that under certain physiological conditions, such as fasting, infection and inflammation, systemic or local concentration of NEFA may reach levels that are conducive to their acting via PPARγ. Furthermore, it seems entirely possible that under most circumstances it is not the native fatty acid, but rather a metabolite that acts as a PPARγ ligand. For example, in our present experiments the effect of exogenous AA treatment on IL-12 production may have been mediated in part through the production of one or more metabolites such as PGE_2 or 15-deoxy-PGJ_2. Others have demonstrated that PGE_2 is a potent inhibitor of human IL-12 production (van der Pouw Kraan *et al.* 1995). In our present study we did not directly test the impact of PGE_2, but rather did show that 15-deoxy-PGJ_2, another metabolite of AA produced by macrophages (Fitzpatrick & Wynalda, 1983; Shibata *et al.* 2002), was quite potent at inhibiting IL-12 production. This eicosanoid may serve as part of an anti-inflammatory negative feedback loop in macrophages. Furthermore, we found that fatty acid oxidation products were much more potent than their native fatty acids in inhibiting IL-12 production.

The major finding presented here is that PUFA-mediated reduction of *in vitro* IL-12 biosynthesis by mouse macrophages appears to be independent of PPARγ. Further, we found little evidence to support a role for PPARα in regulating IL-12 production in macrophages. However, we believe that fatty acids may reduce IL-12 production via interactions with PPARδ (otherwise known as PPARβ). Of the three members of the PPAR family, PPARδ is the least well understood. Recently Lee *et al.* (2003*a*) demonstrated that activation of PPARδ with a synthetic agonist (GW501516) reduces the expression of several pro-inflammatory genes in murine macrophages (i.e. monocyte chemoattractant protein-1, IL-1β, but not TNF-α or matrix metalloproteinase-9). Unfortunately, these researchers did not measure IL-12 production. Welch *et al.* (2003) recently demonstrated that rosiglitazone at concentrations of $50\,\mu M$ inhibit inducible nitric-oxide synthase and IL-12p40 gene expression in murine macrophages in a PPARγ-independent manner. The authors suggest that PPARγ and PPARδ (otherwise known as PPARβ) have overlapping transactivation and transrepression functions in macrophages. Thus, it seems possible that fatty acid modulation of IL-12 biosynthesis may occur through PPARδ and not PPARγ. Unfortunately, at the time that our present studies were conducted, agonists and antagonists specific for PPARδ were not available.

In addition to interaction with PPARδ there are several alternative mechanisms through which *n*-3 PUFA might reduce IL-12 biosynthesis. First, we believe that *n*-3 PUFA-mediated reduction in IFNγ receptor expression may play an important role. We have previously reported that feeding mice a diet high in *n*-3 PUFA was associated with a 25–50 % reduction in IFNγ receptor expression on immune cells (Feng *et al.* 1999). IFNγ signalling via phosphorylation of signal transducers and activators of transcription (STAT1) is essential for early *in vivo* IL-12 biosynthesis following an infection (Flesch *et al.* 1995). We have recently found that macrophages obtained from mice fed a diet with fish oil (i.e. rich in *n*-3 PUFA) have significantly lower levels of phosphorylated STAT1 following treatment with IFNγ compared with macrophages isolated from mice fed a control diet free of *n*-3 PUFA (results not shown). STAT1 plays a central role in the induction of many IFNγ-dependent pro-inflammatory genes, including IL-12 (Ramana *et al.* 2002). Second, recent findings suggest that reactive oxygen species can have an inhibitory effect of STAT1 signalling (Chen, 2003). *n*-3 PUFA and AA treatment may enhance the production of free radicals within activated macrophages and thus oxidative stress might play a role in *n*-3 PUFA-mediated inhibition of IL-12 production. Third, Lee *et al.* (2003*b*) have suggested that PUFA interfere with toll-like receptor signalling pathways in macrophages. Toll-like receptors recognize specific components conserved among micro-organisms and help macrophages and other cells of the innate immune system respond to infection (Takeda *et al.* 2003). Lee *et al.* (2003*b*) showed that *in vitro* treatment of cells with PUFA such as DHA,

EPA, AA and LA diminish, while saturated fatty acids enhance toll-like receptor signalling. Usually, signalling via both toll-like and IFNγ receptors is required for optimal macrophage activation and subsequent IL-12 biosynthesis (Ehrt *et al.* 2001), thus interference with either pathway would be expected to have a negative effect on IL-12 production.

In conclusion, we have confirmed that PPARγ agonists significantly diminish IL-12p70 production by mouse peritoneal macrophages and that this inhibition can be abrogated by pretreatment of cells with GW9662, a PPARγ antagonist. Furthermore, we have demonstrated that fatty acids and their oxidation products can also inhibit IL-12 production in a dose-dependent manner. However, GW9662 does not prevent the inhibitory action of fatty acids on IL-12 production by macrophages. Our present results suggest that fatty acid-mediated inhibition on IL-12 production is independent of PPARγ. Elucidation of the possible involvement of PPARδ or other molecular mechanism(s) through which fatty acids mediate inhibition of IL-12 production awaits further study.

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