Comparison of the effects of the n-3 polyunsaturated fatty acid eicosapentaenoic and fenofibrate on the inhibitory effect of arthritis on IGF1

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Abstract
Adjuvant-induced arthritis is a chronic inflammatory illness that induces muscle wasting and decreases circulating IGF1. Eicosapentaenoic acid (EPA) and fenofibrate, a peroxisome proliferator-activated receptors α agonist, have anti-inflammatory actions and ameliorate muscle wasting in arthritic rats. The aim of this work was to elucidate whether EPA and fenofibrate administration are able to prevent the effect of arthritis on the IGF1–IGFBP system. On day 4 after adjuvant injection control, arthritic rats were gavaged with EPA (1 g/kg) or fenofibrate (300 mg/kg) until day 15 when all rats were killed. Arthritis decreased body weight gain, serum IGF1, and liver Igf1 mRNA, whereas it increased gastrocnemius Igfbp3 mRNA. EPA, but not fenofibrate, administration prevented arthritis–induced decrease in serum IGF1 and liver Igf1 mRNA. In the rats treated with EPA arthritis increased Igfbp5 mRNA in the gastrocnemius. Fenofibrate treatment decreased IGF1 and Igf1 mRNA in the liver and gastrocnemius. In arthritic rats, fenofibrate increased body weight gain and decreased gastrocnemius Igfbp3 and Igfbp5 mRNA. These data suggest that the mechanisms through which EPA and fenofibrate act on the IGF1 system and ameliorate muscle wasting in arthritic rats are different. EPA administration increased circulating levels of IGF1, whereas fenofibrate decreased the Igfbp3 and Igfbp5 in the gastrocnemius muscle.
Journal of Endocrinology (2011) 210, 361–368

Introduction
Chronic inflammation is often associated with skeletal muscle wasting and cachexia (Evans et al. 2008). Inflammatory cachexia has been reported in chronic illnesses such as cancer, heart or renal failure, Crohn’s disease, sepsis, and rheumatoid arthritis. Cachexia can also induce a decrease in body weight that is associated with muscle atrophy and sometimes with fat mass depletion. The increased release of cytokines and other proinflammatory mediators plays a crucial role in inflammatory cachexia (Morley et al. 2006). However, inflammation induces deep modifications in the neuroendocrine system that might also contribute to a decrease in body weight, muscle wasting, and cachexia (Morley et al. 2006). These modifications include an increase in the release of glucocorticoids with a decrease in anabolic hormones such as androgens and insulin-like growth factor 1 (IGF1; Soto et al. 1998).

Adjuvant-induced arthritis is an experimental model of rheumatoid arthritis that is associated with a decrease in body weight and muscle wasting (Castillero et al. 2009a). Arthritic rats have low serum IGF1 levels that are inversely related to body weight gain (López-Calderón et al. 1999), whereas chronic GH administration to arthritic rats increases body weight and serum IGF1 levels (Ibañez de Cáceres et al. 2000). Similarly, GH administration to juvenile RA patients increases growth (Touati et al. 1998). Furthermore, exogenous IGF1 administration to arthritic rats increases body and skeletal muscle weights (López-Menduiña et al. 2010). All these data indicate that arthritis-induced decrease in circulating IGF1 is an important contributor to the decrease in body weight and skeletal muscle wasting induced by experimental arthritis.

Eicosapentaenoic acid (EPA) is an n-3 polyunsaturated fatty acid that is essential for normal growth and is present in large amounts in fish oil. It is well known that EPA has an anti-inflammatory effect decreasing proinflammatory cytokines (Tashiro et al. 1998, Zhao et al. 2004) and both cyclooxygenase-2 activity and expression (Hurst et al. 2009). Furthermore, EPA is able to decrease joint inflammation in rheumatoid arthritis patients (James & Cleland 1997, Simopoulos 2002) and in arthritic rats (Leslie et al. 1985, Volker et al. 2000, Castillero et al. 2009b). In addition to its anti-inflammatory effect, EPA ameliorates arthritis-induced skeletal muscle wasting by decreasing atrogin-1 and Murf-1 gene expression and also increasing the transcription factors
that regulate myogenesis (Castillero et al. 2009a). Taking into account that the beneficial effect of IGF1 treatment on skeletal muscle in arthritic rats is also associated with decreased atrogin-1 and increased myogenic regulatory factors (López-Menduña et al. 2010), it is possible that EPA administration can prevent arthritis–induced inhibition of IGF1.

EPA has several mechanisms of action, and one of these is through the activation of peroxisome proliferator-activated receptors (PPARs; Forman et al. 1997). PPARs are nuclear transcription factors that regulate genes involved in the control of metabolism and inflammatory responses (Michalik & Wahlì 2008). In hepatocytes, EPA has been reported to activate PPARα (Pawer & Jump 2003) and upregulate Ppara gene expression (Liu et al. 2011). Fenofibrate, a PPARα ligand, inhibits cytokine production from rheumatoid synovial fibroblasts and inhibits the development of adjuvant-induced arthritis in female Lewis rats (Okamoto et al. 2005). In arthritic rats, we have reported that fenofibrate administration increases body weight and decreases skeletal muscle atrophy through inhibition of atrogin-1, Murf-1, and myostatin expression in skeletal muscle (Castillero et al. 2011). These data indicate that EPA and fenofibrate have comparable effects on skeletal muscle in arthritic rats. To clarify whether the effect of EPA can be mediated by PPARα activation, we have compared the response of the IGF–IGF1Ps system to experimental arthritis in rats fed with either EPA or the PPARα activator fenofibrate.

Materials and Methods

Animals

Arthritic and control male Wistar rats weighing 150 g were purchased from Charles River (Barcelona, Spain). Arthritis was induced in the rats by a s.c. injection of 4 mg heat-inactivated Mycobacterium butyricum in the right hind paw, under isoflurane anesthesia (Castillero et al. 2009a). Control animals were injected with vehicle (0.1 ml paraffin oil). After arrival (day 3 after adjuvant injection), rats were housed 3 per cage, and maintained under standardized conditions of temperature (20–22°C) and light (lights on from 0730 to 1930 h). Assessment of arthritis was performed by measuring the arthritis index of each animal, which was clinically scored by grading each paw from 0 to 4. Grading was determined as: 0, no erythema or swelling; 1, slight erythema or swelling of one or more digits; 2, swelling of paw; 3, swelling of entire paw and ankle; and 4, ankylosis, incapacity to bend the ankle. The severity score was the sum of the clinical scores of each limb, the maximum value being 16 (Tanaka et al. 1996). The procedures followed the guidelines recommended by the EU for the care and use of laboratory animals, and were approved by the Complutense University animal care committee.

Treatments

EPA 18 control and 18 arthritic rats were divided into two groups on day 4 after adjuvant injection. The first group received a daily dose of 1 g/kg BW highly purified ethyl ester of EPA (E-EPA) containing 90% EPA and 0-02% vitamin D3 (Oy Bio–Vita Ab, Espoo, Finland) by oral gavage. The other group received 1 g/kg BW of coconut oil to ensure isocaloric intake. Coconut oil has a high concentration of short- (8–10 carbons) and medium-chain saturated fatty acids (12–16 carbons). Since arthritis decreases food intake, a pair-fed group of nine rats treated with coconut oil was also included. Pair-fed rats received the same amount of food (g/100 g BW) eaten on the previous day by arthritic rats treated with coconut oil.

Fenofibrate On day 4 after adjuvant injection, 18 control and 18 arthritic rats were each divided into two groups. The first group received fenofibrate (300 mg/kg BW, suspended in 500 μl 1% carboxymethylcellulose (CMC), Sigma–Aldrich) daily by oral gavage. The second group was gavaged with vehicle (500 μl 1% CMC). A pair-fed group of nine rats treated with vehicle was also included. All rats were killed on day 15 after adjuvant injection and after 12 days of treatment. Trunk blood was collected into cooled tubes, allowed to clot, centrifuged and the serum was stored at −20°C until IGF1 and IGFBP3 assays were performed. Immediately after decapitation, left gastrocnemius and liver were dissected, frozen in liquid nitrogen and stored at −80°C until RNA extraction. Isolation and manipulation of tissues were always performed under sterile conditions.

RNA extraction and real-time PCR

Gastrocnemius or liver (100 mg) was homogenized, and total RNA was extracted using Ultraspec (Biotecx Laboratories, Inc., Houston, TX, USA), following the manufacturer’s protocol. The final concentration of RNA was determined (260 nm) with a BioPhotometer (Eppendorf, Germany), and the integrity of the RNA was confirmed by agarose gel electrophoresis. First-strand cDNA synthesis was performed by 1 μg of total RNA with a Quantscript Reverse Transcription kit (Qiagen).

Real-time PCR for quantification of mRNA was performed on a SmartCycler (Cepheid, Sunnyvale, CA, USA) using a SYBR Green protocol on the fluorescence temperature cycler. Each real-time PCR consisted of 10 ng total RNA equivalents, 1X Takara SYBR Green Premix Ex Taq (Takara BIO, Inc., Otsu, Shiga, Japan), and 300 nM forward and reverse primers in a reaction volume of 25-5 μl. Primers for real-time PCR (Table 1), were obtained from Roche. The thermal cycling profile consisted of a preincubation step at 95°C for 10 s followed by 40 cycles of 95°C denaturation steps for 15 s, 60°C annealing steps for 30 s, and 72°C extension steps for 30 s. Results were
was expressed as the percentage of the mean density of sera obtained. The signals of the film were quantified by intensifying screens for 1–4 days according to the signal (Kodak X-Omat AR, Eastman Kodak) and to two

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers (5′–3′) F:</th>
<th>Product (bp)</th>
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<tbody>
<tr>
<td>18S</td>
<td>GGTGCATGGCCGCCTTCA</td>
<td>60</td>
</tr>
<tr>
<td>R:</td>
<td>TCGTITCGTATCGGAAATAC</td>
<td></td>
</tr>
<tr>
<td>Igfbp3</td>
<td>GAAAGAGCAGGCTGATGG</td>
<td>78</td>
</tr>
<tr>
<td>R:</td>
<td>GCGTATGTCAGCTCCAGGT</td>
<td></td>
</tr>
<tr>
<td>Igfbp5</td>
<td>GCCCGAGAAACCAAGATAGA</td>
<td>75</td>
</tr>
<tr>
<td>R:</td>
<td>GGTCTCTCGAGCAGCATTC</td>
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F, forward; R, reverse.

expressed relative to the control animals treated with coconut oil or vehicle, where the relative mRNA abundance has been arbitrarily set to 1, using cycle threshold 2(ΔΔCt) method, with 18S as reference gene. PCR products were separated using agarose gel electrophoresis to confirm the product presence and size.

IGF1 and corticosterone determinations

Serum IGF1 was measured using the antisera to human IGF1 (UB2–495) from Dr Underwood and Dr Van Wik, and is distributed by the NIDDK Hormone Distribution Program through the National Hormone and Pituitary Program. Levels of IGF1 were expressed in terms of rat IGF1 from Gropep Ltd (Adelaide, SA, Australia). The in-assy coefficient of variation was 8%. All samples from the same experiment were run in the same assay. Serum corticosterone was analyzed by a commercial kit from MP Biomedicals, LLC (New York, NY, USA).

Ligand blot

Serum concentrations of IGFBP3 were measured by western blot. Serum (2 μl) was diluted in sample buffer and boiled for 2 min at 90 °C, loaded onto 1% SDS–12.5% polyacrylamide gels, and electrophoresed under non-reducing conditions. Proteins were transferred onto nitrocellulose sheets (Hybond-C extra, Amersham, UK). The membranes were dried and blocked for 1 h with 5% non-fat dry milk, 0.1% Tween (Sigma), in Tris-buffered saline. Membranes were probed overnight at 4 °C with 125I-labeled IGF1 (1·5 × 106 c.p.m./ml). The nitrocellulose sheets were then washed, dried, and exposed at K

Results

Effect of EPA administration

Arthritis decreased body weight gain to values lower than in pair-fed rats (Table 2). EPA administration was unable to modify body weight gain in arthritic or control rats. However, EPA treatment decreased the external signs of arthritis (Table 2). Figure 1 shows the effect of arthritis and EPA administration on serum concentration of IGF1, IGFBP3, and corticosterone. In rats treated with coconut oil, arthritis decreased serum concentrations of IGF1, whereas it increased corticosterone levels. In addition, pair-fed rats had IGF1 serum concentrations similar to arthritic rats, and corticosterone concentrations slightly lower but not significantly different. EPA administration did not modify serum concentrations of these hormones in control rats, but prevented the inhibitory effect of arthritis on serum concentrations of IGF1. Arthritis did not modify serum concentrations of IGFBP3 or its gene expression in the liver (Figs 1B and 2B). Igf1 mRNA in the liver was also decreased in pair-fed and in arthritic rats treated with coconut oil (Fig. 2A). EPA administration did not modify Igf1 expression in the liver of control rats, but increased Igf1 mRNA levels in the liver of arthritic rats to levels similar to control rats (Fig. 2A).

Table 2 Effect of eicosapentaenoic acid (EPA) or fenofibrate administration, from day 4 to day 15 after adjuvant injection, on body weight gain and arthritis score in control and arthritic (AA) rats. Data are mean ± S.E.M. for 8–9 rats

<table>
<thead>
<tr>
<th></th>
<th>Body weight gain days 4–15 g</th>
<th>Arthritis score</th>
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<tbody>
<tr>
<td>Control – coconut oil</td>
<td>62 ± 3·4</td>
<td>10·2 ± 1·1</td>
</tr>
<tr>
<td>AA-coconut oil</td>
<td>18 ± 3·8a</td>
<td></td>
</tr>
<tr>
<td>Pair-fed</td>
<td>31 ± 1·7a</td>
<td></td>
</tr>
<tr>
<td>Control-EPA</td>
<td>62 ± 4·0</td>
<td>6·2 ± 4·0d</td>
</tr>
<tr>
<td>AA–EPA</td>
<td>19 ± 5·6bc</td>
<td></td>
</tr>
<tr>
<td>Control – vehicle</td>
<td>67 ± 2·5</td>
<td></td>
</tr>
<tr>
<td>AA-vehicle</td>
<td>19 ± 3·6a</td>
<td>11·1 ± 1·2</td>
</tr>
<tr>
<td>Pair-fed</td>
<td>38 ± 1·5a</td>
<td></td>
</tr>
<tr>
<td>Control-fenofibrate</td>
<td>70 ± 2·7</td>
<td>6·4 ± 1·2d</td>
</tr>
<tr>
<td>AA-fenofibrate</td>
<td>32 ± 7·6bd</td>
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aDifferent from control rats treated with coconut oil or vehicle.
bDifferent from control rats treated with EPA or fenofibrate.
cDifferent from pair-fed rats.
dDifferent from arthritic rats treated with coconut oil or vehicle.
Neither arthritis nor EPA administration significantly modified Igf1 expression in the gastrocnemius, although arthritis tended to increase it (Fig. 3A). However, arthritis induced a significant increase in Igfbp3 gene expression in the gastrocnemius muscle in rats treated with coconut oil or EPA (Fig. 3B). Arthritis also tended to increase Igfbp5 in the gastrocnemius of rats treated with coconut oil, but this increase was not significant. EPA administration potentiated this effect, since the arthritic rats treated with EPA had higher Igfbp5 mRNA levels than control rats (Fig. 3C).

**Effect of fenofibrate administration**

In arthritic rats treated with fenofibrate body weight gain was higher and arthritis scores were lower than in arthritic rats treated with vehicle (Table 2). In control rats body weight gain was similar in the rats treated with either fenofibrate or vehicle. Fenofibrate administration to control rats decreased serum concentrations of IGF1 to similar levels found in arthritic or pair-fed rats (Fig. 4A). As expected, arthritis increased serum concentrations of corticosterone in the rats treated with vehicle. In addition, serum corticosterone levels in the control rats treated with fenofibrate were similar to those of the arthritic or pair-fed rats (Fig. 4C). Liver Igf1 mRNA showed responses that were similar to serum concentration of IGF1, since it was decreased by arthritis and fenofibrate treatment (Fig. 5A). Neither arthritis nor fenofibrate administration modified serum concentration of IGFBP3 or its gene expression in the liver (Figs 4B and 5B).

The effect of fenofibrate treatment on the IGF system is shown in Fig. 6. Arthritis or pair-feeding the rats did not modify Igf1 mRNA in the gastrocnemius. Fenofibrate treatment decreased Igf1 mRNA levels in the gastrocnemius, but this decrease was only statistically significant in the arthritic rats treated with fenofibrate (Fig. 6A). Arthritis increased both Igfbp3 and Igfbp5 mRNA in the gastrocnemius (Fig. 6B and C). Fenofibrate treatment prevented the stimulatory effect of arthritis on Igfbp3 and Igfbp5 expression in the gastrocnemius.

**Discussion**

Our data suggest that EPA administration can prevent the inhibitory effect of chronic inflammation on serum IGF1 and liver Igf1. In contrast, treatment with the PPARα agonist, fenofibrate, did not only ameliorate arthritis-induced inhibition of IGF1, but it also decreased serum IGF1 as well as Igf1 expression in control rats.

The decrease in liver Igf1 mRNA and serum IGF1 in pair-fed rats on day 15 after adjuvant injection suggests that the inhibitory effect of arthritis on IGF1 is secondary to the decrease in food intake. However, on day 22 after adjuvant injection, food intake in arthritic rats is similar to that of control rats, but serum IGF1 and liver Igf1 mRNA are still lower in arthritic rats than in control or in pair-fed rats (Castillero et al. 2009a). These data indicate that on day 15, the inhibitory effect of arthritis on IGF1 is due to two factors; inflammation and the lower food intake. EPA administration prevented the inhibitory effect of arthritis on IGF1 and on its expression in the liver, without increasing body weight gain. The absence of effect of EPA on body weight gain can be due to the fact that EPA does not modify food intake in arthritic rats (Volker et al. 2000, Castillero et al. 2009b). A lack of
The stimulatory effect of EPA on body weight together with a protective effect on muscle has also been reported in dystrophic muscle degeneration (Machado et al. 2011). All these data suggest that the protective effect of EPA on IGF1 is not related to modifications in food intake. Furthermore, fenofibrate, which increased food intake in arthritic rats (Castillero et al. 2011) and body weight gain, was unable to prevent the inhibitory effect of arthritis on serum concentrations of corticosterone. The lack of effect of EPA on arthritis-induced corticosterone secretion can be due to the treatment length or to a difference in species.

Control rats treated with fenofibrate had decreased liver Igf1 and serum IGF1 and serum corticosterone levels were similar to those of arthritic rats. The stimulatory effect of fenofibrate has been reported after treatments longer than that in this study, 14 months in mice (Bonnet & Ferrari 2010) and 45 days in cattle (Childs et al. 2008). Therefore, arthritic rats with lower serum IGF1 and liver Igf1 levels seem to be more sensitive to the stimulatory effect of EPA than control rats.

The positive effect of EPA on liver Igf1 gene expression can also be secondary to its effect on liver cells. In this sense, EPA is able to prevent LPS-induced increase in TNF expression in both liver cells alone or in liver cells cocultured with macrophages (Hao et al. 2010), and LPS directly inhibits Igf1 expression in hepatocyte cultures and in cocultures with Kupffer cells (Priego et al. 2006, Granado et al. 2008). Furthermore, EPA induces cell proliferation in primary hepatocyte cultures (Liu et al. 2011), and IGF1 has been reported to be an important factor in proliferation and in the response to damage in liver cell (Gatto et al. 2008).

Several studies in humans have shown that long-term treatment with fish oil increases lean body mass whereas it decreases fat mass (Su & Jones 1993, Noreen et al. 2010). The aforementioned authors reported that EPA decreases free cortisol levels, and that the decrease in cortisol can be the cause of a decrease in muscle proteolysis (Noreen et al. 2010). In our data, EPA administration was not able to prevent the stimulatory effect of arthritis on serum concentrations of corticosterone. The lack of effect of EPA on arthritis-induced corticosterone secretion can be due to the treatment length or to a difference in species.
on serum corticosterone has been previously reported (Chen et al. 2008). Taking into account the inhibitory effect of glucocorticoids on liver Igf1 and on serum IGF1 (Luo & Murphy 1989), the increased corticosterone secretion can be one of the mechanisms through which fenofibrate inhibits IGFl and Igf1 expression in control rats.

To our knowledge, there is no data about the effect of fenofibrate on liver Igf1 and serum IGFl, whereas data on Igf1 expression in organs other than the liver are contradictory. Activation of PPARα by fenofibrate has an anti-cancer effect and attenuates signaling responses of IGFlR in medulloblastoma and glioma cell lines (Urbanska et al. 2008, Drukala et al. 2010). In contrast, PPARα activation in the heart, through Wy-14643 administration, results in upregulation of Igf1 expression in myocytes and subsequent protection against ischemia/reperfusion-induced apoptosis (El Azzouzi et al. 2011). A possible explanation is that either fenofibrate or Wy-14643 acts on the IGFl system in a PPARα-independent manner.

Similarly to fenofibrate, thiazolidinediones such as rosiglitazone and pioglitazone are PPARγ ligands that have an antiproliferative effect and block IGFl responses in cancer cells (Freudlsperger et al. 2006, He et al. 2006). Furthermore, rosiglitazone administration reduces serum IGFl without modifying IGFBP3 levels in humans or mice, and also reduces Igf1 expression in liver cells, both in vivo and in vitro (Lecka-Czernik et al. 2007).

The inhibitory effect of fenofibrate on liver Igf1 and serum IGFl observed in the present data contrasts with its protective action against muscle wasting that we found in arthritic rats (Castillero et al. 2011). A possible explanation can be the effect of fenofibrate on the Igfbp genes in the gastrocnemius. As previously reported (Castillero et al. 2009a), arthritis increased Igfbp3 in the gastrocnemius, but not in the liver. These data suggest that Igf1 and Igfbp3 regulation in the skeletal muscle is different to its regulation in the liver. The increased expression of Igfbp3 in the gastrocnemius of arthritic rats has been related to muscle atrophy. This hypothesis is based on the fact that IGFBP3 prevents IGFl from binding to its receptor and it is able to inhibit cell proliferation by an IGF1-independent mechanism (Pampusch et al. 2003). IGFBP5 can also inhibit IGFl action by preventing its binding to its receptors in the skeletal muscle (Mukherjee et al. 2008). Therefore, the inhibitory action of fenofibrate on Igfbp3 and Igfbp5 expression in the gastrocnemius muscle can explain the observed effect of fenofibrate attenuating muscle wasting in arthritic rats (Castillero et al. 2011).

In summary, our data indicate that EPA and fenofibrate have different effects on the IGF–IGFBPs in arthritic rats, which suggests that EPA actions on this pathway are not mediated by its PPARα agonism. EPA administration prevented the inhibitory effect of serum IGFl and on Igf1 gene in the liver, whereas fenofibrate administration prevented the stimulatory effect of arthritis on gastrocnemius Igfbp3 and Igfbp5 expression. The effects of both compounds on the IGF–IGFBPs system can explain their previously reported protective effects against muscle wasting, although these actions are exerted through different mechanisms.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

**Funding**

This work was supported by grants from FIS no PS09/00753 and Fundación Mutua Madrileña, fellowships from Gobierno Vasco to E C (BF06.31) and from Ministerio de Educación y Ciencia to M L-M (BES-2007-16001).
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www.endocrinology-journals.org


Received in final form 13 June 2011
Accepted 29 June 2011
Made available online as an Accepted Preprint 29 June 2011