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Protective effects of dietary EPA and DHA on ischemia–reperfusion-induced intestinal stress☆

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Abstract

The immunoregulatory effects of dietary omega-3 fatty acids are still not fully characterized. The aim of this study was to determine whether dietary eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) intake limits intestinal ischemia–reperfusion (IR) injury. To test this, rats were fed either control or EPA/DHA supplemented diet for 3 weeks following which they underwent either a sham or an IR surgical protocol. A significant reduction in mucosal damage was observed after EPA/DHA supplemented diet as reflected by maintenance of total protein content. To address the underlying mechanisms of protection, we measured parameters of oxidative stress, intestinal and serological cytokines and intestinal eicosanoids. Interestingly, EPA/DHA fed animals displayed a higher activity of oxidative stress enzyme machinery, i.e., superoxide dismutase and catalase in addition to a reduction in total nitrate/nitrite content. While no changes in cytokines were observed, eicosanoid analyses of intestinal tissue revealed an increase in metabolites of the 12-lipoxygenase pathway following IR. Further, IR in EPA/DHA fed animals was accompanied by a significant increase of 17,18-epoxyeicosatetraenoic acid, 8-Iso prostaglandin $F_{3\alpha}$ and thromboxane B_3, by more than 12-, 6-, 3-fold, respectively. Thus, the data indicate that EPA/DHA supplementation may be able to reduce early intestinal IR injury by anti-oxidative and anti-inflammatory mechanisms.

Keywords: Eicosapentaenoic acid; Docosahexaenoic acid; Omega-3; Ischemia-reperfusion; Eicosanoids; Oxidative stress; Nutrition

1. Introduction

Intestinal inflammation and injury as a result of ischemia and subsequent reperfusion (IR) plays a key role in complications of various adult surgical procedures and neonatal clinical conditions, i.e., solid organ transplantation, septic shock and necrotizing enterocolitis [1–6]. Numerous lines of evidence suggest that generation of reactive oxygen species (ROS) and ROS-mediated damage plays a central role in intestinal IR injuries. The intestine is believed to be the highest source of oxidants as compared to other organs [7]. Various mechanisms, such as mitochondrial leakage, xanthine oxidase metabolism and neutrophil activation might be involved in the initial and continued formation of ROS. Apart from an increase in ROS production, intestinal IR leads to ROS-dependent and independent activation of complex downstream cascades which are not well understood. The outcome of this activation is an increased expression of neutrophil adhesion molecules, cytokines and arachidonic acid (AA) metabolites, i.e., eicosanoids. The overwhelming inflammatory response leads to mucosal injury causing increased barrier permeability which is partly responsible for distant organ damages and subsequent morbidity/mortality.

Dietary intervention with omega-3 (n-3) fatty acids, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), has been shown to be important for brain development and for reduction of cardiovascular disease deaths. The current worldwide intake of EPA and DHA varies greatly and correlates well with burden of diseases that can potentially be modified by increasing EPA and DHA intake [8]. Recent studies investigating the role of EPA and DHA in acute critical care settings have also demonstrated a benefit with increased EPA and DHA dietary intakes [9,10]. However, the precise conditions and mechanisms governing the advantages of EPA and DHA intake are unknown [11]. In acute inflammatory conditions, the proinflammatory role of AA metabolites, such as prostaglandin E_2 (PGE_2), leukotriene B_4 (LTB_4) and thromboxane A_2 (TXA_2), is well established [12]. One of the mechanisms by which EPA and DHA are thought to act is through substrate competition with AA for enzymes that generate these proinflammatory compounds, namely cyclooxygenases (COX) and lipoxygenases (LOX). The increased availability and usage of EPA and DHA as alternative substrates leads to formation of corresponding metabolites having different biological properties. For instance, when EPA instead of AA is used as a substrate in the COX pathway, prostaglandin E_3 (PGE_3) instead of PGE_2 is generated; similarly

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leukotriene B_5 (LTB_5) instead of LTB_4 is generated via the 5-LOX pathway. Biologically, it has been shown for some of these compounds that they have decreased proinflammatory properties while some beneficial properties are retained. For example, the chemoattractant capacity of LTB_5 is diminished by up to 10-fold as compared with LTB_4 [13], while the anti-aggregatory properties of prostaglandin I_2 (PGI_2) are retained by the corresponding EPA derived prostaglandin I_3 (PGI_3) [14]. Alternatively, it has been proposed that EPA and DHA may modulate the secretion of proinflammatory cytokines such as Tumor necrosis factor-α (TNFα) and Interleukin-1β (IL-1β) [15] by innate immune cells. In vitro studies also suggest that EPA and DHA may reduce neutrophil adhesion molecules such as intercellular adhesion molecule and vascular cell adhesion molecule [16]. Thus, via multiple mechanisms, it is likely that in intestinal IR injury, which is an acute inflammatory situation, an increase in EPA and DHA might be beneficial. However, to the best of our knowledge this has not been fully evaluated yet.

Herein, we report the effects of dietary intervention with an increased intake of EPA and DHA for 3 weeks on mucosal damage following intestinal IR injury induced by temporary occlusion of mesenteric artery. We demonstrate that increased intake of EPA and DHA can prevent some features of early mucosal damage without having an effect on neutrophil infiltration. In order to investigate the underlying mechanisms, we measured changes in oxidative stress mediators and cytokines. Further, for the first time, we utilize lipidomic methodologies following intestinal IR injury and after intervention with EPA and DHA to characterize the impact of our dietary intervention on eicosanoid generation. The results of our study show a beneficial effect on mucosal damage and suggest that it might be mediated via a reduction in oxidative stress and generation of anti-inflammatory eicosanoids.

2. Methods and materials

2.1. Animals and study design

Eight-week-old male Sprague–Dawley rats (Charles-River –France) were used. Animals were received 2 weeks prior to dietary intervention for acclimatization in our animal facility. All animals were kept under 12-h light/dark cycles at constant room temperature. They had free access to a commonly used basal diet (AIN-93M) and tap water. After 2 weeks, rats were randomly assigned to one of three different experimental groups ($n=8$ per group), i.e., (i) Sham group—this group was provided control diet for 3 weeks and underwent a sham surgical procedure; (ii) I/R group—this group was provided control diet for 3 weeks followed by an IR surgical protocol and (iii) I/R+EPA/DHA group—this group was provided diet supplemented with EPA and DHA for 3 weeks and underwent IR surgical protocol at the end of dietary intervention. At the end of the third week, animals underwent either a sham procedure or an adapted IR surgical protocol. At the end of the reperfusion period, animals were sacrificed, following which sera and organs were collected. The surgical procedures were staggered over 4 days. The experimental protocols were approved by the cantonal veterinary office of Vaud (authorization VD1076) and Nestle Research Center ethical committee. The lipid composition of the control and experimental diets are described in Section 2.2 and the surgical IR protocol is discussed in Section 2.3.

2.2. Lipid composition of the experimental diets

The fatty acid composition as a percentage of total fatty acids for the control and the experimental diets are provided in Table 1. For the control diet, this was achieved using a mixture of soybean oil (50%), corn oil (35%) and cocoa butter (15%). For the experimental diet, this was achieved using a mixture of soybean oil (50%), sunflower oil (23%), fish oil (20%) and cocoa butter (7%). The final lipid composition for both the diets was 4%. The oil mix were further homogenized with a standard AIN-93M mix. Pellets were made and stored in small sachets under vacuum at −20°C. The diets were changed twice a week in each animal cage. These special precautions were taken to avoid oxidative degradation of lipids.

2.3. IR surgical protocol

Intestinal IR protocol was performed by occlusion of the superior mesenteric artery (SMA) as described before [17,18], with a few modifications. Briefly, animals anaesthetized with isoflurane underwent laparotomy, and SMA was occluded with an atraumatic clamp. After 30 min of ischemia, the clamp was removed and followed by 3 hours of reperfusion under anesthesia. Sham-operated controls underwent the same surgery procedures without clamping. Sham and IR procedures were incremented in parallel along a week of experiment. Two animals per group per day were staggered over a total of 4 days. This allowed us to evaluate the intra- and inter-day variability of the surgical interventions in our final statistical result analysis.

Table 1
Fatty acid methyl esters analysis of control and experimental diets

	Control diet(%)	Experimental diet(%)
14:0	0.31	1.46
16:0	14.74	13.50
16:1	0.15	1.63
18:0	8.25	6.60
18:1	26.99	21.53
18:2 n-6	44.10	43.26
18:3 n-3	3.06	3.08
20:5n3 EPA	0.00	3.00
22:6n3 DHA	0.00	1.98

Compositions in % of main total fatty acids are shown.

2.4. Protein extraction and quantification by immunoassays

Total small intestine was snap frozen and stored at −80°C until protein and RNA extraction. Protein extraction was done using RIPA buffer (Sigma, St. Louis, MO, USA). Protein was quantified using the RC DC Protein assay kit (Bio Rad, Benicia, CA, USA). Interleukin (IL)-1β, IL-4, IL-5, IL-6, growth-regulated protein/keratinocyte-derived chemokine (GRO/KC, i.e., rat IL-8), IL-13, Interferon-γ (IFNγ) and TNFα cytokine expression were measured in the intestine protein extracts using rat IL-6 or 7-Plex ultra-sensitive kits (Meso Scale Discovery, Gaithersburg, MD, USA). Cytokine contents were expressed as pg/100 mg of intestine. Same methods were also used for seric cytokine assessments and results expressed in pg/ml of serum. Myeloperoxidase (MPO) content of the small intestine protein extracts was determined with an MPO Elisa kit (HYCULT- HK210). The MPO contents were expressed as ng/mg of intestinal tissue. All these measurements were done following the manufacturer's instructions.

2.5. Determination of lipid metabolites by HPLCMSMS

AA, EPA, DHA and AA- and EPA-derived metabolites were quantified by methods described in [19]. Briefly, snap frozen intestinal tissue that had been pulverized was Polytron-homogenized in 5 ml ethanol, and centrifuged. A portion of the supernatant equivalent to 50 mg tissue was transferred to a siliconized glass tube and spiked with a mixture of deuterated internal standards of eicosanoids (1 ng each, Cayman Chemicals, USA). The ethanol was taken to dryness with a stream of nitrogen gas. Residue was extracted with ethyl acetate (2 ml)/acidified water (1 ml, pH 3) and the organic layer was transferred and washed once with water to neutrality and evaporated. The dry residue was transferred with acetonitrile/water (1/1, v/v) to small siliconized vials (100 μl volume) for analysis by high pressure liquid chromatography coupled with tandem mass spectrometry (HPLCMSMS-AB Sciex QTRAP 5500). An Agilent HPLC 1290 was at the front end, equipped with a short Zorbax SB-phenyl column (3.0×50 mm, 3.5-μm spherical size, Agilent, Technologies, Santa Clara, CA, USA). Compounds were separated by high-performance liquid chromatography (HPLC) with a direct inlet into the mass spectrometry (MS) source. HPLC solvents were acetonitrile and water each containing 2 μl propionic acid/l mixed according to the following program: 80:20 water-acetonitrile (v/v) at sample injection and maintained for 2 min, 75:25 (v/v) for 0.5 min, 50:50 (v/v) by 5 min, 45:55 (v/v) by 6.2 min and 0:100 (v/v) by 11 min, where this was maintained for 1.5 min. The solvent was then recycled to 80:20 (v/v) for the next run. The flow rate was at 400 μl/min. The MS source temperature was maintained at 500°C, and the atmospheric pressure chemical ionization (APCI) ion source voltage, at 4500 V. MS parameters were established through infusion (20 μl/min) of each authentic standard separately. The full scan spectra (Q1) were first obtained, followed by selection of the M-1 precursor ions or the ions of the highest intensity followed by recording of product ion spectra (Q3) after collision induced decomposition (CID). This established the conditions to be used in the MRM ion monitoring assay for each compound. Optimization of parameters was carried out manually. The CID gas was nitrogen [19]. Calibration curves were generated from amounts (1–10 ng) of undeuterated standards (Cayman Chemical, Ann Arbor, MI, USA) and a fixed quantity of deuterated internal standards (1 ng) for each analyte under MRM conditions. Quantitation of analytes in the samples utilized the Analyst (1.5.1) software.

2.6. Fatty acid methyl ester analysis in diet, red blood cells (RBC) and bowel tissues

Homogenized intestinal tissue (200mg) or 50 μl of RBC pellet was used for fatty acid analysis by gas chromatographic analyses as described earlier [20]. Hundred micrograms of heneicosanoic acid was added as the internal standard. Briefly, lipid extraction was performed by a modified Bligh and Dyer method. The extracts were then mixed with a methanolic solution of hydrochloric acid (2 ml, 1.5 N; Supelco, Bellefonte, PA, USA) and

n-hexane (1 ml) in a test tube. Capped test tubes were heated at 100°C for 60 minutes and shaken occasionally. After cooling down to room temperature, a solution of saline (2 mL at 20% w/v) was added, and tubes were centrifuged at 2000 rpm for 4 min. The organic phase was taken for gas chromatography analysis. Analysis of total fatty acid methyl esters was performed on a 7890 Agilent gas chromatograph (Agilent Technologies), equipped with a fused-silica BPX-70 capillary column (10×0.1 mm I.D., 0.2-μm film thickness; SGE, Melbourne, Australia). Split injector (25:1) and flame ionization detection systems were operating at 250°C. Oven temperature programming was 50°C isothermal for 0.2 minutes, increased to 180°C at 120°C/min, isothermal for 1 min at this temperature then increased to 220°C at 20°C/min and then to 250°C at 50°C/min. The carrier gas (H_2) was maintained constant at 0.6 mL/min and the acquisition of the flame ionization detection signal at 100 Hz. Of note, the complete lipid blend used and diets were also characterized by the same methodology and confirmed theoretical ratio of DHA and EPA introduced.

2.7. Total nitrate/nitrite content and enzyme activity measurements

After specific preparation of either small intestinal tissue or serum, the nitrate/nitrite content was evaluated using a commercially available colorimetric kit (Cayman Chemical) following supplier's protocol. The catalase (CAT) and superoxide dismutase (SOD) enzymatic activities were evaluated using commercially available kits (Cayman Chemical) by following supplier's instructions.

2.8. ROS production measurement

Production of superoxide anion upon fMLP stimulation was measured by flow-cytometry as described previously [21]. Briefly, blood samples were collected in syringes containing heparin (final concentration 20 U/ml). Twentyμl of blood from each animal were diluted in 980 μl of phosphate-buffered saline (PBS) then stimulated with 1 μM fMLP (Sigma) for 30 min at 37°C. Cells were PBS-washed then stained with 1μM of Hydroethidine (HE; Molecular Probes) for 15 min at 37°C. HE is directly oxidized by $O_2^{\bullet-}$ to ethidium bromide (EB), which fluoresces after intercalating with nucleic acids. Cell suspensions are washed with cold PBS and kept in ice to stop reaction. Then, erythrocytes were depleted from whole blood with lysing buffer (i.e., NH_4Cl 155mM, $KHCO_3$ 10mM, EDTA 0.1mM, pH7.4) and then PBS washed prior to flow-cytometry analysis. Based on their light scattering profile neutrophils were gated to assess the production of $O_2^{\bullet-}$ by measuring emissions at 590 nm (FL2) for EB ($O_2^{\bullet-}$). Data were expressed as mean fluorescence intensity detected in FL2 channel.

2.9. Statistical analysis

Protein content, analysis of fatty esters of EPA and DHA in RBC and small intestine and the analysis of AA, EPA and DHA in intestine were performed using one-way analysis of variance (ANOVA). All other outcomes were analyzed with the Wilcoxon rank sum test. Differences with a P value <.05 were considered to be statistically significant. Note however, that no correction for multiplicity of tests was applied. It should further be noted that for data analyzed by one-way ANOVA the mean value is given, while for the outcomes analyzed by Wilcoxon rank sum test the median value is provided.

3. Results

3.1. Fatty acid composition of RBC and small intestine

First, we wanted to confirm that our dietary intervention does indeed increase the content of EPA and DHA in animals. Thus, EPA and DHA were measured as a percentage of total fatty acids in RBC and in the target tissue, i.e., small intestine. As shown in Table 2, dietary intervention increased the amounts of both EPA and DHA in the RBC as well as in the small intestine. From initially undetectable levels, EPA increased to a mean value of 1.6% of total fatty acids in the RBC (data not shown) and 0.8% in the small intestine (Table 2). Simultaneously, we also observed a 2–3-fold increase in DHA levels rising to 4% and 1.5% in RBC (data not shown) and small intestine (Table 2), respectively.

3.2. Effects of dietary EPA/DHA on small intestinal tissue integrity

Mucosal damage is one of the hallmarks of IR injury to the small intestine [22]. Since total small intestine protein measures can be used to assess epithelial sloughing [23], IR-mediated epithelial loss was confirmed by measuring total protein (Fig. 1A). Animals receiving dietary intervention with EPA and DHA have increased levels of total tissue protein (88.9 vs. 98.0 μg/mg tissue, P=.007, Fig. 1A) suggesting a reduction in global mucosal injury. Neutrophil infiltration following IR was also confirmed through an increase in tissue MPO content (Fig. 1B). While our dietary intervention protected against IR-mediated mucosal injury, no reduction in neutrophil infiltration was observed, as the tissue MPO content remains unchanged in both, the non-supplemented and the EPA/DHA supplemented, I/R groups (87.2 vs. 84 ng/mg tissue, P=.959, Fig. 2B).

Table 2
Fatty acid methyl esters analysis of small intestines

Fatty acid	SHAM	I/R	I/R+EPA/DHA
14:0	1.23±0.112	1.38±0.050	1.65±0.051
14:1	0.11±0.016	0.12±0.011	0.15±0.012
15:0	0.30±0.036	0.26±0.025	0.23±0.008
16:0	24.81±0.690	26.00±0.347	26.92±0.341
16:1 n-7	5.47±0.560	5.86±0.379	6.72±0.427
17:0	0.22±0.019	0.21±0.015	0.23±0.009
18:0	12.12±1.261	10.07±0.642	9.85±0.376
18:1 n-9	22.90±1.449	24.58±0.938	22.67±0.571
18:2 n-6	20.15±0.320	20.11±0.458	19.78±0.350
18:3 n-3	0.54±0.101	0.71±0.044	0.73±0.024
20:0	0.51±0.066	0.47±0.042	0.44±0.029
20:1 n-9(a,b)	0.25±0.016	0.26±0.016	0.35±0.021
20:3 n-3	0.49±0.069	0.42±0.041	0.42±0.026
20 3 n-6	0.61±0.086	0.62±0.066	0.68±0.042
20:4 n-6	8.44±1.142	7.23±0.758	5.47±0.321
20:5 n-3 (a,b)	N.D.	N.D.	0.86±0.042
22 6 n-3 (a,b)	0.83±0.097	0.72±0.062	1.94±0.112
24:0	0.49±0.067	0.44±0.048	0.39±0.024
24:1 n-9	0.28±0.032	0.30±0.033	0.29±0.019

Composition in % of total fatty acids are shown. The mean values are depicted ±S.D. (a) denotes a P<.05 (Sham vs. I/R+EPA/DHA) and (b) denotes a P value<.05 (I/R vs. I/R +EPA/DHA) as measured by one-way ANOVA. N.D., not detected.

3.3. Effects of dietary EPA/DHA on small intestinal oxidative stress parameters

As observed, EPA/DHA reduced mucosal injury. The role of oxidative stress in mediating tissue damage following IR injury is well established [7,24] and it is thought that EPA/DHA may improve anti-oxidative defence mechanisms [25,26]. Thus, we tested whether the beneficial effects of EPA/DHA may be due to an impact on oxidative stress cell machinery. Under normal conditions, superoxide is neutralized by sequential catalysis by SOD and CAT [27]. Moreover, recent studies have demonstrated that fish oil increases activity of these enzymes [25,26]. Hence, the activities of SOD and CAT were measured. As shown in Fig. 1C and D, while there was no change in either SOD or CAT activity with I/R compared to the sham group, EPA/DHA intervention significantly increased the activities of both of these enzymes, which might explain at least partly its intestinal protective properties. Next, we assessed changes in the total nitrate/nitrite as well as the tissue content of superoxide (Fig. 1E-F). In time frame of our model, no significant change was observed in the total nitrate/nitrite upon comparing the I/R group to sham. However, EPA/DHA intervention significantly reduced the total nitrate/nitrite content as compared to I/R group (10.6 vs. 35.9 μM/g of tissue, P=.022, Fig. 1E). In contrast to the effect on nitrate/nitrite contents, superoxide levels are significantly increased in the I/R group (47.8 vs. 40.1 mean fluorescence intensity (MFI) in I/R vs. sham, P=.041, Fig. 1F). In addition, the dietary EPA/DHA intervention showed a strong trend towards a reduction in superoxide (41.2 vs. 47.8 MFI, P=.065, Fig. 1F).

3.4. Effects of dietary EPA/DHA on proinflammatory cytokines in tissue and sera

Cytokine involvement in early inflammatory processes such as ROS production and neutrophil recruitment is well documented [28]. We hypothesized that EPA/DHA dietary intervention could alter the

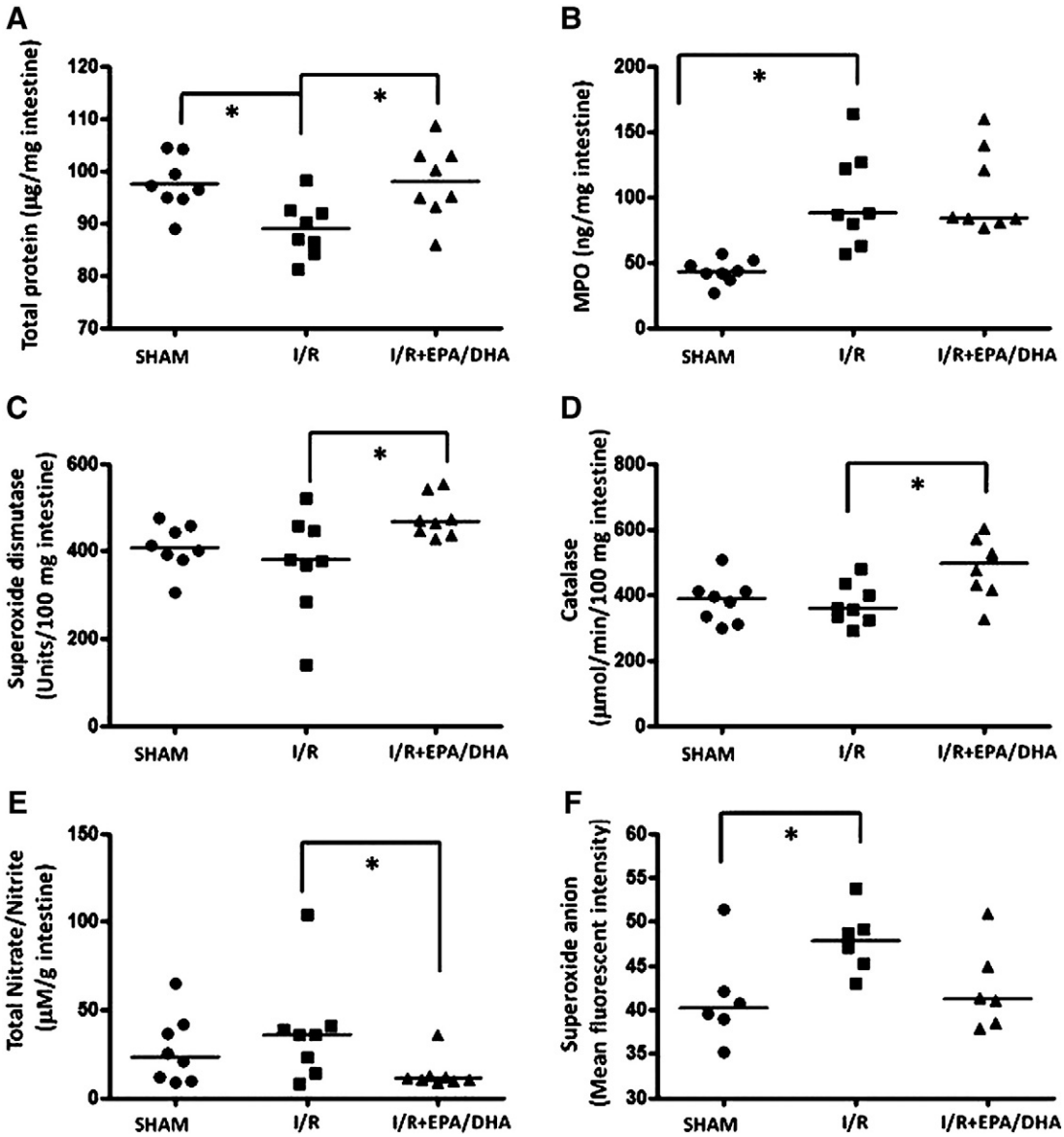

Fig. 1. Evaluation of mucosal integrity and antioxidative mechanisms by colorimetric (A,C,D and E), ELISA (B) or flow cytometry (F) methods as described in the methods section. The individual values and either the mean values (A) or the median values (B–F) are depicted. (*) denotes a $P<.05$ as measured either by one-way ANOVA (A) or the Wilcoxon rank sum test (B–F).

cytokine profile subsequent to IR stress and could provide an explanation of its beneficial effects. To test this, we monitored changes in cytokine levels in both the sera and bowel tissue of our experimental groups. As depicted in Fig. 2A, significant increase in serological levels of early proinflammatory cytokines namely IL-8 (GRO/KC) and TNFα were observed in I/R group. Furthermore, along with an increase in IL-6 and IL-8 (GRO/KC), we observed a decrease in IFNγ in the intestinal tissue of animals undergoing IR stress (Fig. 2B). However, dietary intervention with EPA/DHA did not modulate the above cytokine profile, which remains unchanged as compared to I/R controls. Interestingly, the levels of proinflammatory cytokines were higher in the intestine compared to sera, which reflects a largely local process with limited early systemic impact.

3.5. Effects of dietary EPA/DHA on small intestinal eicosanoids

The benefits of EPA and DHA supplementation are thought to be partly mediated through generation of an alternative series of metabolites when these substrates are used in the AA pathway e.g. PGE_3 rather than PGE_2 is formed when COX utilizes EPA rather than AA as a substrate. These EPA and DHA metabolites are believed to favour a reduction in inflammation [11]. However, a detailed analysis of these metabolites under conditions of IR is not available. Thus next, we performed a quantitative analysis of major AA- and EPA- derived metabolites arising from different enzymatic and non enzymatic pathways in the intestinal tissue of the three experimental groups by HPLCMSMS analyses.

First of all, the results comparing I/R and I/R+EPA/DHA groups showed that non-esterified EPA and DHA were significantly increased in small intestine by >6-fold and >1.5 fold respectively, while a trend in reduction of free AA was observed (Fig. 3). These findings are in agreement with results observed for esterified EPA and DHA (Table 2).

Comparing the AA metabolites during I/R showed significant increases in the hepoxilin metabolites, Trioxilin A3 (2.1 vs. 4.8 pg/mg intestine, $P=.038$) and Trioxilin B3 (0.3 vs. 1 pg/mg intestine,

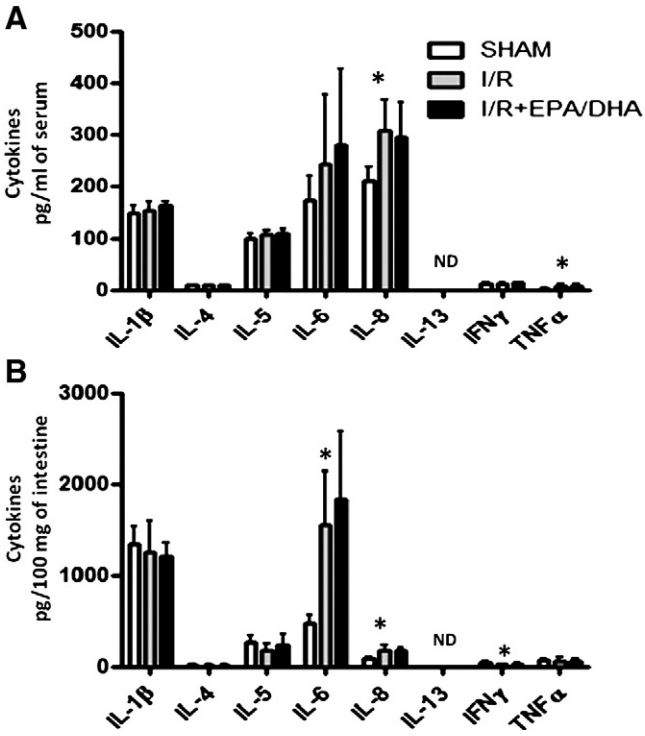

Fig. 2. Cytokine measurements in serum and intestinal tissue (median+RSD). (*) denotes a $P<.05$ between SHAM and I/R groups as measured by Wilcoxon rank sum test.

$P=.001$) (Fig. 4B). Since, hepoxillins are generated through the 12-LOX pathway; it suggests an increase in 12-LOX activity following IR. Indeed, in support of this we also observed an increase in 12-HETE, which however, did not reach statistical significance (29.4 vs. 63 pg/mg intestine, $P=.053$). Furthermore all three 12-LOX metabolites showed a reduction during EPA/DHA supplementation. As depicted in Fig. 4, other AA metabolites appear to be either unaffected or in fact decreased (e.g. PGD2, LTB4).

On the other hand, apart from the above-mentioned changes in free EPA and DHA, the comparison between I/R and I/R+EPA/DHA showed a significant increase in some EPA-derived metabolites namely, thromboxane B_3 (TXB_3), 17,18-epoxyeicosatetraenoic acid (17,18-EEP) and 8-Iso prostaglandin $F_{3\alpha}$ (8-Iso $PGF_{3\alpha}$) (Fig. 5) by more than 12-, 6- and 3- fold, respectively. LTB_5 and PGE_3 showed a ~2 fold increase in I/R+EPA/DHA as compared to I/R, however these changes did not reach statistical significance (data not shown).

Thus overall, the AA metabolites that increased with IR belong to the 12 LOX pathway, while the EPA metabolites show an increase in COX and cytochrome P450 (CYP450) mediated pathways.

4. Discussion

Our data demonstrate that dietary intervention with EPA/DHA has a beneficial effect on mucosal inflammation and injuries following IR stress. It should also be noted that due to our preventive experimental settings the effects of EPA/DHA metabolites on mucosal repair and survival were not fully addressed.

Intestinal IR injury is an acute inflammatory condition wherein oxidative stress is thought to play a central role. In accordance, utilization of anti-oxidants such as N-acetyl cysteine and SOD mimetics reduce intestinal IR injuries. The primary measures for oxidative stress that were included in our study were nitrate/nitrite and superoxide measurements. The specific role of nitric oxide (NO) in intestinal IR is not clearly defined. In our model, we observe a more than 6 fold up-regulation of nitric oxide synthase 2 (NOS2) mRNA at the end of 3 hr of reperfusion (data not shown). Thus an increase in NO would be expected. However, IR conditions did not show an increase in the total nitrate/nitrite. This discrepancy could result from different factors like kinetic variability between NOS2 expression and tissue end-product generation. By comparing the changes between I/R+EPA/DHA and I/R groups, we observed that while there was no change in mRNA levels of NOS2 the overall total nitrate/nitrite content was surprisingly decreased under EPA/DHA diet. This may represent a decrease in overall production of NO. It is well established that under oxidative conditions, NO can combine with superoxide to form peroxynitrite which damage the reperfused tissue [29]. Evidence from iNOS knock-out mice [30] and the use of FeTMPS (a peroxynitrite decomposing compound) [31], suggests that a decrease in NO is beneficial in IR settings. We believe that our results support these earlier observations. As mentioned earlier, superoxide can combine with NO to form peroxynitrite. Further, superoxide can also be converted to hydrogen peroxide, which in turn can be converted to other ROS such as the hydroxyl radicals. We demonstrate that EPA/DHA intervention increases the activity of SOD and CAT which results in a trend towards a decrease in superoxide. SOD and CAT are involved in the detoxication process of ROS. An earlier study using a SOD mimetic, showed that decomposition of superoxide is beneficial in an splanchnic artery occlusion model of intestinal IR injury [32]. However, in another model of intestinal IR, intravenous intervention with MnSOD and CAT 15 min before starting reperfusion did not change histological scoring of the intestinal mucosa but was able to decrease neutrophil infiltration in

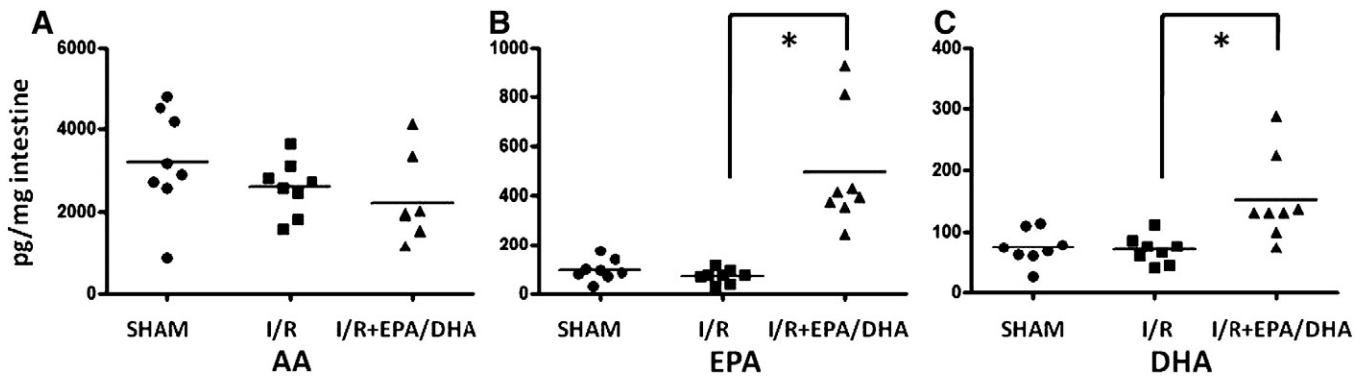

Fig. 3. Changes in arachidonic acid (AA) (A), eicosapentaenoic acid (EPA) (B) and docosahexaenoic acid (DHA) (C) in intestinal tissue. The individual values and the mean values are depicted. (*) denotes a $P<.05$ as measured by one-way ANOVA.

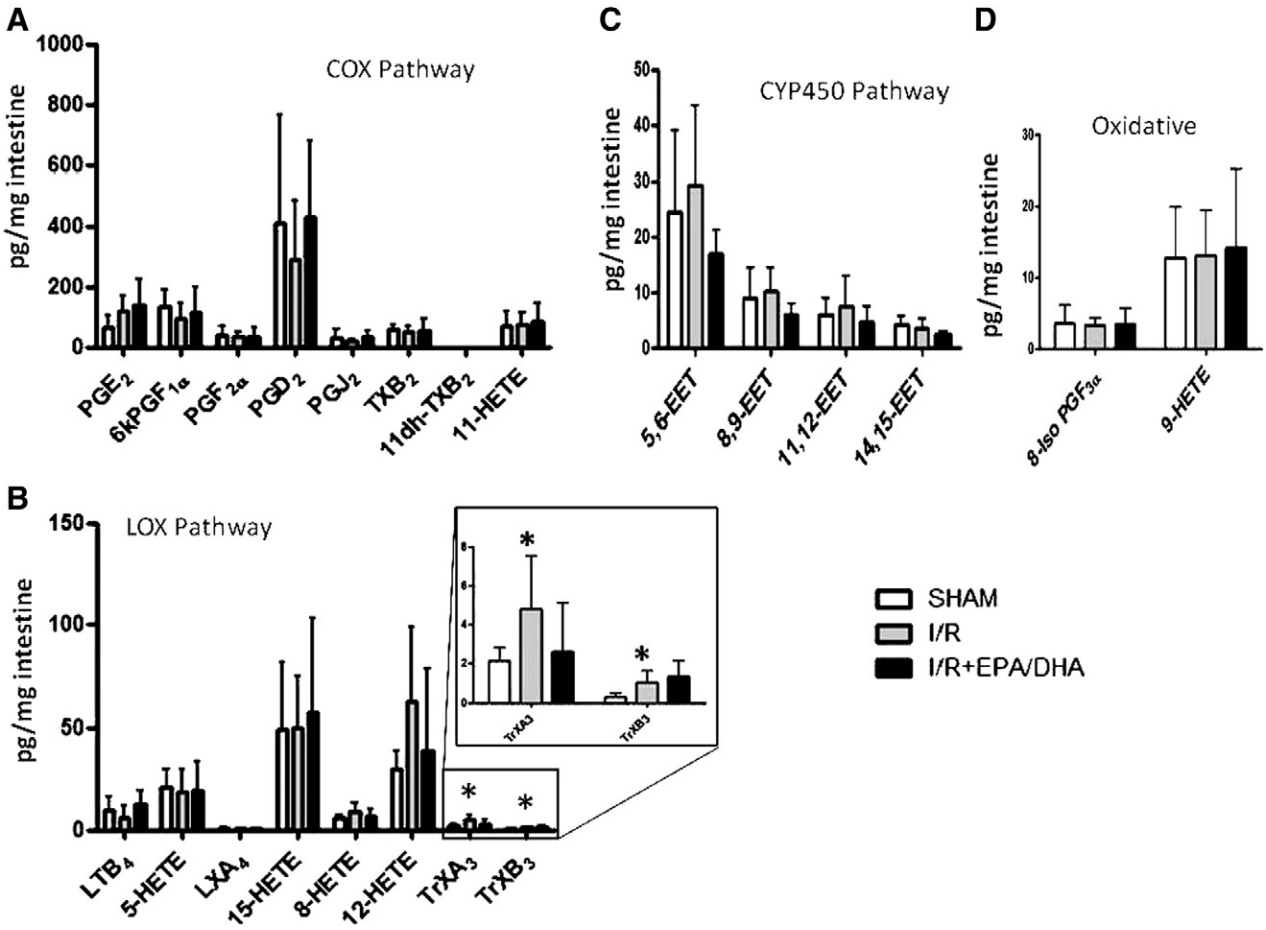

Fig. 4. IR-induced changes in AA metabolites measured in the intestinal tissue. The median + RSD values of AA-derived metabolites derived from COX pathway (A), LOX pathway (B), CYP450 pathway (C) and by oxidation (D) are depicted. The inset shows in B shows the values for $TrXA_3$ and $TrXB_3$. (*) denotes a $P<.05$ between the SHAM and I/R groups as measured by Wilcoxon rank sum test.

intestine and had beneficial effects on distal organ injury [33]. Altogether our results show that increased EPA/DHA intake enhances basal SOD and CAT activity. Thus an interesting idea to consider is whether dietary EPA/DHA creates a preconditioning effect which is beneficial under intestinal IR conditions. In support of this, it is known that increased anti-oxidant enzyme activities play a crucial role in the preconditioning effects of isoflurane and sevoflurane [34,35]. Further, brief intestinal ischemia also preserves SOD and CAT activities in the kidney [36] and finally, dietary intervention with EPA/DHA has also been demonstrated to increase SOD and CAT activities in the liver [26]. However we did not completely address this issue which deserves further investigation.

The ability of EPA and DHA to influence inflammatory response has been recognized in many recent reports and has generated an

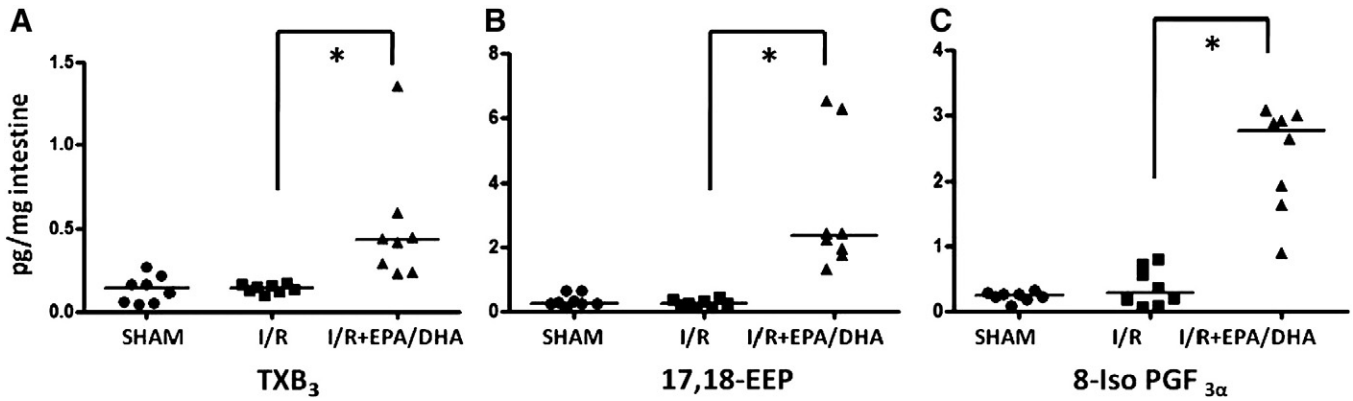

Fig. 5. Changes in EPA-derived metabolites following dietary intervention. The individual values and the median values of EPA-derived metabolites, thromboxane B3 (TXB_3) (6A), 17,18-epoxyeicosatetraenoic acid (17,18-EEP) (6B) and 8-Iso prostaglandin $F_{3\alpha}$ (8-Iso$PGF_{3\alpha}$) (6C) are depicted. (*) denotes a P value <.05 as measured by Wilcoxon rank sum test between I/R and I/R+EPA/DHA groups.

increased interest in the design of alternative immunomodulatory strategies based on dietary interventions. However, how EPA and DHA suppress inflammatory immune response is not yet fully understood. Current available evidences indicate that dietary n-3 PUFA mainly EPA and DHA could modulate immune responses by an alteration of cell membrane composition and function and/or a modification of eicosanoid production and/or an alteration of cytokine synthesis and secretion. Thus, we addressed whether positive effects of an EPA/DHA supplemented diet could be driven by changes in proinflammatory cytokine expression in IR settings either in the tissue or serum as early as 3 hour post reperfusion. Interestingly, known early proinflammatory cytokines, IL-6 and IL-8 (GRO/KC) were up-regulated and might explain the concomitant attraction of neutrophils in bowel tissue. Our dietary intervention did not reduce the expression of these cytokines. Therefore we ruled out the hypothesis that dietary EPA/DHA mediate their positive effects on IR injury by mitigating early proinflammatory cytokine secretion. However, we cannot exclude that EPA/DHA may either lower proinflammatory cytokine expression in the late phase (>3h) of IR injury or that other proinflammatory cytokines not studied herein participate towards better outcomes.

Intestinal IR injury has been shown to induce COX-2 [37,38], which was also confirmed in our study (data not shown). Additionally, increases in prostaglandins, thromboxanes and leukotrienes (LTB_4, PGE_2 and thromboxane B_2 (TXB_2) as a stable metabolite of TXA_2) arising from AA metabolism have also been identified [39]. These metabolites may play a detrimental role in intestinal IR due to bioactive properties ascribed to these metabolites such as increased vascular permeability, neutrophil chemoattraction and platelet aggregation [12]. However, the metabolite changes with IR that we observed have not been previously reported. We demonstrated an increase in early AA metabolites, the trioxilins $TrXA_3$ and $TrXB_3$ representative of the hepoxilins A3 and B3. Additionally, a trend in increase of 12-HETE was also observed, thus suggesting that IR injury activates the intestinal 12-LOX pathway. Hepoxillins were identified in the mid 80's from the rat pancreatic islets of Langerhans and shown to stimulate the release of insulin, however, information regarding their possible role in inflammation is limited [40]. HxA_3 has been shown to be equipotent to LTB_4 in terms of its neutrophil chemoattractant properties [41]. In a recent study performed in a mouse model of lyme arthritis, an early proinflammatory role of hepoxillins has been proposed [42]. Moreover, 12-LOX antagonists have also been shown to reduce neutrophil migration across intestinal epithelium [40,43]. It will be very interesting to pursue the role of this pathway in intestinal IR injury in the future and test whether specific abrogation of hepoxillin signaling can limit or suppress the inflammatory loop in IR. To our surprise, some of the AA metabolites that have previously been shown to increase under intestinal IR conditions were not significantly altered when considering their changes in absolute amounts. However, when absolute values were normalized to AA values to generate a product/precursor ratio, new observations emerged. Apart from confirmation of the previously mentioned changes, PGE_2, 5,6-EET and 8-HETE also show significant changes. These observations indicate increases in their respective enzymatic pathways, namely, COX, CYP450 epoxygenases and LOX.

The proinflammatory properties of AA metabolites in intestinal IR are well appreciated. Consistent with this, studies have shown a reduction of intestinal IR injuries upon inhibition of either the COX or the 5-LOX pathways [44,45]. However on the flip side, it is believed that COX or 5-LOX derived metabolites could also play a protective role in IR injury. Indeed for instance, the role of PGE_2 in mucosal repair is well known [46]. Moreover, other AA metabolites such as PGI_2, 15-dehydro-PGJ_2 and $LipoxinA_4$ by virtue of their effects on vasodilatation, peroxisome proliferator-activated receptor-γ(PPARγ) agonistic and pro-resolution properties, respectively, may also exert beneficial effects. Thus, in our study, the diet was designed to increase EPA and DHA while maintaining the AA levels. This allowed us to tease out the differences related solely to EPA/DHA increase. The changes observed upon comparing IR with or without EPA/DHA dietary intervention were as follows: (i) for AA and AA metabolites few changes were observed except for a reduction in the hepoxilins by EPA/DHA diet; (ii) as expected the levels of EPA and DHA were increased; (iii) EPA-derived metabolites, 17,18-EEP, 8-Iso $PGF_{3\alpha}$ and TXB_3 were increased, and finally; (iv) trends towards increases in LTB_5 and PGE_3 were observed. Thus, we were able to retain the IR mediated changes on AA and AA metabolites after dietary intervention and the changes were hence related to increased incorporation of EPA and DHA. We believe that the increases observed in TXB_3, LTB_5 and PGE_3 are beneficial under inflammatory situation and could either limit or influence the resolution of inflammatory processes. However, the highest increase observed was in 17,18-EEP (>12 fold). While recent data have demonstrated an increase in 17,18-EEP with EPA intervention [47], reports on its role in inflammation are limited. A study by Morin et al [48], showed that 17,18-EEP is anti-inflammatory. It mediated its action via activation of PPARγ. As PPARγ activation has been shown to reduce intestinal IR injury [49], it is possible that formation of 17,18-EEP might be beneficial and provide a relevant biomarker for efficient anti-inflammatory treatment. However, with limited literature addressing the functional relevance of 17,18-EEP, this needs to be investigated further. Given that our diet consisted of both EPA and DHA and that docosanoids possess anti-inflammatory and pro-resolution properties [12], these may also contribute to the beneficial effects observed. However, since we do not directly address this issue in our investigation, further studies will be needed.

In conclusion, we show that dietary intake of EPA/DHA has positive effects on different features of IR associated inflammation and injuries. Herein, we extend the body of knowledge around health benefits of n-3 PUFA to an intestinal IR model and show how AA and EPA derived eicosanoids are modulated by IR stress as well as dietary intake of EPA/DHA. These findings support the hypothesis that dietary n-3 PUFA contained in fish oil produces metabolites with potentially protective activity under acute inflammatory conditions.

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