Long chain poly-unsaturated fatty acids attenuate the IL-1β-induced pro-inflammatory response in human fetal intestinal epithelial cells

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Abstract

Background—Evidence suggests that excessive inflammation of the immature intestine may predispose premature infants to necrotizing enterocolitis (NEC). We investigated the anti-inflammatory effects of docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and arachidonic acid (ARA) in human fetal and adult intestinal epithelial cells (IEC) in primary culture.

Methods—Human fetal IEC in culture were derived from a healthy fetal small intestine (H4) or resected small intestine of a neonate with NEC (NEC-IEC). Intestinal cell lines Caco2 and NCM460 in culture were used as models for mature IEC. IEC in culture were pre-treated with 100µM palmitic acid (PAL), DHA, EPA, ARA or ARA+DHA for 48 hrs and then stimulated with pro-inflammatory IL-1β.

Results—DHA significantly attenuated IL-1β induced pro-inflammatory IL-8 and IL-6 protein and mRNA in fetal H4, NEC-IEC and mature Caco2, NCM460 IEC, compared to control and PAL treatment. DHA down regulated IL-1R1 (IL-1β receptor) and NFκB mRNA expression in fetal and adult IEC. ARA had potent anti-inflammatory effects with lower IL-8 and IL-6 (protein and mRNA) in fetal H4 but not in NEC-IEC or adult IEC.
Conclusion—The present study provides evidence that DHA and ARA may have important anti-inflammatory functions for prevention of NEC in premature infants.

Introduction

Necrotizing enterocolitis (NEC) is the most devastating gastrointestinal disease in neonates, especially affecting very low birth weight premature infants (1). The pathogenesis of NEC is not well defined but evidence strongly suggests that it is multi-factorial (2,3). Prematurity and enteral feeding are major risk factors for NEC (1,2). An excessive inflammatory response by the immature intestine to external stimuli, impaired intestinal barrier integrity and/or abnormal bacterial colonization are key factors implicated in the pathophysiology of NEC (1–3). Current evidence strongly suggest that immaturity of innate immunity mediated via TLR4 and NFκB signaling pathway may contribute to excessive intestinal inflammation in NEC (4–6).

Breast milk protects the immature intestine against excessive inflammation underlying NEC (1,3). The long chain polyunsaturated fatty acids (LC-PUFA, ≥20 carbon PUFA) docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (ARA, 20:4n-6) present in human breast milk (7) may have important anti-inflammatory functions during early development (8,9). A recent meta-analysis study of randomized clinical trials reported that DHA supplementation reduced the risk of NEC in premature infants with gestational age ≤32 wks (8). Carlson, et al also (9) reported a decrease in the incidence of NEC in premature infants fed a pre-term formula supplemented with DHA and ARA compared to a standard formula without DHA and ARA. However, Fewtrell, et al (10) did not find any effect of DHA and ARA supplementation on the incidence of NEC in premature infants. Further, the mechanisms underlying the anti-inflammatory functions of DHA and ARA in immature intestine during early fetal development are currently not well defined.

Accordingly, the primary goal of the present study was to determine the anti-inflammatory effects of DHA, eicosapentaenoic acid (EPA, 20:5n-3) and ARA and delineate their mechanisms of action in human fetal intestinal epithelial cells (IEC) in primary culture as a model for fetal (immature) human intestine. The specific objectives of the study were to determine the effects of DHA, EPA and ARA, within physiological ranges present in human breast milk (7), on i) the pro-inflammatory cytokines IL-8 and IL-6 response and ii) NFκB and IL1-R1 gene expression in human fetal and adult IEC in culture, after pro-inflammatory IL-1β insult.

Results

Fatty acid enrichment of human fetal (H4, NEC-IEC) and adult (Caco2) intestinal epithelial cells in culture after supplementation

Fatty acid enrichment measured in human fetal H4 IEC, NEC-IEC and adult Caco2 after 48 hrs of supplementation with palmitic acid (PAL), ARA, EPA, DHA and ARA+DHA, is shown in Table 1. All fatty acids supplemented showed enrichment in H4, NEC-IEC and Caco2 IEC in culture. In fetal IEC, DHA increased by 10 and 12 fold in H4 and NEC-IEC respectively compared to un-supplemented control IEC. EPA was 103 fold higher in H4
IEC, but only 8 fold higher in NEC-IEC. ARA increased by 2-fold in H4 and NEC-IEC. In adult Caco2, ARA, EPA and DHA increased by 10, 57 and 23 fold. The fatty acid composition (% total fatty acids) of fetal and adult IEC in culture is shown in Supplementary Table S1 (online). ARA supplementation significantly lowered DHA in IEC. Similarly, DHA supplementation led to a significant decrease in ARA in IEC.

Significant 2 carbon elongation of 20-carbon PUFA ARA and EPA (as indicated by 22:4n-6/20:4n-6 and 22:5n-3/20:5n-3 ratios, respectively) was evident in fetal H4 IEC (Figure 1). In contrast, in NEC-IEC, 22:4n-6/20:4n-6 ratio and 22:5n-3/20:5n-3 ratios were 2-fold and 15-fold lower respectively compared to H4 IEC.

ARA or EPA supplementation did not increase the levels of their down-stream desaturation products namely, 22:5n-6 or DHA in fetal (H4, NEC) or adult (Caco2) IEC, indicating a low fatty acid desaturase 2 (FADS2) activity in IEC.

**Fatty acid effects on pro-inflammatory cytokine response after IL-1β challenge in human fetal (H4 and NEC) and adult (Caco2 and NCM460) intestinal epithelial cells**

IL-8 and IL-6 concentration (ng/mg protein) in cell fee supernatant (CFS) of un-supplemented (control) fetal and adult IEC after IL-1β exposure is shown in Table 2. IL-8 and IL-6 protein concentration (ng/mg protein) was highest in fetal NEC-IEC, followed by H4 and lowest in adult Caco2 and NCM400 enterocytes (Table 2). In NEC-IEC, IL-8 and IL-6 concentrations (ng/mg) in CFS after IL-1β exposure were 14 fold- and 7 fold greater compared to H4 IEC. IL-6 concentration was not detectable in adult Caco2 or NCM CFS after IL-1β challenge.

IL-8 protein (ng/mg protein, Figure 2) and its mRNA expression (Figure 3) was measured in fetal and adult IEC supplemented with PAL, ARA, EPA, DHA or ARA+DHA compared to control IEC after IL-1β exposure. DHA significantly attenuated the IL-8 protein response to IL-1β in fetal H4, NEC-IEC and adult Caco2, NCM460 IEC compared to control and PAL treated IEC. In NEC-IEC, DHA decreased IL-8 levels by 41% compared to control after IL-1β challenge. IL-8 mRNA expression was also significantly lower in DHA treated fetal H4 and adult Caco2 cells compared to control and PAL. EPA significantly decreased the IL-8 response to IL-1β in H4 IEC, but not in NEC-IEC or adult Caco2 or NCM460 IEC. ARA had potent anti-inflammatory effects on fetal H4 IEC with significantly lower IL-8 protein and mRNA compared to control and PAL treated IEC. ARA decreased IL-8 levels in H4 IEC by 58% compared to control. However, ARA did not attenuate pro-inflammatory IL-8 response to IL-1β in NEC-IEC or in Caco2 or NCM460 IEC. ARA+DHA decreased IL-8 response to IL-1β in fetal H4 IEC compared to control and PAL.

LC-PUFA effects on pro-inflammatory IL-6 protein concentration (ng/mg protein) (Figure 4) and mRNA expression (Figure 5) in fetal H4 and NEC-IEC were similar to their effects on the IL-8 response. DHA significantly decreased IL-6 levels (ng/mg protein) in the CFS of H4 and NEC-IEC and its mRNA in H4 IEC compared to control and PAL. ARA significantly lowered the IL-6 response (protein and mRNA) in H4 cells but not in NEC-IEC.
Fatty acid effects on gene expression of IL-1R1 and NFκβ1 in H4 and Caco2 IEC after IL-1β exposure

Expression levels of the IL-1β receptor (IL-1R1) and the down-stream signaling molecule NFκβ1 mRNA were 7- and 5-fold higher respectively in H4 cells compared to Caco2 cells (Figure 6). Relative gene expression (normalized to control un-supplemented IEC) of the IL-1β receptor (IL-1R1) and its down-stream pro-inflammatory signaling mediator NFκβ1 in H4 and Caco2 enterocytes 4 hrs after IL-1β challenge is shown in Figure 7. DHA significantly lowered IL-1R1 and NFκβ1 mRNA in H4 and Caco2 IEC compared to control and PAL. In H4 IEC, DHA down regulated IL-1R1 and NFκβ1 by 2 fold- and 1.5 fold compared to control. ARA or EPA did not significantly alter IL-1R1 or NFκβ1 expression compared to control in H4 or Caco2 cells.

Discussion

The present study provides evidence that DHA and ARA may have significant anti-inflammatory functions in the human fetal intestinal epithelium. To our knowledge this is the first report investigating DHA, EPA and ARA effects, at a physiological range present in human breast milk, on the inflammatory response in human fetal IEC. Previous studies from our laboratory have well established the human fetal H4 and NEC-IEC in primary culture, used in the present study, as a valid model for immature human intestine to investigate the mechanisms underlying NEC (4,11,12).

Results from the current study demonstrate a significant enrichment of human fetal and adult IEC with DHA, EPA and ARA, 48 hrs after supplementation of the respective LC-PUFA. These findings are consistent with previous in-vivo animal studies (13,14). Fetal and adult IEC showed preferential enrichment of n-3 LC-PUFA EPA and DHA. Further, the present study provides evidence for significant elongation of EPA and ARA to 22 carbon LC-PUFA in healthy human fetal H4 IEC. Significant 22 carbon LC-PUFA enrichment in H4 IEC supplemented with EPA and ARA suggests a relatively high activity of elongases involved in the elongation of n-3 and n-6 LC-PUFA, such as Elovl2, Elovl4 and/or Elovl5, in human fetal intestinal epithelial cells. The 22 carbon LC-PUFA and their very long chain PUFA (VLC-PUFA, ≥24 carbon fatty acids) derivatives are essential components of membrane lipids (15,16) and may enhance intestinal barrier integrity. Preliminary data from this study suggest that elongation of EPA and ARA to 22 carbon LC-PUFA may be impaired in NEC-IEC compared to healthy fetal H4 IEC. Impairment in elongation of precursors EPA and ARA observed in NEC IEC may alter down-stream VLC-PUFA synthesis and potentially contribute to intestinal barrier dysfunction. These are preliminary observations and only suggest that failure of PUFA elongation in NEC-IEC may contribute to NEC inflammation indirectly through barrier dysfunction. Additional in-depth dose response studies are needed before specific conclusions can be drawn. Future in-vivo animal and clinical studies are needed to determine the functional role of intestinal elongases involved in the elongation of n-3 and n-6 LC-PUFA and their clinical significance in the pathogenesis of NEC.

DHA supplementation, at a physiological concentration present in human breast milk (100µM), decreased pro-inflammatory IL-8 and IL-6 secretion (ng/mg protein) and their mRNA expression in both fetal and adult human IEC, after IL-1β challenge,. A combination
of DHA with ARA (ARA+DHA at 50µM conc each) also lowered the IL-8 and IL-6 response in immature human enterocytes, suggesting a potentially increased effect in immature enterocytes. It is interesting to note that anti-inflammatory effects of DHA were more pronounced in human NEC-IEC compared to H4 IEC and mature enterocytes (Caco2, NCM 460), suggesting a functionally significant role for DHA in attenuating severity of inflammation in immature intestine and possibly in attenuating NEC. Previous human clinical (8,9) and in-vivo animal (13,17,18) studies have shown DHA to attenuate immature intestinal inflammation and lower the incidence of NEC. Findings from this study confirm that DHA has direct anti-inflammatory effects on human fetal and adult intestinal epithelial cells, but a greater effect in the fetal enterocytes, again supporting the potential preventative effect of DHA with NEC.

An interesting finding on the mechanism underlying the observed anti-inflammatory effects of DHA is that DHA decreased the IL-1R1 receptor expression and the down-stream pro-inflammatory signaling mediator NFκβ1 gene expression in fetal and adult human IEC compared to control and PAL supplemented IEC. IL-1R1 and NFκβ1 expression after IL-1β exposure in fetal H4 IEC were 5–7 fold higher than their expression in adult Caco2 IEC, indicating exaggerated innate immune inflammatory response in human fetal IEC. IL-1R1, the trans-membrane receptor for IL-1β, is similar to toll like receptors (TLR4) in activating NFκβ1 signaling pathway involved in innate immunity (19). Current evidence strongly suggest that an exaggerated TLR4 and NFκβ1 expression in immature and NEC intestine may be a major factor underlying the excessive pro-inflammatory response in NEC (4,6,20). Further, a recent report on genome wide analysis of human NEC intestinal tissues identified aberrations in the NFκβ1 signaling pathway as one possible major functional pathway contributing to the pathogenesis of NEC (5). The present study provides evidence that in human immature and mature intestinal epithelial cells, DHA attenuated inflammation by a down-regulation of pro-inflammatory NFκβ1 gene expression. In-vivo animal studies have shown that DHA and/or fish oil containing DHA down-regulated the intestinal TLR4 and NFκβ1 signaling pathway in rat models of NEC (17,18) and neonatal piglets after LPS challenge (13). Thus, the mechanism underlying anti-inflammatory effects of DHA in fetal and adult intestinal inflammation may in part involve the common axis of down-regulating NFκβ1-mediated innate immunity. Others have shown that DHA/PUFAs can modulate other inflammatory pathways (8,18,21) suggesting that DHA/PUFAs affect multiple pathways in intestinal inflammation. We plan transcription profiling of H4 and NEC-IEC cells exposed to DHA and IL-1β to determine the extent of these pathways.

The n-6 LC-PUFA ARA had potent anti-inflammatory effects in healthy fetal H4 IEC but not in NEC-IEC or adult Caco2 or NCM460 IEC. Further, ARA did not modulate the IL1-R1 or NFκβ1 gene expression in fetal or adult enterocytes. Thus, ARA and DHA likely modulate fetal intestinal inflammation via different mechanisms. Additional cellular studies are needed to determine these mechanisms. Recent evidence demonstrates that ARA and its eicosanoid derivatives are essential for the enhanced structural and functional integrity of intestinal barrier during early development (14,22). Innis, et al (14) reported that supplementation of the maternal diet with ARA during early gestation led to enrichment of ARA in fetal colon epithelium and protected the offspring against adult onset colitis, suggesting programming of intestinal barrier integrity by ARA during early development.
Our finding that ARA supplementation attenuated the pro-inflammatory IL-8 and IL-6 response to IL-1β stimulus in human immature H4 IEC only, but not in adult Caco2 or NCM460 IEC, provide additional evidence for the developmental regulation of intestinal inflammation by ARA.

EPA had modest anti-inflammatory effects on healthy fetal H4 IEC, but no effects on NEC-IEC or adult IEC in culture. Significant enrichment of EPA and its elongation product 22:5n-3 in healthy fetal H4 IEC suggests that EPA, similar to ARA, may play an important role in structural and functional integrity of fetal intestinal cell membrane during early human development. A recent study (23) in human macrophage cells demonstrated that 22:5n-3 derived from EPA and 22:4 n-6 derived from ARA potently inhibited pro-inflammatory eicosanoid synthesis from ARA by shunting ARA to an anti-inflammatory lipoxigenase pathway. Developmental regulation of ARA and EPA metabolism to shunt these eicosanoid precursors towards the anti-inflammatory lipoxigenase pathway and/or to utilize these LC-PUFA for membrane lipid synthesis and structural integrity of the intestinal barrier in the fetus may help protect the immature intestine from an excessive inflammatory response. Further studies are needed to investigate the regulation of ARA and EPA metabolism in relation to intestinal inflammation during early development.

In conclusion, the present study demonstrated that DHA, at a physiological concentration present in human breast milk, significantly attenuated the pro-inflammatory cytokine response in healthy fetal, NEC and adult human intestinal epithelial cells after IL-1β challenge. In addition, DHA down-regulated exaggerated NFκB1 and IL-1R1 gene expression in fetal IEC, which may partly contribute to its observed anti-inflammatory effects. Further, ARA had potent anti-inflammatory effects in healthy fetal IEC, but not in NEC or adult IEC. Thus, data from this study supports the notion that DHA may be an important anti-inflammatory nutrient for prevention and treatment of NEC during early human development. Well controlled randomized clinical trials are clearly needed to further investigate the clinical significance and appropriate ratios of DHA, EPA and ARA supplementation for prevention of NEC in premature infants.

Methods

Human fetal and adult IEC in culture were supplemented with 100μM PAL or ARA or EPA or DHA or ARA+DHA for 48 hrs in the media containing delipidized fetal bovine serum (FBS). Control IEC were treated with fatty acid free BSA only in the media. PAL, a saturated fatty acid present in human breast milk, was included as a positive control. Fatty acid composition of human IEC in culture was determined 48 hrs after fatty acid supplementation. Cytokine concentrations and gene expression in control and fatty acid supplemented IEC were determined 6 hrs and 4 hrs after IL-1β challenge, respectively. All experiments were done in triplicates.

Human fetal and adult intestinal epithelial cell lines

Human fetal intestinal epithelial cells isolated and cultured from a 20 wk gestation healthy fetal ileum (H4 cells) and a resected ileum of a 25wk gestation neonate diagnosed with NEC (NEC-IEC) were used as models for human fetal IEC. These cells were prepared after
obtaining an informed consent according to the Partners IRB #1999P003833 at Massachusetts General Hospital. Details on H4 and NEC-IEC in primary culture have been described previously (4,11,20,24). Briefly, H4 cells were cultured in Dulbecco’s modified Eagle medium (DMEM, Life Technologies, Grand Island, NY) supplemented with 10% heat inactivated fetal bovine serum (FBS, Life Technologies, Grand Island, NY), 1% hepes buffer (Life Technologies, Grand Island, NY), 1% glutamax (Life Technologies, Grand Island, NY), 1% non essential amino acids (Life Technologies, Grand Island, NY), 0.2U/mL insulin (Eli Lilly and Company, Indianapolis, IN) and 1% antibiotic-antimycotic (Life Technologies, Grand Island, NY) in a sterile cell culture humidifier at 37°C and 5% CO₂. NEC-IEC were cultured in Opti-Minimal Essential Medium (Opti-MEM, Life Technologies, Grand Island, NY) supplemented with 10% heat inactivated FBS (Life Technologies, Grand Island, NY), 0.2U/mL insulin (Eli Lilly and Company, Indianapolis, IN), 20ng/mL Epidermal Growth Factor (Life Technologies, Grand Island, NY) and 1% antibiotic-antimycotic (Life Technologies, Grand Island, NY) in a sterile cell culture humidifier at 32°C and 5% CO₂. Adult human colonic cell lines namely, Caco2 derived from human colonic carcinoma, purchased from American Type Culture Collection (ATCC, Manassas, VA) and NCM460, a primary cell line derived from normal human colon, purchased from INCCELL Corp (SA, Texas) were used as models for adult IEC and were cultured as previously published (24). Briefly, Caco2 IEC were cultured in DMEM (Life Technologies, Grand Island, NY) supplemented with 10% heat inactivated FBS (Life Technologies, Grand Island, NY), 1% Hepes buffer (Life Technologies, Grand Island, NY), 1% glutamax (Life Technologies, Grand Island, NY), 1% non essential amino acids (Life Technologies, Grand Island, NY) and 1% antibiotic-antimycotic (Life Technologies, Grand Island, NY), in a sterile cell culture humidifier at 37°C and 5% CO₂. The NCM 460 IEC were cultured in M3:Base A (M3A, Incell, SA, Texas) media supplemented with 10% heat inactivated FBS (Life Technologies, Grand Island, NY) and 1% antibiotic-antimycotic (Life Technologies, Grand Island, NY), in a sterile cell culture humidifier at 37°C and 5% CO₂.

Fatty acid supplementation of human fetal and adult IEC in culture

Fatty acids (≥99% purity) including PAL, ARA, EPA and DHA were purchased from Nu-Chek prep, Inc (Waterville, MN). When human fetal and adult IEC in culture reached 70% confluence, they were supplemented with 100µM PAL (positive control) or ARA or EPA or DHA or ARA+DHA (50µm each) bound to fatty acid free bovine serum albumin (BSA, Sigma Aldrich, St. Louis, MO) at 3:1 ratio (25) in media containing delipidized FBS (Gemini Bio-Products, Sacramento, CA) for 48 hrs. Control IEC were treated with fatty acid free BSA only in the media. IEC cell viability in culture after 48 hrs of fatty acid supplementation was determined by alamar blue (Life Technologies, Grand Island, NY) and LDH assay (Cayman Chemical, Ann Arbor, MI) according to manufacturer’s protocol. Cell viability was not affected by any of the fatty acid supplementation compared to control un-supplemented H4, NEC-IEC, Caco2 or NCM460 IEC in culture (data not shown).

Fatty acid analysis of human IEC in culture

Forty eight hrs after fatty acid treatment, culture media was aspirated and IEC in culture were washed three times with PBS (pH 7.0) buffer (Sigma Aldrich, St. Louis, MO). Washed IEC cells were harvested by trypsinization and collected in 5mL deionized distilled water.
IEC suspended in sterile, deionized water were centrifuged at 4000 rpm and supernatant was aspirated after centrifugation; IEC were re-suspended in 800µL deionized water and frozen immediately at −80°C for fatty acid analysis.

Fatty acid analysis of human IEC harvested after 48 hrs of fatty acid supplementation was done at the laboratory of Dr. JT Brenna (Cornell University, Ithaca, NY), according to methods described in detail previously (25,26). Enrichment of fatty acids in IEC after supplementation (Table 1) is expressed as a ratio of fatty acids (% total fatty acids) in supplemented IEC to un-supplemented control IEC.

**IL-8 and IL-6 protein analysis**

IEC in culture were stimulated with 1ng/mL of IL-1β after 48 hrs of fatty acid supplementation. Six hrs after IL-1β exposure, cell free supernatant (CFS) from IEC in culture were collected, gently vortexed and frozen immediately in aliquots at −80°C for IL-8 and IL-6 analysis. IL-8 and IL-6 concentration in CFS was determined by ELISA using human IL-8 and IL-6 Elisa kits (R&D Systems, Minneapolis, MN). Protein content of IEC in culture were determined by lysing cells in monolayer with protein lysis buffer (Thermo Scientific, Rockford, IL) and measuring protein content of cell lysate by nanometer. IL-8 and IL-6 concentration in CFS was expressed per mg protein in cell lysates (ng/mg protein).

**Gene expression analysis**

The fatty acid effects on gene expression was determined only in fetal H4 IEC (since human NEC-IEC was a primary cell line with limited availability and DHA had consistent effects in H4 and NEC-IEC). Similarly, mRNA expression was measured only in adult Caco2 cells since fatty acids effects were similar between adult Caco2 and NCM 460 IEC in culture.

H4 and Caco2 IEC were exposed to 1ng/mL of IL-1β after 48 hrs of fatty acid supplementation. Four hrs after IL-1β exposure, IL-8, IL-6, IL-1R1 and NFκβ1 mRNA expression, relative to GAPDH as a house-keeping gene, was determined in IEC by quantitative qRT-PCR, as described in detail previously (4,11). Briefly, total RNA from IEC were extracted using the Qiagen RNA kit (Qiagen Inc, Valencia, CA), according to manufacturer’s instruction. RNA was reverse transcribed using a Qiagen RNA reverse transcriptase kit (Qiagen Inc, Valencia, CA), according to manufacturer’s instructions.

mRNA expression was quantified using Sybergreen master mix system (BioRad, Hercules, CA), following the manufacturer’s protocol and as described in detail previously (4,11). Primers for IL-8, IL-6, IL-1R1 NFκβ1 and GAPDH (house-keeping gene) were synthesized at the MGH genomics core facility. Fatty acid effects on mRNA expression of IL-8, IL-6, IL-1R1 and NFκβ1 are expressed as a relative ratio of respective mRNA expression in the control (un-supplemented) IEC (arbitrarily set as 1).

**Statistical Analysis**

All experiments were done in triplicates. Fatty acid effects on IL-8 (ng/mg protein and mRNA), IL-6 (ng/mg protein and mRNA) and IL-1R1 and NFκβ1 mRNA were determined by one way ANOVA and post-hoc Tukey test using Excel 2013 software. Fatty acid fold
enrichment (Table 1) and ARA, EPA elongation (Figure 1) data were log transformed for statistical analysis. Data is reported as mean ± standard deviation (Mean±SD).

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Elongation of ARA and EPA to 22 carbon products in human fetal H4, NEC and adult Caco2 IEC. a) 22:4n-6/20:4n-6 ratio in H4, NEC and Caco2 IEC after ARA supplementation and b) 22:5n-3/20:5n-3 ratio in H4, NEC and Caco2 IEC after EPA supplementation. *p ≤0.05
Figure 2.
IL-8 protein concentration (ng/mg protein) in cell free supernatant of human a) fetal H4 IEC, b) NEC-IEC, c) adult Caco2 and d) adult NCM 460 IEC after fatty acid supplementation and IL-1β stimulation. a) p ≤ 0.05, ARA vs. control, PAL; EPA vs. all other groups; DHA vs. control, PAL, ARA+DHA vs. control and PAL; b) p ≤ 0.05, DHA vs. all other groups; c) p ≤ 0.05 PAL vs. control and ARA; EPA vs. PAL; DHA vs. all other groups, ARA+DHA vs. PAL.
Figure 3.
IL-8 mRNA expression (relative to control IEC arbitrarily set as 1 in human fetal H4 and adult Caco2 IEC) after fatty acid supplementation and IL-1β stimulation. IL-8 mRNA in a) fetal H4 and b) adult Caco2 IEC. *p ≤0.05 a) fetal H4 IEC, ARA vs. all other groups; EPA vs. control and PAL; DHA vs. control and PAL; ARA+DHA vs. control and PAL; b) adult Caco 2 IEC, PAL vs. control; ARA vs. PAL; EPA vs. PAL; DHA vs. all other groups except ARA+DHA.
Figure 4.
IL-6 protein concentration (ng/mg protein) in cell free supernatant of human fetal H4 and NEC-IEC after fatty acid supplementation and IL-1β stimulation. IL-6 (ng/mg protein) in a) fetal H4 and b) NEC-IEC. *p ≤0.05 a) fetal H4IEC, PAL vs. control; ARA vs. all other groups except ARA+DHA; EPA vs. control and PAL; DHA vs. control and PAL; ARA +DHA vs. all other groups except ARA; b) adult Caco 2 IEC, DHA vs. all other groups.
**Figure 5.**
IL-6 mRNA expression (relative to control IEC arbitrarily set as 1 in human fetal H4 IEC) after fatty acid supplementation and IL-1β stimulation. *p ≤0.05 PAL vs. control; ARA vs. control, PAL and EPA; EPA vs. control and PAL; DHA vs. control and PAL; ARA+DHA vs. all other groups except ARA.
Figure 6.
IL-1R1 and NFκB1 mRNA fold induction (expressed as a ratio of house-keeping gene GAPDH) in human fetal H4 and adult Caco2 control IEC after IL-1β stimulation. □ H4 ■ Caco2 *p ≤0.05 vs H4.
Figure 7.

IL-1R1 and NFκβ1 mRNA expression (relative to control IEC arbitrarily set as 1) in a) human fetal H4 and b) adult Caco2 IEC after fatty acid supplementation and IL-1β stimulation. □ Control ■ PAL □ ARA □ EPA □ DHA □ ARA+DHA * a) fetal H4 IEC, IL-R1 mRNA *p ≤ 0.05 DHA vs. control and PAL; NFκB1 mRNA *p ≤ 0.05 PAL vs. control; ARA vs. PAL; DHA vs. control, PAL and ARA; ARA+DHA vs. control and PAL. b) fetal H4 IEC. IL-R1 mRNA, *p ≤ 0.05 PAL vs. control; DHA vs. all other groups. Fetal H4 IEC NFκB1 mRNA, *p ≤ 0.05 PAL vs. control; DHA vs. control, PAL and ARA.
|                  | Fold Enrichment of Supplemented Fatty Acids<sup>a,b</sup> |          |          |          |
|------------------|---------------------------------------------------------|----------|----------|----------|
|                  | H4           | NEC-IEC   | Caco2    |          |
| PAL 16:0         | 1.7±0.11     | 2.6±0.05  | 1.5±0.15 |          |
| ARA 20:4n-6      | 2.2±0.63     | 2.3±0.26  | 10.2±1.06<sup>†</sup> |          |
| EPA 20:5n-3      | 103.1±61.9   | 8.1±3.2<sup>‡</sup> | 57.1±20.1 |          |
| DHA 22:6n-3      | 9.9±4.7      | 11.6±5.8  | 22.9±5.8<sup>†</sup> |          |
| ARA+DHA 20:4n-6  | 1.7±0.63     | 1.6±0.52  | 7.6±0.70<sup>‡</sup> |          |
|                  | 4.6±1.5      | 2.4±0.17  | 12.8±3.1<sup>‡</sup> |          |

<sup>a</sup> Fold enrichment is the ratio of fatty acid (% wt) in supplemented IEC to control (un-supplemented) IEC

<sup>b</sup> Mean±STD

<sup>†</sup> p ≤0.05 vs NEC-IEC

<sup>‡</sup> p ≤0.05 vs Caco2
## Table 2

Baseline IL-8 and IL-6 concentrations (ng/mg protein) in H4, NEC, Caco2 and NCM460 IEC after IL-1β challenge

| Cytokines | H4     | NEC-IEC | Caco2 | NCM 460 |
|-----------|--------|---------|-------|---------|
| IL-8 (ng/mg protein) | 4.42±0.43 | 65.99±20.63 * ‡§ | 0.47±0.09 *§ | 0.86±0.22 * |
| IL-6 (ng/mg protein) | 3.76±0.52 | 26.55±7.72 * | ND    | ND      |

* p ≤ 0.05 vs H4
† p ≤ 0.05 vs NEC-IEC
‡ p ≤ 0.05 vs Caco2
§ p ≤ 0.05 vs NCM 460
ND – Not detectable