Long-chain polyunsaturated fatty acids attenuate the IL-1β-induced proinflammatory response in human fetal intestinal epithelial cells

Vasuki Wijendran[1], J.T. Brenna[2], Dong Hao Wang[2], Weishu Zhu[1], Di Meng[1], Kriston Ganguli[1], Kumar S.D. Kothapalli[2], Pilar Requena[3], Sheila Innis[4] and W. Allan Walker[1]

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 pretreated with 100 μmol/l palmitic acid (PAL), DHA, EPA, ARA, or ARA+DHA for 48 h and then stimulated with proinflammatory IL-1β.

RESULTS: DHA significantly attenuated IL-1β induced proinflammatory 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 downregulated IL-1R1 (IL-1β receptor) and NFkβ1 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.

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 multifactorial (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 NFkβ1 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 wk (8). Carlson *et al.* (9) also reported a decrease in the incidence of NEC in premature infants fed a preterm 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 ant-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 proinflammatory cytokines IL-8 and IL-6 response and (ii) NFkβ1 and IL1-R1 gene expression in human fetal and adult IEC in culture, after proinflammatory 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 h of supplementation with palmitic acid (PAL), ARA, EPA, DHA and ARA+DHA, is shown in **Table 1**. All fatty acids supplemented showed enrichment in

[1]Mucosal Immunology and Biology Research Center, Massachusetts General Hospital for Children, Boston, Massachusetts; [2]Division of Nutritional Sciences, Cornell University, Ithaca, New York; [3]Liverpool School of Tropical Medicine, Liverpool, UK; [4]Department of Pediatrics, University of British Columbia, Vancouver, British Columbia, Canada. Correspondence: W. Allan Walker (wwalker@partners.org)

Table 1. Fatty acid enrichment in H4, NEC, and Caco2 IEC in culture after supplementation

	Fold enrichment of supplemented fatty acids[a,b]		
	H4	NEC-IEC	Caco2
Palmitic acid; 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*,[†]
Eicosapentaenoic acid; 20:5n-3	103.1 ± 61.9	8.1 ± 3.2*,[‡]	57.1 ± 20.1
DHA; 22:6n-3	9.9 ± 4.7	11.6 ± 5.8	22.9 ± 5.8*,[†]
ARA+DHA; 20:4n-6, 22:6n-3	1.7 ± 0.63; 4.6 ± 1.5	1.6 ± 0.52; 2.4 ± 0.17	7.6 ± 0.70*,[†]; 12.8 ± 3.1*,[†]

[a]Fold enrichment is the ratio of fatty acid (% wt) in supplemented IEC to control (un-supplemented) IEC. [b]Mean ± SD.
*$P \leq 0.05$ vs. H4. [†]$P \leq 0.05$ vs. NEC-IEC. [‡]$P \leq 0.05$ vs. Caco2.
ARA, arachidonic acid; DHA, docosahexaenoic acid; IEC, intestinal epithelial cells; NEC, necrotizing enterocolitis.
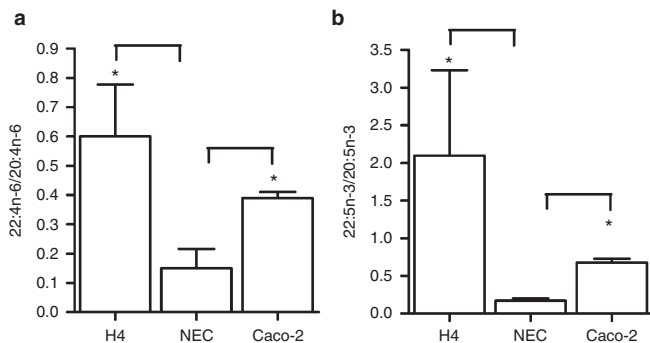

Figure 1. Elongation of arachidonic acid (ARA) and eicosapentaenoic acid (EPA) to 22 carbon products in human fetal H4, necrotizing enterocolitis (NEC) and adult Caco2 IEC. (**a**) 22:4n-6/20:4n-6 ratio in H4, NEC, and Caco2 IEC after ARA supplementation and (**b**) 22:5n-3/20:5n-3 ratio in H4, NEC, and Caco2 IEC after EPA supplementation. *$P \leq 0.05$.

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

*$P \leq 0.05$ vs. H4.
[†]$P < 05$.
[†]$P \leq 0.05$ vs. NEC-IEC.
[‡]$P \leq 0.05$ vs. Caco2.
[§]$P \leq 0.05$ vs. NCM 460.
IEC, IEC, intestinal epithelial cells; ND, not detectable; NEC, necrotizing enterocolitis

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 unsupplemented control IEC. EPA was 103-fold higher in H4 IEC, but only eightfold higher in NEC-IEC. ARA increased by twofold 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- 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 Proinflammatory 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 unsupplemented (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- 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 proinflammatory 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
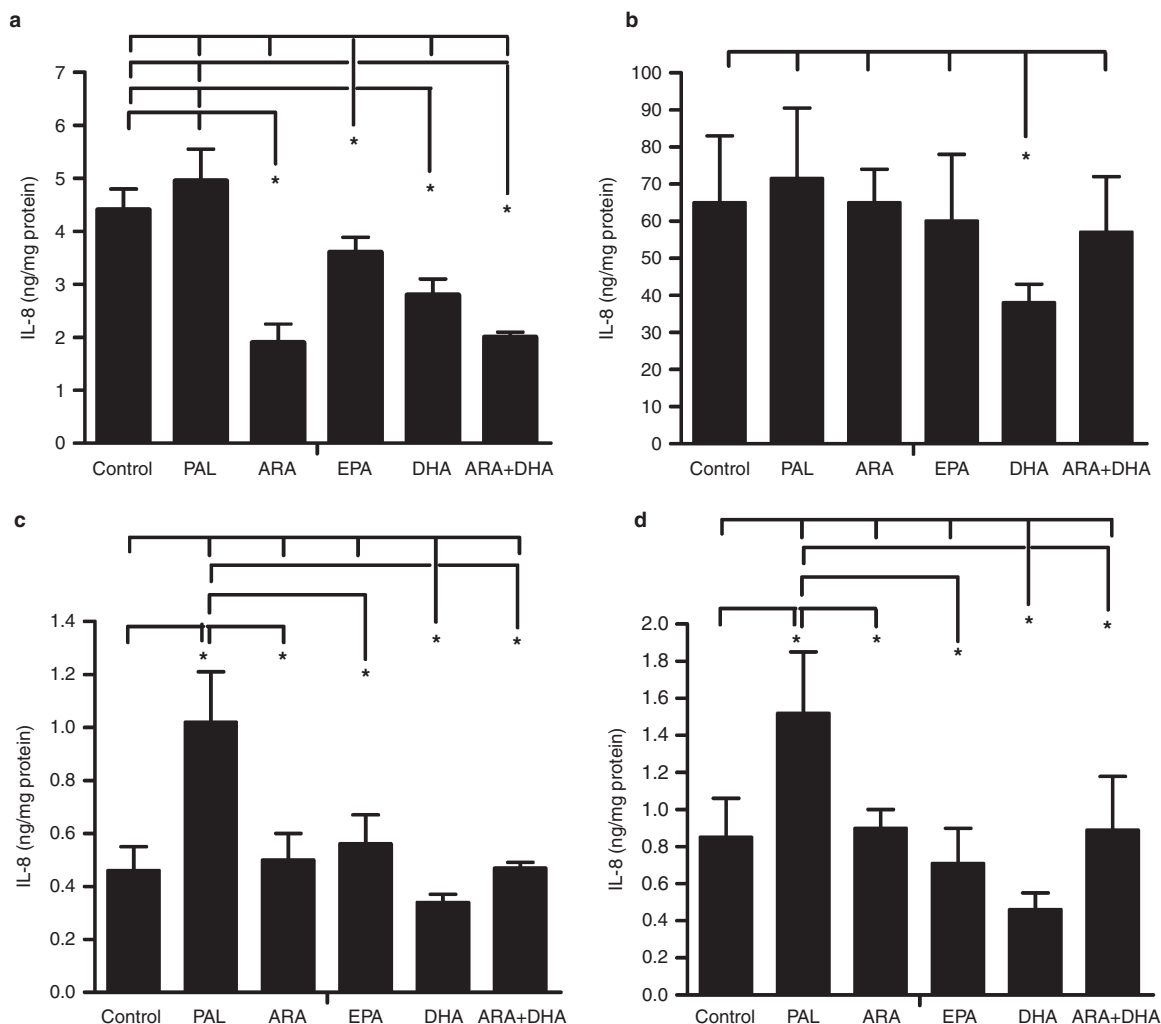
Figure 2. IL-8 protein concentration (ng/mg protein) in cell-free supernatant of human (**a**) fetal H4 necrotizing enterocolitis (IEC), (**b**) NEC-IEC, (**c**) adult Caco2, and (**d**) adult NCM 460 IEC after fatty acid supplementation and IL-1β stimulation. (**a**) *$P \leq 0.05$, arachidonic acid (ARA) vs., control, palmitic acid (PAL); eicosapentaenoic acid (EPA) vs. all other groups; docosahexaenoic acid (DHA) vs. control, PAL, ARA+DHA vs. control and PAL; (**b**) *$P \leq 0.05$, DHA vs. all other groups; (**c**) *$P \leq 0.05$ PAL vs. control and ARA; EPA vs. PAL; DHA vs. all other groups, ARA+DHA vs. PAL.
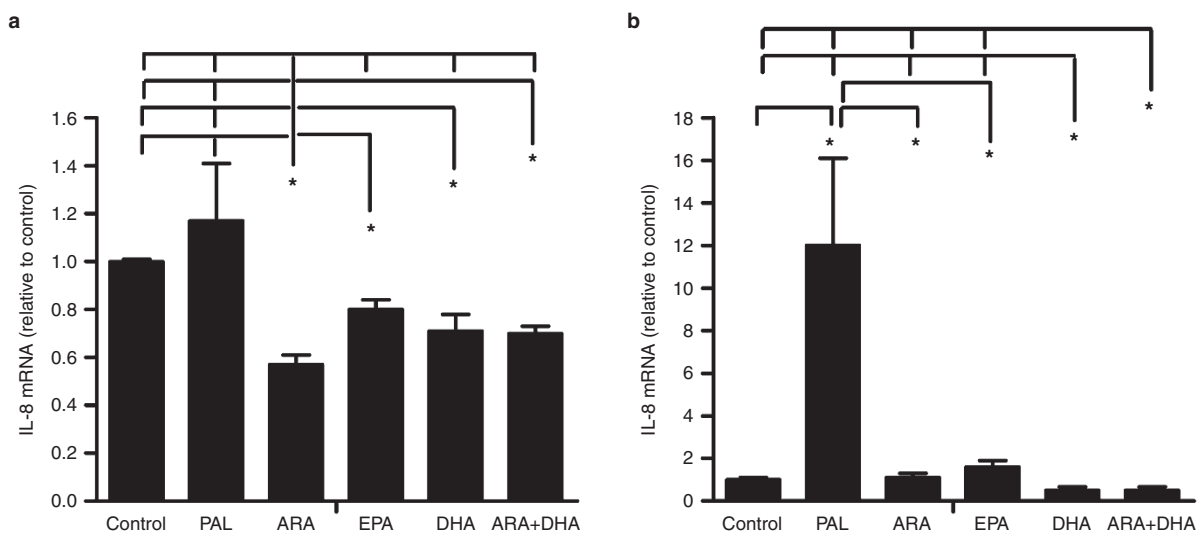

Figure 3. IL-8 mRNA expression (relative to control IEC arbitrarily set as 1 in human fetal H4 and adult Caco2 necrotizing enterocolitis (IEC)) after fatty acid supplementation and IL-1β stimulation. IL-8 mRNA in (**a**) fetal H4 and (**b**) adult Caco2 IEC. *$P \leq 0.05$; (**a**) fetal H4 IEC, ARA vs. all other groups; eicosapentaenoic acid (EPA) vs. control and palmitic acid (PAL); docosahexaenoic acid (DHA) vs. control and PAL; ARA+DHA vs. control and PAL; (**b**) adult Caco 2 IEC, PAL vs. control; arachidonic acid (ARA) vs. PAL; EPA vs. PAL; DHA vs. all other groups except ARA+DHA.
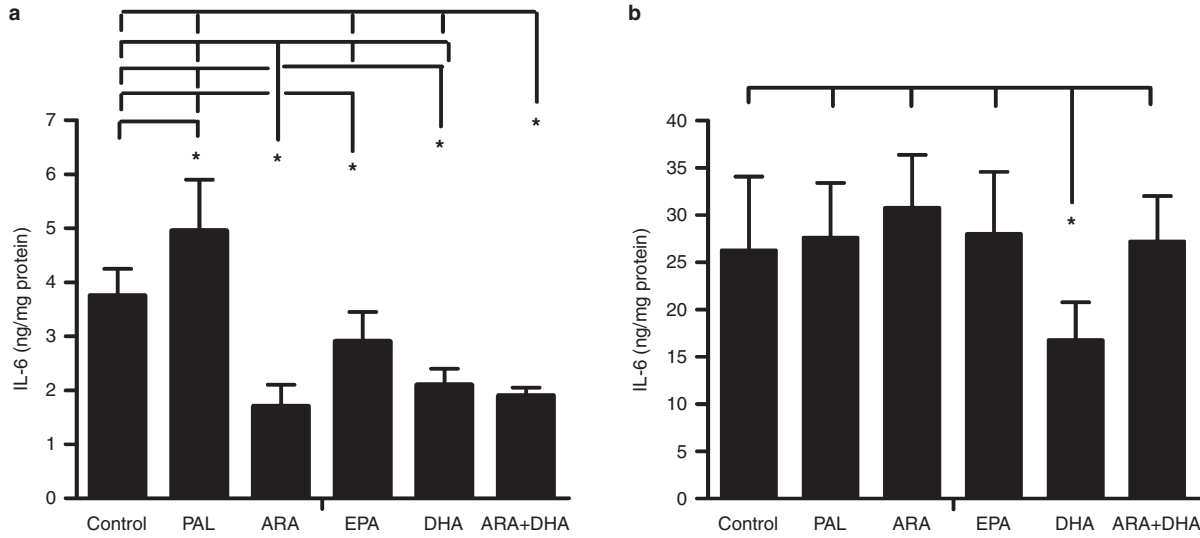
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b

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. *$P \leq 0.05$; (**a**) fetal H4IEC, palmitic acid (PAL) vs. control; ARA vs. all other groups except ARA+DHA; eicosapentaenoic acid (EPA) vs. control and PAL; docosahexaenoic acid (DHA) vs. control and PAL; ARA+DHA vs. all other groups except arachidonic acid (ARA); **b**) adult Caco 2 IEC, DHA vs. all other groups.
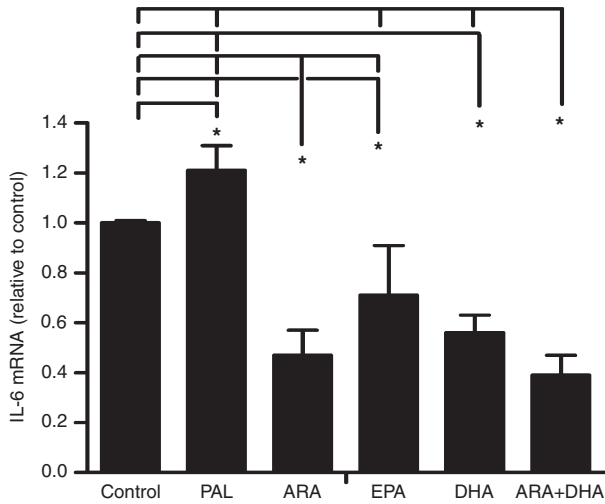

Figure 5. IL-6 mRNA expression (relative to control IEC arbitrarily set as 1 in human fetal H4 IEC) after fatty acid supplementation and IL-1β stimulation. *$P \leq 0.05$ palmitic acid (PAL) vs. control; arachidonic acid (ARA) vs. control, PAL and eicosapentaenoic acid (EPA); EPA vs. control and PAL; docosahexaenoic acid (DHA) vs. control and PAL: ARA+DHA vs. all other groups except ARA.

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 NFkβ1 in H4 and Caco2 IEC After IL-1β Exposure
Expression levels of the IL-1β receptor (IL-1R1) and the down-stream signaling molecule NFkβ1 mRNA were seven- and fivefold higher respectively in H4 cells compared to Caco2 cells (**Figure 6**). Relative gene expression (normalized to control unsupplemented IEC) of the IL-1β receptor (IL-1R1) and its down-stream proinflammatory signaling mediator NFkβ1
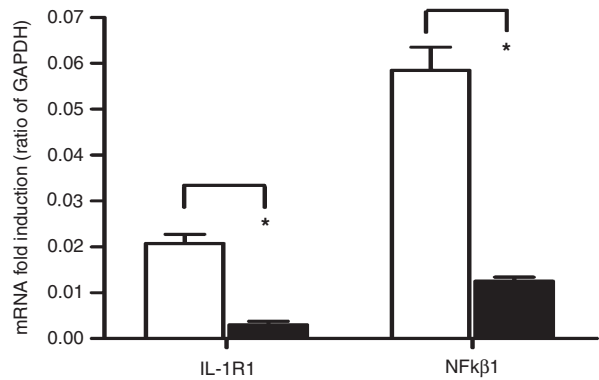

Figure 6. IL-1R1 and NFkβ1mRNA fold induction (expressed as a ratio of house-keeping gene GAPDH) in human fetal H4 and adult Caco2 control IEC after IL-1β stimulation. □ H4 ■ Caco2. *$P \leq 0.05$ vs. H4.

in H4 and Caco2 enterocytes 4 h after IL-1β challenge is shown in **Figure 7**. DHA significantly lowered IL-1R1 and NFkβ1 mRNA in H4 and Caco2 IEC compared to control and PAL. In H4 IEC, DHA downregulated IL-1R1 and NFkβ1 by two- and 1.5-fold compared to control. ARA or EPA did not significantly alter IL-1R1 or NFkβ1 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).
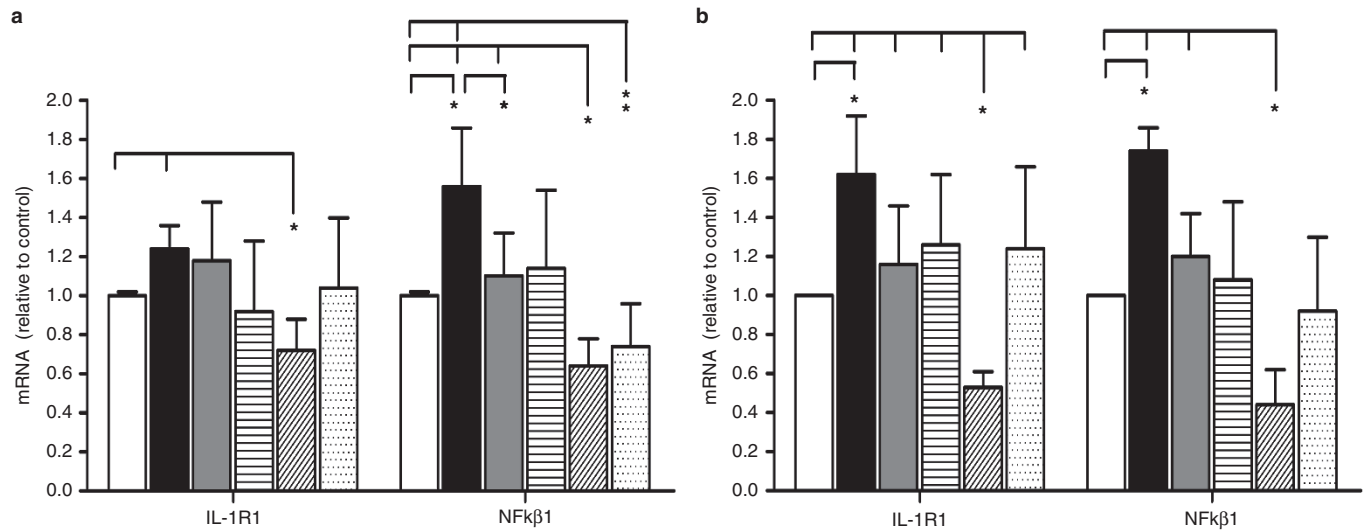
Figure 7. IL-1R1 and NFkβ1mRNA expression (relative to control IEC arbitrarily set as 1) in (**a**) human fetal H4 and (**b**) adult Caco2 IEC after fatty acid supplementation and IL-1β stimulation. □ Control ■ PAL ▨ ARA ▤ EPA ▨ DHA ⋮ ARA+DHA*. (**a**) Fetal H4 IEC, IL-R_1mRNA *$P \leq 0.05$ DHA vs. control and palmitic acid (PAL); NFκB1 mRNA; *$P \leq 0.05$ PAL vs. control; ARA vs. PAL; DHA vs. control, PAL and arachidonic acid (ARA); ARA+DAH vs. control and PAL. (**b**) Fetal H4 IEC. IL-R_1mRNA, *$p \leq 0.05$ PAL vs. control; DHA vs. all other groups. Fetal H4 IEC NFκB1 mRNA, *$P \leq 0.05$ PAL vs. control; DHA vs. control, PAL, and ARA.

Results from the current study demonstrate a significant enrichment of human fetal and adult IEC with DHA, EPA, and ARA, 48 h 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 downstream 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 (100 µmol/l), 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 50 µmol/l concentration 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 downstream proinflammatory signaling mediator NFkβ1 gene expression in fetal and adult human IEC compared to control and PAL supplemented IEC. IL-1R1 and NFkβ1 expression after IL-1β exposure in fetal H4 IEC were five- to sevenfold 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 NFkβ1 signaling pathway involved in innate immunity (19). Current evidence strongly suggest that an exaggerated TLR4 and NFkβ1 expression in immature and NEC intestine may be a major factor underlying the excessive proinflammatory response in NEC (4,6,20). Further, a recent report on genome-wide analysis of human NEC intestinal tissues identified aberrations in the NFkβ1 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 downregulation of proinflammatory NFkβ1 gene expression. *In vivo* animal studies have shown that DHA and/or fish oil containing DHA downregulated the intestinal TLR4 and NFkβ1 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 downregulating NFkβ1-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 NFkβ1 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 proinflammatory 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 proinflammatory eicosanoid synthesis from ARA by shunting ARA to an anti-inflammatory lipoxygenase pathway. Developmental regulation of ARA and EPA metabolism to shunt these eicosanoid precursors toward the anti-inflammatory lipoxygenase 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 proinflammatory cytokine response in healthy fetal, NEC, and adult human intestinal epithelial cells after IL-1β challenge. In addition, DHA downregulated exaggerated NFkβ1 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 support 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 100 μmol/l PAL or ARA or EPA or DHA or ARA+DHA for 48 h in the media containing delipidized fetal bovine serum (FBS). Control IEC were treated with fatty acid–free bovine serum albumin 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 h after fatty acid supplementation. Cytokine concentrations and gene expression in control and fatty acid supplemented IEC were determined 6 and 4 h 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 25-wk 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 (Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated FBS (Life Technologies), 1% hepes buffer (Life Technologies), 1% glutamax (Life Technologies), 1% nonessential amino acids (Life Technologies), 0.2 U/ml insulin (Eli Lilly and Company, Indianapolis, IN), and 1% antibiotic–antimycotic (Life Technologies) in a sterile cell culture humidifier at 37 °C and 5% CO_2. NEC-IEC were cultured in Opti-Minimal Essential Medium (Opti-MEM, Life Technologies) supplemented with 10% heat-inactivated FBS (Life Technologies), 0.2 U/ml insulin (Eli Lilly and Company), 20 ng/ml epidermal growth factor (Life Technologies), and 1% antibiotic–antimycotic (Life Technologies) in a sterile cell culture humidifier at 32 °C and 5% CO_2. Adult human colonic cell lines namely, Caco2 derived from human colonic carcinoma, purchased from American Type Culture Collection (Manassas, VA) and NCM460, a primary cell line derived from normal human colon, purchased from Incell (SA, Texas) were used as models for adult IEC and were cultured as previously published (24). Briefly, $Caco_2$ IEC were cultured in Dulbecco's modified Eagle medium (Life Technologies) supplemented with 10% heat-inactivated FBS (Life Technologies), 1% Hepes buffer (Life Technologies), 1% glutamax (Life Technologies), 1% nonessential amino acids (Life Technologies) and 1% antibiotic–antimycotic (Life Technologies), in a sterile cell culture humidifier at 37 °C and 5% CO_2. The NCM 460 IEC were cultured in M3:Base A (M3A, Incell) media supplemented with 10% heat-inactivated FBS (Life Technologies), and 1% antibiotic–antimycotic (Life Technologies), in a sterile cell culture humidifier at 37 °C and 5% CO_2.

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 (Waterville, MN). When human fetal and adult IEC in culture reached 70% confluence, they were supplemented with 100 µmol/l PAL (positive control) or ARA or EPA or DHA or ARA+DHA (50 µm each) bound to fatty acid–free bovine serum albumin (Sigma Aldrich, St. Louis, MO) at 3:1 ratio (25) in media containing delipidized FBS (Gemini Bio-Products, Sacramento, CA) for 48 h. Control IEC were treated with fatty acid–free bovine serum albumin only in the media. IEC cell viability in culture after 48 h of fatty acid supplementation was determined by alamar blue (Life Technologies) 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 unsupplemented H4, NEC-IEC, Caco2, or NCM460 IEC in culture (data not shown).

Fatty Acid Analysis of Human IEC in Culture

Forty-eight hours after fatty acid treatment, culture media was aspirated and IEC in culture were washed three times with phosphate buffered saline (pH 7.0) buffer (Sigma Aldrich). Washed IEC cells were harvested by trypsinization and collected in 5 ml deionized distilled water. IEC suspended in sterile, deionized water were centrifuged at 4,000 rpm and supernatant was aspirated after centrifugation; IEC were resuspended in 800 µl deionized water and frozen immediately at −80 °C for fatty acid analysis.

Fatty acid analysis of human IEC harvested after 48 h of fatty acid supplementation was done at the laboratory of Dr. JT Brenna (Cornell University, Ithaca, NY), according to the 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 unsupplemented control IEC.

IL-8 and IL-6 Protein Analysis

IEC in culture were stimulated with 1 ng/ml of IL-1β after 48 h of fatty acid supplementation. Six hours after IL-1β exposure, 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 enzyme-linked immunosorbent assay 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 were 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 1 ng/ml of IL-1β after 48 h of fatty acid supplementation. Four hours after IL-1β exposure, IL-8, IL-6, IL-1R1, and NFkβ1 mRNA expression, relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a house-keeping gene, was determined in IEC by quantitative reverse transcription PCR, as described in detail previously (4,11). Briefly, total RNA from IEC were extracted using the Qiagen RNA kit (Qiagen, Valencia, CA), according to manufacturer's instruction. RNA was reverse transcribed using a Qiagen RNA reverse transcriptase kit (Qiagen), 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 NFkβ1, 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 NFkβ1 are expressed as a relative ratio of respective mRNA expression in the control (unsupplemented) 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 NFkβ1 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 are reported as mean ± SD.

AUTHOR CONTRIBUTIONS

V.W.: Contributed significantly to concept, experimental design, experiments, and manuscript preparation; J.T.B.: Contributed significantly to experimental design, technical approach, and manuscript preparation; D.H.W.: helped with experiments and sample analysis; W.Z.: helped with experiments and sample analysis; D.M.: contributed to experiments; K.G.: contributed to experiments; K.S.D.K.: contributed to experiments; P.R.: contributed to experiments; S.M.I.: contributed to technical approach; W.A.W.: contributed significantly to concept, design, and manuscript preparation.

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