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Impact of the ω-3 to ω-6 Polyunsaturated Fatty Acid Ratio on Cytokine Release in Human Alveolar Cells

Short title: Fatty acids and alveolar cell cytokine release

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Précis: The study investigated the effects of different DHA/AA ratios on membrane composition of alveolar cells and LPS-induced balance between pro/anti-inflammatory cytokines. The supply of 1:1 and 1:2 DHA/AA ratios reversed the predominance of ω-6 over ω-3 in cell membranes, decrease TNF-α, IL-6, and IL-8 release, and increase IL-10 release.

Keywords: acute lung injury; acute respiratory distress syndrome; docosahexaenoic acid; inflammation; nutritional therapy; A549 cells

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ABSTRACT. *Background*: ω-3 polyunsaturated fatty acids (PUFAs) and ω-6 PUFAs have opposing influences upon inflammation. Our objective was to determine whether lipopolysaccharide (LPS)-induced cytokine release by human alveolar cells was affected by changes of the ω-3/ω-6 ratio in cell membranes induced by different PUFA supplies.

Methods: After LPS challenge, PUFAs were added to alveolar cells as docosahexaenoic acid (DHA, ω-3) plus arachidonic acid (AA, ω-6) in four different DHA/AA ratios (1:1, 1:2, 1:4, and 1:7) and cytokine release was measured.

Results: The supply of 1:1 and 1:2 DHA/AA ratios reversed the baseline predominance of ω-6 over ω-3 in the ω-3/ω-6 PUFA ratio of cell membranes. The release of pro-inflammatory cytokines (TNF-α, IL-6, and IL-8) was reduced by 1:1 and 1:2 DHA/AA ratios ($p < .01$ to $< .001$), but increased by 1:4 and 1:7 DHA/AA ratios ($p < .01$ to $< .001$) vs. control. The 1:1 and 1:2 ratios increased the release of anti-inflammatory IL-10 ($p < .001$). The balance between pro- and anti-inflammatory cytokines showed an anti-inflammatory response with 1:1 and 1:2 ratios and a pro-inflammatory response with 1:4 and 1:7 ratios ($p < .001$).

Conclusions: This study showed that pro-inflammatory cytokine release was dependent on the proportion of ω-3 in ω-3/ω-6 ratio in alveolar cell membranes, being reduced with the supply of high proportion of DHA and increased with high proportion of AA, respectively. Our results support the biochemical basis for current recommendations to shift the PUFA supply from ω-6 to ω-3 in nutrition of acute lung injury patients.

Acute lung injury (ALI) is characterized by an intense inflammatory response within the alveolar spaces,[1] with accumulation of pro- and anti-inflammatory cytokines.[2] Several studies have been carried out to find strategies for reducing the severity of lung inflammatory process, e.g. by reducing the release of pro-inflammatory mediators; however, few studies have demonstrated a significant effect on mortality in patients with acute respiratory distress syndrome (ARDS).[3] Treatment of patients with ALI/ARDS includes nutritional support with lipids; usually, soybean oil-based lipid emulsions are used. These emulsions are rich in ω-6 polyunsaturated fatty acids (PUFAs) (i.e. linoleic acid) and poor in ω-3 PUFAs: thus, the ratio between ω-3 and ω-6 is quite low (between 1:5 and 1:7). An equally low ω-3/ω-6 PUFA ratio is typical of most enteral formulas. Consequently, ALI/ARDS patients are usually exposed to a relatively large amount of ω-6 PUFAs compared with ω-3,[4] although administration of high amounts of linoleic acid appears to be undesirable in ARDS patients.[5]

Several studies clearly demonstrated in lung cells that ω-6 PUFAs (e.g. arachidonic acid/AA) are precursors of pro-inflammatory mediators, as well as the pivotal role of AA and its metabolites as mediators of injury.[6,7] Conversely, administration of ω-3 PUFAs (e.g. eicosapentaenoic acid/EPA and docosahexaenoic acid/DHA) has been demonstrated to decrease alveolar production of pro-inflammatory mediators and to reduce organ failure in lung animal models.[7-9] Based on these data, several reviews suggested that ω-3 PUFAs could modulate the pulmonary inflammatory response[4,10] and represent a non-ventilatory therapeutic tool for ARDS.[11] Three studies showed that enteral nutrition with EPA, gamma-linolenic acid, and antioxidants reduced alveolar inflammatory mediators and improved clinical outcomes in ALI/ARDS patients,[12,13] as well as in ventilated severe septic patients.[14] Parenteral nutrition (PN) with an ω-3 enriched lipid emulsion in ARDS patients showed selective anti-inflammatory effects.[15] However, in contrast to ω-3 enteral administration,[16] to date no randomized controlled clinical trial using ω-3 enriched parenteral lipid emulsions has shown clear evidence of beneficial effects on clinical end-points in ALI/ARDS patients.[17]

Evidence is accumulating that the ω-3/ω-6 PUFA ratio in nutritional support may influence inflammation. Optimal ω-3 administration is not only dose-related but is also independently affected by the ω-3/ω-6 ratio.[18] Though ω-3/ω-6 PUFA ratios from 1:1 to 1:4 have been proposed,[4,19-25] the impact of ω-3/ω-6 ratio on the inflammatory response is still an unresolved question. Indeed, PN with the 1:2 ω-3/ω-6 PUFA ratio did not affect inflammation or clinical outcomes, compared to PN with a MCT/LCT emulsion in unselected critically ill medical patients.[26]

No previous study has investigated the effects of supply of different ω-3/ω-6 ratios on phospholipid composition of cell membranes and cytokine release in the presence of a pro-inflammatory stimulus in alveolar cells. The aim of our study was to determine whether changes of the ω-3/ω-6 PUFA ratio in cell membrane phospholipid composition induced by PUFA supply may have effects upon the release of pro-inflammatory cytokines (TNF-α, IL-6, and IL-8) and one anti-inflammatory cytokine (IL-10) from human alveolar cells after endotoxin challenge.

Materials and Methods

Fatty acids (FAs) and LPS from *Escherichia coli* 055:B5 were obtained from Sigma Chemical Co. (St. Louis, MO). A human lung carcinoma cell line (A549 cells, ATCC, Rockville, MD) was used. A549 are alveolar epithelial cells with type II pneumocyte properties. The A549 cell cultures were treated as previously described.[27]

Preliminary tests

The study was preceded by the following preliminary tests performed in triplicate to design the experimental model.

Test 1. Analysis of baseline A549 cell FA composition. The FA percentage content was determined in neutral and polar lipids as previously described.[28] Briefly, total lipids were isolated by the Folch[29] method and separated by thin-layer chromatography. FA methyl esters from phospholipids were prepared following the Metcalfe[30] method and separated by gas-liquid chromatography (CP 9002

Chrompack). Internal standard (methyl heptadecanoate) was added to each preparation to determine recovery.

Test 2. Effects of PUFA addition (10, 25, and 50 μM) without LPS stimulation on the cytokine release and the phospholipid composition of A549 cells.

Test 3. Effect of LPS challenge on A549 cells. Cells were exposed to various doses of LPS (100, 200, and 400 μg/ml) to determine the dose- and time-dependence of LPS on cytokine release as assessed by TNF-α release.

Experimental study

Six different cultures of A549 cells were prepared: 1) baseline, non-stimulated cells; 2) control, cells stimulated with LPS at time 0; 3) DHA/AA1:1, cells stimulated with LPS and exposed to DHA(25 μM)/AA(25 μM) ratio; 4) DHA/AA1:2, cells stimulated with LPS and exposed to DHA(17 μM)/AA(33 μM) ratio; 5) DHA/AA1:4, cells stimulated with LPS and exposed to DHA(10 μM)/AA(40 μM) ratio; 6) DHA/AA1:7, cells stimulated with LPS and exposed to DHA(6.5 μM)/AA(43.5 μM) ratio. Three h after LPS challenge, DHA/AA ratios (50 μM) were added to cell cultures for 4 h. After 7 h from time 0, the release of TNF-α, IL-6, IL-8, and IL-10 in supernatant and the phospholipid composition of A549 cell membranes were determined (four independent experiments). All culture supernatants were harvested and stored at -80°C for cytokine measurement via ELISA kits (Euroclone, Paignton-Devon, UK) according to the manufacturer instructions).

Statistical analysis

Data were expressed as mean ± SD. Multiple comparisons were carried out using one-way ANOVA, followed by Bonferroni post hoc test. SPSS 14 (SPSS Inc., Chicago, IL) was used for analyses. Significance was defined as $p < .05$.

Results

Preliminary tests

Test 1. The FA percentage content in phospholipids of A549 cell membranes was shown in table 1.

Test 2. Both AA and DHA addition (50 μM) changed respective percentage content in phospholipids and ω-3/ω-6 PUFA ratio of A549 cells at 7 h ($p < .001$ vs. baseline) (Table 2). The TNF-α and IL-6 constitutive production was decreased by DHA (50 μM) ($p < .05$ and $< .001$, respectively) and increased by AA (50 μM) ($p < .01$ and $< .001$, respectively) at 7 h. Addition of 10 μM PUFA did not induce significant modifies of the cytokine release and the phospholipid composition, whereas no difference between addition of 25 and 50 μM PUFA was found at 7 h.

Test 3. A dose- and time-dependent effect of LPS on TNF-α release was observed. Concentration and exposure-time of LPS that induced the most significant TNF-α release were 400 μg/ml and 7 h, respectively ($p < .001$).

As previously demonstrated, A549 cell growth was decreased in a concentration- and time-dependent manner by addition of AA[27] and DHA;[28] however, at 7 h no difference on A549 cell proliferation and viability versus baseline was found after 50 μM AA or DHA addition, as well after LPS challenge. Based on the results of these preliminary tests, we designed for the experimental study the LPS challenge of 400 μg/ml for 7 h and the addition of DHA/AA ratios in a total final concentration of 50 μM.

Experimental study

Effect of LPS and DHA/AA ratios on phospholipid composition of A549 cell membranes (Table 1)

In control cells, we observed a remarkable decrease of AA percentage content ($p < .001$ vs. baseline), while DHA did not change in phospholipids of cell membranes. Following addition of different DHA/AA ratios, the DHA ($p < .001$ vs. all) and AA ($p < .05$ to $< .001$ vs. all, except 1:4 vs. 1:7) contents were changed. Finally, the ω-3/ω-6 PUFA ratio in cells exposed to 1:1 and 1:2 DHA/AA ratios was markedly changed ($p < .001$ vs. all).

Effect of DHA/AA ratios on LPS-induced cytokine release from A549 cells (Fig. 1 A-B-C-D)

The release of TNF-α, IL-6, IL-8, and IL-10 from control was considerably increased ($p < .001$) compared with baseline. The 1:1 DHA/AA ratio decreased TNF-α, IL-6, and IL-8 release ($p < .001$) compared with control, as well as, the 1:2 DHA/AA ratio decreased TNF-α ($p < .001$), IL-6 ($p < .01$), and IL-8 ($p < .01$), but less than the 1:1 ratio ($p < .001$). Exposure of cell cultures to 1:4 DHA/AA and 1:7 DHA/AA ratios increased the release of TNF-α ($p < .01$), IL-6 ($p < .001$), and IL-8 ($p < .001$) vs. control. Such increase in release of pro-inflammatory cytokines was also significant vs. 1:1 and 1:2 ratios ($p < .001$). The release of IL-10 was increased by 1:1 and 1:2 ratios ($p < .001$), while it was not affected by 1:4 and 1:7 ratios.

Effect of DHA/AA ratios on balance between pro- and anti-inflammatory cytokines (Fig. 2 A-B-C)

The balance between pro- and anti-inflammatory cytokines was evaluated by three cytokine ratios (TNF-α/IL-10, IL-6/IL-10, and IL-8/IL-10): a reduction of such ratios was considered as an anti-inflammatory response, while an increased ratio was considered a further amplification of the pro-inflammatory response. The three cytokine ratios showed the same pattern. An anti-inflammatory response was observed with 1:1 and 1:2 ratios ($p < .001$), due to the net effect of a reduction of TNF-α, IL-6, and IL-8 plus an increase of IL-10 concentrations. On the contrary, a pro-inflammatory response was observed when 1:4 and 1:7 ratios ($p < .001$).

Discussion

The main finding of this study was that the supply of 1:1 and 1:2 DHA/AA ratios reversed the baseline predominance of ω-6 over ω-3 in the ω-3/ω-6 PUFA ratio of cell membranes, decreasing the release of TNF-α, IL-6, and IL-8 in alveolar cells. In contrast, the supply of DHA/AA ratios with an ω-6 prevalence (i.e. 1:4 and 1:7) caused itself a further significant increase in pro-inflammatory cytokine release compared with LPS alone. Thus, the balance between the release of

pro- and anti-inflammatory cytokines showed a relevant anti-inflammatory response with 1:1 and 1:2 ratios and a pro-inflammatory response with 1:4 and 1:7 ratios.

The reason some patients with ALI develop ARDS whereas others recover remains unclear. Inflammatory cytokines are key elements in the pathogenesis of ARDS and they appear to have concentration-dependent biologic effects. Persistently alveolar elevated levels of pro-inflammatory cytokines associated with decreased production of those anti-inflammatory correlate with the severity of lung injury, and the degree of this cytokine imbalance leads to additional non-pulmonary organ dysfunction and increased mortality rates in ARDS patients.[2,31,32] Previous studies suggested that the lung itself can be an important cytokine-producing organ and the type II pneumocytes have a central position in the pathophysiology of the alveolar space.[33]

Lipids are known to have immune-modulatory properties and their administration could influence the prognosis of ALI/ARDS patients.[17] Indeed, after PUFA challenge many cell properties are shown to be modified, mainly the inflammatory response (e.g. eicosanoid and cytokine productions).[23,25,34,35] In general, ω-3 PUFAs are regarded to be anti-inflammatory whereas ω-6 are pro-inflammatory.[34] The differential impact of ω-3 vs. ω-6 supply on cytokine generation provoked by various stimuli was demonstrated in respiratory cells,[35] as well as in septic and ALI murine models.[36,37] In septic patients, ω-3 PUFA parenteral administration influenced lipid mediator generation and reduced endotoxin-elicited monocyte pro-inflammatory cytokine generation, while cytokine generation was markedly amplified by ω-6 PUFA infusion.[38] The alveolar cells have an intense lipid metabolism and, as demonstrated both in A549 cells and lung models, EPA/DHA are rapidly incorporated in the phospholipids of lung cell membranes, inducing rapidly (from 5 min to 4 h) changes in cell membrane FA composition[9] and lipid-derived inflammatory mediator generation.[8,9,39]

A complex network of factors regulates the relation between ω-3 and inflammation[25,34,40] and the mechanisms underlining the anti-inflammatory effects of DHA are not completely clear; however, DHA seems to be more effective than EPA in alleviating LPS-induced pro-inflammatory cytokine

production in macrophages.[41] One of the goal of EPA/DHA supplementation is to reduce the severity of inflammatory processes by reducing the availability of AA in cell membranes.[10] Moreover, DHA can decrease the release of AA from membrane phospholipids by decreasing phospholipase A2 activity.[42] A decrease in AA leads to reduced release of pro-inflammatory mediators (prostaglandin E_2 and leukotriene B_4).[34] Our findings indicate that the baseline predominance of ω-6 over ω-3 was reversed following the supply of 1:1 and 1:2 ratios, with the DHA exceeding the AA content nearly 4-8-fold. Thus, a remarkable change in the ω-3/ω-6 PUFA ratio in membranes of alveolar cells occurred, with the ratio raised from 1:5 to 6:1 and 3:1, respectively. According to numerous available data, we believe that a manipulation of PUFA supply modifies the cell membrane structure and consequently its function.[9,10,20,38,43] Indeed, there is a close association between the change of membrane-associated protein function linked to ω-3 supply and the LPS-stimulated cytokine response[44] (e.g. DHA decreases the responsiveness of TLR-4 to LPS).[42] The ω-3 fatty acid decreases the production of cytokines (TNF-α, IL-1β, IL-6, and IL-8) acting both directly, by replacing AA in cell membranes, and indirectly by decreasing activation of pro-inflammatory transcription factors (e.g. NFκ-B) and increasing activation of anti-inflammatory transcription factors (e.g. peroxisome proliferator activated receptor γ).[34]

An increased IL-10 (a potent anti-inflammatory cytokine) concentration was detected in supernatant of alveolar cells treated with 1:1 and 1:2 ratios. Moreover, in alveolar cells treated with the 1:1 ratio we found that the TNF-α/IL-10 ratio was 0.79, which is similar to that (i.e. 0.85) found in the bronchoalveolar lavage of patients who did not develop ARDS.[45] It was demonstrated that IL-10 suppresses LPS-induced production of TNF-α, IL-1β, and IL-6 *in vitro*, as well as reduces TNF-α concentrations post-LPS challenge in animals.[46] Thus, we hypothesized that the increased release of IL-10 could be another factor contributing to the reduction of pro-inflammatory cytokine release observed in our study.

In the alveolar spaces the balance between pro- and anti-inflammatory responses is a critical element for progression of ALI, and early activation of an anti-inflammatory response through an ω-3 supplementation could provide a mechanism for limiting the inflammatory response. However, the ω-3 supplementation is not completely without risk due to a potential excessive reduction in pro-inflammatory response, which could induce immunosuppression.[21] The ω-3/ω-6 ratio of 1:2.1 was considered 'neutral' in terms of 'immunosuppressive' effects.[19] In our study, both 1:1 and 1:2 ratios shifted the balance between pro- and anti-inflammatory cytokines towards an anti-inflammatory response; however, we believe that the 1:2 ω-3/ω-6 ratio should be preferred because it could combine efficacy and low risk of 'immunosuppressive' effects.

The novelties of this study are the use of different PUFA ratios and the use of the alveolar cell line; the limit is that we do not investigate what transcriptional mechanisms are at work in our experimental model. In fact, the study was designed as an explorative investigation and further studies are necessary to offer more mechanistic insights.

In conclusion, this study has shown that shifting the PUFA supply from AA to DHA reduced significantly the release of pro-inflammatory cytokines in human alveolar cells undergoing inflammatory stress. Unexpectedly, we found that in the presence of a PUFA ratio with an AA predominance there was a cytokine balance more oriented to a pro-inflammatory response than with LPS alone. Our results suggest that the change of the ω-3/ω-6 ratio in PUFA supply could be an important factor affecting the alveolar cytokine release. Finally, these data support the biochemical basis for current recommendations[47,48] to shift the lipid supply from ω-6 to ω-3 PUFA in nutritional support of ALI/ARDS patients.

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Figure legends

Figure 1

Effect of ω-3/ω-6 PUFA ratios on LPS-induced cytokine release from A549 cells. The results are expressed as picograms of released cytokines *per* 10^6 adherent cells (pg/10^6 cells). Data are presented as median with interquartile ranges (n = 4 experiments). *(A)* TNF-α: **p* < .001 vs. all; §*p* < .01 vs. control. *(B)* IL-6: **p* < .001 vs. all; ***p* < .01 vs. control; §*p* < .001 vs. control and 1:2. *(C)* IL-8: **p* < .001 vs. all; ***p* < .01 vs. control; §*p* < .001 vs. control and 1:2. *(D)* IL-10: **p* < .001 vs. all.

Figure 2

Effect of ω-3/ω-6 PUFA ratios on balance between pro- and anti-inflammatory cytokines. The balance was evaluated by three cytokine ratios (TNF-α/IL-10, IL-6/IL-10, and IL-8/IL-10). The results are expressed as $\log_{10}$ picograms of released cytokines *per* 10^6 adherent cells ($\log_{10}$ pg/10^6 cells). Data are presented as median with interquartile ranges (n = 4 experiments). **p* < .001 vs. all; §*p* < .001 vs. control.

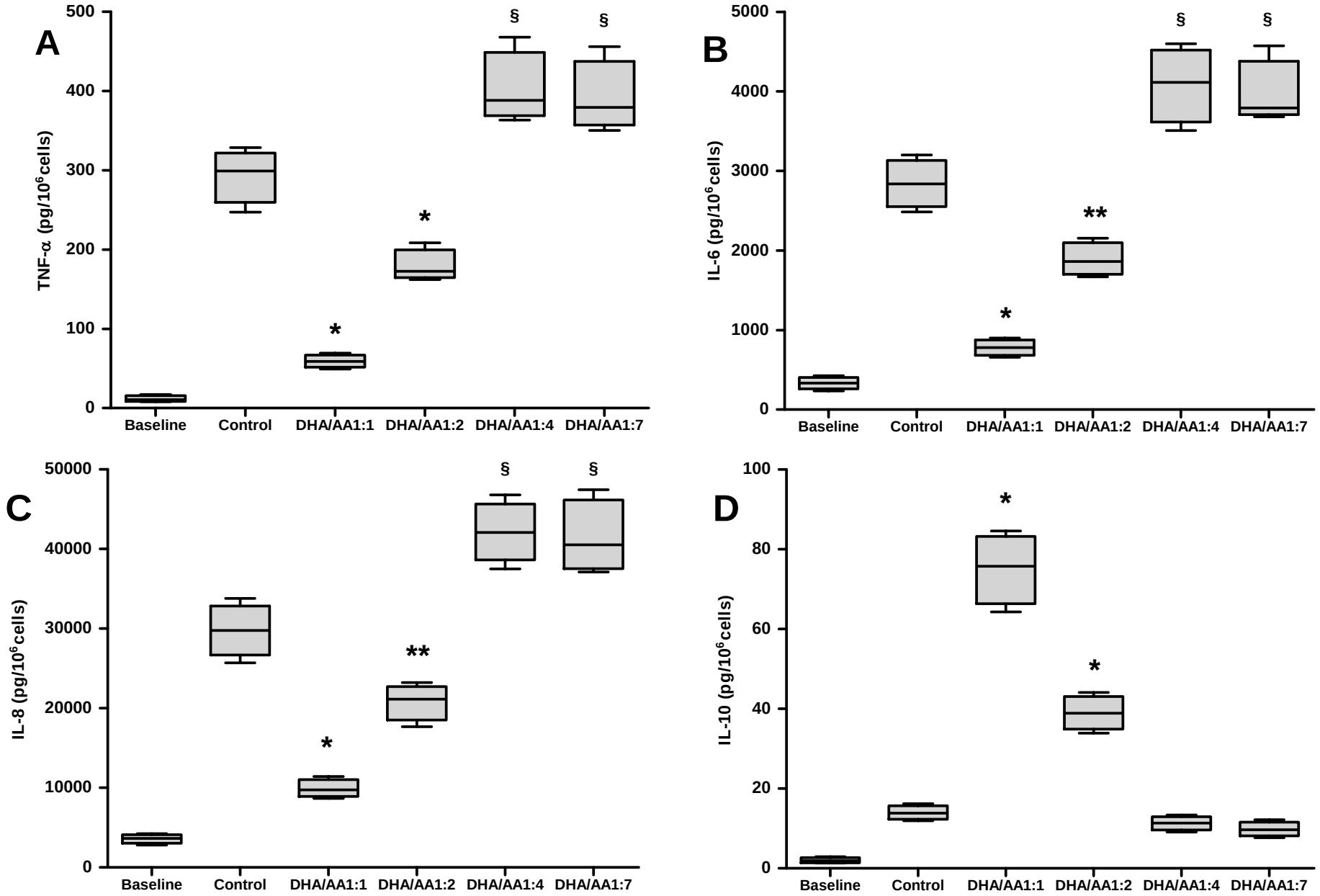
A
TNF-α (pg/10^6 cells)
500
400
300
200
100
0
§
§
*
*
Baseline
Control
DHA/AA1:1
DHA/AA1:2
DHA/AA1:4
DHA/AA1:7
B
IL-6 (pg/10^6 cells)
5000
4000
3000
2000
1000
0
§
§
*
**
Baseline
Control
DHA/AA1:1
DHA/AA1:2
DHA/AA1:4
DHA/AA1:7
C
IL-8 (pg/10^6 cells)
50000
40000
30000
20000
10000
0
§
§
*
**
Baseline
Control
DHA/AA1:1
DHA/AA1:2
DHA/AA1:4
DHA/AA1:7
D
IL-10 (pg/10^6 cells)
100
80
60
40
20
0
*
*
Baseline
Control
DHA/AA1:1
DHA/AA1:2
DHA/AA1:4
DHA/AA1:7

Figure 1

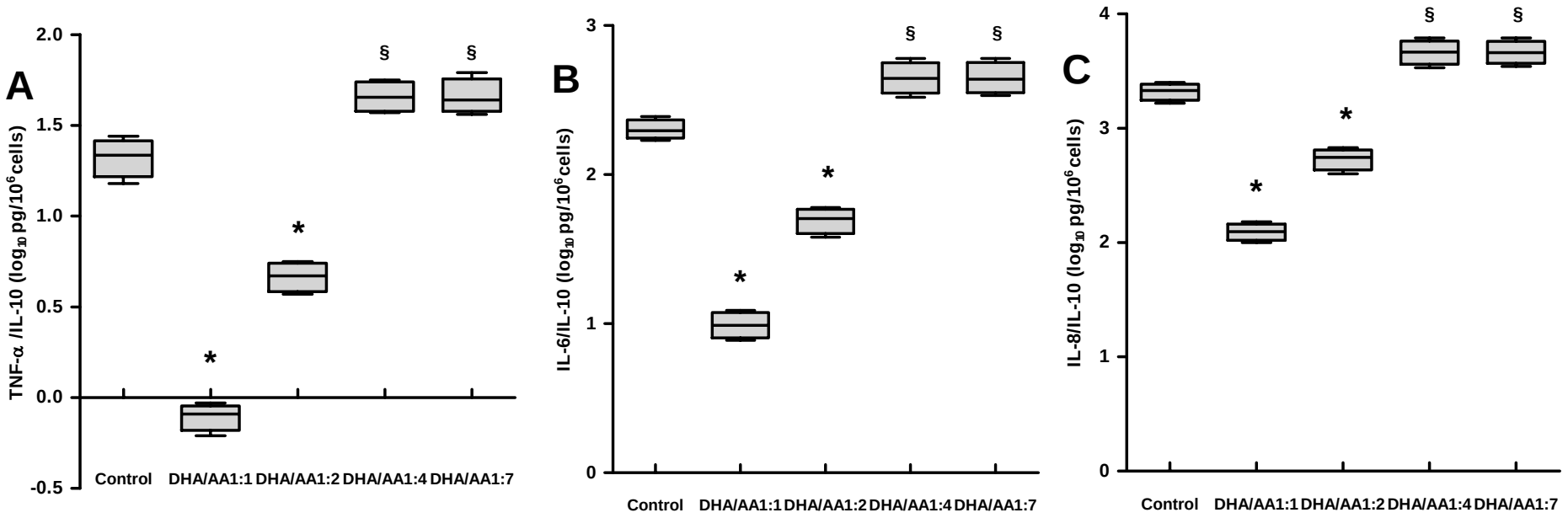
A
TNF-α /IL-10 (log10 pg/10^6 cells)
2.0
1.5
1.0
0.5
0.0
-0.5
*
*
§
§
Control
DHA/AA1:1
DHA/AA1:2
DHA/AA1:4
DHA/AA1:7
B
IL-6/IL-10 (log10 pg/10^6 cells)
3
2
1
0
*
*
§
§
Control
DHA/AA1:1
DHA/AA1:2
DHA/AA1:4
DHA/AA1:7
C
IL-8/IL-10 (log10 pg/10^6 cells)
4
3
2
1
0
*
*
§
§
Control
DHA/AA1:1
DHA/AA1:2
DHA/AA1:4
DHA/AA1:7

Figure 2

Table 1. Percentage content of fatty acids in phospholipids of A549 cell membranes at 7 h

Fatty acid	Baseline	Control	DHA/AA 1:1	DHA/AA 1:2	DHA/AA 1:4	DHA/AA 1:7
C14:0	3.7	7.2	7	6.9	7.4	7.5
C16:0	31.9	56.5	51.2	52	58.2	59.7
C16:1	9.6	1.8	1.6	1.9	2	2.2
C18:0	16.8	26.6	17.2	18.1	15.2	14.2
C18:1	27.3	4.3	3.5	3.9	4.8	5
C18:2	2.8	0.5	0.6	0.8	1.6	1.8
C20:4 (AA)	6.2[§]	0.9[§]	2.2[§]	3.5[§]	4.5[§]	4.9[§]
C22:6 (DHA)	1.7	2.2	16.7*	12.9*	6.3*	4.7*
ω-3/ω-6 PUFA ratio	*1:5*	*1.6:1*	*6:1**	*3:1**	*1:1*	*1:1.4*

PUFA, Polyunsaturated Fatty Acid; AA, arachidonic acid; DHA, docosahexaenoic acid. Data are expressed as percentage of fatty acids and are means of 4 experiments. SD (not shown) was below 10% in all cases. Percentage content of fatty acids less than 0.5% were not reported. [§]$p < .05$ to $< .001$ vs. all, except 1:4 vs. 1:7. *$p < .001$ vs. all.

Table 2. Percentage content of fatty acids in phospholipids of A549 cell membranes at 7 h

Fatty acid	Baseline	AA (50 μM)	DHA (50 μM)
C20:4 (AA)	6.1	13.5*	3.5[§]
C22:6 (DHA)	1.8	1.3	9.6*
ω-3/ω-6 PUFA ratio	*1:5*	*1:12**	*2:1**

PUFA, Polyunsaturated Fatty Acid; AA, arachidonic acid; DHA, docosahexaenoic acid. Data are expressed as percentage of fatty acids and are means of 3 experiments. SD (not shown) was below 10% in all cases. [§]$p < .05$ vs. baseline, *$p < .001$ vs. baseline.