Original Article

Inflammatory cytokine release is modified by the ratio of omega-3 to omega-6 polyunsaturated fatty acid in human alveolar cells

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ABSTRACT: ***Objective.*** *The aim of the study was to determine whether lipopolysaccharide (LPS)–induced cytokine release by human alveolar cells is modified by addition of omega (n)-3 and n-6 polyunsaturated fatty acids (PUFAs).*
Materials and methods: *After LPS challenge, PUFAs were added to A549 cells as docosahexaenoic acid (DHA, n-3) plus arachidonic acid (AA, n-6) in four different n-3/n-6 ratios (1:1, 1:2, 1:4, and 1:7), then tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), IL-8, and IL-10 concentrations were measured.*
Results: *The release of proinflammatory cytokines (TNF-α, IL-6, and IL-8) was reduced by 1:1 and 1:2 n-3/n-6 PUFA ratios ($p < 0.05$ to $p < 0.001$), but increased by 1:4 and 1:7 n-3/n-6 ratios ($p < 0.05$ to $p < 0.001$). The 1:1 and 1:2 n-3/n-6 ratios induced the release of the antiinflammatory cytokine IL-10 ($p < 0.001$). The 1:1 ratio had the greatest effect on cytokine release ($p < 0.001$).*
Conclusion: *Our results confirm that n-3 PUFAs are able to modify the balance between proinflammatory and antiinflammatory forces also via modulation of cytokine release. (Nutritional Therapy & Metabolism 2008; 26: 36-44)*

KEY WORDS: A549 cells, Acute lung injury, Acute respiratory distress syndrome, Nutrition, Omega-3 fatty acids, Polyunsaturated fatty acids

INTRODUCTION

Critically ill patients with acute lung injury (ALI) receive a nutritional support including lipids (1). In daily practice, omega (n)-6 polyunsaturated fatty acid (PUFA)–rich lipid infusions (i.e., soybean oil–based fat emulsions) are used. Both in parenteral and enteral formulas, the n-3/n-6 PUFA ratio is low (i.e., between 1:5 and 1:7). Consequently, standard artificial nutrition in ALI patients is richer in n-6 than in n-3 PUFAs.

Over the past few years, there has been an increased understanding of the pathophysiology and the mechanisms of action of PUFAs (2-4). Evidence is accumulating that the n-3/n-6 PUFA ratio may influence inflammation. In fact, mediators originating from n-3 PUFAs and those from n-6 PUFAs have opposing influences upon inflammatory processes. Though the potential beneficial effects of n-3 PUFA-enriched lipid formulas in acute respiratory distress syndrome (ARDS) have been stressed in several papers (5-7), the pharmacological effects of n-3 as modulators of lung inflammatory process have been investigated only in a few studies (8-12). Moreover, the most favorable n-3/n-6 PUFA ratio in ALI or ARDS patients is yet to be identified (13). Authors have recommended different PUFA ratios from 1:1 to 1:4 (13-15). Also, the administration of n-3 is not completely without risk, due to the possibility of excessive immunosuppression (16).

To our knowledge, no previous study has addressed the effect of different n-3/n-6 PUFA ratios on cytokine release, either in clinical conditions or in experimental models of lung cells after an inflammatory stimulus. In the present study, our main goal was to determine whether lipopolysaccharide-induced proinflammatory and antiinflammatory cytokine release from human alveolar cells might be modulated by the addition of n-3 and n-6 PUFAs to the cells.

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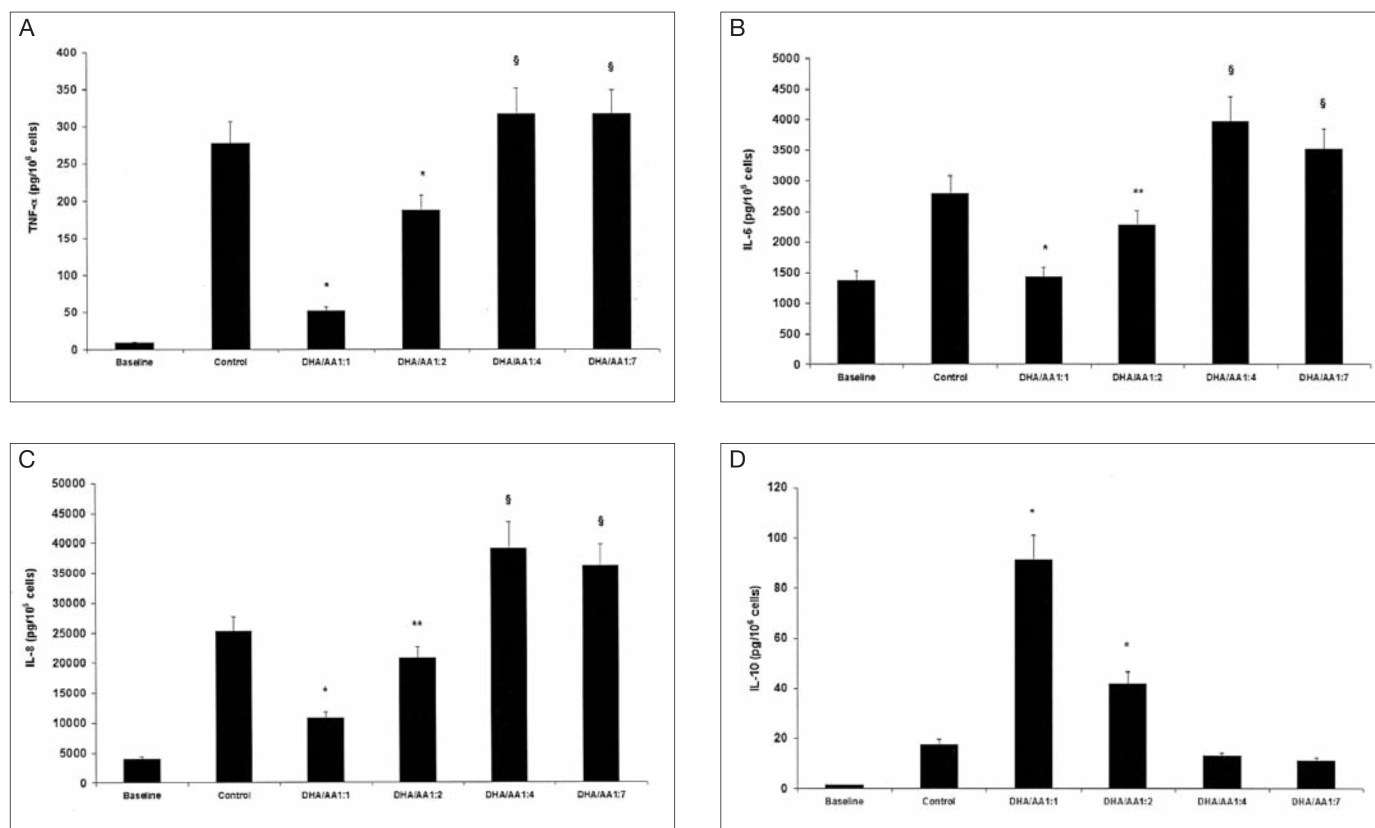

Fig. 1 - *Effect of n-3/n-6 PUFA ratios on lipopolysaccharide (LPS)-induced cytokine release from A549 cells. LPS (400 µg/mL) was added to A549 cells (except in baseline). Three hours later, docosahexaenoic acid (DHA) and arachidonic acid (AA) were added to A549 cells in 4 different PUFA ratios: 1:1, 1:2, 1:4, and 1:7 (50 µM) (except in baseline and control). The results are expressed as picograms of released cytokines per 10^6 adherent cells (pg/10^6 cells). Data are means ± SD (from 4 independent experiments for each of 4 n-3/n-6 ratios). A) TNF-α: *$p < 0.001$, vs. all; §$p < 0.05$, vs. control. B) IL-6: *$p < 0.001$, vs. all; **$p < 0.01$, vs. control; §$p < 0.001$, vs. control and 1:2. C) IL-8: *$p < 0.001$, vs. all; **$p < 0.05$, vs. control; §$p < 0.001$, vs. control and 1:2. D) IL-10: *$p < 0.001$, vs. all.*

MATERIALS AND METHODS

Ham F-12K medium, fetal bovine serum (FBS), phosphate-buffered saline (PBS), PUFAs, and lipopolysaccharide (LPS) were obtained from Sigma Chemical Company (St. Louis, MO, USA). Enzyme-linked immunosorbent assay (ELISA) kits for determination of tumor necrosis factor-α (TNF-α), interleukin (IL)-6, IL-8, and IL-10 levels were obtained from Euroclone (Paingnton-Devon, UK).

Culture of cells

A human lung carcinoma cell line (A549 cells) obtained from American Type Culture Collection (Rockville, MD, USA) was used. A549 cells are alveolar epithelial cells with the characteristics of normal type II epithelial cells (17). The A549 cells have been frequently used as a model to study functions of human alveolar epithelial cells. The A549 cells were grown as a confluent monolayer in culture plates in Ham F-12K medium containing 2 mM L-glutamine, 1% (v/v) antibiotic/antimycotic solution, 1% non-essential amino acids, and 10% (v/v) FBS, and maintained at 37°C in 5% CO_2. After 24 hours of cell seeding (30,000 cells/cm^2), the medium was removed and replaced with the same Ham F-12K medium supplemented with 2 mM L-glutamine, 1% (v/v) antibiotic/antimycotic solution, 1% non-essential amino acids, 0.4% albumin, 1% insulin, transferrin, and sodium selenite, and 1% vitamin solution. Cells were trypsinized, harvested, and centrifuged at 600*g* for 10 minutes, and cell proliferation was evaluated using a Burker chamber as number of cells present in the monolayer and in the culture medium (18).

Experimental study

After 24 hours of cell seeding, LPS was added to the A549 cells to obtain final concentrations equal to 400 µg/mL, except in non-stimulated cells (baseline) (time 0). Baseline cells received an equal quantity of PBS. Three hours after the start of LPS treatment, docosahexaenoic acid (DHA; 22:6, n-3) and arachidonic acid (AA; 20:4, n-6) were added to cell cultures at the final concentration of 50 µM, except in baseline and LPS alone cells (control). Baseline and control received an equal quantity of FBS. DHA and AA were supplied in four different n-3/n-6 PUFA ratios: one balanced (1:1) and three with an increasing n-6 to n-3 predominance (1:2, 1:4, and 1:7). Treatment period with n-3/n-6 ratios was 4 hours. After 7 hours from time 0, all culture supernatants were harvested and stored at -80°C for cytokine measurements. The release of cytokines (TNF-α, IL-6, IL-8, and IL-10) in culture media was evaluated with ELISA kits according to the manufacturer's instructions. The results were expressed as picograms of released cytokine per 10^6 adherent cells (pg/10^6 cells). Four independent experiments for each of the four different n-3/n-6 PUFA ratios were carried out.

Statistical analysis

Data are expressed as means ± SD. Multiple comparisons were carried out using one-way analysis of variance (ANOVA), followed by the Student-Newman-Keuls post hoc test. The level of significance was defined as p value < 0.05.

RESULTS

Effect of n-3/n-6 PUFA ratios on LPS-induced cytokine release from A549 cells

After 7 hours of LPS stimulation (400 µg/mL), the release of TNF-α, IL-6, IL-8, and IL-10 from control was significantly increased by 30, 2, 6, and 12 times, respectively ($p < 0.001$) (Fig. 1A-D). The 1:1 n-3/n-6 ratio induced an 81%, 49%, and 57% reduction of TNF-α, IL-6, and IL-8, respectively ($p < 0.001$), as well as a 414% increase of IL-10 ($p < 0.001$). The 1:2 n-3/n-6 ratio decreased TNF-α, IL-6, and IL-8 (32%, $p < 0.001$; 19%, $p < 0.01$; and 18%, $p < 0.05$, respectively), but less than the 1:1 n-3/n-6 ratio ($p < 0.001$), and induced a 235% increase of IL-10 ($p < 0.001$). Exposure of cell cultures to a 1:4 and 1:7 n-3/n-6 ratio induced a significant increase for TNF-α (14%, $p < 0.05$), IL-6 (42% and 26%, respectively; $p < 0.001$), and IL-8 (55% and 43%, respectively; $p < 0.001$) vs. control. The increase of proinflammatory cytokine release was substantial (69% to 508%, 55% to 177%, and 74% to 264% for TNF-α, IL-6, and IL-8, respectively) and always extremely significant also in comparison with the 1:1 and 1:2 n-3/n-6 ratios ($p < 0.001$). The 1:4 and 1:7 ratios did not affect release of IL-10.

DISCUSSION

The main purpose of our study was to investigate how LPS-stimulated cytokine release is affected by changes in the n-3/n-6 PUFA ratio. The results can be briefly summarized as follows. The addition of PUFAs in 1:1 and 1:2 n-3/n-6 ratios to the cell culture media produces a significant reduction in proinflammatory cytokines. Notably, proinflammatory cytokine release is remarkably greater with a n-6 predominance over n-3 (1:4 and 1:7). The 1:1 and 1:2 n-3/n-6 ratios are associated with the release of the antiinflammatory cytokine IL-10. The 1:1 ratio had the most relevant effects on cytokine release.

PUFAs and lung inflammation

Accepted guidelines recommend providing 30%-35% of non-protein calories as lipids in ALI or ARDS patients (13). Administration of lipid emulsions has many advantages. Compared with glucose, lipids provide a richer source of calories in a small volume with a lower CO_2 load. Moreover, lipid administration is required to avoid essential fatty acid (FA) deficiency. Conventional intravenous fat emulsions contain large quantities of n-6 PUFAs (i.e., more than 50% of linoleic acid) that can be metabolically converted to AA (n-6 PUFAs), and only 7% α-linolenic acid that forms eicosapentaenoic acid (EPA; 20:5, n-3 PUFAs) and DHA (n-3 PUFAs). Thus, parenteral lipid emulsions have a very low n-3/n-6 PUFAs ratio, ranging between 1:5 and 1:9. Similarly, the n-3/n-6 ratio in enteral products may be as low as 1:50. As a result, ALI or ARDS patients – whether on standard parenteral or enteral nutrition – are usually exposed to relatively large amounts of n-6 compared with n-3 (13, 19), which is associated with high amounts of AA, whose main functional role is to be a substrate for synthesis of bioactive proinflammatory mediators known as eicosanoids (above all, prostaglandin [PG]E_2, thromboxane [TX]A_2, and leukotriene [LT]B_4) and platelet activating factor (PAF) which modulates the intensity and duration of inflammatory responses (2, 3).

Since the 1980s, the effect of intravenous lipid administration on the pulmonary function of ventilated patients has been controversial (20, 21). Potential negative

effects have been explained by composition and/or infusion rate of fat emulsions. Indeed, many authors have suggested that these adverse effects might be related to the administration of soybean oil–based fat emulsions containing a n-3/n-6 PUFA ratio of 1:7, and they have suggested that AA-derived synthesis of eicosanoids may be the cause of worsened gas exchange and pulmonary hemodynamics in the critically ill (22-30). Eicosanoids are important pathogenetic factors of ARDS, and their adverse effects on lung function (vasoconstriction, bronchoconstriction, increase in permeability, edema formation, polymorphonuclear activation, and thrombocyte aggregation) have been widely studied (8, 9, 31-33). As a rule, practically all experimental studies with isolated lungs and culture of different pulmonary cells have showed a pivotal role of AA and its metabolites as mediators of injury (9).

In recent years, many editorials have stressed the possibility of manipulating the inflammatory response in ALI or ARDS patients using specific nutrients as pharmacological agents – e.g., n-3 PUFAs (5, 6, 13, 19). The effects of n-3 PUFAs (EPA and DHA) on the lung inflammatory process have been tested in many experimental and clinical studies. Administration of enteral formulas containing increased n-3 PUFAs reduced organ failure in a model of septic lung injury (9) and decreased alveolar production of proinflammatory mediators in an animal model of sepsis-induced ARDS (34). Data from Gadek et al (10) support the ability of an enteral formula with a high n-3/n-6 PUFA ratio (1:1) to reduce pulmonary inflammation and improve clinical outcomes (oxygenation, mechanical ventilation requirements, length of stay in intensive care unit, and new incidence of organ failure) in patients with or at risk of developing ARDS. Singer et al (11) showed that an enteral diet enriched with EPA and gamma-linolenic acid (GLA), may be beneficial for gas exchange, respiratory dynamics, and requirements for mechanical ventilation in ventilated patients with ALI. Pontes-Arruda et al (12) demonstrated that the administration of the same enteral diet was associated with lower mortality rates in ARDS ventilated patients with severe sepsis and septic shock. However, enteral administration of n-3 PUFAs may require some days before effectively influencing the cellular FA composition and the lipid-mediated inflammatory responses. In contrast, parenteral administration of PUFAs induced rapid changes in lipid-derived responses in healthy humans (35). In ALI there is the need for promptly active treatments to early modulate the net inflammatory response in the alveolar spaces (36). Currently, no clinical studies have been performed on parenteral infusion effects of n-3–enriched lipid emulsions in ALI or ARDS patients.

Timing of effect of PUFA addition

As demonstrated in previous studies in lung models, the addition of PUFAs induce rapid changes in cell membrane FA composition and lipid-derived inflammatory mediator generation. Breil et al (8) showed that a short-term n-3 supplementation induced a significant increase in the n-3 composition of perfusate (after 5 minutes) and lung tissue (after 3 hours). Yamazaki et al (37) observed a significant increase in DHA concentration in the lung phospholipid fraction 1 hour after DHA infusion. Yang et al (38) reported that PGE_3 was produced within 5 minutes of A549 cells being exposed to EPA and had a peak at 4 hours. The authors concluded that the rapid increase of PGE_3 may result from the metabolism of EPA by cyclo-oxygenases in the A549 cells. Changes in inflammatory mediator synthesis after a combined short-term intravenous administration of n-3 and an inflammatory stimulus were also examined. After as little as 3 hours of lung perfusion with an n-3 PUFA–enriched emulsion, significant alterations in the spectrum of eicosanoid generation were found in an isolated lung model (8). Grimminger et al (9) obtained the same results after 10 minutes in a similar model. Finally, Novak et al (39) reported that a 3-hour treatment period with n-3 PUFAs was enough to significantly decrease TNF-α production after LPS stimulation in a murine macrophage cell culture.

Effect of n-3/n-6 PUFA ratios on balance of cytokine release from A549 cells

ARDS is an inflammatory disease and considerable evidence indicates that an aggressive inflammatory response is determinant in its pathogenesis and progression (40, 41). Current theories suggest that it is not neutrophil and macrophage number but their proinflammatory cytokine secretion that determines injury (42). ARDS involves an intense inflammatory response within the alveolar air spaces, with accumulation of both proinflammatory and antiinflammatory cytokines (36). Cytokines appear to have concentration-dependent biologic effects (43). At low concentrations they control tissue response to insults. At higher concentrations they mediate proportionally more intense local and systemic responses. If these responses are not regulated, they may result in excessive amplification of the inflammatory process and the overproduction of proinflammatory mediators (44). The importance of a hyperinflammatory response, characterized by overproduction of TNF-α, IL-6, and IL-8, in the progression of ALI patients to ARDS is recognized. In fact, several studies have described increased mortality in patients who reveal elevated cytokine (TNF-α, IL-6, and IL-8) levels in the bronchoalveolar lavage (BAL) at

the onset of ARDS or persistent elevated levels over time (days 3-10) (40, 41, 45-48).

Increased or persistence pulmonary inflammation in patients with ARDS leads to additional non-pulmonary organ dysfunction that contributes to excess mortality rates (44). Physiologically, the critical dependency on the presence of the inflammatory ligand (e.g., PAF) and free AA may help to restrict the burst of lipid-mediator formation to sites of inflammatory events (33). In contrast, compartmentalization of alveolar cytokines can be lost in injured alveolar barrier. Cytokines produced in the lungs may enter into systemic circulation leading to initiation or propagation of a systemic inflammatory response (49, 50).

The reason some patients with ALI develop ARDS whereas others recover remains unclear. In ARDS-developing patients, it was supposed that, at some level, regulation of the inflammatory response must be deficient, but why this regulation is ineffective or insufficient in this clinical situation is an unanswered question (51). The investigation of the cytokine balance between proinflammatory and antiinflammatory cytokines could be of greater relevance to understanding the pathophysiology of ALI than a single cytokine concentration, because the degree of this cytokine imbalance may be a contributing factor to disease severity (36, 42).

Data from the literature suggest that elevated levels of proinflammatory cytokines associated with decreased production of those that are antiinflammatory correlate with the severity of lung injury and its outcome. Pugin et al (52) suggested that the proinflammatory activity is dependent on the balance between the cytokine and its inhibitor(s). In the early phase of ARDS, the measurement of low concentrations of antiinflammatory cytokines (i.e., IL-10 and IL-1ra) in the BAL is associated with increased patient mortality (53). The ratio TNF-α to IL-10 in the BAL fluid was significantly higher (3.52 vs. 0.85) in patients with ARDS than in patients at risk of developing ARDS (54).

Mechanisms of PUFA modulation of cytokine release

Our results provide adequate evidence that cytokine release can be modified by varying the n-3/n-6 PUFA ratio. However, our study was designed as an explorative investigation and limited details of mechanisms of action may be inferred. In recent years, many different mechanisms for the interaction between n-3 PUFAs and inflammation or immune response have been demonstrated (2-4, 7). Besides, the mechanisms underlying the effect of n-3 PUFA on cellular event modulation are still yet to be completely elucidated.

In the present study, several mechanisms of action which have been related to n-3 regulatory potential may have contributed to modulate the inflammatory response of alveolar cells. After LPS challenge, when as secondary stimulus, alveolar cells were incubated with PUFAs, many functions were shown to be rapidly modified. These functions include mainly eicosanoid and cytokine productions. We assume that PUFA administration induced the eicosanoid production in our model, and this aspect was not investigated within the scope of the present study. This assumption is supported by experimental and clinical studies in lung tissues (8, 9, 29, 33, 34, 38, 55, 56). The alveolar cells are able to synthesize eicosanoids from PUFAs (54). Which eicosanoids are synthesized by A549 cells depends upon the availability of precursors in cell membranes (56). Moreover, studies on PUFA supply have related to the differential synthesis of lipid-derived inflammatory mediators to a critical dependence on exogenous free FA provided, both in human alveolar cells (55) and in perfused lungs (33).

Previous findings demonstrated that shifting PUFA supply from an n-6 to an n-3 prevalence changed the composition of phospholipids in many different cells. Consequently, during LPS challenge, the competing n-3 and n-6 PUFAs incorporated in membranes are hydrolyzed by various phospholipases resulting in different bioactive mediators, LTB_5 vs. LTB_4 and PGE_3 vs. PGE_2, respectively. The changes in the nature of inflammatory mediator production have relevant consequences. For instance, a higher concentration of n-3 in the membranes results in the formation of smaller amounts of LTB_4 if compared with LTB_5, which is at least 10-fold less potent as an inflammatory mediator (3).

In our experimental study, n-3 PUFAs was given as DHA, which is the most abundant n-3 PUFAs in the A549 cells as well as in most tissues. Although DHA is not able to give rise to leukotrienes, it has a pivotal role because it can be incorporated unchanged into membrane lipids or retroconverted into EPA (13). In A549 cells treated with the 1:1 n-3/n-6 ratio, PGE_2 levels were significantly reduced compared with those cells treated with AA alone; also, EPA and DHA were equally able to inhibit PGE_2 production (38). Animal studies suggest that both EPA and DHA have immunomodulatory effects. Evidence suggests that the antiinflammatory and immunosuppressive effects of n-3 supply may be attributed mainly, or even exclusively, to DHA supplementation (35, 57-59).

Some studies have shown that n-3 PUFA administration results in reduced proinflammatory cytokine production (15, 57, 60). The conclusions of these studies are that because proinflammatory cytokine production is regulated by AA-derived eicosanoids (61) and because n-3 PUFAs modify eicosanoid production, it is expected that n-3 PU-

FAs modulate cytokine production. For instance, LTB_4 enhances production of proinflammatory cytokines (TNF-α, IL-1, IL-6, IL-2, and IFN-γ) (3). Furthermore, it was demonstrated that an eicosanoid-dependent autoamplification loop controls TNF-α and IL-1β production by TXA_2, a potent AA-derived eicosanoid (35). A clear autoregulatory relationship between TNF-α and proinflammatory PG_2 was reported (39). Based on these data, we suggest that reducing the availability of AA in A549 cell membranes may explain the reduction in TNF-α, IL-6, and IL-8 levels.

An interesting finding of this study was that a considerable IL-10 concentration was detected in the cell cultures treated with 1:1 and 1:2 n-3/n-6 PUFA ratios. It is known that there is a close association between the change in membrane FA composition and the cell functions, e.g., binding of cytokines to receptors and the lipid-dependent signal transduction pathway that leads to cytokine production (3, 4, 30). Recent studies suggested that n-3 change the sensitivity of the toll-like receptor 4 for LPS, consequently modifying transduction of proinflammatory signals into the cell interior (39). Our data support but do not prove the hypothesis that the increased IL-10 concentration may be one of the further factors contributing to significantly reduce proinflammatory cytokine release. Indeed, the IL-10 inhibition of cytokine release by different mechanisms is well demonstrated (62, 63). Studies in animals also showed that the inhibition of endogenous IL-10 results in enhanced lung injury (64). IL-10 has been shown to reduce mortality rates and the development of ALI when administered to rabbits with pancreatitis (65).

CONCLUSION

The balance between proinflammatory and antiinflammatory cytokines is more important for the outcome than the absolute concentration of proinflammatory cytokine(s). In ALI, cytokine balance is oriented toward inflammation. Nowadays, strategies for redressing the balance of the lung inflammatory response are therefore of great importance.

The present study is the first to demonstrate that the LPS-induced release of several cytokines can be differently modulated by different PUFA ratios in human alveolar cells. We found that the n-6 PUFAs – the conventional lipids that are commonly delivered in nutrition of ALI or ARDS patients – can themselves lead to an increase in proinflammatory cytokine release. On the other hand, our data indicate that lowering the n-3/n-6 ratio in the PUFA supply, thus increasing n-3 over n-6, correlates with a significant reduction of proinflammatory cytokine release.

There are some limitations to this study. It is obvious that our in vitro model does not fully represent the in vivo alveolar environment. Although A549 cells have been used in many studies of lung pathophysiology, we can not infer that these results obtained in human lung cells may be reproducible in vivo. However, this is a general comment for all findings resulting from cell line studies (66). Also, in this paper we did not show data on transcriptional mechanisms of our experimental model. In ALI, the balance of proinflammatory to antiinflammatory cytokines is regulated by a highly intricate network of transcription factors (67).

Despite these limitations, two conclusions can be drawn from the present study. First, our data show that the n-3/n-6 PUFA ratio per se may be an important factor in determining the net cytokine balance in alveolar cells. Second, our study supports the hypothesis that one of the most relevant effects of n-3 is related to their ability in restoring the balance between proinflammatory and antiinflammatory forces via modulation of cytokine release.

The conclusions from this study show clear evidence of the need for considerable further work. Additional studies, testing intravenous administration of different n-3/n-6 PUFA ratios in animal models as well as in ALI or ARDS patients, are required to evaluate their effects on inflammatory response as well as on clinical end points. Until then, these data could support the expert recommendation to reduce amounts of n-6 PUFAs in artificial nutrition prescribed for these patients.

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