Alteration of n-3 fatty acid composition in lung tissue after short-term infusion of fish oil emulsion attenuates inflammatory vascular reaction

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Abstract

Objectives

To investigate whether modulation of the fatty acid profile can be achieved by the short-term infusion of a fish oil emulsion which may attenuate the pulmonary response to inflammatory stimulation. Changes of fatty acid pattern in lung tissue and perfusate were analyzed and correlated with physiologic data after a 3-hr infusion of fish oil in comparison with a soybean oil preparation.

Design

Prospective, randomized, controlled trial.

Setting

Experimental laboratory in a university teaching hospital.

Subjects

Forty standard breed rabbits of either gender.

Interventions

Isolated lungs from anesthetized rabbits were ventilated and recirculation-perfused (200 mL/min) with 200 mL of cell-free buffer solution to which either 2 mL of saline (control, n = 6), 2 mL of a 10% soybean oil preparation (n = 6), or 2 mL of a 10% fish oil emulsion (n = 6) were added. Samples of perfusate and lung tissue were collected for analysis of fatty acid composition. Tissue and perfusate fatty acid composition were analyzed by capillary gas chromatography. To study metabolic alterations in states of inflammatory stimulation, lungs of each group were stimulated with small doses of the calcium ionophore, A23187 (10 sup -8 M), during the 180-min lipid perfusion period and again after washing out the lipids by exchanging the perfusion fluid. Pulmonary arterial pressure and lung weight gain were monitored, and eicosanoids were analyzed in the perfusate.

Measurements and Main Results

Free eicosapentaenoic acids increased several-fold in lung tissue and perfusate during a 3-hr infusion with fish oil. The intravenously administered n-3 fatty acids were rapidly hydrolyzed, as indicated by the appearance of substantial quantities of eicosapentaenoic acid in the perfusate free fatty acid fraction. This increase of perfusion levels of eicosapentaenoic acid was paralleled by an attenuated pressure increase and edema formation due to calcium ionophore challenge and an altered eicosanoid spectrum determined in the perfusate compared with soybean oil-treated lungs.

Conclusion

Short-term n-3 lipid application (fish oil emulsion) exerts anti-inflammatory effects on lung vasculature, which may be due to the metabolism of eicosapentaenoic acid resulting in the generation of less potent inflammatory eicosanoids.

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Key Words: n-3 fatty acids; fish oil; eicosapentaenoic acid; arachidonic acid; metabolization; lung; inflammation; permeability; vascular resistance; eicosanoids

Dietary supplementation with fish oil, a source of highly polyunsaturated n-3 fatty acids, has been demonstrated to exert beneficial effects in a number of inflammatory diseases [1-4]. When n-3 fatty acids are included in the diet, eicosapentaenoic acid (C20:5n-3) competes with arachidonic acid (C20:4n-6) on the cyclo-oxygenase and on the 5-lipoxygenase levels. This circumstance causes modulation of the products of diene prostanoids (e.g., prostaglandin E_2, prostaglandin I_2, thromboxane A_2) and of tetraene leukotrienes (e.g., leukotriene C_4), derived from arachidonic acid in favor of the corresponding triene prostanoids (e.g., prostaglandin E_3, prostaglandin I_3, thromboxane A_3) and pentaene leukotriene (leukotriene C_5) derived from eicosapentaenoic acid [5]. Examination of the eicosapentaenoic/arachidonic acid ratio in several cells, i.e., erythrocytes [6], neutrophils [7,8], platelets [9,10], endothelial cells, monocytes, and brain and liver cells [11]

demonstrated that n-3 fatty acids are preferentially incorporated into membranes, resulting in an enhanced release of eicosapentaenoic acid-derived products with an attenuated spectrum of biological activity instead of those products derived from arachidonic acid [12-15].

Profiting from this knowledge, n-3 fatty acids-enriched lipid infusions have been used for combining parenteral nutrition with an anti-inflammatory intervention, by shifting the arachidonic acid/eicosapentaenoic acid ratio toward predominance of the latter lipid mediator precursor [16-18]. Although these studies [16-18] investigated the dietary intake of n-3 fatty acids following long feeding periods, the main question posed in this study is whether omega-3 fatty acids would be metabolized and integrated into the phospholipid pool of cell membranes, even during short-term infusion (3 hrs), thus leading to dampened inflammatory events.

We specificallly studied the influence of a 3-hr fish oil supplementation in comparison with a classic soybean oil preparation on the incorporation of the long-chain fatty acids into plasma lipids and in lung tissue in an experimental rabbit lung model. Moreover, we investigated the impact of lipid infusions on 2- vs. 3-series lung eicosanoid generation, as well as on vascular reaction and edema formation in response to challenge with the calcium ionophore A23187. As an unspecific inflammatory stimulus, the calcium ionophor A23187 was used to stimulate an inflammatory response with enhanced eicosanoid synthesis. A23187 induces an intracellular calcium influx, thereby activating the enzyme phospholipase A_2, which liberates the precursor fatty acids required for eicosanoid synthesis from membrane phospholipids.

MATERIALS AND METHODS

The Lung Model.

The techniques of preparing and perfusing isolated rabbit lungs have been previously described in detail [19,20]. Standard breed rabbits of either gender, weighing 3150 +/- 186 (SD) g, were anesthetized with sodium pentobarbital (60 to 80 mg/kg) and anticoagulated with heparin-sodium 1000 U/kg body weight. The isolated lungs were suspended from an electronic weight balance (Baldwin Me beta technik Type U1, Hottinger, Darmstadt, Germany) in a tempered (37 degrees C) and humidified chamber, and were perfused with cell- and plasma-free Krebs-Henseleit-hydroxy-ethyl-starch buffer solution at a constant flow of 200 mL/min in a recirculating system (circulating volume 200 mL). Ventilation was performed with 4% CO_2 in air (frequency 25 breaths/min, tidal volume 30 mL, positive end-expiratory pressure 0.5 to 1.0 cm H_2O). The pulmonary arterial pressure, airway pressure, and weight of the isolated lung were recorded continuously by means of pressure and weight transducers. Due to a constant perfusion flow, alterations of perfusion pressure directly reflected alterations of pulmonary vascular resistance. Samples of perfusate were taken at 30-min intervals for measurements of PO_2, PCO_2, oxygen saturation (ABL 330, Radiometer Copenhagen), and oncotic pressure (Onkometer BMT 921, Dr. Karl Thomae GmbH, Biberach, Germany). Initially, the lungs were perfused with Krebs-Henseleit-hydroxy-ethyl-starch buffer solution, using low flow rates in the open circulatory system to remove remaining blood in the vascular bed. The perfusion fluid was then exchanged for fresh buffer via two separate perfusion circuits 2 mins after the beginning of the extracorporeal circulation and 30 mins later, after the flow was increased to 200 mL/min. The Krebs-Henseleit-hydroxy-ethyl-starch buffer perfusion was able to maintain the integrity of the microcirculation for >5 hrs in our model, verified by ultrastructural studies. Homogeneous capillary organ perfusion and the absence of structural endothelial damage (e.g., vacuolization, mitochondrial disintegration, or hydropic swelling of endothelial cells) could be confirmed by light- and electronmicroscopic controls. No relevant alterations in vascular tone (< +/- 2 mm Hg), permeability (weight increase <1.5 g) or mediator (histamine, thromboxane) release occurred during this observation period. Criteria for acceptance in the present study were that the lungs had a homogeneous white appearance, without

signs of hemostasis or edema formation (weight gain 0 g/min), without changes in vascular resistance (+/- 1 mm Hg) during the 30-min equilibration period.

Experimental Protocol.

The lung preparations were randomly assigned to groups of six lungs each. Following a 30-min equilibration period, the first perfusate sample was drawn for measurements of baseline values. To study the incorporation and metabolization of the lipids, either 2 mL saline (controls, n = 6), 2 mL of soybean oil preparation (Lipovenos Registered Trademark 10%, Fresenius AG, Bad Homburg, Germany) (n = 6), or 2 mL of a fish oil emulsion (Omegavenos Registered Trademark 10%, Fresenius AG) (n = 6), corresponding to 70 mg lipids/kg body weight, were added to the perfusate. During the following 180-min perfusion period, the lungs were stimulated with lower doses of calcium ionophore (10 sup -8 M) three times at 30, 90, and 150 mins after commencing lipid perfusion to induce an enhanced eicosanoid turnover. After 180 mins of lipid perfusion, the lipids were washed out by exchanging the perfusion fluid (CP) with lipid-free Krebs-Henseleit-hydroxy-ethyl-starch buffer solution. After another 30-min equilibration period at 210 mins, the lungs were stimulated with a calcium ionophore A23187 (10 sup -7 M) dose that was ten times higher. Samples for determination of free and total plasma fatty acids, as well as for histamine and arachidonic acid metabolites, were taken at short intervals. Since arachidonic acid and eicosapentaenoic acid are precursors of metabolites, which differ in one double bond, lipoxygenase products were differentiated as to the tetraene and pentaene leukotrienes. The same protocol was used in four experimental settings for leukotriene analysis in which glutathione was added to the perfusate, yielding a final concentration of 2.5 mg/mL in the perfusion fluid to prevent conversion of leukotriene C to leukotrienes D and E. When the experiments were finished, lipidcontaining perfusate was removed and subsequently lung tissue samples were taken to find out whether polyunsaturated fatty acids had accumulated in the lungs after elimination of the circulating lipids.

The dosage of A23187 was selected to investigate acute effects due to unspecified inflammatory stimulation on pulmonary vascular resistance and permeability and mediator release. Pilot studies (n = 8) show that this dose was able to induce inflammatory reactions, like significant histamine release and eicosanoid generation, a marked activation of granulocytes with elastase release, and a moderate increase in pulmonary vascular resistance and microvascular permeability. Higher doses resulted in overwhelming pressure increases followed by immediate alveolar flooding and severe barotrauma of the lung, whereas lower doses produced an insufficient mediator release, hardly detectable in the perfusate by the methods used.

In additional pilot experiments (n = 10), we tested whether the perfusion with either soybean oil or fish oil per se leads to vascular reactions and mediator release in our model. Since increasing doses from 1 mL up to 10 mL per 200 mL perfusate of each lipid emulsion did not induce alterations in vascular tone and permeability or mediator generation, the observed effects after A23187 stimulation could be attributed to the different spectrum of metabolites.

Analysis of Fatty Acid Content Within Lung Tissue and Perfusate.

Samples of perfusate were frozen at -80 degrees C until they were required for measurement of fatty acid composition. Perfusate (100 micro Liter) samples were analyzed with heptadecanoic acid (C:17) as an internal standard. Free fatty acids were converted to methylester by mixing them with methyliodide; methylation was achieved over a solid phase of potassium carbonate and products extracted in the presence of chloroform [21]. The probes were dried, redissolved in chloroform, and transferred to gas chromatography. Gas chromatography analysis was performed in a gas chromatograph (CP HP 5890, Hewlett Packard, Palo Alto, CA) with 60 m times 0.32 mm internal diameter glass columns. The temperature was increased to 250 degrees C and maintained for 45 mins. The fatty acid methylesters were detected by use of a flame ionization detector, and peak area integration was carried out.

For quantifying of total plasma and lung tissue fatty acids, 200 micro Liter of perfusate or 150 mg of lung tissue were subjected to hydrolysis in hydrochloric acid (6 hydrochloric acid, 2 hrs, 120 degrees C) and then extracted in chloroform/methanol (2:1, volume/volume). After evaporation, the residues were redissolved in methanol/hydrochloric acid (1.5 hrs, 120 degrees C), then evaporated once more to dryness, and finally redissolved in chloroform and subjected to gas chromatographic analysis.

Radioimmunoassay of Thromboxane B sub 2 and 6-keto Prostaglandin F sub 1 alpha.

Thromboxane B_2 and 6-keto-Prostaglandin F_1 alpha were assayed serologically from 100 micro Liter of recirculating Krebs-Henseleit-hydroxy-ethyl-starch buffer solution as stable hydrolysis products of thromboxane A_2 and prostacyclin by radioimmunoassay, according to a method described by Peskar et al [22]. Radioactivity was quantified with a Philips PW 4700 liquid scintillation counter (2000 Ca, Canberra Packard, Frankfurt, Germany). Results were obtained by standard constructed doseresponse curves. The cross-reactivity of thromboxane B_2-antiserum with prostaglandin D_2 was 2.7%, and 0.05% with 6-ketoprostaglandin F_1 alpha prostaglandin E_2, prostaglandin E_1, 6-ketoprostaglandin F_1 alpha, 13,14-dihydro-15-keto prostaglandin E_2, and 13,14-dihydro-15-keto prostaglandin E_2 alpha, respectively. The cross-reactivity of 6-ketoprostaglandin F_1 alpha antiserum was 0.05% with thromboxane B_2, as well as with the above-mentioned prostaglandins.

Radioimmunoassay of Histamine.

Histamine was measured by means of a commercially available solid phase radioimmunoassay test kit (see Materials and Methods section). The acylated histamine in the sample and the acylated^{125} I-histamine compete for the limited monoclonal antibody binding-sites, which were coated on the surface of the test tubes. Cross reactivities of the monoclonal antibodies used in the test kit with the following substances were determined: acylated histamine (100%), acylated t-methylhistamine (69 times 10 sup -4 %), acylated histidine (4 times 10 sup -4 %), acylated serotonin (<10 sup -4 %), cimetidine (6 times 10 sup -4 %), ranitidine (<10 sup -4 %), and histamine (5 times 10 sup -4 %).

Reversed-Phase High-Pressure Liquid Chromatography.

Leukotriene C_4 and leukotriene C_5 released into the perfusion fluid were determined using reversed-phase high-pressure liquid chromatography followed by radioimmunoassay of the eluted fractions [23,24]. Aliquots of perfusate were acidified with 1 N hydrochloric acid to a pH of 3.0 and extracted using SEP-PAK C sub 18 cartridges (Waters Associates, Milford, MA). Adsorbed products were purified by consecutive elution with 0.1 M of sodium phosphate buffer (pH 7.4), distilled water, hexane, and methyl formate (10 mL each). Leukotriene C was then eluted with 10 mL of methanol, and the solvent was evaporated. The residues were taken up in 0.05 mL of 30% methanol in distilled water and chromatographed using reversed-phase high-pressure liquid chromatography (Nucleosil C_{18}, particle size 5 micro meter, mobile phase: methanol/water/acetic acid 68:32:0.01 [pH 5.5, adjusted with ammonium hydroxide]). The eluates were collected in 1-mL fractions and then evaporated. The residues were taken up in 0.5 mL of 0.01 M Tris (hydrochloric acid buffer [pH 7.4]) containing 1 mg of gelatin per mL, and were analyzed for leukotriene C by radioimmunoassay [23]. The antiplasma recognizes leukotriene C_4 to the same extent as it does leukotriene C_5. The retention times of immunoreactive materials were compared with the retention times of authentic standard leukotriene C_4 and leukotriene C_5 (kindly donated by Dr. A.W. Ford-Hutchinson, Merck Frosst Laboratories, Pointe Claire, Dorval, PQ, Canada). Prostaglandin B_2 (10 ng, Sigma, St. Louis, MO) serves as the internal standard.

Materials.

A cell- and plasma-free perfusion medium was used in the present study in order to avoid the complex interactions with different circulating cells, which may mask direct effects on vascular tone and mediator release. For perfusion, a Krebs-Henseleit buffer solution was mixed with human albumin (Rhodalbumin Registered Trademark 20%, Merieux GmbH, Leimen, Germany) in order to keep the colloid oncotic pressure between 23 and 25 mm Hg. The used buffer solution yielded final concentrations in the perfusate of: albumin 20 g/L; sodium 138 mmol/L; potassium 4.5 mmol/L; magnesium 1.33 mmol/L; chloride 135 mmol/L; ionized calcium 2.38 mmol/L; glucose 12 mmol/L; and bicarbonate 12 mmol/L. The osmolality was approximate 330 mosm/kg (Mikro-Osmometer, Roebling Me beta technik, Berlin, Germany). The pH of the buffer solution was adjusted to 7.4 with 1 M of sodium bicarbonate. Effects due to endotoxin contamination of the plasmafree perfusate can be excluded in our model as assessed in previous experiments. No hemodynamic reactions, thromboxane generation, or histamine release were observed following endotoxin addition to the perfusate in the absence of plasma complement components.

Rabbit anti-thromboxane B_2 and rabbit anti-6-keto-prostaglandin F_1 alpha were purchased from Paesel (Frankfurt, Germany),^{3} H-labeled thromboxane B_2 and^{3} H-labeled-6-keto-prostaglandin F_1 alpha from New England Nuclear (Dreieich, Germany), and precipitating goat antirabbit antibodies from Calbiochem-Behring (Frankfurt, Germany). A radioimmunoassay test kit from Immunotech (Hermann Biermann GmbH, Bad Nauheim, Germany) was used for determining histamine concentrations. Glutathion was purchased from Merck (Darmstadt, Germany).

The lipid emulsions prepared from fish oil and soybean oil were a generous gift from Fresenius AG (Bad Homburg, Germany). Both are 10% fatty acid emulsions. The used batches of the 10% fish oil emulsion, rich in long-chain n-3-fatty acids, yielded the following final concentrations in the emulsion: eicosapentaenoic acid (C20:5n3) 19 mg/mL, docosahexaenoic acid (C22:6n3) 25 mg/mL, palmitic acid (C16:0) 12 mg/mL, stearic acid (C18:0) 2.5 mg/mL, oleic acid (C18:1n9) 14 mg/mL, linoleic acid (C18:2n6) 2.7 mg/mL, linolenic acid (C18:3n3) 0.7 mg/mL, arachidonic acid (C20:4n6) 2.2 mg/mL, and other fatty acids 27.3 mg/mL. The major components of the soybean oil preparation, rich in n-6 fatty acids, were: linoleic acid 51 mg/mL, linolenic acid 7.2 mg/mL, oleic acid 22.4 mg/mL, palmitic acid 12 mg/mL, stearic acid 4 mg/mL, and other fatty acids 10 mg/mL.

Statistical Analysis.

Data are presented as mean +/- SEM. Differences between groups were tested by one-way analysis of variance followed by a Scheffe's multiple comparison procedure. Significance was accepted at $p < .05$.

This study was approved by the Animal Subject Protection Committee of the University of Giessen. The care and handling of animals were in accordance with the National Institutes of Health guidelines.

RESULTS

After an isogravimetric equilibration period, either saline, soybean oil, or fish oil was given to the perfusate.

Fatty Acid Composition in Plasma and Lung Tissue.

The 3-hr supplementation of n-3 fatty acids resulted in a marked change of the fatty acid composition of perfusate and lung tissue.

Zero concentrations of docosahexaenoic acid and eicosapentaenoic acid, and only small amounts of arachidonic acid of total plasma content, as well as of free plasma acids, were detected in the perfused lungs before starting the perfusion with lipid emulsion. The amount of palmitic acid varied from 32 mol% to 34 mol%, linoleic acid from 18 mol% to 20 mol%, stearic acid from 9 mol% to 10 mol%, and oleic acid from 30 mol% to 32 mol%. The evolution of the most relevant fatty acids in this study, arachidonic acid, eicosapentaenoic acid, docosahexaenoic acid, palmitic acid, linoleic acid, oleic acid, and stearic acid are presented in Table 1.

Table 1:
Fatty acid pattern of perfusate lipids (mean +/- SEM)

Five minutes after commencement of the n-3 polyunsaturated fatty acid infusion, the total perfusion concentrations of eicosapentaenoic acid and docosahexaenoic acid dramatically increased, surpassing those concentrations of arachidonic acid several times Figure 1. Despite continuation of fish oil supplementation via the intravenous route, the total plasma content of eicosapentaenoic acid and docosahexaenoic acid decreased to values of 9.1 +/- 1.5 and 9.4 +/- 1.7 mol%, respectively, whereas arachidonic acid concentrations remained roughly unaltered. This significant decrease of the n-3 fatty acids was accompanied by a three- to ten-fold increase in the corresponding nonesterified plasma fatty acid concentrations, eicosapentaenoic acid and docosahexaenoic acid, during the 3-hr perfusion period (4.0 +/- 0.5 mol% and 5.0 +/- 0.8 mol%, respectively). At the expense of this increased content of n-3 fatty acids, oleic and linoleic acid tended to decrease from 31.2 +/- 0.8 mol% and 20.2 +/- 1.9 mol%, respectively, after 5 mins of infusion, to 22.4 +/- 0.8 mol% and 13.4 +/- 1.2 mol%, respectively, after 180 mins. Palmitic and stearic acid did not substantially change. However, regarding total amounts, the n-6 fatty acids significantly ($p < .001$) decreased during the fish oil infusion period.

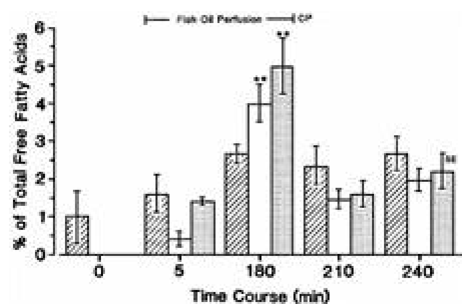

Figure 1:
Time course of detection of arachidonic acid (reverse-hatched bars), eicosapentanoic acid (open bars), and docosahexeanoic acid (dotted bars) in the perfusate lipids. Perfusate was collected during and after fish oil emulsion supplementation. Values at 210 and 240 mins were obtained after elimination of circulating lipids by exchanging the perfusion fluid (CP). Fatty acids linked to complex lipids were hydrolyzed as described. The quantities of arachidonate, eicosapentanoate, or docosahexeanoate acid are expressed as a percentage of the total amount of free fatty acids. Data are presented as mean +/- SEM from six lungs. **$p < .001$ vs. corresponding baseline values (0 min), as well as vs. values after 5 mins of lipid infusion.

In accordance with the high intake of linoleic acid provided by the soybean oil preparation, there was a 197% increase of this free fatty acid in the perfusate triglyceride fraction. Palmitic and oleic acid concentrations decreased during the perfusion period; this decrease was more pronounced

after soybean oil application compared with fish oil infusion. Arachidonic acid and stearic acid values remained unchanged.

(Figure 2) shows the incorporation of various fatty acids into the rabbit lungs. Lungs receiving saline, which represented the control lungs, demonstrated a preponderance of n-6 incorporation with high concentrations of palmitic acid (40.0 +/- 0.8 mol%), linoleic acid (10.0 +/- 0.2 mol%), and arachidonic acid (7.5 +/- 0.3 mol%), while almost undetectable amounts of the n-3 fatty acids, eicosapentaenoic acid, and docosahexaenoic acid were found in the perfusate. When the lungs were perfused with fish oil for 3 hrs, there was a marked ($p < .001$) increase in lung n-3 fatty acid incorporation. At the end of the experiments, eicosapentaenoic acid and docosahexaenoic acid accounted for 6.22% and 6.17% of the total fatty acids content, respectively, paralleled by a corresponding reduced content of n-6 fatty acids compared with control lung tissue. When lungs were perfused with the soybean oil preparation containing high concentrations of n-6 fatty acids, an increase in linoleic acid was seen along with a significantly ($p < .001$) smaller portion of arachidonic, palmitic, and stearic acid. The perfusate of soybean oil-treated lungs was completely free of eicosapentaenoic acid, and only low concentrations of docosahexaenoic acid were detected.

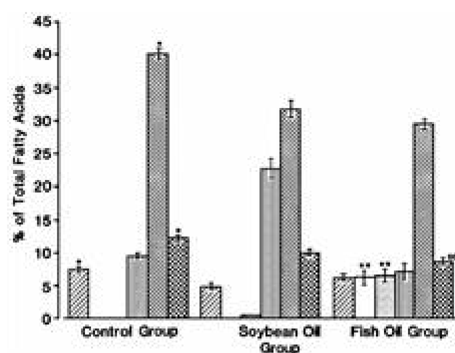

Figure 2:
Influence of lipid infusion on fatty acid content of lung tissue. Lung tissue samples were collected from rabbit lungs following treatment with saline (control, n = 6), soybean oil preparation (Soybean Oil Group) (n = 6), or fish oil emulsion (Fish Oil Group) (n = 6). *$p < .05$; **$p < .001$ vs. all other groups. Data are expressed as mean +/- SEM. Reverse-hatched bars, arachidonate acid; open bars, eicosapentanoate acid; dotted bars, docosahexeanoate acid; vertical bars, linoleic acid; hatched bars, palmitic acid; criss-crossed bars, stearic acid.

After 3 hrs of lipid infusion, intravenous lipid administration was finished and circulating lipids were completely washed out by exchanging the perfusion fluid in all groups. The total elimination of circulating lipids in the perfusate was verified in exemplary measurements of the lipid composition using gas chromatography, which revealed the exchanged perfusion fluid to be free of fatty acids.

Measurements of the content of fatty acids in the perfusion fluid 30 and 60 mins after exchanging the perfusate demonstrated that concentrations of all measured fatty acids were restored in amounts of approximate 35% (eicosapentaenoic acid and docosahexaenoic acid) and 89% (arachidonic acid) of the peak values during the perfusion period, reflecting the exchange rate between tissue and the plasma free fatty acid pool Figure 1. Even in this lipid-free perfusing period, higher concentrations of eicosapentaenoic acid and docosahexaenoic acid were found in the perfusate of fish oil-treated lungs compared with soybean oil-treated and control-treated lungs.

Generation of Mediators.

The lipoxygenase pathway products, leukotriene C_4 and leukotriene C_5, were quantified three times (at 30, 60, and 90 mins) before and after activation of the lungs by the calcium ionophore A23187 (10 sup -8 M) in the lipid-perfusion phase. Additionally, leukotrienes were determined 30 mins after calcium ionophore stimulation in the lungs, following removal of the circulating lipids. Figure

3 shows representative experiments where tissues were perfused with either fish oil (n = 2) or soybean oil (n = 2).

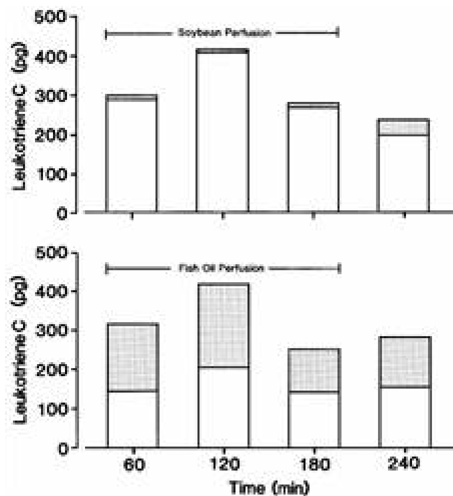

Figure 3:
Leukotriene C_4 (open bars) and leukotriene C_5 (dotted bars) generation in a representative experiment perfused with either 10% fish oil emulsion (bottom) or 10% soybean oil preparation (top). Samples were taken 30 mins after calcium-ionophore challenge. Values at 60, 120, and 180 mins represent leukotriene synthesis in the presence of either soybean oil preparation or fish oil emulsion in the perfusion fluid, whereas the value at 240 mins was obtained after cessation of infusion.

Eicosapentaenoic acid-derived leukotrienes are not normally produced in measureable quantities by lung tissue after A23187 stimulation. This phenomenon was assessed by only small amounts of leukotriene C_5 measured in soybean oil-perfused lungs, whereas four-series leukotrienes were detectable in large amounts. Since the total amount of leukotrienes was not substantially changed in experiments when fish oil was perfused, calcium ionophore injection already resulted in a marked production of leukotriene C_5 after only 1 hr of fish oil infusion. Likewise, A23187 provoked a steep increase in the generation of leukotriene C_5 and a decrease in leukotriene C_4 generation in the absence of circulating n-3 fatty acids.

The stable products of thromboxane A_2 and prostacyclin, thromboxane B_2 and 6-keto prostaglandin F_1 alpha, as well as histamine, were detected by radioimmunoassay before final A23187 (10 sup -7 M) injection and after this stimulation (215 mins). Three-hour administration of the soybean oil preparation resulted in an increase of thromboxane B_2 obtained on calcium ionophore stimulation. In contrast, perfusing the lungs with fish oil resulted in a significant ($p < .05$) suppression in thromboxane B_2 generation. Peak values of 590 +/- 124 pg/mL in the fish oil group and 1025 +/- 213 pg/mL in the soybean oil group were detected after final calcium ionophore administration. Stimulation of the soybean oil-treated lungs with A23187 exhibited generation of histamine and prostacyclin, measured at the end of observation. When compared with the corresponding n-3 fatty acid-treated-lungs, no significant differences in prostacyclin and histamine production were observed between the lungs perfused with either the fish oil or soybean oil preparation Figure 4. There was an increase in histamine from 95.8 +/- 17.5 nmol/L before A23197 challenge rising up to 328.6 +/- 57.8 nmol/L 30 mins following A23187 injection in the soybean oil group, compared with 91.3 +/- 22.3 nmol/L up to 404.2 +/- 13.4 nmol/L in the fish oil group. Corresponding to this increase in histamine, the prostacyclin concentrations increased from 599 +/- 87 to 2366 +/- 563 pg/mL in the soybean oil group, and from 509 +/- 101 to 2455 +/- 458 pg/mL in the fish oil treated lungs.

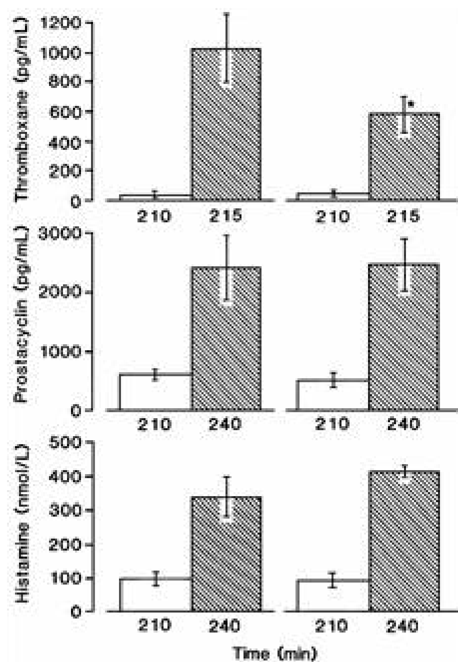

Figure 4:
Maximum release of thromboxane, prostacyclin, and histamine due to the last calcium ionophore challenge after cessation of fish oil emulsion (n = 6) (right panel) and soybean oil preparation (n = 6) (left panel) infusion. Data are mean +/- SEM. *$p < .05$ vs. corresponding values in the soybean oil preparation group. Open bars, before A23187 challenge; hatched bars, after A23187 challenge.

Vascular Reactions.

Pulmonary artery injection of A23187 caused an increase in pulmonary arterial pressure, with maximum pressure increases of 13.5 +/- 3.1 mm Hg and 17.5 +/- 2.2 mm Hg occurring 5 mins and 10 mins, respectively, after calcium ionophore challenge in the soybean oil group. In contrast to this increase in pulmonary arterial pressure, infusion of fish oil resulted in a significantly ($p < .05$) attenuated pressure reaction following A23187 stimulation. After elimination of circulating lipids and renewed stimulation with A23187, both emulsions-treated lungs exhibited an increase in pulmonary arterial pressure and lung edema. The pressure reaction was most pronounced after 15 mins and 20 mins, reaching values of 16 mm Hg in the soybean oil-treated lungs. However, fish oil infusion was associated with a significantly ($p < .05$) suppressed increase in pulmonary arterial pressure (2 and 3 mm Hg 15 mins and 20 mins after A23187 challenge, respectively), and weight gain evoked by calcium ionophore challenge. Furthermore, edema formation was significantly less marked in the fish oil group than in the soybean oil group Figure 5.

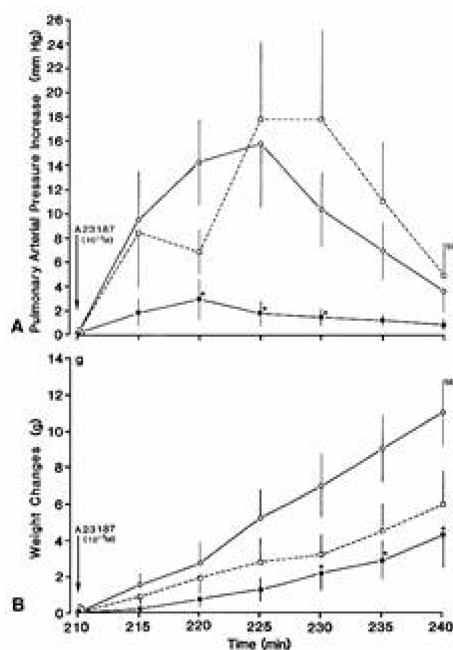

Figure 5:
Top: Increase in pulmonary arterial pressure after calcium ionophore (10 sup -7 M) application after a preceding 3-hr perfusion period with saline (control, n = 6) (open squares), 10% soybean oil preparation (n = 6) (open circles), or 10% fish oil emulsion (n = 6) (solid circles). Data are mean +/- SEM. *p < .05 vs. corresponding values in the soybean oil preparation group. Bottom: Weight changes following A23187 (10 sup -7 M) injection after pretreatment with saline (control, n = 6) (open squares), 10% soybean oil preparation (n = 6) (open circles), or 10% fish oil emulsion (n = 6) (solid circles). Data are mean +/- SEM. *p < .05 vs. corresponding values in the soybean oil preparation group.

DISCUSSION

In this study, we evaluated the efficacy of short-term n-3 fatty acid supplementation with respect to the inflammatory pulmonary vascular reaction in isolated perfused rabbit lungs. Administration of fish oil for 3 hrs induced significant alterations in the fatty acid composition of perfusate and lung tissue, as well as in the spectrum of the biologically active prostaglandins and leukotrienes. Modification of vascular reaction after fish oil perfusion in terms of an attenuation of pulmonary vascular resistance increase and edema formation due to calcium ionophore challenge may be related to the observed changes in fatty acid profile and to the altered mediator profile.

Fatty Acid Changes in Perfusate and Lung Tissue.

Dietary fish oil has been shown to alter fatty acid composition of cell membranes of different blood cells (i.e., erythrocytes [6], neutrophils [7,8], and platelets [9,10]), whereas, very little is known about the changes in fatty acid composition in organs after infusion of n-3 fatty acids. The data Figure 1 illustrate that nonesterified eicosapentaenoic acid as well as docosahexaenoic acid were significantly increased during 3-hr infusion and sustained within 60 mins after stopping intravenous lipid supplementation. Eicosapentaenoic acid reached significantly higher concentrations compared with those concentrations determined immediately after the beginning of the lipid infusion (time point of 5 mins). It is likely that a large amount of eicosapentaenoic acid and docosahexaenoic acid offered by the lipid infusion was hydrolyzed by lipoprotein lipase, shown by a rapid increase in free fatty acids and a concomitant decrease of hydrolysates Table 1. During inflammation, the lipoprotein lipase is known to detach itself from the endothelial cell surface and to translocate from its cellular binding sites into the vascular compartment, thus inducing hydrolysis of the esterified mediator precursors [25-27]. Additionally, the artificial lipid aggregates

infused with fish oil and soybean oil increase the plasma lipolytic activity. Effective concentrations of free eicosapentaenoic acid are liberated in this way into the circulating perfusion fluid, therefore becoming preferentially available for eicosanoid formation, even before incorporation into membrane phospho-lipids can occur [27]. Contrarily, when n-3 lipids are orally administered, direct incorporation and subsequent biological neutralization results in very low plasma contents of free fatty acids. Only a small amount of the ingested n-3 fatty acids is liberated in the relevant pool of nonesterified fatty acids [28]. Hence, it appears that the intravenous administration of eicosapentaenoic acid- and docosahexaenoic acid-containing triglycerides is the most successful method of achieving rapid availability of n-3 fatty acids, and presumably, of providing increased plasma concentrations of the direct lipid precursor fatty acid, eicosapentaenoic acid, in nonesterified form. The increase of eicosapentaenoic acid in the plasma free fatty acid fraction may modify arachidonic acid metabolism by competition with arachidonic acid in the cyclooxygenase and lipoxygenase pathways.

The influence of dietary fatty acid supplementation on lung tissue fatty acid content was evaluated by collecting lung tissue samples at the end of each experiment and after elimination of lipid-containing perfusate by rinsing the tissue. Docosahexaenoic acid and eicosapentaenoic acid concentrations were increased in lung tissue Figure 2 after a single infusion of fish oil emulsion. Since native lung tissue does not contain eicosapen-taenoic acid or docosahexaenoic acid, their presence must be due to the short-term fish oil infusion. In view of the high concentrations of free fatty acids (eicosapentaenoic acid and docosahexaenoic acid) in the perfusate during lipid infusion, the lung tissue probably took up nonesterified eicosapentaenoic acid and docosahexaenoic acid from the perfusate. The cessation of lipid perfusion and thorough elimination of circulating lipids were followed by the reappearance of the previously observed fatty acids in the perfusate. Due to the incorporation of eicosapentaenoic acid and docosahexaenoic acid in lung tissue, fatty acids measured in the perfusate may originate from the lung tissue. Arachidonic acid returns to a greater extent than eicosapentaenoic acid and docosahexaenoic acid in the perfusate Figure 1, indicating that they are distinct with regard to exchange rate between perfusate and lung tissue. The mechanism that regulates the pool size and profile of these fatty acids was not evaluated in our study. Two possibilities arise: arachidonic acid is the preferred substrate for the lipoprotein lipase, responsible for the liberation of nonesterified fatty acids, and arachidonic acid is integrated in phospholipid types more accessible to the activated phospholipase than eicosapentaenoic acid.

Inflammatory Lipid Mediator Generation and Vascular Reaction.

We estimated eicosanoid biosynthesis in response to an inflammatory stimulus via measurement of the stable hydrolysis products of prostacyclin and thromboxane A_2, 6-keto-prostaglandin F_1 alpha and thromboxane B_2, as well as of the leukotrienes C_4 and C_5 in the perfusate of the isolated, cell-free perfused rabbit lungs. Eicosanoids are essentially involved in the pathogenesis of pulmonary lesions after shock, trauma, and sepsis by changing vascular tone and disturbing epithelial and endothelial barrier function in the microcirculation of the lung and other organs. Since the generation of eicosanoids seems to be a common pathophysiologic mechanism, being of significance in various species, including humans, experimental studies on animal models may contribute to elucidate the complex interactions involved in the pathogenesis of acute respiratory distress syndrome and multiple organ failure. In particular, it could be demonstrated that the pattern of the generated eicosanoids in the pulmonary circulation and their vascular reaction is comparable in rabbits and human beings [29,30]. These mediators derived from membrane phospholipid-associated arachidonic acid are liberated by the activation of phospholipase A_2 by increased intracellular ionized calcium concentrations. In this study, a synthetic calcium ionophore, A23187, was used as stimulus of ionized calcium-influx by forming artificial pores into the cell membranes resembling the effects of pore-forming bacterial toxins (e.g., Escherichia coli hemolysin, staphylococcal alpha-toxin [31,32]). In control lungs, systemic administration of calcium ionophore elicited an increase of pulmonary arterial pressure as well as lung edema formation

Figure 5 and 6. In contrast to these effects, pressure increase and weight gain were effectively attenuated when A23187 was injected into lungs that had been circulated with fish oil for only 3 hrs. These findings paralleled the observation of a shift in A23187-stimulated leukotriene synthesis from leukotriene C_4 to leukotriene C_5, as well as of a reduced throboxane A_2 biosynthesis [33]. This discovery is in agreement with the suggested mechanism of eicosapentaenoic acid to reduce thromboxane A_2 synthesis. Eicosapentaenoic acid competes with the thromboxane A_2 precursor, arachidonic acid, for binding at the active site of cyclooxygenase, which in consequence reduces thromboxane A_2 formation [34]. However, thromboxane A_3, the n-3 eicosapentaenoic acid homologue of thromboxane A_2, is supposed to have less effect on the vasculature than thromboxane A_2[35]. Taken into account that thromboxane A_2 is known to be the predominant mediator of the arachidonic acid-induced pressure response in rabbit lungs, surpassing the vasodilator properties of the simultaneously liberated prostacyclin, this assumption might explain the reduced vascular reaction brought about by pretreatment with fish oil [36,37].

The reduced edema formation may be due to the decreased leukotriene C sub 4 production and shift to leukotriene C_5 during and after dietary supplementation with fish oil. As mentioned, eicosapentaenoic acid-synthesized leukotriene C_5 has minimal vasoconstrictive potency in the lung vasculature compared with leukotriene C_4. The cysteinyl leukotrienes C_4, D_4, and E_4 affect the permeability of postcapillary venules [38], thereby causing fluid extravasation and tissue edema. The influence of histamine on edema formation could be excluded, since generation of histamine is not suppressed in fish oil-treated lungs, whereas simultaneously edema formation is significantly reduced. The observed increase in edema formation and concomitant increase in vascular resistance due to A23187 challenge in soybean oil-treated and control lungs implicate that the lung edema may be provoked by an increase in microvascular filtration pressure caused by thromboxane. To what extent the generation of four-series leukotrienes contribute to the edema formation in the soybean oil-treated lungs remains to be elucidated.

The protective effect of n-3 fatty acid supplementation is supposed to be complex. Besides the shift from synthesis of bioactive leukotriene C_4 to less proinflammatory active leukotriene C_5 and reduction of thromboxane A_2, it has to be considered that the production of platelet activating factor is markedly inhibited in human leukocytes by n-3 fatty acids. The suppression of platelet activating factor production in leukocytes is presumably caused by incorporation of fish oil-derived polyunsaturated fatty acids in membrane phospholipids, replacing arachidonic acid in phosphatidylcholin, the precursor molecule for platelet activating factor formation [39]. In addition, consideration must also be given to the effects of n-3 fatty acids on blood viscosity, erythrocyte deformability, production of endothelium-derived relaxing factor and platelet-derived growth factor, vascular compliance, and response to catecholamines [40].

Conclusion.

The present study provided evidence of a close correlation between the type of fatty acid accumulation in plasma and lung tissue and important inflammatory reactions. An increase in eicosapentaenoic acidderived metabolites induced by a calcium ionophore appears to be related to rapid change of fatty acid composition in the perfusate and lung tissue. Although dietary fish oil exerts a variety of metabolic changes (e.g., inhibition of oxygen radical generation and release of platelet activating factor and cytokines [39,41]), under our experimental conditions, the obviously successful suppression of inflammatory reactions induced by calcium ionophore with fish oil suggests that the efficiency of fish oil may be attributed to rapid accumulation of the direct lipid precursor fatty acid, eicosapentaenoic acid, and subsequent modulation of eicosanoid pattern. The observed beneficial effects lasted for the entire infusion period and were reversible within 1 hr after ceasing fish oil intake. This effect indicates that increased eicosapentaenoic acid content of lung tissue, even after short-term fish oil infusion, may serve as a storage pool for the nonesterified fatty

acid, eicosapentaenoic acid, as a precursor of the formation of triene prostanoids and pentaene leukotrienes in the circulating liquid. In view of the pivotal role of lipid mediators in mediating inflammatory reactions after shock, trauma, and sepsis, this study may suggest short-term n-3 fatty acid supplementation as an additional acute anti-inflammatory therapeutical approach in patients with inflammatory disorders.

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