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ω-3 vs. ω-6 lipid emulsions exert differential influence on neutrophils in septic shock patients: impact on plasma fatty acids and lipid mediator generation

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Abstract *Objective:* To compare the effects of a conventional ω-6 lipid infusion and a fish oil based (ω-3) lipid infusion for parenteral nutrition on neutrophil function, lipid mediators, and plasma free fatty acids. *Design and setting:* Open-label, randomized, pilot study in a university hospital medical intensive care unit and experimental laboratory. *Patients and participants:* Ten patients with septic shock and eight healthy controls. *Interventions:* Patients (five per group) requiring parenteral nutrition received intravenously either a ω-3 or a ω-6 lipid emulsion for a 10-day period. *Measurements and results:* At baseline levels of plasma free fatty acids were elevated several-fold, including high concentrations of the ω-6 lipid precursor arachidonic acid (AA). Neutrophils isolated from septic patients displayed markedly reduced responsiveness to ex vivo stimulation, including lipid mediator generation [leukotrienes (LT), PAF], respiratory burst, and phosphoinositide hydrolysis signaling. Under the ω-6 lipid infusion regimen abnormalities in plasma free fatty acids and impairment of neutrophil functions persisted or worsened. In contrast, a rapid switch in the plasma free fatty acid fraction to predominance of the ω-3 acids eicosapentaenoic acid and docosahexaenoic acid over AA occurred in response to ω-3 lipid infusion. LTB_5, in addition to LTB_4, appeared upon neutrophil stimulation originating from these patients, and neutrophil function was significantly improved in the ω-3 lipid group. *Conclusions:* ω-3 vs. ω-6 lipid emulsions differentially influence the plasma free fatty acid profile with impact on neutrophil functions. Lipid-based parenteral nutrition in septic patients may thus exert profound influence on sequelae and status of immunocompetence and inflammation.

Keywords Septic shock · Neutrophils · Leukotrienes · Fish oils · Thromboxanes · Total parenteral nutrition

Introduction

Despite advances in critical care medicine sepsis and septic shock continue to be associated with high mortality rates, ranging between 30% and 60% [1, 2, 3], and sepsis is the major cause of death in critical care units worldwide. Uncontrolled generation of a plethora of proinflammatory and potentially autotoxic mediators has been noted both in experimental models of sepsis and in clinical settings [4, 5, 6]. The fact that such a systemic inflammatory reaction may not only be triggered by microbial invasion but is also encountered in response to various kinds of tissue injury is reflected in the term "systemic inflammatory response syndrome" (SIRS). Polymorphonuclear granulocytes (PMN) are intimately involved in these events in a dual way as

they represent the first line of defense against microbial invasion but can cause most serious tissue destruction [5, 7].

To the present, however, clinical trials aiming to antagonize specific inflammatory sequelae (e.g., tumor necrosis factor α antibodies) in human sepsis have failed to result in increased survival from this disease. Moreover, recent evidence suggests that, in parallel to the inflammatory response to the inciting injury, the body mounts an anti-inflammatory reaction termed "compensatory anti-inflammatory response syndrome" [8]. Upregulation of anti-inflammatory cytokines, impairment of neutrophil function, and monocyte deactivation have been implicated in this phenomenon, which is linked with impaired host defense and enhanced susceptibility to secondary infections [9, 10, 11, 12, 13, 14].

Eicosanoids have long been implicated in both pro- and anti-inflammatory events occurring in sepsis [6, 15]. They are synthesized from arachidonic acid (AA) via multiple metabolic pathways. The family of ω-6 fatty acids, including AA, is the predominant polyunsaturated fatty acids in the common Western diet and current nutritional regimes. In contrast, ω-3 fatty acids [in which the last double bond is located between the third and fourth carbon atoms from the methyl end, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)] make up an appreciable part of the fat in cold-water fish and seal meat. They serve as alternative lipid precursors for both cyclooxygenase and lipoxygenase pathways, with the formation of trienoic prostanoids (instead of the 2-series originating from AA) and 5-series leukotrienes (LT), instead of the 4-series LTs derived from AA [16]. Interestingly, many of the ω-3 fatty acid derived metabolites, including 5-series cysteinyl LTs, LTB_5 and thromboxane (Tx) A_3, possess markedly less inflammatory and vasomotor potency than AA-derived lipid mediators and may even exert antagonistic functions.

Against this background, employment of diets with specific fat composition has long attracted interest for modulating inflammatory and immunological processes ("immunonutrition"). Via the enteral route, however, administration for several days to weeks of ω-3 lipids such as fish oil is required effectively to influence fatty acid composition of membrane (phospho)-lipids and subsequently lipid mediator profile in humans [17, 18]. Notwithstanding these limitations a diet for gastric tube feeding containing ω-3 fatty acids was recently reported to significantly reduce infectious complications and mortality rate in intensive care patients [19, 20, 21]. As this regime for enteral nutrition is additionally enriched with glutamine and arginine, no definite conclusion about the role of the ω-3 lipids can be drawn. Experimental prefeeding animals with ω-3 lipids in place of ω-6 lipids without further additives enhanced survival in subsequently provoked septic events [22, 23]. Injection of EPA, in contrast to AA, reduced organ failure in a model of septic lung injury [24]. We recently administered a fish oil based lipid emulsion intravenously in patients with inflammatory bowel and skin disease and observed rapid changes in cell membrane fatty acid composition and lipid mediator generation [25, 26].

In the present study septic shock patients requiring parenteral nutrition received in a randomized fashion a ω-3 fatty acid rich lipid emulsion (fish oil based) for intravenous alimentation in comparison with a conventional (ω-6 fatty acid rich) lipid emulsion. Marked impact of the fish oil based regime on plasma free fatty acid composition towards predominance of ω-3 over ω-6 fatty acids was noted, together with the appearance of the respective prostanoids and leukotrienes. Most interestingly, neutrophil functions, found to be consistently depressed in the septic shock patients, were partially restored in response to the ω-3 but not the ω-6 lipid emulsion. Lipid emulsions with ω-3 vs. ω-6 fatty acid predominance may thus allow combination intravenous alimentation with differential influence on plasma fatty acids, lipid mediator generation, and function of immunocompetent cells in septic patients.

Materials and Methods

Study design

Ten patients fulfilling the criteria of sepsis and septic shock and and intolerant of enteral nutrition were recruited in the Intensive Care Unit of the Department of Internal Medicine, Justus Liebig University, Giessen. The study was approved by the University Ethics Committee, and written informed consent was obtained from each patient, next of kin, or legal guardian. The protocol was in agreement with the recommendations of the Declaration of Helsinki. The study was conducted as an open-label, randomized trial. Patients were randomly assigned to receive total parenteral nutrition employing either the standard ω-6 lipid infusion (Lipoven) or the ω-3 lipid infusion (fish oil based; Omegaven). The fatty acid composition of these different lipid emulsions is presented in Table 1. Infusion of glucose, amino acid, and electrolyte solutions did not differ between the two groups. Via central venous catheter, 400 ml of a 10% ω-6 or ω-3 lipid emulsion was infused each day, divided into three parts (200 ml from 8 a.m. to 12 a.m., 100 ml from 4 p.m. to 8 p.m., 100 ml from midnight to 4 a.m.). The patients were followed for 10 days with this infusion regimen. Before the onset of the study none of the patients had received lipid emulsions.

The Acute Physiology and Chronic Health Evaluation (APACHE) II score was calculated from data obtained at baseline [27]. The Glasgow Coma Scale item of the APACHE II score in sedated patients was considered according to the clinical situation before initiation of sedation. Sepsis and septic shock were defined according to the criteria developed by Bone et al. [28]. Cultures reported as positive were defined as the identification of bacterial/fungal growth from normally sterile body fluids. An organ was defined as focus by positive culture and clinical signs of infection.

Table 1 Fatty acid composition of the ω-6 and the ω-3 lipid emulsion (g/l). The ω-6 (Lipoven®) and the ω-3 lipid emulsion (Omegaven®) were manufactured with identical techniques and additives. Repetitive gas chromatographic controls of both lipid emulsions revealed less than 0.3% free EPA or AA as related to the esterified amounts of these fatty acids

Fatty acid (g/l)	ω-6 lipid emulsion	ω-3 lipid emulsion
C14:0	–	4.9
C16:0	12.4	10.7
C16:1ω-7	–	8.2
C18:0	5.0	2.4
C18:1ω-9	24.1	12.3
C18:2ω-6	52.2	3.7
C18:3ω-3	8.2	1.3
C20:4ω-6	–	2.6
C20:5ω-3	–	18.8
C22:5ω-3	–	2.8
C22:6ω-3	–	16.5
Others	–	16.1

Study subjects

Patient selection

Enrolled patients had to satisfy the clinical suspicion of infection (including introduction or change in systemic antimicrobial therapy), systemic inflammatory response, and acute onset of end-organ dysfunction within the previous 24 h [28]. Septic shock was defined as a syndrome characterized by severe sepsis associated with *either* a drop in mean arterial pressure of less than 70 mmHg for at least a 30-min period despite fluid resuscitation *or* the need for vasopressors for at least a 30-min period to maintain MAP at 90 mmHg. Patients were excluded if they were under 18 years old or with known or suspected pregnancy. Other exclusion criteria included: treatment with corticosteriods (equivalent of ≥1 mg prednisone/kg) within the previous 48 h, treatment with other major immunosuppressive drugs, infection with the human immunodeficiency virus, neutropenia due to causes other than sepsis, participation in an other ongoing investigational clinical trial, and the presence of irreversible underlying disease anticipated to be rapidly fatal.

Blood sampling

Blood was collected by venipuncture on day 1 before the start of the infusion therapy and on days 3, 7, and 10 at 8.00 a.m. after a 4-h period without lipid infusion.

Control group

Eight healthy adults (aged 23–66 years, four men, four women) served as control group. Medical history, physical examination, and routine laboratory investigation were completely normal in all subjects. In the month prior to the study the control group did not use medications (especially nonsteroidal anti-inflammatory drugs) and had no febrile disease. After an overnight fast blood was collected at 8.00 a.m. by antecubital venipuncture.

Patients' clinical characteristics

Baseline demographic data and clinical features of the study patients are presented in Table 2. All patients required vasopressor therapy at study entry, according to the criteria of septic shock. All presented with markedly elevated leukocyte counts, C-reactive protein (CRP) values, and plasma lactate levels (Table 3). In each group four out of five patients were mechanically ventilated. The two groups did not differ significantly at the beginning of the study in terms of age, gender, APACHE II score, CRP, lactate, leukocyte count, or body core temperature. Due to the severity of the disease all patients received total parenteral nutrition during the 10-day study period. None of the patients received corticosteroids.

Experimental procedures

Materials

Superoxide dismutase, cytochrome *c* type IV, bovine serum albumin (BSA), and hydroxyethylpiperazine ethanesulfonic acid (HEPES) were purchased from Sigma (Deisenhofen, Germany). RPMI 1640 medium, Hank's balanced salt solution, and fetal calf serum were

Table 2 Demographic and clinical data (*APACHE* Acute Physiology and Chronic Health Evaluation, *MOF* multiple-organ failure, *ARDS* adult respiratory distress syndrome, *CABG* coronary artery bypass grafting, *CRF* chronic renal failure, *COPD* chronic obstructive pulmonary disease)

Patient no.	Sex	Age (years)	Underlying diagnosis	Secondary diagnosis	Causative Organism	APACHE II score
ω-6 group						
1	M	59	Pneumonia, MOF	Diabetes	None identified	23
2	F	71	Gangrene dig. V left feet	Diabetes, hypertension	*Streptococcus* group A	9
3	F	50	ARDS after emergency CABG	Hypertension	None identified	14
4	F	62	ARDS after CABG	Hypertension	*Pseudomonas aeruginosa*	10
5	M	40	ARDS, MOF		*Streptococcus viridans*	19
ω-3 group						
1	M	71	Pneumonia, MOF	COPD, CRF	*Candida albicans*	34
2	M	57	ARDS after CABG	COPD	*Serratia* species	14
3	F	31	Pneumonia, MOF		None identified	21
4	M	59	Infected ulcus cruris, MOF	Diabetes, CRF		24
5	F	37	Pneumonia	None identified	12	

Table 3 basic laboratory and clinical data over the 10 day lipid infusion period. Mean ±SEM (*n*=5 each). The groups did not differ significantly at base (day 1). (*CRP* C-reactive protein, *APACHE* Acute Physiology and Chronic Health Evaluation)

Day	Leukocytes (G/I)		CRP (mg/l)		Lactate (mmol/l)		APACHE II	
	ω-6	ω-3	ω-6	ω-3	ω-6	ω-3	ω-6	ω-3
1	21,800±4,300	25,000±8,300	196.0±42.0	168.2±36.0	3.4±1.4	2.6±0.7	16.3±3.0	23.3±4.2
3	22,400±4,500	12,600±3,600	205.0±53.0	89.0±20.0*	1.8±0.6	1.5±0.5	14.8±3.5	16.5±5.1
7	15,700±2,700	14,500±3,000	124.4±38.0	85.0±18.8	1.1±0.2	1.3±0.5	11.6±2.0	12.8±3.2
10	11,600±1,200	9,300±1,500	135.0±20.8	112.4±38.8	1.3±0.2	1.2±0.4	11.0±2.8	13.3±2.3

*p=0.08

from Gibco Laboratories (Grand Island, N.Y., USA). Percoll was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). The 4-series and 5-series LTs were obtained from Paesel (Frankfurt, Germany). All LTs were checked for purity and quantified spectrophotometrically before use, as described [29]. Tritiated LTs, used as internal standards, were obtained from New England Nuclear (Boston, Mass., USA). Tritiated inositol phosphates (IP) and myo-[2-^{3}H]inositol were purchased from Amersham (Dreieich, Germany). Chromatographic supplies included silica gel 5-µm column packing (Machery & Nagel, Düren, Germany) and HPLC-grade solvents distilled in glass (Fluka, Heidelberg, Germany). All other biochemicals were obtained from Merck (Darmstadt, Germany).

Preparation of human granulocytes

Heparinized blood from patients and healthy donors was centrifuged in a discontinuous Percoll gradient [30] to yield a PMN fraction of approximately 97% purity. Prior to experiments PMN were kept in RPMI 1640 with 10% fetal calf serum for 30–60 min at 37°C. Immediately before stimulus application cells were washed twice and suspended in Hanks' HEPES buffer to obtain PMN concentrations of 10×10^6 PMN/ml. Cell viability, as assessed by trypan blue exclusion, ranged above 96%, and lactate dehydrogenase release was consistently below 3%.

Measurement of leukotrienes

PMN were stimulated with 1 µmol/l A23 187. LTs were extracted from cell supernatants by octadecylsilyl solid-phase extraction columns, as described [29, 31]. Conversion into methyl esters was performed by addition of freshly prepared diazomethane in ice-cold diethyl ether. Reversed-phase HPLC of nonmethylated compounds was carried out on octadecylsilyl columns (Hypersil, 5-µm particles) [29]. In addition to the conventional UV detection at 270 nm (LTs) and 237 nm (HETEs), a photodiode array detector (Waters model 990) was used. This provided full UV spectra (190–600 nm) of eluting compounds, allowing peak purity and subtraction of possible coeluting material to be checked. Straight-phase HPLC of methylated compounds was carried out using a modification of the method of Nadeau et al. [26, 29, 32]. All data obtained by the various analytical procedures were corrected and are presented here in picomoles per milliliter. Recovery was determined by separate recovery experiments and radioactive standards using different quantities of the individual compounds in the appropriate concentration range [29].

Measurement of platelet-activating factor

Neutrophil production of platelet-activating factor (PAF) was quantified by post-HPLC liquid scintillation counting using the radiochromatogram imaging system (5LS Raytest). PMN (10^7) were stimulated in the presence of 50 µCi [^{3}H]acetate (7.75 Ci/mmol) with 1 µmol/l A23 187 in a total volume of 1 ml according to Tessner et al. [33] measuring cell bound and secreted PAF. After termination of PMN incubation in Hanks' balanced salt solution containing 20 mM HEPES and 0.25% BSA reactions were stopped by the addition of 3 vol. chloroform/methanol (1:2, v/v). Then extraction was performed according to the method of Bligh and Dyer [34].

Superoxide generation

PMN O_2^- generation was measured as superoxide dismutase inhibitable reduction of cytochrome *c* as described [35]. Duplicate reaction mixtures containing neutrophils (5×10^6 PMN/ml) and 75 µM ferricytochrome *c* were incubated at 37°C. The mixtures were in the presence or absence of 10 µg/ml superoxide dismutase and stimulated with 1 µmol/l formyl-methionyl-leucyl-phenylalanine (fMLP).

Phosphoinositide metabolism

The phosphatidylinositol turnover of stimulated neutrophils was investigated by measuring the accumulation of IPs according to Berridge et al. [36]. Three procedures for prelabeling of cellular phospholipid pools were performed. First, PMN were resuspended to 1×10^7 cells/ml with medium 199 containing 2% fetal calf serum plus 40 mM HEPES buffer (pH 7.4). Second, myo-[^{3}H]inositol (50 µCi/ml) was added. Finally, cells were incubated at 37°C for 2 h on a shaking water bath. Before experimental use cells were washed twice and resuspended in Hanks' balanced salt solution containing 20 mM HEPES and 10 mM LiCl (an inhibitor of IP_1-phosphatase). At different times after stimulus application (fMLP, 1 µmol/l) samples were quenched with trichloracetic acid (final concentration 7.5%), kept on ice for 15 min, and extracted four times with diethylether. The aqueous phase was neutralized with sodium tetraborate to pH 8.0 and processed to separate IP on Dowex anion exchange columns as described by Berridge et al. [36]. Under current assay conditions cyclic inositmonophosphate decomposes quantitatively to generate IP_1. All IP are summarized as IP_x.

Platelet thromboxane generation

Blood was collected in 3-ml plastic tubes containing 700 µl of a 7.5% EDTA solution. After centrifugation at 200 *g* for 10 min the platelet-rich plasma was decanted and spun again (1500 *g*, 10 min). Pelleted platelets were washed with isotonic PBS (pH 7.4), recentrifuged and resuspended as described [37]. Platelet count was adjusted to 10^8/ml. Stimulation was performed with 5 U/ml thrombin (vehicle PBS). Platelet purity was greater than 99% throughout the study. Incubation was terminated after 15 min. TxA_2 and TxA_3 in the supernatant were measured as their

stable hydrolysis products TxB_2 and TxB_3. The analytes were extracted from the buffer solution by solid-phase extraction, subjected to reversed-phase RP-HPLC separation, and quantified by post-HPLC enzyme-linked immunosorbent assay as detailed [37]. A monoclonal mouse antibody against TxB_2 with established cross-reactivity to TxB_3 (90%) was used.

Plasma free fatty acids

Nonesterified plasma fatty acids were quantified by one-step rapid extractive methylation for gas chromatographic analysis [38], as described previously [25, 26]. First, citrate plasma was spiked with heptadecanoic acid as internal standard. Next, free fatty acids were converted to methyl esters by mixing with ethereal diazomethane. Lastly, the ethereal layer was dried, redissolved in chloroform, and transferred to the gas chromatograph. The fatty acid methyl esters were detected by use of a flame ionization detector, and peak area integration was performed.

Statistics

The values are given as the mean ±SEM. Because the study was a planned pilot study, explorative data analysis was performed. All statistical analyses were performed with the SigmaStat computing package. In the case of LTB_4, elastase, IP_x, and PAF the highest value of each study group was compared to the control value using analysis of variance and Student's/Newman-Keul post-hoc test. For the ratio of TxB_2 to TxB_3, free AA, and free EPA the same procedure was carried out for the lowest value. To assess the influence of treatment, we performed repeated-measures analysis with Student-Newman-Keul post-hoc test. To test the hypothesis that use of vasopressors and ventilation days differed between study groups the Cochran-Armitage trend test was performed. Probability of randomness (p) values of 0.05 or less were considered to indicate statistical significance.

Results

On the third day of the lipid infusion regimen CRP levels increased in the ω-6 lipid infusion group (205.0±53.0 mg/l) and decreased in the ω-3 group (89.0±20.0 mg/l), although the difference between the groups did not reach the level of statistical significance (p=0.08). In parallel, leukocyte counts decreased in the ω-3 group (12,600±3,600 G/l) and increased in the ω-6 lipid group (22,400±4,500 G/l; p=0.09). In both treatment groups temperature, lactate, CRP, leukocyte count, and APACHE II score decreased during the further course of the 10-day lipid infusion period. All patients survived the 10 day observation period. On day 10 two patients in each group still needed vasopressor support and all were extubated. Patients infused with n-6 lipids exhibited a trend (p=0.07) towards a longer ventilation time.

Leukotriene generation

Ionophore-induced secretion of LTB_4 from PMN in both treatment groups was markedly lower than in healthy controls (Fig. 1a). However, it did not differ significantly

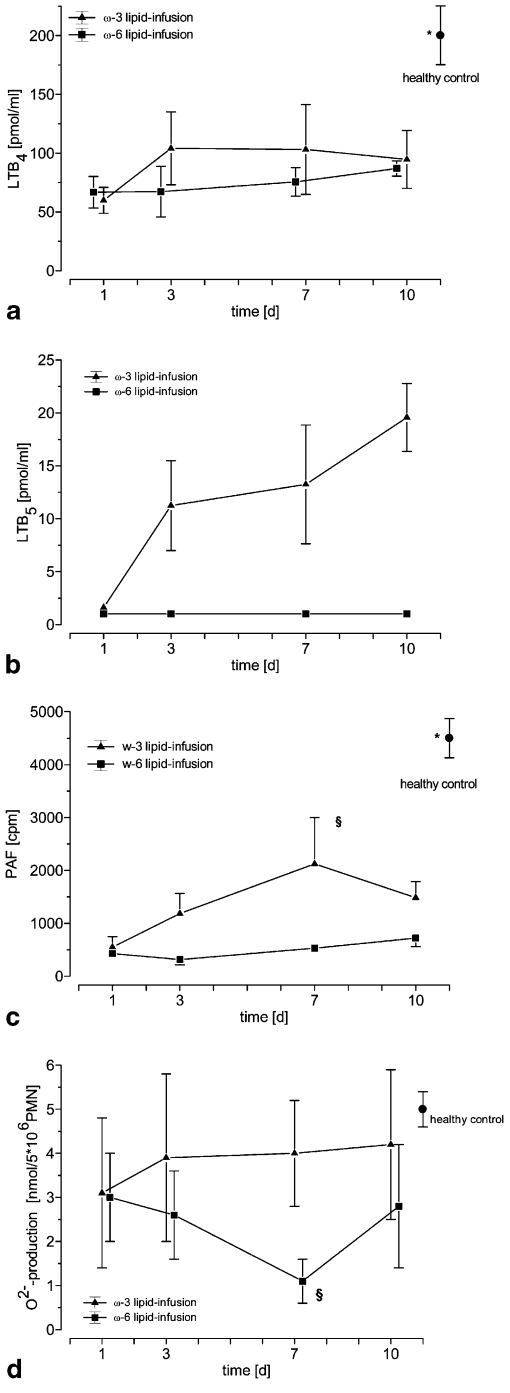

Fig. 1 Generation of leukotriene (*LT*) B_4 (**a**), LTB_5 (**b**), synthesis of platelet-activating factor (*PAF*, **c**), and respiratory burst (**d**). PMN were isolated on days 1, 3, 7, and 10 of the lipid infusion regimen and subsequently stimulated with A23 187 (LTs and PAF) or fMLP (respiratory burst) as described. Generation of LTB_4 and PAF in PMN of septic patients was markedly lower than in healthy controls (*p<0.05). LTB_4 release did not differ significantly between the treatment groups. LTB_5 generation was detected only in the ω-3 lipid infusion group (note different scales for LTB_4 and LTB_5). On day 7 PAF generation and respiratory burst in the ω-3 lipid infusion group were significantly higher than in the ω-6 lipid infusion group (§p<0.05, mean ±SEM are given)

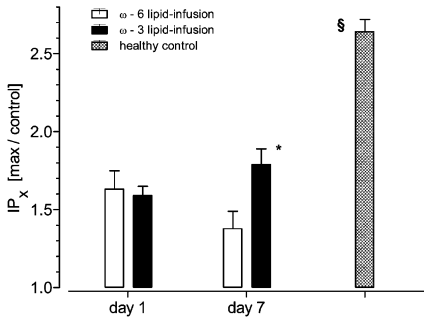

Fig. 2 Neutrophil inositol phosphate generation. Neutrophil inositol phosphate (*IP*) generation evoked by fMLP. IP_3, IP_2, and IP_1 are summarized as IP_x. Mean ±SEM is given. The IP_x generation of the ω-3 lipid infusion group on day 7 differed significantly from that in the ω-6 lipid infusion group at this time (*p<0.05) and was significantly increased over baseline (day 1). The control group was significantly higher than all other groups (§p<0.05)

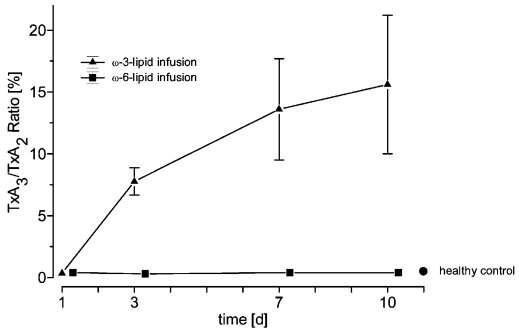

Fig. 3 Platelet thromboxane (*Tx*) generation in response to thrombin. TxA_2/TxA_3 generation of isolated platelets was measured by post-HPLC enzyme-linked immunosorbent assay as described. Mean ±SEM of the TxA_3/TxA_2 ratio is given for the different groups

between the two treatment groups throughout lipid infusion period. LTB_5 was not detectable on day 1, and then rapidly appeared only in the ω-3 group, approaching a LTB_5/LTB_4 ratio of nearly 20% at the end of the infusion period (Fig. 1b).

Phosphatidyl-inositol generation

In response to fMLP challenge all septic patients exhibited significantly lower IP_x synthesis than healthy controls (Fig. 2). When reassessed on day 7, a further decline in IP_x generation was noted in the ω-6 group but an increase in the ω-3 group (p<0.05).

PAF generation

Ionophore-induced PAF secretion was markedly lower in PMN from septic patients than in those from controls (Fig. 1c). In the ω-3 lipid infusion group PAF synthesis increased over time. It was significantly higher on day 7 than in the ω-6 lipid infusion group, in which hardly any change was noted in PAF synthesis over the 10 day-observation period.

Respiratory burst

fMLP-induced neutrophil superoxide generation was lower in the septic patients than in the healthy volunteers (Fig. 1d). Values further decreased in the ω-6 group but slightly increased in the ω-3 group (statistically significant on day 7).

TxA_2/TxA_3 generation

In healthy controls and patients receiving ω-6 lipid infusion the TxA_3/TxA_2 ratio remained below 1% throughout (Fig. 3). Increasing quantities of TxA_3 became detectable in the ω-3 group, and the TxA_3/TxA_2 ratio reached 17.8±7.4% at the end of the infusion period.

Plasma free fatty acids

In comparison to the healthy controls and before onset of lipid infusion therapy all plasma free fatty acids quantified by gas chromatography were increased several-fold in septic patients (Table 4, Electronic Supplementary Material). The overall sum of plasma free fatty in the septic patients on day 1 surpassed that in the controls more than fourfold. AA was markedly higher in both infusion groups than healthy controls at baseline. Overall quantities and fatty acid profile remained largely unchanged in the ω-6 lipid infusion group. In contrast, a marked increase in the ω-3 fatty acids EPA and DHA was noted in the patients receiving fish oil based lipid infusion (Table 4, Electronic Supplementary Material). Both ω-3 fatty acids plateaued after 7 days, and the sum of EPA and DHA surpassed the AA level nearly twofold.

Discussion

As previously reported in patients suffering from sepsis [39, 40], overall levels of plasma free fatty acids were markedly elevated in all patients in this study, even prior to onset of any lipid infusion therapy. The magnitude of metabolic changes that we found (from more than twofold to tenfold elevated values for all single fatty acids)

may reflect the severity of disease resulting from shock in all patients. Several factors may underlie this metabolic response: (a) Plasma free fatty acid elevation is a component of the general metabolic response syndrome to stress [41]. (b) Lipolysis from adipocytes and hepatic de novo lipogenesis are increased in sepsis [42, 43, 44, 45], and muscle fatty acid oxidation is reduced [46]. (c) Secretory phospholipase A_2 is elevated in sepsis [47]. (d) Vasopressors such as epinephrine and norepinephrine, presently administered to all patients, preferentially increase the plasma levels of polyunsaturated free fatty acids by activating lipoprotein lipase and the hormone-sensitive lipoprotein lipase of adipose tissue [48, 49]. (e) Heparin, used in low doses in all patients, is another activator of the lipoprotein lipase [50]. Overall, these metabolic changes resulted in the most impressive appearance of free concentrations of the eicosanoid precursor AA between 25 and more than 100 µmol/l. In contrast, approximately 11 µmol/l free AA was detected in healthy controls. Moreover, measurements of free extracellular AA within an inflammatory focus suggested levels range between 20 and 50 µmol/l [51, 52, 53].

After commencing fish oil based lipid infusion a rapid increase in free EPA and DHA levels was noted. This averaged approx. 120 µmol/l EPA and 200 µmol/l DHA. In consequence the (EPA+DHA)/AA ratio reached nearly 2:1. The immediate appearance of free ω-3 fatty acids indicates rapid hydrolysis of the EPA- and DHA-containing triglycerides in septic patients. This is in line with the notion that synthetic lipid aggregates activate endothelial lipoprotein lipases, including a translocation of this enzyme from its cellular binding sites into the vascular compartment. Resulting from activation, plasma free fatty acids increase due to escape from local cellular uptake mechanisms [54]. Kinetics and extent of plasma ω-3 lipid increase thus exceed corresponding alterations in response to conventional dietary fish oil uptake by orders of magnitude [55, 56]. Interestingly, an increase in AA was also noted in the ω-3 lipid infusion group. However, this rise is much less impressive than the increase in EPA and DHA levels in these patients. It may not yet be decided whether the increase in free AA results from release of the free fatty acids from the ω-3 lipid infusion (which contains AA to 2.6%) or is secondarily cleaved from endogenous AA-containing lipid pools due to substitution by EPA and/or DHA.

In parallel with the plasma free EPA and DHA increase, substantial quantities of LTB_5 were detected. Determined in neutrophils ex vivo after challenge with calcium ionophore, the LTB_5/LTB_4 ratio was greater than 20% in these experiments. Notably, the PMN stimulation was performed in the absence of plasma. It may therefore concluded that some EPA-containing membrane lipid pool(s), providing precursor fatty acids for the neutrophil 5-lipoxygenase pathway, had rapidly exchanged with plasma EPA. Moreover, in the physiological microenvironment the LT metabolism of neutrophils is strongly influenced by the levels of exogenous free precursor fatty acids [57, 58]. Also, the dramatic impact of free EPA has been demonstrated, representing preferred substrate over free AA for the 5-lipoxygenase pathway [57]. Thus, when considering the very high values of free EPA and free DHA in the plasma of the patients undergoing ω-3 lipid infusion, the appearance of an even higher percentage of LTB_5 may well be anticipated for PMN activation in vivo. Such reasoning may also hold true for the platelet thromboxane generation. The TxA_3/TxA_2 ratio of approx. 18% noted in this study upon in vitro stimulation of washed thrombocytes may even be surpassed in vivo when platelet activation takes part in an environment of free EPA and DHA concentrations supervening those of free AA.

Under inflammatory conditions such as sepsis both enhanced and suppressed neutrophil superoxide generation in response to various protocols of ex vivo stimulation have been reported. However, evidence is growing that impairment of neutrophil function, including respiratory burst, becomes increasingly clear the more severe and prolonged the course of sepsis is. In a series of earlier studies in neutrophils isolated from patients with severe sepsis, Solomkin and coworkers [11, 59] demonstrated suppressed fMLP-induced superoxide generation, although fMLP receptor density and internalization were found to be unaffected. In the same line, Vespasiano et al. [60] and Pascual et al. [61] recently reported reduced superoxide release from neutrophils isolated from patients in septic shock, regardless of the stimulus used for provocation of respiratory burst. Consistent with these observations, decreased PMN superoxide generation was noted in all patients at study entry. Suppression of respiratory burst became even more prominent over subsequent days in the group receiving a conventional ω-6 lipid infusion regimen. Moreover, all other features of neutrophil function addressed here were found to be suppressed in PMN originating from septic shock patients: lipid mediator generation including LTB_4 and PAF, and the phosphatidylinositol response. The latter, representing an important neutrophil signal transduction pathway, may offer an underlying mechanism causally related to impairment of other neutrophil functions observed. It is in this line that Burke et al. [62] recently noted decreased Ca^{2+} fluxes in response to fMLP challenge in neutrophils isolated from septic patients. Further detailed studies addressing the signal transduction abnormalities in neutrophils of septic shock patients are required.

The pathogenetic sequelae resulting in impairment of neutrophil function in septic shock have not as yet been resolved. The concept of compensatory anti-inflammatory response syndrome favors the assumption that an initial hyperinflammatory phase of the disease is followed by a counterregulatory period with predominance of anti-inflammatory mediators, with impact on the

function of the immunocompetent cells [8]. Alternatively, ongoing involvement of neutrophils in inflammatory processes and microcirculatory disturbances may result in downregulation or even "exhaustion" of their responsiveness to further (ex vivo) stimulation. Irrespective of these open questions, the present study strongly suggests that these sequelae are differentially influenced by ω-6 vs. ω-3 lipid emulsions employed for parenteral nutrition. In patients receiving the conventional lipid formulation the PMN hyporesponsiveness either persisted (LTB and PAF generation) or became even more prominent in the further course (phosphoinositide hydrolysis, and superoxide). In contrast, partial restoration of a more "normal" responsiveness was noted in the patients infused with ω-3 lipid emulsions. Given the fact that the mechanisms of neutrophil impairment in septic shock are unresolved, as discussed above, speculations as to the kind of intervention upon employment of ω-3 lipids are clearly too early. However, the basic features known for the EPA-derived mediators and the more prominent (although not significant) decrease in CRP in the ω-3 group suggest some dampening of ongoing inflammatory events in the presence of high plasma ω-3 fatty acid levels. A corresponding mechanism was has been proposed for the clinical benefit of intravenous fish oil in severe ulcerative colitis and acute, extended guttat psoriasis [15, 63]. Supporting the idea of suppressed innate immunity due to inflammatory mediators in sepsis, Czermak and coworkers [64] have reported beneficial effects of a C5a blockade in a rat model. Inhibition of C5a-induced activation of PMN in vivo restored suppressed neutrophil respiratory burst in vitro, accompanied by increased survival of septic rats and improved bacterial clearance.

Conclusions

The power of the present study in a limited number of septic shock patients was clearly insufficient to evaluate possible impact of the ω-3 vs. the ω-6 lipid infusions on clinical course of disease, including development of organ failure and death. However, the two alternative regimens for lipid-based parenteral nutrition exerted differential influence on key variables of inflammatory mediator generation and neutrophil function, both intimately involved in pathogenetic sequelae of sepsis. Under ω-6 lipid infusion both the predominance of high plasma free AA levels and impairment of neutrophil functions persisted or became even more prominent. In contrast, the lipid mediator precursor profile in the plasma was rapidly shifted towards predominance of ω-3 fatty acids EPA and DHA in patients receiving a fish oil based lipid emulsion. Concomitantly, partial restoration of ex vivo analyzed neutrophil functions was noted, significantly different from the ω-6 infusion group. Lipid emulsions enriched in ω-3 instead of ω-6 fatty acids may thus offer the possibility to combine parenteral nutrition with differential impact on inflammatory variables and neutrophil function in septic patients.

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References

1. Friedman G, Silva E, Vincent JL (1998) Has the mortality of septic shock changed with time? Crit Care Med 26:2078–2086
2. Wheeler AP, Bernard GR (1999) Treating patients with severe sepsis. N Engl J Med 340:207–214
3. Bone RC, Grodzin CJ, Balk RA (1997) Sepsis: a new hypothesis for pathogenesis of the disease process. Chest 112:235–243
4. Dinarello CA (1997) Proinflammatory and anti-inflammatory cytokines as mediators in the pathogenesis of septic shock. Chest 112:321S-329S
5. Chabot F, Mitchell JA, Gutteridge JM, Evans TW (1998) Reactive oxygen species in acute lung injury. Eur Respir J 11:745–757
6. Heller A, Koch T, Schmeck J, van Ackern K (1998) Lipid mediators in inflammatory disorders. Drugs 55:487–496
7. Yao YM, Redl H, Bahrami S, Schlag G (1998) The inflammatory basis of trauma/shock-associated multiple organ failure. Inflamm Res 47:201–210
8. Bone RC (1996) Sepsis, SIRS and CARS. Crit Care Med 24:1125–1128
9. Docke WD, Randow F, Syrbe U, Krausch D, Asadullahn K, Reinke P, Volk HD, Kox W (1997) Monocyte deactivation in septic patients: restoration by IFN-gamma treatment. Nat Med 3:678
10. Kox WJ, Bone RC, Krausch D, Docke WD, Kox SN, Wauer H, Egerer K, Querner S, Asadullah K, Baehr R, Volk HD (1997) Interferon gamma-1b in the treatment of compensatory anti-inflammatory response syndrome. A new approach: proof of principle. Arch Intern Med 157:389–393
11. Solomkin JS, Jenkins MK, Nelson RD, Chenoweth D, Simmons RL (1981) Neutrophil dysfunction in sepsis. II. Evidence for the role of complement activation products in cellular deactivation. Surgery 90:319–327
12. Solomkin JS, Cotta LA, Brodt JK, Hurst JW, Ogle CK (1984) Neutrophil dysfunction in sepsis. III. Degranulation as a mechanism for nonspecific deactivation. J Surg Res 36:407–412
13. Sorrell TC, Sztelma K, May GL (1994) Circulating polymorphonuclear leukocytes from patients with gram-negative bacteremia are not primed for enhanced production of leukotriene B4 or 5-hydroxyeicosatetraenoic acid. J Infect Dis 169:1151–1154
14. Pascual C, Karzai W, Meier-Hellmann A, Bredle DL, Reinhart K (1997) A controlled study of leukocyte activation in septic patients. Intensive Care Med 23:743–748
15. Mayer K, Seeger W, Grimminger F (1998) Clinical use of lipids to control inflammatory disease. Curr Opin Clin Nutr Metab Care 1:179–184

16. Calder PC (1998) Immunoregulatory and anti-inflammatory effects of n-3 polyunsaturated fatty acids. Braz J Med Biol Res 31:467–490
17. Vognild E, Elvevoll EO, Brox J, Olsen RL, Barstad H, Aursand M, Osterud B (1998) Effects of dietary marine oils and olive oil on fatty acid composition, platelet membrane fluidity, platelet responses, and serum lipids in healthy humans. Lipids 33:427–436
18. Guarini P, Bellavite P, Biasi D, Carletto A, Galvani S, Caramaschi P, Bambara LM, Corrocher R 1998. Effects of dietary fish oil and soy phosphatidylcholine on neutrophil fatty acid composition, superoxide release, and adhesion. Inflammation 22:381–391
19. Galban C, Montejo JC, Mesejo A, Marco P, Celaya S, Sanchez-Segura JM, Farre M, Bryg DJ (2000) An immune-enhancing enteral diet reduces mortality rate and episodes of bacteremia in septic intensive care unit patients. Crit Care Med 28:643–648
20. Kudsk KA, Minard G, Croce MA, Brown RO, Lowrey TS, Pritchard E, Dickerson RN, Fabian TC (1996) A randomized trial of isonitrogenous enteral diets after severe trauma. Ann Surg 224:531–543
21. Senkal M, Mumme A, Eickhoff U, Geier B, Späth G, Wulfert D, Joosten W, Frei A, Kemen M (1997) Early postoperative enteral immunonutrition: clinical outcome and cost-comparison analysis in surgical patients. Crit Care Med 25:1489–1496
22. Barton RG, Wells CL, Carlson A, Singh R, Sullivan JJ, Cerra FB (1991) Dietary omega-3 fatty acids decrease mortality and Kupffer cell prostaglandin E_2 production in a rat model of chronic sepsis. J Trauma 31:768–774
23. Johnson III, JA, Griswold JA, Muakkassa FF (1993) Essential fatty acids influence survival in sepsis. J Trauma 35:128–131
24. Grimminger F, Wahn H, Mayer K, Kiss L, Walmrath D, Seeger W (1997) Impact of arachidonic versus eicosapentaenoic acid on exotonin-induced lung vascular leakage. Am J Respir Crit Care Med 155:513–519
25. Mayser P, Mayer K, Mahloudjian M, Benzing S, Krämer H-J, Schill W-B, Seeger W, Grimminger F (2002). A double-blind, randomized, placebo-controlled trial of n-3 vs. n-6 fatty acid based lipid infusion in atopic dermatitis. JPEN J Parenter Enteral Nutr 26:151–158
26. Grimminger F, Führer D, Papavassilis C, Schlotzer E, Mayer K, Heuer K, Kiss L, Walmrath D, Piberhofer D, Lübbecke F, Krämer HJ, Stevens J, Schütterle G, Seeger W (1993) Influence of intravenous n-3 lipid supplementation on fatty acid profiles and lipid mediator generation in a patient with severe ulcerative colitis. Eur J Clin Invest 23:706–715
27. Knaus WA, Draper EA, Wagner DPO, Zimmermann JE (1985) APACHE II: a severity of disease classification system. Crit Care Med 13:818–829
28. Bone RC, Balk RA, Cerra FB, Dellinger RP, Fein AM, Knaus WA, Schein RM, Sibbald WJ (1992) Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM consensus conference committee. American college of chest physicians/society of critical care medicine. Chest 101:1644–1655
29. Grimminger F, Becker G, Seeger W (1988) High yield enzymatic conversion of intravascular leukotriene A4 in blood-free perfused lungs. J Immunol 141:2431–2436
30. Hjorth R, Jonsson AK, Vretblad P (1981) A rapid method for purification of human granulocytes using Percoll: a comparison with dextran sedimentation. J Immunol Methods 43:95–101
31. Grimminger F, Menger M, Becker G, Seeger W (1988) Potentiation of leukotriene production following sequestration of neutrophils in isolated lungs: indirect evidence for intercellular leukotriene A_4 transfer. Blood 72:1687–1692
32. Nadeau M, Fruteau de Laclos B, Picard S, Braquet P, Corey EJ, Borgeat P (1984) Studies on leukotriene B_4-oxidation in human leukocytes. Can J Biochem Cell Biol 62:1321–1326
33. Tessner TG, O'Flaherty JT, Wykle RL (1989) Stimulation of platelet-activating factor synthesis by a nonmetabolizable bioactive analog of platelet-activating factor and influence of arachidonic acid metabolites. J Biol Chem 264:4794–4799
34. Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37:753–757
35. Cohen HJ, Chovaniec ME (1978) Superoxide generation by digitonin-stimulated guinea-pig granulocytes. J Clin Invest 61:1081–1087
36. Berridge MJ, Dawson RM, Downes CP, Heslop JP, Irvine RF (1983) Changes in the level of inositol phosphates after agonist-dependent hydrolysis of membrane phosphoinositides. Biochem J 212:473–482
37. Krämer HJ, Stevens J, Seeger W (1993) Analysis of 2- and 3-series prostanoids by post-HPLC ELISA. Anal Biochem 214:535–543
38. Pace-Asciak CR (1989) One-step rapid extrachive methylation of plasma nonesterified fatty acids for gas chromatographic analysis. J Lipid Res 30:451–454
39. Bursten SL, Federighi DA, Parsons P, Harris WE, Abraham E, Moore EE Jr, Moore FA, Bianco JA, Singer JW, Repine JE (1996) An increase in serum C18 unsaturated free fatty acids as a predictor of the development of acute respiratory distress syndrome. Crit Care Med 24:1129–1136
40. Robin APJ Askanazi J, Greenwood MRC, Carpentier YA, Gump FE, Kinney JM (1981) Lipoprotein lipase activity in surgical patients: influence of trauma and sepsis. Surgery 90:401–408
41. Weissman C (1990) The metabolic response to stress: an overview and update. Anesthesiology 73:308–327
42. Forse RA, Leibel R, Askanazi J, Hirsch J, Kinney JM (1987) Adrenergic control of adipocyte lipolysis in trauma and sepsis. Ann Surg 206:744–751
43. Spitzer JA, Leach GJ, Palmer MA (1980) Some metabolic and hormonal alterations in adipocytes isolated from septic dogs. Adv Shock Res 4:55–62
44. Kiuchi S, Matsuo N, Takeyama N, Tanaka T (1993) Accelerated hepatic lipid synthesis in fasted septic rats. Eur Surg Res 25:146–154
45. Winter BK, Fiskum G, Gallo LL (1995) Effects of L-carnitine on serum triglyceride and cytokine levels in rat models of cachexia and septic shock. Br J Cancer 72:1173–1179
46. Mela-Riker L, Bartos D, Vlessis AA, Widener L, Muller P, Trunkey DD (1992) Chronic hyperdynamic sepsis in the rat. Characterization of liver and muscle energy metabolism. Circ Shock 36:83–92
47. Guidet B, Piot O, Masliah J, Barakett V, Maury E, Bereziat G, Offenstadt G (1996) Secretory non-pancreatic phospholipase A2 in severe sepsis: relation to endotoxin, cytokines and thromboxane B2. Infection 24:103–108
48. Gavino VC, Gavino GR (1992) Adipose hormone-sensitive lipase preferentially releases polyunsaturated fatty acids from triglycerides. Lipids 27:950–954
49. Samra JS, Simpson EJ, Clark ML, Forster CD, Humphreys SM, Macdonald IA, Frayn KN (1996) Effects of epinephrine infusion on adipose tissue: interactions between blood flow and lipid metabolism. Am J Physiol 34:E834–E839

50. Jaume JC, Mendel CM, Frost PH, Greenspan FS, Laughton CW (1996) Extremely low doses of heparin release lipase activity into the plasma and can thereby cause artifactual elevations in the serum-free thyroxine concentration as measured by equilibrium dialysis. Thyroid 6:79–83
51. Hammarström S, Hamberg M, Samuelsson B, Duell EA, Stawiski M, Voorhees JJ (1975) Increased concentrations of non-esterified arachidonic acid, 12L-hydroxyeicosatetraenoic acid, prostaglandin E_2 and prostaglandin F_{2alpha} in the epidermis of psoriasis. Proc Natl Acad Sci USA 72:5130
52. Unterberg A, Wahl M, Hammersen F, Baethmann A (1987) Permeability and vasomotor response of cerebral vessels during exposure to arachidonic acid. Acta Neuropathol (Berl) 73:209
53. Ullrich V, Hecker G, Schatz-Mundig M (1991) Platelet-neutrophil interactions. In: Sies H, Flohé L, Zimmer G (eds) Molecular aspects of inflammation. Springer, Berlin Heidelberg New York, pp 59–71
54. Peterson J, Bihain BE, Bengtsson-Olivecrona G, Deckelbaum RJ, Carpentier YA, Olivecrona T (1990) Fatty acid control of lipoprotein lipase: a link between energy metabolism and lipid transport. Proc Natl Acad Sci USA 87:909
55. Rustan AC, Hustvedt BE, Drevon CA (1998) Postprandial decrease in plasma unesterified fatty acids during n-3 fatty acid feeding is not caused by accumulation of fatty acids in adipose tissue. Biochim Biophys Acta 1390:245–257
56. Lovegrove JA, Brooks CN, Murphy MC, Gould BJ, Williams CM (1997) Use of manufactured foods enriched with fish oil as a means of increasing long-chain n-3 polyunsaturated fatty acid intake. Br J Nutr 78:223–236
57. Grimminger F, Scholz C, Bhakdi S, Seeger W (1991) Subhemolytic doses of Escherichia coli hemolysin evoke large quantities of lipoxygenase products in human neutrophils. J Biol Chem 266:14262–269
58. Grimminger F, Hattar K, Papavassilis C, Temmesfeld B, Csernok E, Gross WL, Seeger W, Sibelius U (1996) Neutrophil activation by anti-proteinase 3 antibodies in Wegener's granulomatosis: role of exogenous arachidonic acid and leukotriene B4 generation. J Exp Med 184:1567–1572
59. Solomkin JS, Brodt JK, Antrum RM (1985) Suppressed neutrophil oxidative activity in sepsis: a receptor-mediated regulatory response. J Surg Res 39:300–304
60. Vespasiano MC, Lewandoski JR, Zimmerman JJ (1993) Longitudinal analysis of neutrophil superoxide anion generation in patients with septic shock. Crit Care Med 21:666–672
61. Pascual C, Karzai W, Meier-Hellmann A, Bredle DL, Reinhart K (1997) A controlled study of leukocyte activation in septic patients. Intensive Care Med 23:743–748
62. Burke PA, Canning CM, Chartier S, Lazo S, Daley J, Forse RA, Ritz J (1994) Alterations of Ca2+ signal transduction in critical ill patients. Surgery 116:378–387
63. Grimminger F, Mayser P (1995) Lipid Mediators, free fatty acids and psoriasis. Prostaglandins Leukot Essent Fatty Acids 521–15
64. Czermak BJ, Sarma V, Pierson CL, Warner RL, Huber-Lang M, Bless NM, Schmal H, Friedl HP, Ward PA (1999) Protective effects of C5a blockade in sepsis. Nat Med 5:788–792