

In vitro mimicry of essential fatty acid deficiency in human endothelial cells by TNF α impact of ω -3 versus ω -6 fatty acids

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Abstract Severe endothelial abnormalities are a prominent feature in sepsis with cytokines such as tumor necrosis factor (TNF) α being implicated in the pathogenesis. As mimic to inflammation, human umbilical vascular endothelial cells (HUVEC) were incubated with TNF α for 22 h, in the absence or presence of the ω -6 fatty acid (FA), arachidonic acid (AA), or the alternative ω -3 FA eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). TNF α caused marked alterations in the PUFA profile and long chain PUFA content of total phospholipids (PL) decreased. In contrast, there was a compensatory increase in mead acid [MA, 20:3(ω -9)], the hallmark acid of the essential fatty acid deficiency (EFAD) syndrome. Corresponding changes were noted in phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol, but not in the sphingomyelin fraction. Supplementation with AA, EPA, or DHA markedly increased the respective FA contents in the PL pools, suppressed the increase in MA, and resulted in a shift either toward further predominance of ω -6 or predominance of ω -3 FA. We conclude that short-term TNF α incubation of HUVEC causes an EFAD state hitherto only described for long-term malnutrition, and that endothelial cells are susceptible to differential influence by ω -3 versus ω -6 FA supplementation under these conditions.—Mayer, K., R. Schmidt, M. Muhly-Reinholz, T. Bögeholz, S. Gokorsch, F. Grimminger, and W. Seeger. **In vitro mimicry of essential fatty acid deficiency in human endothelial cells by TNF α impact of ω -3 versus ω -6 fatty acids.** *J. Lipid Res.* 2002. 43: 944–951.

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Sepsis and septic shock continue to represent the major cause of death in critical care medicine worldwide, with unacceptably high mortality rates still ranging between 30% and 60% (1–3). Liberation of a large number of proinflammatory mediators has been demonstrated under clinical conditions as well as in experimental sepsis models (4–6). The term “systemic inflammatory response syndrome” (SIRS) reflects the fact that microcir-

culatory abnormalities and a systemic inflammatory reaction may not only be triggered by microbial toxins, but are also encountered in response to different kinds of severe tissue injury. Loss of endothelial function with prominent vascular leakage, perfusion abnormalities, disturbances of coagulation homeostasis, and enhanced endothelial-leukocyte interaction are hallmark characteristics of both sepsis and SIRS (7). Such endothelial abnormalities are mostly attributed to the impact of proinflammatory mediators, with cytokines such as tumor necrosis factor α (TNF α) assumed to play a major role (8, 9).

In addition to cytokines, eicosanoids have long been implicated in inflammatory events occurring in sepsis (6, 10). Arachidonic acid (AA) is metabolized via multiple metabolic pathways including cyclooxygenase and various lipoxygenases to prostanoids, leukotrienes, and other lipoxygenase products (6, 10). Both pro- and anti-inflam-

Abbreviations: AA, 20:4 ω -6, all-cis-5,8,11,14-eicosatetraenoic acid, arachidonic acid; CL, cardiolipin; DHA, 22:6 ω -3, all-cis-4,7,10,13,16,19-docosahexaenoic acid; EFAD, essential fatty acid deficiency syndrome; EPA, 20:5 ω -3, all-cis-5,8,11,14,17-eicosapentaenoic acid; FA, fatty acids; HUVEC, human umbilical venous endothelial cells; LT, leukotriene; OA, 18:1 ω -9, cis-9-octadecenoic acid, oleic acid; PAF, platelet-activating factor; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, prostaglandin; PI, phosphatidylinositol; PL, phospholipid; PS, phosphatidylserine; SPH, sphingomyelin; TNF, tumor necrosis factor; 14:0, tetradecanoic acid, myristic acid; 16:0, hexadecanoic acid, palmitic acid; 16:1 ω -7, cis-9-hexadecenoic acid, palmitoleic acid; 18:0, octadecanoic acid, stearic acid; 18:2 ω -6, all-cis-9,12-octadecadienoic acid, linoleic acid; 20:0, eicosanoic acid, arachidic acid; 20:1 ω -9, cis-11-eicosenoic acid; 20:2 ω -6, all-cis-11,14-eicosadienoic acid; 20:3 ω -9, all-cis-5,8,11-eicosatrienoic acid, mead acid; 20:3 ω -6, all-cis-8,11,14-eicosatrienoic acid; 20:3 ω -3, all-cis-11,14,17-eicosatrienoic acid; 22:0, docosanoic acid, behenic acid; 22:1 ω -9, cis-13-docosenoic acid, erucic acid; 22:4 ω -6, all-cis-7,10,13,16-docosatetraenoic acid; 22:5 ω -3, all-cis-7,10,13,16,19-docosapentaenoic acid; 24:0, tetracosanoic acid, lignoceric acid; 24:1 ω -9, cis-15-tetracosenoic acid, nervonic acid.

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matory functions have been ascribed to the various AA metabolites. The family of ω -6 fatty acids including AA represents the predominant PUFA in common Western diet. In contrast, ω -3 fatty acids make up an appreciable part of the fat in cold-water fish and seal meat. In this family of fatty acids (FA), the last double bond is located between the third and fourth carbon atom from the methyl end, with eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) being prominent representatives. They serve as alternative lipid precursors for all metabolic pathways hitherto recognized for AA, 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) (11). Interestingly, many of the ω -3 fatty acid-derived metabolites, including 5-series cysteinyl-LTs, LTB₅, and TxA₃, possess markedly reduced inflammatory and vasomotor potencies as compared with the AA-derived lipid mediators and may even exert antagonistic functions.

In addition to being precursors for different eicosanoids, ω -3 versus ω -6 fatty acid incorporation into membrane (phospho)-lipid pools was suggested to have impact on lipid-related intracellular signaling events (12–17). Phosphatidylinositol and sphingomyelin pools, but also subclasses of phosphatidylcholine such as the platelet-activating factor (PAF)-precursor pool, may be particularly relevant in this respect. This is of interest in view of the fact that diets with specific fat composition have attracted interest for modulating inflammatory and immunological processes (immunonutrition) (18–20). When offering ω -3 lipids such as fish oil via the enteral route it does, however, take several days to weeks to influence effectively the fatty acid composition of membrane (phospho)-lipids and thereby the lipid mediator profile in humans (21, 22). Pre-feeding animals with ω -3 lipids instead of ω -6 lipids was indeed found to enhance the survival in subsequently provoked septic events (23, 24). As an alternative approach, intravenous lipid emulsions, as used for parenteral nutrition under intensive care conditions, might be employed for rapid manipulation of the ω -3/ ω -6 balance, by choosing either conventional preparations (ω -6 predominance) or a fish oil-based lipid emulsion (ω -3 predominance). High levels of the respective free fatty acids are readily detectable in septic patients undergoing parenteral nutrition with lipids (25). The lipid emulsions cause an activation of the endothelial lipoprotein lipase and the release of free fatty acids (26–28), which is greatly enhanced in septic patients where levels of free AA may surpass 100 μ mol/l (25). The impact of these interventions on the fatty acid composition of endothelial (phospho)-lipid pools is, however, largely unknown.

Against this background, the current study employed human endothelial cells under TNF α challenge as a mimic of systemic inflammatory conditions *in vitro*. We sought first putative changes in the fatty acid composition of relevant endothelial phospholipid pools in response to the cytokine challenge, and second for the impact ω -3 versus ω -6 fatty acid supplementation under these conditions. Most interestingly, both loss of PUFAs in the various

phospholipid pools and enhanced appearance of the hallmark FA eicosatrienoic acid [20:3(ω -9), mead acid] indicated that 22 h of TNF α incubation sufficed to provoke a severe essential fatty acid deficiency (EFAD) state in the endothelial cells, hitherto only recognized during long-term malnutrition (29) or under conditions of long-term lipid-deprived cell culture (30). When offered under these conditions, both ω -3 and ω -6 fatty acids were readily incorporated into the various phospholipid pools, with a corresponding shift of the ω -3/ ω -6 balance. We speculate that the endothelial essential fatty acid homeostasis may be disturbed under conditions of severe inflammation and sepsis, which may have impact on lipid-dependent signaling pathways and mediator generation and is susceptible to differential influence by ω -3 versus ω -6 fatty acid supplementation.

METHODS

Cell culture

Primary cultures of human umbilical vein endothelial cells (HUVEC) were prepared by a modification of the method of Jaffe et al. (31) as previously described (32). For incorporation experiments, cells were washed and then incubated for 6 h in a 5% CO₂ atmosphere with MCDB 131 (Gibco, Karlsruhe, Germany) containing 1% FBS enriched with 10 μ M fatty acids (free acids of [³H]AA, [³H]EPA, and [³H]DHA, Amersham Biosciences, Freiburg, Germany). Reactions were terminated by removal of the incubation medium and washing the cells twice with medium. For counting and extraction, HUVEC were harvested with trypsin (Gibco). Cells were washed and immediately frozen at -80° C under nitrogen.

Experimental protocol

In control experiments HUVEC were incubated in the absence of TNF α and fatty acids in MCDB 131 supplemented with 1% FCS for 22 h. In all other groups, the incubation medium was supplemented with 10 ng/ml TNF α (R & D, Wiesbaden, Germany) for 16 h after a pre-incubation of HUVEC for 6 h with either sham (+TNF-group) or 10 μ mol/l of either AA, EPA, or DHA (free fatty acids from Sigma, Deisenhofen, Germany; TNF + AA, TNF + EPA, TNF + DHA groups, respectively). All experiments were performed in $n = 6$ independent cell cultures, unless otherwise stated.

Lipid analysis

Following treatment with ultrasound, cellular lipids were extracted using methanol-chloroform containing 0.01% butylated hydroxytoluene (BHT, *m/v*) according to the method of Bligh and Dyer (33). Total phospholipids (PL) were calculated using a colorimetric phosphorus assay (34). The distribution of PL classes was determined using high performance TLC as previously described (35). The one-dimensional system allows the separation of eight phospholipid classes: lyso-phosphatidylcholine (LPC), sphingomyelin (SPH), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidic acid (PA), and cardiolipin (CL). Staining was performed using molybdenum blue reagent according to Gustavsson (36). Quantification was done by means of densitometric scanning at 700 μ m using a TLC-scanner II (Camag).

FA analysis of PL classes

Choline, serine, inositol, and ethanolamine phospholipids and sphingomyelin were separated on silica 60 thin layer plates (Merck, Darmstadt, Germany) with chloroform-methanol-acetic acid-water (50:37.5:3.5:2, v/v/v/v) as developing solvent. Following nondestructive visualization of individual and standard PLs with primuline (37), the corresponding gel compartments as well as blanks were eluted after addition of 10 µg pentadecanoic acid as an internal standard with 10 ml chloroform-methanol (v/v). The ester bound fatty acids were converted to fatty acid methyl ester (FAME) using acid-catalyzed transmethylation with 2 N HCl in methanol and analyzed using gas chromatography as described (38, 39).

Analysis of PC subclasses

Phosphatidylcholine subclasses were isolated by means of HPLC subsequent to phospholipolytic cleavage of the polar headgroup with phospholipase C and conversion of the resulting diradylglycerols (DRG) with 1-naphthylisocyanate, with slight modifications of the method originally described by Ruestow et al. (40, 41). For fatty acid analysis, the alkyl-acyl subclass was subjected to gas chromatography as described (37).

Statistics

For statistical comparison, one-way ANOVA was performed. A level of $P < 0.05$ was considered to be significant.

RESULTS

Kinetics of free fatty acid uptake by endothelial cells

To determine the kinetics of free FA incorporation into endothelial cells, HUVEC were incubated with 10 µmol/l AA, EPA, or DHA, each spiked with radiolabeled FA. Within 2 h about 50–60%, and within 6 h about 80–90% of the label was found to be incorporated into the cells (data not given in detail).

Profile of phospholipid classes

Analysis of the phospholipid profile revealed no substantial differences between cells in the absence and presence of TNF challenge and those additionally incubated with the different PUFAs (Table 1). The most abundant PL was phosphatidylcholine (PC, ~40%), followed by phosphatidylethanolamine (PE, ~30%), phosphatidylserine (PS, ~12%), and sphingomyelin (SPH, ~9%). Phosphatidylinositol (PI, ~5%) and cardiolipin (CL, ~4%) appeared as minor compounds of total phospholipids. Other phos-

pholipid classes (lyso-PC, phosphatidic acid) ranged below detection limits.

Fatty acid profile of total phospholipids

The FA profile of total endothelial PL under control conditions was examined first. Next to palmitic acid (~22%) and oleic acid (24%), stearic acid (~18%) and arachidonic acid (~9%) represented major compounds, with only a minor contribution of ω-3 fatty acids (~10% total). Pre-incubation of cells with TNF (+TNF) caused marked alterations in the FA profile of total phospholipids. PUFA content decreased in response to the cytokine challenge, as evident for 18:2, 20:3(ω-6), 20:4, 20:5, 22:5(ω-3), and 22:6(ω-3). In contrast, the eicosatrienoic acid 20:3(ω-9) (mead acid) increased under these conditions (2.3%; control: 0.8%, $P < 0.01$). As a consequence, the 20:3(ω-9) to 20:4 ratio increased from 1:11.4 to 1:2.8. When the TNF challenge was undertaken in the presence of AA, the decrease in the percentage of this FA was fully reversed, and the increase in 20:3(ω-9) was reduced. In addition, an increase in the content of docosatetraenoic acid [22:4(ω-6)], the product of AA chain elongation, was noted, suggesting PUFA metabolism. Corresponding results were obtained for EPA and DHA incubation of TNF-treated HUVEC; the percentage of the respective fatty acids markedly increased, even above control levels, concomitant with a limitation of the upregulation of 20:3(ω-9). In addition, docosapentaenoic acid [22:5(ω-3)], the elongase metabolite of EPA, rose from 3.2% (TNF) to 10.6% (TNF + EPA). A minor increase in EPA was noted also in the DHA-exposed cells, further suggesting interconversion of the ω-3 fatty acids in the endothelial cells (data not given in detail).

Fatty acid profile of phosphatidylcholine

The main fatty acids in the PC fraction of control HUVEC were palmitic acid (~38%), oleic acid (~30%), and stearic acid (~9%) (Table 2). Compared with the fatty acids of total PL, the PC fraction contained relatively small amounts of long chain PUFA (3.6% AA, 0.6% EPA, and 1.7% DHA). Similar to total PL, TNF treatment of HUVEC resulted in an approximately 50% reduction of AA, EPA, and DHA, concomitant with an approximately 3-fold increase of 20:3(ω-9). In the presence of exogenous AA, the percentage of this fatty acid as well as 22:4(ω-6) was increased. Supplementa-

TABLE 1. Profile of phospholipid classes

	PC	PE	PS	SPH	PI	CL
	%					
Control	40.1 ± 2.3	30.4 ± 0.4	13.0 ± 0.9	8.0 ± 0.9	3.7 ± 0.1	4.6 ± 0.6
+TNF	42.5 ± 1.3	28.2 ± 0.9	11.5 ± 0.6	8.3 ± 0.2	4.9 ± 0.2	4.6 ± 0.2
+AA +TNF	42.9 ± 1.8	27.8 ± 1.1	12.4 ± 1.1	9.2 ± 1.1	5.0 ± 0.7	2.7 ± 0.2
+EPA +TNF	40.3 ± 0.7	30.3 ± 0.3	12.1 ± 0.3	9.5 ± 0.3	4.9 ± 0.0	2.9 ± 0.3
+DHA +TNF	40.8 ± 1.0	29.9 ± 1.1	12.4 ± 0.7	8.7 ± 0.5	4.9 ± 0.5	3.3 ± 0.1

The percentage (wt/wt) of each phospholipid class is shown (mean ± SD).

CL, cardiolipin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SPH, sphingomyelin.

TABLE 2. Fatty acid profiles of phosphatidylcholine originating from the different experimental groups

	Ctrl	+TNF	+AA +TNF	+EPA +TNF	+DHA +TNF
14:0	3.4 ± 0.8	4.6 ± 0.6	4.6 ± 0.6	4.0 ± 0.8	4.6 ± 0.3
16:0	37.7 ± 1.9	40.7 ± 2.0	39.3 ± 1.5	36.8 ± 0.5	39.4 ± 2.1
16:1	3.7 ± 1.3	5.3 ± 0.8	4.8 ± 0.3	3.2 ± 0.9	4.5 ± 0.4
18:0	9.2 ± 1.6	7.9 ± 1.1	6.6 ± 1.5	5.8 ± 1.9	6.7 ± 1.4
18:1 (n-9)	30.2 ± 2.9	31.5 ± 1.0	29.1 ± 1.4	26.5 ± 3.7	30.0 ± 1.4
18:2 (n-6)	2.9 ± 1.5	1.6 ± 0.1	2.0 ± 0.3	2.7 ± 0.7	2.1 ± 0.4
20:1 (n-9)	0.6 ± 0.1	0.8 ± 0.2	0.4 ± 0.2	0.4 ± 0.2	0.4 ± 0.2
20:3 (n-9)	0.4 ± 0.2	1.2 ± 0.1 ^b	0.4 ± 0.3 ^d	0.3 ± 0.2 ^d	0.3 ± 0.2 ^d
20:3 (n-6)	1.6 ± 0.3	0.7 ± 0.2 ^a	1.5 ± 0.3 ^d	1.3 ± 0.3 ^c	1.1 ± 0.2
20:4 (n-6)	3.6 ± 0.4	2.0 ± 0.3 ^b	5.0 ± 0.5 ^e	2.6 ± 0.3 ^c	2.1 ± 0.1
20:5 (n-3)	0.7 ± 0.1	0.3 ± 0.1 ^a	0.3 ± 0.1	4.3 ± 1.3 ^e	1.7 ± 0.7 ^d
22:1 (n-9)	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.1
22:4 (n-6)	0.9 ± 0.5	0.5 ± 0.1	1.9 ± 0.2 ^e	0.9 ± 0.2 ^c	0.4 ± 0.1
22:5 (n-3)	1.9 ± 0.7	1.1 ± 0.2	1.5 ± 0.1	8.5 ± 2.9 ^e	1.8 ± 0.3 ^c
22:6 (n-3)	1.7 ± 0.5	0.9 ± 0.2 ^a	1.1 ± 0.2	1.2 ± 0.3	3.5 ± 1.0 ^e
24:0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.1	0.2 ± 0.1
MA/AA	0.1 ± 0.0	0.6 ± 0.0 ^b	0.1 ± 0.0 ^e	0.1 ± 0.1 ^e	0.2 ± 0.1 ^e
(EPA + DHA)/AA	0.7 ± 0.0	0.7 ± 0.1	0.3 ± 0.0 ^d	2.1 ± 0.3 ^e	2.4 ± 0.4 ^e

The relative content of each fatty acid is given in percent (wt/wt; mean ± SD) of all fatty acids analyzed in the PC fraction of cellular lipid extract from the different experimental groups. In addition, the ratio of mead acid to AA (MA/AA) and the (EPA + DHA)/AA ratio are given.

^a $P < 0.01$ and ^b $P < 0.001$, as compared to control.

^c $P < 0.05$, ^d $P < 0.01$, and ^e $P < 0.001$, as compared to TNF-challenge (+TNF).

tion of HUVEC with EPA markedly augmented the EPA and the 22:5(ω-3) content in the TNF-exposed HUVEC, and supplementation with DHA was reflected by an increase of this fatty acid and of EPA in the PC fraction.

Fatty acid profile of phosphatidylethanolamine

Phosphatidylethanolamine of control HUVEC contained large amounts of long chain PUFA (AA, ~17%; EPA, ~3%; DHA, ~12%) (Table 3). As found in PC, the pretreatment with TNF resulted in marked reduction of

PUFAs in the PE fraction [18:2, -30%; 20:3(ω-6), -35%; 20:4, -20%; 20:5, -27%; 22:4(ω-6), -25%; 22:5(ω-3), -17%; 22:6(ω-3), -18%], whereas 20:3(ω-9) increased from ~0.8% to ~3.8%. The responses to supplementation with AA, EPA, or DHA largely corresponded to those in the PC fraction.

Fatty acid profile of phosphatidylserine

Control cell PS was composed mainly of stearic acid (~36%), oleic acid (~21%), palmitic acid (~6%), and lig-

TABLE 3. Fatty acid profiles of phosphatidylethanolamine originating from the different experimental groups

	Ctrl	+TNF	+AA +TNF	+EPA +TNF	+DHA +TNF
14:0	0.4 ± 0.0	0.5 ± 0.8	0.4 ± 0.1	0.5 ± 0.0	0.6 ± 0.0
16:0	7.3 ± 1.1	8.8 ± 1.1	9.5 ± 0.6	9.2 ± 0.9	9.3 ± 0.5
16:1	1.1 ± 0.6	1.9 ± 0.3	1.2 ± 0.2	1.6 ± 0.0	1.2 ± 0.2
18:0	21.2 ± 1.4	22.0 ± 2.2	23.4 ± 2.8	22.5 ± 0.5	22.6 ± 1.0
18:1 (n-9)	19.4 ± 2.4	22.6 ± 2.5	21.4 ± 2.1	20.4 ± 0.4	20.2 ± 0.1
18:2 (n-6)	1.3 ± 0.6	0.9 ± 0.3	1.0 ± 0.3	1.4 ± 0.2	1.3 ± 0.1
20:1 (n-9)	0.4 ± 0.1	0.9 ± 0.4	0.4 ± 0.3	0.8 ± 0.1	0.8 ± 0.1
20:3 (n-9)	0.8 ± 0.5	3.8 ± 1.1 ^b	1.1 ± 0.5 ^d	0.2 ± 0.0 ^e	0.3 ± 0.1 ^e
20:3 (n-6)	2.2 ± 0.2	1.4 ± 0.2 ^a	1.8 ± 0.4	1.9 ± 0.4	1.7 ± 0.1
20:4 (n-6)	17.0 ± 2.2	13.7 ± 1.4	17.2 ± 1.2 ^c	8.9 ± 0.2 ^d	7.8 ± 0.2 ^d
20:5 (n-3)	3.1 ± 0.4	2.3 ± 0.7	1.2 ± 0.2 ^c	7.9 ± 1.0 ^e	4.5 ± 0.1 ^d
22:1 (n-9)	0.8 ± 0.1	0.8 ± 0.2	0.3 ± 0.1 ^c	0.1 ± 0.0 ^d	0.1 ± 0.1 ^d
22:4 (n-6)	3.6 ± 1.4	2.7 ± 0.5	5.3 ± 0.2 ^e	2.5 ± 0.4	1.3 ± 0.2 ^d
22:5 (n-3)	8.6 ± 0.7	7.2 ± 0.8	5.8 ± 0.6	16.0 ± 1.3 ^e	5.4 ± 0.7
22:6 (n-3)	11.5 ± 0.8	9.4 ± 1.4	7.1 ± 0.6 ^c	5.2 ± 0.6 ^d	21.1 ± 1.0 ^e
24:0	0.4 ± 0.2	0.5 ± 0.1	0.3 ± 0.0	0.4 ± 0.0	0.4 ± 0.1
MA/AA	0.05 ± 0.01	0.27 ± 0.05 ^b	0.07 ± 0.04 ^d	0.02 ± 0.00 ^e	0.04 ± 0.00 ^e
(EPA + DHA)/AA	0.9 ± 0.0	0.9 ± 0.1	0.5 ± 0.0 ^d	1.5 ± 0.0 ^d	3.3 ± 0.0 ^e

The relative content of each fatty acid is given in percent (wt/wt; mean ± SD) of all fatty acids analyzed in the PE fraction of cellular lipid extract from the different experimental groups. In addition, the ratio of mead acid to AA (MA/AA) and the (EPA + DHA)/AA ratio are given.

^a $P < 0.05$ and ^b $P < 0.001$, as compared to control.

^c $P < 0.05$, ^d $P < 0.01$, and ^e $P < 0.001$, as compared to TNF-challenge (+TNF).

TABLE 4. Fatty acid profiles of phosphatidylserine originating from the different experimental groups

	Ctrl	+TNF	+AA +TNF	+EPA +TNF	+DHA +TNF
14:0	0.3 ± 0.1	0.6 ± 0.0 ^a	0.8 ± 0.5	0.8 ± 0.4	0.9 ± 0.2
16:0	5.6 ± 1.7	9.0 ± 0.8 ^a	11.7 ± 3.2	9.8 ± 5.4	10.4 ± 0.6
16:1	0.9 ± 0.4	1.5 ± 0.4	1.0 ± 0.6	0.7 ± 0.4	0.6 ± 0.4
18:0	36.4 ± 1.1	35.2 ± 1.2	34.4 ± 2.4	33.1 ± 1.4	34.0 ± 1.4
18:1(n-9)	21.2 ± 4.1	22.1 ± 1.2	20.0 ± 2.7	19.7 ± 0.4	22.2 ± 1.6
18:2(n-6)	1.1 ± 0.5	0.9 ± 0.3	1.1 ± 0.3	1.1 ± 0.5	0.8 ± 0.3
20:0	0.7 ± 0.9	0.6 ± 0.1	0.4 ± 0.2	0.6 ± 0.3	0.4 ± 0.3
20:1(n-9)	0.5 ± 0.2	0.4 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
20:3(n-9)	0.1 ± 0.1	0.2 ± 0.0	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
20:3(n-6)	3.0 ± 0.3	3.0 ± 0.9	2.0 ± 0.5	1.9 ± 0.5	2.6 ± 0.2
20:4(n-6)	2.1 ± 1.2	1.1 ± 0.2	2.0 ± 0.4 ^c	1.2 ± 0.3	1.5 ± 0.3
20:5(n-3)	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.7 ± 0.4 ^c	0.1 ± 0.1
22:0	2.7 ± 0.6	2.3 ± 0.4	2.5 ± 0.6	2.4 ± 0.8	3.0 ± 0.2
22:1(n-9)	0.8 ± 0.2	0.5 ± 0.1	0.6 ± 0.2	0.5 ± 0.4	0.5 ± 0.1
22:4(n-6)	8.2 ± 1.7	7.6 ± 1.1	9.2 ± 1.6	7.7 ± 2.9	7.1 ± 2.7
22:5(n-3)	3.7 ± 1.1	3.5 ± 0.7	2.0 ± 0.3	8.7 ± 2.4 ^e	2.6 ± 0.9
22:6(n-3)	3.8 ± 1.3	2.0 ± 1.2	1.9 ± 0.3	2.7 ± 1.2	2.4 ± 0.3
24:0	8.8 ± 1.5	9.4 ± 1.4	10.0 ± 0.7	8.0 ± 0.2	9.2 ± 2.0
MA/AA	0.05 ± 0.01	0.20 ± 0.02 ^b	0.11 ± 0.02 ^d	0.15 ± 0.02 ^c	0.13 ± 0.02 ^c
(EPA + DHA)/AA	1.9 ± 0.2	1.9 ± 0.1	0.9 ± 0.2 ^e	2.8 ± 0.3 ^d	1.7 ± 0.2

The relative content of each fatty acid is given in percent (wt/wt; mean ± SD) of all fatty acids analyzed in the PS fraction of cellular lipid extract from the different experimental groups. In addition, the ratio of mead acid to AA (MA/AA) and the (EPA + DHA)/AA ratio are given.

^a $P < 0.05$ and ^b $P < 0.001$, as compared to control.

^c $P < 0.05$, ^d $P < 0.01$, and ^e $P < 0.001$, as compared to TNF-challenge (+TNF).

noceric acid (~9%). Again, treatment with TNF caused reduction in PUFAs and an increase in 20:3(ω-9). Very low values of AA (~2.1%) and DHA (~3.8%) were found in the PS fraction of control HUVEC, further decreasing upon TNF challenge, whereas EPA was near the detection limit (Table 4). Supplementation with AA, EPA, and DHA increased the percentages in these fatty acids.

Fatty acid profile of sphingomyelin

Sphingomyelin from nontreated HUVEC mainly contained palmitic acid (~42%), lignoceric acid (24:0, ~18%), nervonic acid (24:1, ~26%), stearic acid (~5%), and behenic acid (22:0, ~6%; Table 5). Only saturated and monoenoic FAs were found in this PL. Pretreatment of HUVEC with TNF resulted in no major alteration of the FA profile. No detectable amounts of PUFA were found upon supplementation of HUVEC with AA, EPA, or DHA.

Fatty acid profile of phosphatidylinositol

The main FA in the PI fraction of control HUVEC was stearic acid (~43%), followed by arachidonic acid (~26%) and oleic acid (~10%, Table 6). Pretreatment of cells with TNF again resulted in a pronounced decrease of PUFA including AA and EPA, whereas 20:3(ω-9) increased from ~4% to ~11%. The response to AA, EPA, or DHA supplementation largely corresponded to that in the PC and PE fraction.

Distribution of phosphatidylcholine subclasses

Analysis of the phosphatidylcholine subclasses by HPLC revealed a predominance of the diacyl compounds (~95%; data not given in detail), with small amounts of alkyl-acyl (PAF-precursor, ~2.5%) and alkenyl-acyl com-

pounds (plasmalogens, ~2.5%). The relative distribution of PC subclasses did not significantly change in response to TNF treatment and AA/EPA/DHA supplementation. Because of its significance for signaling events, analysis of the fatty acid content of the PAF-precursor pool was undertaken; however, quantification was hampered by the very limited amount of material. AA was detected only in the TNF/AA challenged HUVEC, and the same was true for EPA (experiments with TNF/EPA) and DHA (experiments with TNF/DHA).

DISCUSSION

In the present study, incubation of human endothelial cells with TNFα for 22 h caused marked alterations in the fatty acid profile of total phospholipids. Long chain PUFA content decreased, as evident for 18:2, 20:3(ω-6), 20:4, 20:5,

TABLE 5. Fatty acid profiles of sphingomyelin originating from the different experimental groups

	Ctrl	+TNF	+AA +TNF	+EPA +TNF	+DHA +TNF
14:0	1.5 ± 0.9	2.0 ± 1.1	1.9 ± 0.8	2.2 ± 0.6	1.3 ± 0.1
16:0	41.9 ± 2.9	40.9 ± 3.5	42.1 ± 4.3	43.2 ± 4.2	45.4 ± 4.1
18:0	4.9 ± 1.6	7.0 ± 2.9	5.8 ± 2.6	5.1 ± 1.0	5.3 ± 1.6
18:1(n-9)	0.4 ± 0.3	0.8 ± 0.5	1.3 ± 0.8	1.0 ± 0.7	0.6 ± 0.3
20:0	0.7 ± 0.3	0.9 ± 0.6	0.9 ± 0.4	0.8 ± 0.5	0.6 ± 0.4
22:0	5.7 ± 1.1	6.3 ± 1.7	6.2 ± 1.1	5.6 ± 1.2	5.6 ± 0.9
24:0	18.1 ± 2.1	16.4 ± 2.6	16.0 ± 2.7	15.3 ± 0.6	17.3 ± 1.9
24:1	26.3 ± 3.5	25.0 ± 4.1	25.1 ± 3.2	26.3 ± 3.6	23.4 ± 3.3

The relative content of each fatty acid is given in percent (wt/wt; mean ± SD) of all fatty acids analyzed in the sphingomyelin fraction of cellular lipid extract from the different experimental groups.

TABLE 6. Fatty acid profiles of phosphatidylinositol originating from the different experimental groups

	Ctrl	+TNF	+AA +TNF	+EPA +TNF	+DHA +TNF
14:0	0.2 ± 0.1	0.3 ± 0.3	0.5 ± 0.1	0.5 ± 0.3	0.3 ± 0.1
16:0	3.1 ± 0.4	4.2 ± 0.5	4.4 ± 0.6	4.3 ± 0.7	4.3 ± 0.8
16:1	0.5 ± 0.2	0.8 ± 0.2	0.5 ± 0.2	0.5 ± 0.4	0.4 ± 0.2
18:0	41.7 ± 1.1	39.8 ± 1.0	39.6 ± 1.7	38.8 ± 0.8	44.5 ± 0.6 ^d
18:1 (n-9)	9.8 ± 2.7	13.4 ± 1.4	11.6 ± 0.8	11.2 ± 0.8	11.5 ± 0.9
18:2 (n-6)	0.7 ± 0.3	0.6 ± 0.1	0.6 ± 0.1	0.9 ± 0.1	0.7 ± 0.1
20:2 (n-6)	0.4 ± 0.3	1.0 ± 0.5	0.2 ± 0.1	0.1 ± 0.1 ^d	0.0 ± 0.0 ^d
20:3 (n-9)	3.7 ± 0.6	11.0 ± 4.0 ^b	2.8 ± 1.6 ^d	2.5 ± 1.8 ^d	3.6 ± 2.0 ^d
20:3 (n-6)	5.0 ± 1.8	3.4 ± 1.1	2.7 ± 0.5	2.5 ± 0.4	3.6 ± 0.8
20:4 (n-6)	26.1 ± 3.2	17.9 ± 2.9 ^a	27.1 ± 5.1 ^c	20.6 ± 3.0	18.7 ± 2.5
20:5 (n-3)	0.5 ± 0.4	0.4 ± 0.1	0.1 ± 0.1 ^d	6.2 ± 2.3 ^e	2.0 ± 1.0 ^d
22:4 (n-6)	1.9 ± 0.1	1.5 ± 0.2	3.2 ± 0.3 ^e	1.2 ± 0.2	0.9 ± 0.4
22:5 (n-3)	3.5 ± 0.5	2.7 ± 0.6	3.6 ± 0.7	8.1 ± 2.6 ^d	2.9 ± 0.2
22:6 (n-3)	2.3 ± 0.8	1.7 ± 0.6	2.5 ± 0.4	1.4 ± 0.2	6.0 ± 1.1 ^e
MA/AA	0.15 ± 0.01	0.66 ± 0.13 ^b	0.13 ± 0.09 ^d	0.15 ± 0.13 ^d	0.22 ± 0.15 ^e
(EPA + DHA)/AA	0.11 ± 0.01	0.13 ± 0.01	0.10 ± 0.01	0.36 ± 0.06 ^e	0.42 ± 0.03 ^e

The relative content of each fatty acid is given in percent (wt/wt; mean ± SD) of all fatty acids analyzed in the PI fraction of cellular lipid extract from the different experimental groups. In addition, the ratio of mead acid to AA (MA/AA) and the (EPA + DHA)/AA ratio are given.

^a $P < 0.05$ and ^b $P < 0.001$, as compared to control.

^c $P < 0.05$, ^d $P < 0.01$, and ^e $P < 0.001$, as compared to TNF-challenge (+TNF).

22:5(ω-3), and 22:6(ω-3), whereas mead acid [20:3(ω-9)] increased. Corresponding changes were noted in the phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol pools, but not in the sphingomyelin fraction. Supplementation with AA, EPA, or DHA under these conditions increased the respective fatty acid contents in the various phospholipid pools, with a corresponding shift in the ω-3/ω-6 ratio, and suppressed the increase in 20:3(ω-9).

In contrast to measurements in leukocytes (42, 43), only limited data on the fatty acid composition of the various phospholipid pools of human endothelial cells are currently available (44). Most impressively, 22 h incubation with TNFα in a concentration range found in peak levels in septic shock patients (45) sufficed to provoke marked changes in the fatty acid composition of all, with the exception of sphingomyelin, endothelial phospholipid pools were investigated: a consistent decrease in long-chain PUFAs was noted. These changes correspond to the alterations in fatty acid composition encountered under conditions of essential fatty acid deficiency (EFAD) due to malnutrition (29, 30). It is in line with this view that the eicosatrienoic acid 20:3(ω-9), the hallmark acid of EFAD, was noted to be markedly increased in total phospholipids as well as in the PC, PE, PS, and PI pools. Of note, the 20:3(ω-9)/20:4 ratio, usually taken as parameter for quantifying the severity of EFAD (46), increased from ~1:7 to ~1:1.5 in PC and PI of endothelial cells under TNFα challenge. Mead acid is normally present in animal tissue at very low quantities. It is the main PUFA synthesized by complete endogenous synthesis via elongation and desaturation of 18:1(ω-9), and its formation is upregulated upon lack of essential fatty acids. Mead acid itself is a poor substrate for elongation and desaturation and, due to the lack of a ω-6 double bond, for prostanoid and lipoxigenase product formation (46, 47).

Several mechanisms may underlie the depletion in PUFAs and the compensatory increase in mead acid in the TNFα-exposed endothelial cells. In vitro growth of hepatic cells and HUVEC in delipidated medium was previously noted to mimic essential fatty acid deficiency with upregulation of 20:3(ω-9) within 10 days (30, 48). However, HUVEC were incubated in FCS supplemented medium with normal lipid contents for only 22 h, and control endothelial cells grown under these conditions displayed fully normal PUFA composition and mead acid-AA ratios. Furthermore, TNFα may lead to activation of different phospholipolytic activities in various cell types, and preferential liberation of PUFAs, and in particular AA, was noted under these conditions (49). In addition to enhanced phospholipolytic activities, however, reduced re-incorporation of the PUFAs would be expected to result in such a markedly disturbed PUFA balance of the various phospholipid pools. In addition, TNFα may proceed via increased oxygen radical attack on the sensitive unsaturated domains of PUFAs, whether membrane-incorporated or liberated by phospholipolytic activity, in the TNFα-stimulated HUVEC. To the best of our knowledge, the quantitative relevance of such mechanisms in endothelial cells in the absence of neutrophil attack has hitherto not been addressed in detail (50–52); however, reduced intracellular glutathione levels were previously noted in endothelial cells undergoing TNFα challenge (51, 52). Finally, endothelial cells possess limited ability to synthesize AA from the precursor linoleic acid due to low basal rates of elongases and desaturases (30, 53) adding to the loss of PUFA. Notably, the marked changes in HUVEC phospholipid FA composition in response to the presently used TNFα concentration occurred in the absence of overt cell damage, as ascertained by lactate dehydrogenase release and trypan blue exclusion (data not given in detail).

Offering exogenous AA to the TNFα-activated endothe-


lial cells resulted in a marked increase in the percentage of this FA in the various phospholipid pools as compared with mono-cytokine challenge. Notably, due to near normalization of the AA content in the various phospholipid pools but still reduced EPA and DHA levels under TNF α challenge, the (EPA + DHA)/AA ratio was markedly decreased under conditions of AA supplementation.

Supplementation of TNF α activated HUVEC with EPA or DHA resulted in similarly impressive rates of incorporation of the respective fatty acids into the various phospholipid pools as noted for AA. Calculated for all phospholipids, the percentages of EPA and DHA in the TNF α /EPA and TNF α /DHA groups, respectively, were even manifold increased compared with control EC. This incorporation rate resulted in a dramatic change in the ω -3 (EPA + DHA)/ ω -6 (AA) eicosanoid precursor fatty acid ratio in all phospholipid pools: in PI, the ratio increased from \sim 1:9.4 to \sim 1:2.8–1:2.4, and in the other PL pools the content of EPA plus DHA even surpassed the content of AA. Such an impressive shift of the ω -3/ ω -6 ratio toward the alternative precursor fatty acids was hitherto only noted, for example, in leukocytes after long term dietary intake of fish oil capsules (17). In addition, the increase in 22:5(ω -3) in EPA-treated HUVEC and in 20:5(ω -3) in DHA-treated cells indicated some further conversion of these fatty acids in the endothelial cells. Notably, the increase in the EFAD indicator fatty acid 20:3(ω -9) was similarly suppressed under conditions of EPA or DHA supplementation as observed for supply with AA.

Of note, the concentration of AA, EPA, and DHA used in our experimental protocol is well in the range of these free fatty acids under oral supplementation with fish oil capsules and much lower as achieved under conditions of total parenteral nutrition of septic patients (25).

Although assessment of the functional consequences of the currently observed marked changes in fatty acid composition of the various endothelial phospholipid pools was beyond the scope of the present study, a marked impact of these changes on metabolic and signaling events is to be expected. The appearance of mead acid, representing a poor substrate for many metabolic pathways, in companion with decreased percentages of PUFAs, has been implicated in abnormalities in prostanoid formation and PI-dependent signaling pathways in the EFAD syndrome (54). The ω -3/ ω -6 ratio in pools serving as precursors for eicosanoid formation, in particular the PI pool and the PC pool including the PAF-precursor subpool, will have an impact on prostanoid formation by the endothelial cells themselves (55).

In conclusion, this is the first report to describe that short-term incubation of human endothelial cells with TNF α in doses not provoking cell death reproduce the criteria of essential fatty acid deficiency in vitro, i.e., loss of PUFAs in various phospholipid pools and upregulation of mead acid [20:3(ω -9)] as EFAD hallmark fatty acid. Supplementation with AA, EPA, or DHA under these conditions resulted in rapid incorporation into various phospholipid pools, reversed the increase in mead acid, and at the same time profoundly shifted the ω -3/ ω -6 ratio of the

endothelial cells to further predominance of ω -6 (AA) or to near equimolar presence of ω -3 and ω -6 (EPA, DHA). Both the cytokine-induced essential fatty acid deficiency and the susceptibility to ω -3- versus ω -6-fatty acid supplementation may be relevant for endothelial abnormalities and responses to parenteral nutrition with lipid emulsions in patients suffering from sepsis or SIRS. 

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