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Development and characterization of essential fatty acid deficiency in human endothelial cells in culture

(eicosapentaenoic acid/prostacycline)

RICHARD LERNER*†, PETER LINDSTRÖM*, ANDERS BERG‡, ELSBETH JOHANSSON‡, KERSTIN ROSENDAHL‡, AND JAN PALMBLAD*

Departments of *Medicine and ‡Clinical Chemistry, Karolinska Institute, Stockholm Söder Hospital, S-118 83 Stockholm, Sweden

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ABSTRACT **We induced an essential fatty acid deficiency (EFAD) in human umbilical vein endothelial cells by culture in medium with 20% (vol/vol) delipidated fetal calf serum. EFAD, reflected by decreased cellular linoleic acid (18:2ω6) and arachidonic acid (20:4ω6) and emergence of the oleic acid derivative 5,8,11-eicosatrienoic acid (20:3ω9; Mead's acid), was evident after 1 week of culture and became pronounced after 2 weeks. Beyond that time point, control cells (cultured in 20% normal fetal calf serum) grew deficient of 18:2ω6, and EFAD cells died. 18:2ω6 addition to EFAD cells resulted in dose-dependent increases of 18:2ω6 and 20:4ω6. 20:4ω6 or 5,8,11,14,17-eicosapentaenoic acid (20:5ω3) additions resulted in normalization of these acids, and conversion of 20:5ω3 to 4,7,10,13,16,19-docosahexaenoic acid (22:6ω3) was noted. Agonist-induced increases in concentrations of prostacycline (prostaglandin I_2; PGI_2) and cytosolic Ca^{2+}, $[Ca^{2+}]_i$, were reduced in EFAD cells and not restored by 18:2ω6 or 20:4ω6 additions. Change of the medium in EFAD cultures 1 day before the experiments decreased 20:3ω9 and normalized the PGI_2 production and $[Ca^{2+}]_i$ changes, whereas addition of 20:3ω9 to control cells impaired the $[Ca^{2+}]_i$ response, indicating a suppressive effect of 20:3ω9. Thus, EFAD in endothelial cells is associated with abnormalities of eicosanoid and second-messenger production partly attributable to 20:3ω9 accumulation. Moreover, the gradual emergence of 18:2ω6 deficiency in regularly grown control cells underlines the need for careful analysis of fatty acids in long-term cell cultures.**

Essential fatty acid deficiency (EFAD) is characterized by growth retardation, skin and renal lesions, increased susceptibility to infections, and reduced autoimmune and inflammatory reactions (1–9). EFAD is also associated with alteration of functional responses of neutrophil granulocytes and macrophages that is normalized when linoleic acid (9,12-octadecadienoic acid; 18:2ω6), the major dietary essential fatty acid for mammals, is replenished (10–12).

The biochemical basis for impairment of phagocyte responses in EFAD is assumed to be lack of eicosanoids, metabolites generated from cellular 18:2ω6 and arachidonic acid (5,8,11,14-eicosatetraenoic acid; 20:4ω6) and in particular cyclo- and lipoxygenase products. This hypothesis is supported by a number of observations most importantly that development of decreased endogenous generation of leukotrienes, prostaglandins, and platelet-activating factor coincide with impairment of cellular responsiveness (13–19). Moreover, addition of 18:2ω6, 20:4ω6, or lipoxygenase products to EFAD cells may restore their responsiveness, including production of eicosanoids (18, 19).

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Against this background we asked whether functional responses of endothelial cells, being intimately involved in the inflammatory reaction, might be reduced in EFAD. This report concerns the biochemical characterization of EFAD produced in human umbilical vein endothelial cells (HUVEC) by culturing the cells in a delipidated medium, the generation of prostacycline (PGI_2), and the ability to respond with a rise of cytosolic concentrations of Ca^{2+}, $[Ca^{2+}]_i$.

MATERIALS AND METHODS

Chemicals. 20:4ω6, 5,8,11,14,17-eicosapentaenoic acid (20:5ω3), heparin, human serum albumin (essential fatty acid-free), 18:2ω6, thrombin, and Triton X-100 were obtained from Sigma. Hepes, fetal calf serum (FCS), penicillin, streptomycin, trypsin, RPMI 1640 medium, and Hanks' balanced salt solution (HBSS) were from GIBCO. Collagenase (type 3) was from Worthington, and endothelial cell growth supplement was from Collaborative Research. Fura-2 acetoxymethyl ester (AM) and ionophore A23187 were from Calbiochem. Acetyl chloride, 1-butanol, diisopopyl ether, demthyl sulfoxide, EDTA, methylbenzene, methyl margarate, and potassium carbonate were from Merck. 5,8,11-Eicosatrienoic acid (20:3ω9; Mead's acid) was obtained from Cayman Chemicals (Ann Arbor, MI).

Delipidated FCS. Delipidated serum was obtained from FCS as described (20). Before delipidation the cholesterol content was 0.9 mmol/liter; triglycerides, 0.9 mmol/liter; and free fatty acids, 0.09 mmol/liter. After the procedure cholesterol and triglycerides were not detectable, and the free acid concentration was 0.02 mmol/liter.

HUVEC. Endothelial cells were obtained from human umbilical veins (21, 22). Cells were resuspended in culture medium (RPMI 1640 containing 20% (vol/vol) FCS, 90 μg of heparin per ml, 50 μg of endothelial cell growth supplement per ml, 200 units of penicillin per ml, and 200 μg of streptomycin per ml) and were grown in tissue culture flasks. HUVEC were trypsinized when confluent, counted, and equally resuspended in standard medium or delipidated medium (20% delipidated FCS), grown in flasks, and passaged every week. Photographs were taken every day, and cell growth was calculated from the graphs. For the PGI_2 release assay, HUVEC were transferred to 10-cm^2 Petri dishes. Fatty acid analyses were made every week. Indirect immunofluorescence staining against factor VIII-related antigen was used for characterization of cells in culture. Fatty acids dissolved in

Abbreviations: EFA, essential fatty acid(s); EFAD, EFA deficient/deficiency; $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentrations; HUVEC, human umbilical vein endothelial cells; PGI_2, prostaglandin I_2 (prostacycline); $PGF_{1\alpha}$, prostaglandin $F_{1\alpha}$; 18:2ω6, 9,12-octadecadienoic acid (linoleic acid); 20:3ω9, 5,8,11-eicosatrienoic acid (Mead's acid); 20:4ω6, 5,8,11,14-eicosatetraenoic acid (arachidonic acid); 20:5ω3, 5,8,11,14,17-eicosapentaenoic acid; 22:6ω3, 4,7,10,13,16,19-docosahexaenoic acid; FCS, fetal calf serum.

†To whom reprint requests should be addressed.

Table 1. Growth of HUVEC in control and EFAD media

Days in culture	Cells, no. per mm^2	
	Control	EFAD
2	312 ± 16	172 ± 17
4	581 ± 51	297 ± 20
6	704 ± 32	418 ± 18
8	700 ± 26	427 ± 19

Data are means ± SEM for three separate experiments.

ethanol were added to HUVEC cells, and the solvent at a corresponding concentration was added to control cells.

Fatty Acid Analysis. The direct transestrification method (23) was used with some modifications. Separation of fatty acid was performed in the gas chromatograph (Finnigan-MAT, Sunnyvale, CA). Fatty acids were identified in a mass spectrometer (Model 1020 Finnigan).

PGI_2 Release. HUVEC, grown to confluence in 10-cm^2 Petri dishes, were washed three times and subsequently covered with 1 ml of HBSS containing 10 mM Hepes. Stimuli or HBSS alone (for assessment of spontaneous release) was added. Dishes were incubated for 2 or 10 min, and supernatants were aspirated. In one set of experiments, HBSS-treated cells were lysed by adding 0.1% Triton X-100, and the resulting fluid phase was processed as the supernatants had been. All aspirates were frozen immediately. The remaining HUVEC layers were lysed with distilled water, frozen and thawed repeatedly, and analyzed for total protein content with a Coomassie blue technique (Bio-Rad). PGI_2 was assessed as its stable metabolite 6-keto-$PGF_{1\alpha}$ by a radioimmunoassay (Amersham).

$[Ca^{2+}]_i$ Measurements. HUVEC grown on coverslips were treated with 2–4 μM Fura-2 AM in HBSS containing 10 mM Hepes and 1% human serum albumin for 40 min (24). The coverslips were positioned at 45° to the exciting and emitted light (at 340 and 510 nm, respectively) in a cuvette with HBSS containing 10 mM Hepes. Agonist-induced fluorescence changes were followed in a Hitatchi F-3000 spectrofluorimeter for at least 15 min at 37°C with continuous stirring of the buffer. The calibration of the system and calculations of $[Ca^{2+}]_i$ were done as described (24, 25). Results were expressed as the net increase of the $[Ca^{2+}]_i$ above that of resting cells.

Statistical Calculations. These were carried out with Student's *t* test.

RESULTS

Cell Growth and Morphology. The replication rate of HUVEC grown in EFAD medium was lower than in normal medium (Table 1). This growth retardation was evident already at culture day 2 and became progressively more prominent. After 2–3 weeks of culture, EFA-deprived cells stopped growing, and cell death hampered further evaluation. The normal HUVEC morphology was changed in EFAD cultures. After 1 week, and more prominently after 2 weeks of culture, EFAD cells became more elongated and formed a less dense monolayer relative to controls. Additions of 20:4ω6, 18:2ω6, or 20:5ω3 did not restore the normal HUVEC morphology during the observation time of 3 days. EFAD cells did not differ from control cells in immunofluorescence for factor VIII-related antigen.

Cellular Fatty Acids. Relative contents of fatty acids are shown in Table 2 and Figs. 1–3. In HUVEC grown in delipidated serum, reduced contents of ω3 and ω6 fatty acids could be observed after 1 week of culture and became more evident after 2 weeks. The marker for EFAD, the oleic acid desaturation and elongation product 20:3ω9, appeared after the first week of culture (Fig. 2 *Left*). The mean ratio of 20:3ω9 to 20:4ω6 increased gradually in EFAD cells but not in control cells. Control cells did not exhibit changes of fatty acid concentrations over the first week of culture, but reductions of EFA occurred to some extent during the second week. The saturated fatty acids did not differ between EFAD and control cells on day 10. The change of medium in EFAD cell culture on day 9 was associated with the disappearance of 20:3ω9, but with no other consistent changes of ω6 or ω3 fatty acids (Fig. 4 and data not shown).

Additions of Fatty Acids. In an attempt to reverse the EFAD state, we added 1–30 μM of 18:2ω6, 20:4ω6, or 20:5ω3 acids to the 7-day-old EFAD HUVEC cultures (Figs. 1–3). 18:2ω6

Table 2. Fatty acid (FA) composition of HUVEC cultures for various times in control and EFAD media

	Cellular FA composition, % of all FAs						
	Day 0	Day 7		Day 10		Day 16	
FA	control	Control	EFAD	Control	EFAD	Control	EFAD
Saturated							
16:0	31.84 ± 1.07	33.23 ± 1.57	33.70 ± 1.98	41.03 ± 3.67	41.63 ± 4.41	33.05 ± 1.65	26.00 ± 0.80
18:0	21.54 ± 0.53	20.51 ± 1.12	20.89 ± 1.98	29.20 ± 2.38	28.87 ± 4.44	21.90 ± 0.10	16.90 ± 0.40
Total	55.80 ± 1.36	56.08 ± 2.42	58.81 ± 3.53	68.85 ± 6.60	69.05 ± 8.83	56.61 ± 1.82	46.88 ± 0.48
ω9FA							
18:1ω9	24.17 ± 0.82	22.14 ± 2.03	29.51 ± 2.88	11.50 ± 4.82	15.40 ± 6.09	25.10 ± 0.80	36.65 ± 0.95
20:3ω9	0.10 ± 0.03	0.11 ± 0.06	2.66 ± 0.49	0.07 ± 0.05	1.53 ± 0.67	0.07 ± 0.01	4.50 ± 0.03
Total	24.23 ± 0.83	22.24 ± 2.05	32.18 ± 3.33	15.53 ± 4.86	21.78 ± 6.72	25.17 ± 0.79	41.15 ± 0.98
ω6FA							
18:2ω6	2.66 ± 0.33	2.59 ± 0.15	0.43 ± 0.06	1.50 ± 0.31	0.53 ± 0.06	2.80 ± 0.30	0.36 ± 0.09
20:3ω6	1.54 ± 0.20	1.69 ± 0.41	0.53 ± 0.16	1.32 ± 0.34	0.83 ± 0.45	1.11 ± 0.05	0.07 ± 0.07
20:4ω6	5.08 ± 1.21	4.60 ± 0.80	1.51 ± 0.39	2.60 ± 1.05	1.15 ± 0.69	3.80 ± 0.00	0.84 ± 0.24
Total	9.54 ± 1.27	9.00 ± 0.74	2.64 ± 0.48	6.17 ± 1.01	2.99 ± 0.82	7.75 ± 0.22	1.61 ± 0.56
ω3FA							
18:3ω3	0.09 ± 0.05	0.08 ± 0.03	0.02 ± 0.01	0.39 ± 0.24	0.03 ± 0.01	ND	ND
20:5ω3	1.13 ± 0.16	1.33 ± 0.21	0.11 ± 0.03	0.53 ± 0.46	0.03 ± 0.03	2.33 ± 0.28	0.37 ± 0.23
22:6ω3	4.90 ± 0.27	5.91 ± 0.68	1.37 ± 0.26	3.63 ± 0.49	0.82 ± 0.58	0.05 ± 0.12	0.84 ± 0.24
Total	6.12 ± 0.24	7.32 ± 0.54	1.51 ± 0.27	5.32 ± 0.88	1.06 ± 0.61	7.37 ± 0.16	1.21 ± 0.01
				Ratio20:3ω9/20:4ω9			
	0.09 ± 0.05	0.02 ± 0.01	2.36 ± 0.50	0.01 ± 0.01	4.15 ± 1.26	0.02 ± 0.00	5.85 ± 1.71

Data are means ± SEM for nine experiments on day 0 of culture, eight experiments on day 7, four experiments on day 10, and two experiments on day 16. ND, not detected.

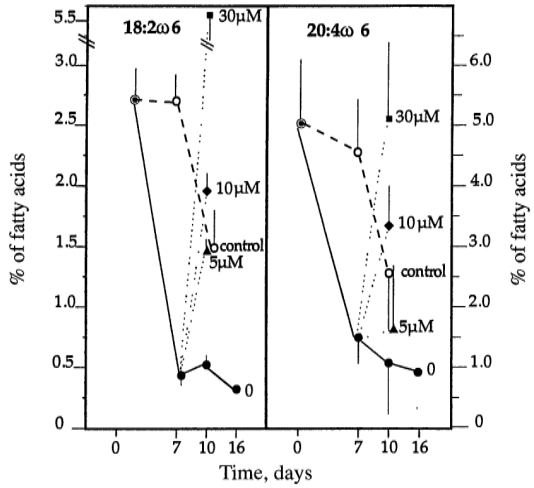

FIG. 1. Effect of addition of 18:2ω6 on cellular concentrations of 18:2ω6 (*Left*) and 20:4ω6 (*Right*) expressed as a percentage of all fatty acids. HUVEC were grown in normal (- - -) or delipidated (——) medium. After 7 days of culture, the indicated amounts of 18:2ω6 were added to the delipidated medium (· · · ·). Data are means ± SEM for four separate experiments.

addition resulted in dose-dependent increases of cellular contents of 18:2ω6 and 20:4ω6 (Fig. 1), except for 1 μM 18:2ω6 that gave no change (data not shown); 5 μM 18:2ω6 sufficed to raise the cellular 18:2ω6 level to that of control cells (Fig. 1 *Left*), and 10 μM was needed to restore dihomo-γ-linolenic acid (8,11,14-eicosatrienoic acid; 20:3ω6) (data not shown) and 20:4ω6 levels (Fig. 1 *Right*). An addition of 30 μM 18:2ω6 tripled 18:2ω6 and doubled 20:4ω6 levels. The levels of 20:3ω9 dropped when 18:2ω6 was added. 20:5ω3 and 4,7,10,13,16,19-docosahexaenoic acid (22:6ω3) concentrations were not affected by 18:2ω6 supplementations (data not shown).

20:4ω6 additions resulted in dose-dependent increases of cellular 20:4ω6 but not of 18:2ω6, γ-linolenic acid (6,9,12-octadecatrienoic acid; 18:3ω6), and 20:3ω6 (Fig. 2 *Right* and data not shown). An addition of 5–10 μM restored 20:4ω6 levels and of 30 μM increased 20:4ω6 levels 4-fold. 20:3ω9 was dose-dependently reduced by 20:4ω6 additions (Fig. 2 *Left*).

When 20:5ω3 was added, the levels of this acid were increased in a manner corresponding to the increase observed

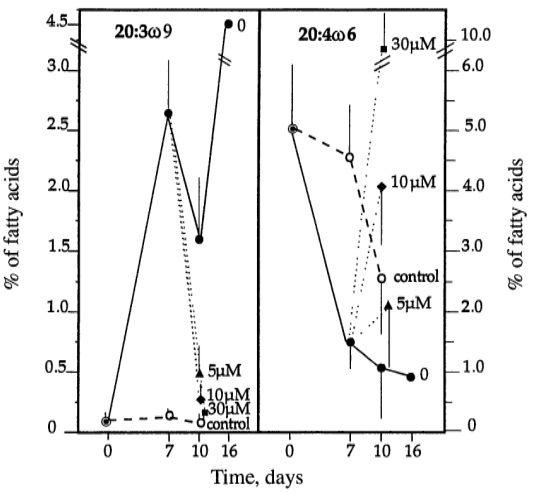

FIG. 2. Effect of addition of 20:4ω6 on cellular concentrations of 20:3ω9 (*Left*) and 20:4ω6 (*Right*), expressed as a percentage of all fatty acids. HUVEC were grown in normal (- - -) or delipidated (——) medium. After 7 days of culture, the indicated amounts of 20:4ω6 were added to the delipidated medium (· · · ·). Data are means ± SEM for four separate experiments.

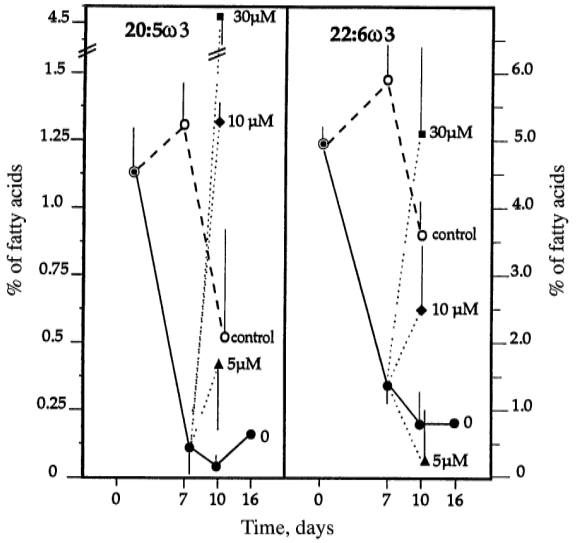

FIG. 3. Effect of addition of 20:5ω3 on cellular concentrations of 20:5ω3 (*Left*) and 22:6ω3 (*Right*) expressed as a percentage of all fatty acids. HUVEC were grown in normal (- - -) or delipidated (——) medium. After 7 days of culture, the indicated amounts of 20:5ω3 were added to delipidated medium (· · · ·). Data are means ± SEM for four separate experiments.

for cellular 18:2ω6 and 20:4ω6 after additions of those acids (Fig. 3 *Left*). 22:6ω3 concentrations became dose-dependently increased after 20:5ω3 additions (Fig. 3 *Right*). 20:3ω9 levels decreased upon 20:5ω3 supplementation (data not shown).

Overnight incubation of control HUVEC with 20:3ω9 (1–200 μM) resulted in a dose-dependent increase of cellular concentrations of this fatty acid. For further experiments we chose to add 100 μM, since this concentration conferred levels of 20:3ω9 close to those observed in EFAD cells (5.4%). No effect on intracellular 18:2ω6 was seen upon the addition of 20:3ω9.

PGI_2 Release. Both EFAD and control cells released PGI_2 into the medium (Fig. 5). However, the spontaneous release was close to zero in EFAD cells, whereas it was readily assessable in control cells. The total cellular content in Triton X-100-lysed cells was also lower for EFAD cells (40% of controls). After stimulation with 1 unit of thrombin per ml or 5 μM of the Ca^{2+} ionophore A23187, control cells increased the PGI_2 release 17-fold and 4-fold, respectively (Table 3). This release was evident 2 min after addition of the stimulus and increased only marginally in samples assessed 8 min later. EFAD cells also increased PGI_2 release upon stimulation, but the maximal release was only 10% of that noted for control cells.

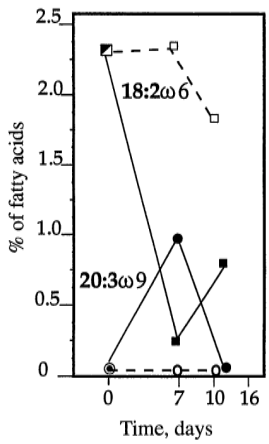

FIG. 4. Effect of exchange of medium on day 9 on cellular concentrations of 18:2ω6 (□, ■) and 20:3ω9 (○, ●), expressed as a percentage of all fatty acids. - - - -, normal medium; ——, delipidated medium.

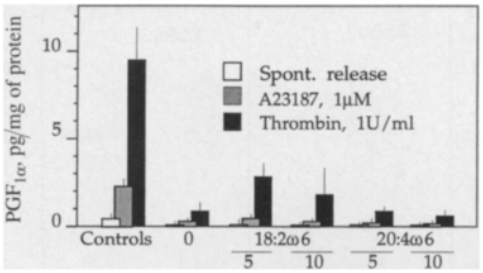

FIG. 5. PGI_2 release, assessed as 6-keto-$PGF_{1\alpha}$ concentrations from HUVEC and the effect of supplementation of EFAD cells with 18:2ω6 and 20:4ω6 on day 7. Open bars depict spontaneous release, whereas shaded and black bars depict release conferred by 1 μM A23187 or 1 unit of thrombin per ml, respectively.

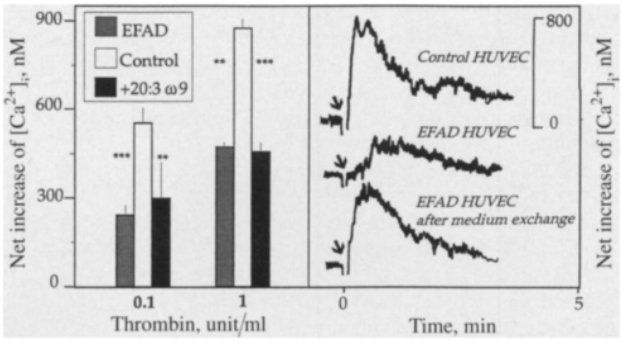

FIG. 6. $[Ca^{2+}]_i$ in EFAD, control HUVEC, and control cells supplemented with 100 μM 20:3ω9 in response to thrombin. (*Left*) Changes in $[Ca^{2+}]_i$ in response to 0.1 or 1 unit of thrombin per ml as indicated. Data are means ± SEM for at least three separate experiments. **, $P < 0.01$, ***, $P < 0.001$. (*Right*) Fura-2 fluorescence changes in control and EFAD HUVEC after addition of thrombin at 1 unit/ml. Thrombin was added at time points indicated by arrows. The figure depicts typical experiments that were repeated with similar results four times. The bracket from 0 to 800 applies to each of the three traces.

When 5–10 μM 18:2ω6 was added to EFAD cells on day 7 and PGI_2 release was assessed on day 10, the stimulated release was doubled (Fig. 5). However, the PGI_2 release did not exhibit full restoration to the levels of control cells. When 20:4ω6 was added to obtain normal cellular level of this acid, no enhancement of PGI_2 production was noted (Fig. 5).

When the medium of EFAD cultures was changed on day 9, the spontaneous and stimulated PGI_2 release from EFAD cells became similar to controls (Table 3).

$[Ca^{2+}]_i$ Levels. When HUVEC were stimulated with thrombin, rapid and transient increases of $[Ca^{2+}]_i$ were noted in control and EFAD cells. However, EFAD cells mounted a response that was 54% of that of controls (Fig. 6 *Left*). This difference ($P < 0.01$) in response was observed over a 10-fold concentration range of thrombin. The kinetics of the $[Ca^{2+}]_i$ response differed in the two cell types. In control HUVEC it consisted of an initial peak that reached its maximum after 20 sec, followed by a gradual decline to a new base level. A second wave of fluorescence was often observed after the first peak, a phenomenon that also has been noted in neutrophils (26). In EFAD cells the initial peak was slower in onset and reached its highest amplitude after ≈30–40 sec; the second wave of fluorescence was not seen (Fig. 6 *Right*). Addition of 10 μM 18:2ω6 to EFAD cells did not alter the $[Ca^{2+}]_i$ response (data not shown). In contrast, $[Ca^{2+}]_i$ response in EFAD cells became identical to that in control cells when the medium was changed 1 day before the Ca^{2+} experiment (Fig. 6 *Right*). Addition of 100 μM 20:3ω9 to control HUVEC significantly ($P < 0.01$–0.001) inhibited the Ca^{2+} responses to stimulation with 0.1 and 1 unit of thrombin per ml (Fig. 6). No change in the kinetics of the response was observed.

DISCUSSION

Culture of human endothelial cells in delipidated serum induced an EFAD state documented by very low intracellular 20:4ω6 and 18:2ω6 contents and the high concentration of 20:3ω9, the marker for EFAD. This high 20:3ω9/20:4ω6 ratio is a generally accepted sign of EFAD (27–29). In addition, the EFAD state was accompanied by marked reductions of release of 20:4ω6 cyclooxygenase metabolite PGI_2 and reduced increases of $[Ca^{2+}]_i$ in response to thrombin. The reduction in PGI_2 release was a consequence of diminished production as shown by the experiments with Triton X-100.

EFAD has previously been induced in cell lines by culturing those in delipidated or synthetic lipid-free media. Laposata *et al.* (30) described the HSDM1C1 cells grown in delipidated serum lacked 20:4ω6 and were unable to synthesize prostaglandin E_2 in response to bradykinin. The prostaglandin E_2 production was restored with 20:4ω6 and 18:2ω6 repletion. Wright *et al.* (31) grew HL-60 cells in synthetic medium, also achieving total depletion of 20:4ω6. The EFAD HL-60 cells synthesized no leukotriene B4 and exhibited reduced functional responses. These responses and leukotriene B4 production were restituted by 18:2ω6 replenishment (31). One difference between those cell lines and HUVEC studied here was that HUVEC did not tolerate the EFAD culture condition for more than 3 weeks. Another difference is the blunted eicosanoid response in HUVEC despite additions of 18:2ω6 and 20:4ω6 to normal cellular levels.

The EFAD state was subject to various manipulations. Additions of 18:2ω6, 20:4ω6, and 20:5ω3, usually at a concentration between 5 and 30 μM, normalized the cellular content of those very acids. In addition, 18:2ω6 supplementation resulted in increases of 20:4ω6, showing that both EFAD and

Table 3. PGI_2 production in endothelial cells, assayed as 6-keto-$PGF_{1\alpha}$

	6-keto-$PGF_{1\alpha}$ from cultured cells, pg/mg of protein			
	Intact medium		Medium exchanged on day 9	
Condition/stimulus	EFAD cells	Control cells	EFAD cells	Control cells
Release				
Spontaneous	0.13 ± 0.06	0.55 ± 0.22	0.74 ± 0.01	0.63 ± 0.04
Induced				
Thrombin (1 unit/ml)	0.90 ± 0.22	9.52 ± 2.14	3.55 ± 0.20	3.65 ± 0.12
A23187 (5 μM)	0.28 ± 0.10	2.19 ± 0.38	0.94 ± 0.11	1.08 ± 0.13
	Total content in lysed cells			
	2.86 ± 0.21	7.10 ± 2.04	7.38 ± 0.46	7.87 ± 1.01

Data are means ± SEM for six experiments with intact medium and 2 experiments with exchanged medium. Results are given as 6-keto $PGF_{1\alpha}$ in supernatants from endothelial cells grown to confluence and 10 days after initiation of EFAD and in Triton X-100-lysed cells.

control HUVEC retained an ability to metabolize 18:2ω6 along the normal pathways for elongation and desaturation. This capacity has been questioned in the past (32). Similarly, 20:5ω3 additions resulted in increases of 22:6ω3, emphasizing that changes of fatty acid concentrations were not due to impairment of enzymatic conversions. Also 20:3ω9 levels became lower upon 18:2ω6, 20:4ω6, and 20:5ω3 additions, showing that replenishments of normal acids made substitution with oleic acid metabolites less necessary for the cell.

The role of 20:3ω9 appears to be complex. It might serve as a substitute for 20:4ω6 in EFAD cells. However, few or no biologically active eicosanoids originating from 20:3ω9 have been described. Instead, claims have been made that 20:3ω9 acts as an inhibitor of normal eicosanoid generation (15, 33, 34). Support for that hypothesis is provided here, since removal of 20:3ω9 by medium exchange restored PGI_2 production in EFAD cells. Thus, the relative inability of EFAD HUVEC to generate PGI_2 upon stimulation might be attributed to the presence of 20:3ω9 as well as deficiency of obligate precursors.

The reason why 18:2ω6 and, particularly, 20:4ω6 supplementation restored cellular levels of these acids but not PGI_2 production is not fully understood. It may be due to the hampering effects of 20:3ω9, but it cannot be ruled out that the added fatty acids were incorporated into cellular pools that were not accessible for conversion into eicosanoids.

The reduction of the $[Ca^{2+}]_i$ response in EFAD HUVEC also points to alterations of the biologically active phospholipid fractions. Thrombin induces a surface receptor-dependent and pertussis toxin-sensitive inositol trisphosphate mobilization, leading to the increase in $[Ca^{2+}]_i$. Since EFAD HUVEC displayed lower $[Ca^{2+}]_i$ peaks more slowly reached then those in control cells, it is suggested that the signal transduction system for $[Ca^{2+}]_i$ increases is impaired. It can be ascribed to accumulation of 20:3ω9, since the $[Ca^{2+}]_i$ response in EFAD cells was corrected by change of medium, and exogenouse 20:3ω9 hampers the Ca^{2+} response in control cells. Our results in HUVEC are different from those in EFAD neutrophils, where $[Ca^{2+}]_i$ increases were normal (10). Thus, effects of EFAD on the stimulus–response coupling differ with cell types.

The cellular concentrations of EFA in control cells became gradually lower during the culture period, indicating a relative deficiency state, although we used regular fetal calf serum at a standard concentration. This relative EFAD, which can be assumed to be progressive with prolonged culture, might eventually influence cellular reactivity. Such EFAD has seldomly been appreciated in research on cultured cells. To the best of our knowledge, this issue has not been addressed systematically in the literature. It might represent a significant pitfall for research on variables dependent on 20:4ω6 metabolites.

EFAD is accompanied by diminished influx of granulocytes into an experimentally induced inflammatory lesion (15). EFAD also prolongs survival of transplanted incompatible kidneys and reduces autoimmune nephritis manifestations and myocardial infarction size (8, 35, 36). Although not studied specifically in those *in vivo* models, endothelial cells of an EFAD organism conceivably may show EFAD characteristics and may interact differently with emigrating leukocytes.

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