Linoleic acid and linolenic acid: effect on permeability properties of cultured endothelial cell monolayers[1-3]

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ABSTRACT High circulating plasma levels of free fatty acids may injure endothelial cells, resulting in decreased barrier function of the vascular endothelium. The effect of media supplementation with varying concentrations of either linoleic (C18:2ω6) or linolenic acid (C18:3ω3) on albumin transfer across cultured endothelial monolayers was studied. A 24-h cell exposure to linoleic but not linolenic acid resulted in a concentration dependent and largely reversible increase in albumin transfer. Both fatty acids and in particular linolenic acid incorporated into cellular phospholipids. In contrast, only supplementation with linoleic but not linolenic acid resulted in an increased incorporation of this fatty acid into cell triglycerides. Similarly, only total cell triglyceride content increased after incubation with linoleic- but not with linolenic-enriched media. These results indicate that cellular enrichment with linoleic but not linolenic acid causes cellular perturbations that may be implicated in atherosclerosis. *Am J Clin Nutr* 1989;49:301-5.

KEY WORDS Endothelial cell, injury, fatty acid, triglyceride, phospholipid, permeability, albumin transfer

Introduction

The average American diet contains ~40% of calories as fat (1). An increase in the consumption of vegetable oils, rich in ω-6 fatty acids such as linoleic (C18:2ω6) and fish oils rich in ω-3 fatty acids such as linolenic (C18:3ω3) and eicosapentaenoic (C20:5ω3), generally has been advocated. Plasma chylomicron levels are elevated in humans after a high-fat meal and hepatic synthesis of very-low-density lipoproteins (VLDLs) is increased when caloric intake is in excess of body needs (2, 3). High levels of circulating triglyceride-rich lipoproteins specifically have been implicated in the injury process of the endothelium (4, 5). Endothelial cell injury may be involved in the pathogenesis of atherosclerosis (4–6). When the plasma triglyceride-rich lipoproteins are elevated, triglyceride hydrolysis by lipoprotein lipase occurs in proximity to the endothelial surface (7, 8). This may expose the endothelium to an excessive local concentration of fatty acid anions and lead to an accumulation of fatty acids within the endothelial cells (5, 9). Furthermore, this excessive liberation of fatty acid anions during lipoprotein triglyceride hydrolysis may cause endothelial injury and in turn allow increased penetration of cholesterol-rich remnant lipoproteins into the arterial wall (4, 5, 9). In support of this possibility, research from this laboratory has shown that exposure of cultured porcine endothelial cells to elevated concentrations of oleic acid (C18:1ω9) increased the transfer of albumin across the endothelium (10). In addition to endothelial injury produced by exposure to high concentrations of fatty acid anions, enrichment of endothelial lipid with selective fatty acids has been shown to alter the morphology of cultured cell monolayers (11).

In the present study, endothelial cells from cultured porcine pulmonary artery were exposed to relatively high concentrations of either linoleic or linolenic acid. An attempt was made to examine whether or not selective changes in fatty acid composition of endothelial cell monolayers would affect their permeability properties.

Materials and methods

Cell culture

Endothelial cells were obtained from porcine pulmonary arteries and cultured in medium 199 (M-199, GIBCO Labora-

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tories, Grant Island, NY) containing 10% fetal bovine serum (HyClone Laboratories, Inc, Logan, UT) (10). Cultures were determined to be endothelial by uniform morphology and by quantitative determination of angiotensin-converting enzyme activity. Cells from passages 5–12 were used in this study. Experiments were performed more than once and results were similar at cell passages 5–12.

Cell viability was determined by lactate dehydrogenase (LDH) release, trypan blue exclusion, and morphological assessment by phase-contrast microscopy. Cells were grown in six-well culture plates (Costar Corp, Cambridge, MA) and LDH activity was assayed in the media and the cell lysate as described by Bergmeyer and Garvehn (12). LDH release was expressed as a percentage of total LDH (LDH in medium + LDH in cells). Correction was made for LDH activity of fetal bovine serum.

The experimental media were composed of M-199 enriched with 5% fetal bovine serum, 100 μmol crystalline fatty acid-free bovine albumin/L (Sigma Chemical Co, St Louis, MO), vitamins, amino acids (GIBCO Laboratories) and various concentrations of fresh aliquots of linoleic or linolenic acid (Nu-Chek-Prep, Elysian, MN; ≥ 99% pure by gas-liquid chromatography). Fatty acids were dissolved in ethanol. After one or two drops of 6 mol NaOH/L were added, the material was dried under high purity N_2, redissolved in a small amount of warm distilled water, and added to the medium containing fetal bovine serum. The pH was adjusted immediately to 7.4.

Albumin transfer

For albumin transfer studies, cells from passages 5–12 were plated on gelatin-impregnated polycarbonate filters (Nucleopore Corp, Pleasanton, CA; 13 mm diameter and 0.8 μm pore size) glued to polystyrene chemotactic chambers (ADAPS, Inc, Dedham, MA). After confluent endothelial monolayers were exposed to the fatty acid enriched media for 24 h, albumin transfer across the endothelium was measured according to the procedure described by Hennig et al (10). Chemotactic chambers with attached monolayers were placed into 24-well plates containing 1.5 mL serum-free and fatty acid-free M-199 and each chemotactic chamber was filled with 0.5 mL M-199 containing 200 μmol albumin/L. After a 1-h incubation, the media within the chemotactic chambers and the surrounding media were sampled and their respective albumin concentrations were determined. This was done by measuring the change in absorbance at 630 nm after adding a reagent solution of bromcresol green (Sigma Chemical Co) to a portion of the sample.

For experiments testing the reversibility of the fatty acid effect, the chemotactic chambers and attached monolayers were removed from the plates used to measure albumin transfer and placed into new 24-well plates where the monolayers were reincubated in M-199 enriched with 10% fetal bovine serum for 8 h. After the end of the reincubation, the chemotactic chambers and monolayers were washed and transferred to new plates and the albumin transfer was again determined.

Lipid analyses

For lipid analytical studies, cells were grown in tissue culture plates (Costar). After a 24-h incubation of endothelial cell cultures with media enriched with albumin-bound fatty acids, total lipids from cells were extracted in chloroform-methanol (2:1, vol:vol) by the method of Bligh and Dyer (13) and separated by thin-layer chromatography with petroleum ether:diethylether:glacial acetic acid, (80:20:1, vol:vol:vol). Methyl esters were prepared after saponification using 14% boron trifluoride (14). The methyl esters were extracted in isooctane for chromatographic analysis by an HP 5890 gas chromatograph equipped with a flame ionization detector and a Nelson Analytical Data System (Hewlett Packard Co, Sunnyvale, CA). A DB225 (25% cyanopropylphenyl) fused silica capillary column (J & W Scientific Co, Rancho Cordova, CA; 30 m × 0.25 mm ID) was used with helium as a carrier gas. The oven temperature was maintained at 190 °C for the first 15 min of the run and increased at a rate of 0.8 °C/min to a final temperature of 208 °C. Total analysis time per sample was 45 min. Both injector port and detector were set at 250 °C. Standards for gas-liquid chromatography and thin-layer chromatography were purchased from Nu-Chek-Prep, Inc, Elysian, MN. Proportions of fatty acids present in each sample were determined as area percent. The triglyceride content of cultured endothelial cells was measured at 2 and 24 h after incubation in experimental media by a colorimetric procedure described by Biggs et al (15).

Statistical analysis

Data were analyzed statistically by analysis of variance and when significant differences occurred, Student's *t* test and Duncan's multiple range test were performed (16). Variation between mean fatty acid values was expressed as pooled SEM.

Results

Fatty acid exposure during culture did not appear to cause a loss in cell viability as established with trypan blue exclusion studies and morphological assessment by phase contrast microscopy. Furthermore, treatment of endothelial cells with supplemental fatty acids did not affect cellular LDH release into the culture medium; this suggested that the fatty acid concentration used in our experiments was not cytotoxic. For example, cellular LDH release after a 24-h exposure to various fatty acid enriched media was 4.8 ± 0.8 ($\bar{x}$ ± SEM, n = 3) for the control, 6.3 ± 1.5 for linoleic acid, and 6.0 ± 1.7 for linolenic acid.

The effect of albumin-bound linoleic acid or linolenic acid on albumin transfer across endothelial cell monolayers is shown in **Figure 1.** Cells were exposed to the fatty acid-enriched media for 24 h before albumin transfer was measured over 1 h. Two hour exposures to the fatty acid-enriched media had no effect on endothelial permeability properties (unpublished observations from this laboratory). The amount of albumin transferred increased in a dependent manner on the linoleic concentration to which the cultures were exposed. However, cell exposure to linolenic acid-enriched media had no effect on albumin transfer even at concentrations as high as 300 μmol/L.

The effect of linoleic acid exposure on albumin transfer was largely reversible within 8 h after removal of medium supplemented with the fatty acid. For control cells, albumin transfer after a 24-h incubation with experimental medium was 4.0 ± 0.4 nmol (n = 6) and albumin transfer after reincubating the same cells in medium without additional fatty acid was 4.7 ± 0.3 nmol. For the cells exposed to 300 μmol linoleic acid/L, albumin transfer after a 24-h incubation period was 13.3 ± 1.1

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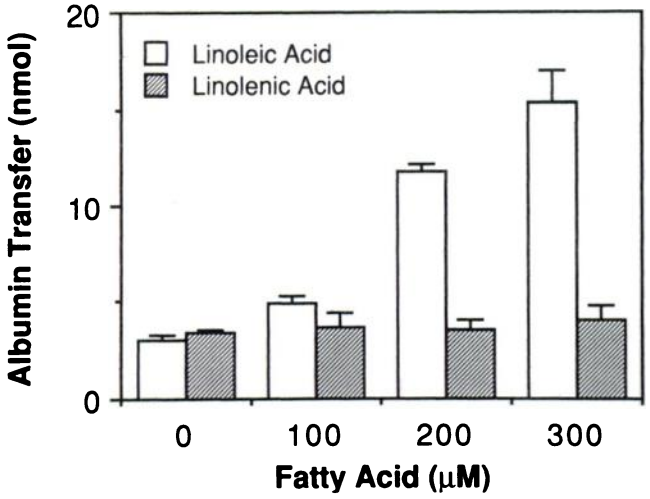

FIG 1. The effect of fatty acid enrichment on subsequent transfer of albumin across cultured endothelial monolayers.

nmol, and albumin transfer after an 8-h reincubation of these same cells in medium without additional fatty acid was 7.1 ± 0.8 nmol.

The effect of fatty acid enrichment on the triglyceride content of cultured endothelial cells is shown in **Figure 2.** Compared with control cultures, enriching cells with linoleic acid for 24 h resulted in an elevated triglyceride content, whereas supplementing linolenic acid in culture media did not affect the triglyceride content of endothelial cells. A 2-h incubation with either linoleic or linolenic acid was not sufficient to appreciably increase the cellular triglyceride content.

The fatty acid composition of the phospholipid fraction after a 24-h incubation with media containing 0 (control), 100, 200, or 300 μmol linoleic acid/L or linolenic acid/L is shown in **Table 1.** The sum of these values do not equal 100% because only the major fatty acids of interest are listed. Compared with control cultures the amount of linoleic acid increased nearly four-fold after incubation with 100 μmol linoleic acid/L and five- and eight-fold when the media contained 200 or 300 μmol linoleic acid/L, respectively. A similar but more dramatic pattern of fatty acid enrichment into the phospholipid fraction of endothelial cells was observed when the media contained supplemental linolenic acid. Compared with control cultures, enriching cells with linolenic acid resulted in an elevated content of linolenic acid in the cellular phospholipid fraction, with an increase of 10-, 14- or 20-fold when the media contained 100, 200, or 300 μmol linolenic acid/L, respectively. After exposure to linoleic acid, elongation products of linoleic acid, such as arachidonic acid (C20:4ω6), were not increased. On the other hand, elongation products of linolenic acid, such as eicosapentaenoic acid were markedly increased after cell exposure to media supplemented with linolenic acid. For example, cell exposure to 100 μmol linolenic acid/L resulted in a 36-fold increase in the formation of eicosapentaenoic acid from linolenic acid compared with control cultures that were not exposed to supplemental fatty acid. The increase in the conversion of linolenic acid to eicosapentaenoic acid after cell exposure to 200 and 300 μmol linolenic acid/L was 22- and 26-fold, respectively.

The fatty acid composition of the cellular triglyceride fraction after a 24-h incubation with media containing 0 (control), 100, 200, or 300 μmol linoleic/L or linolenic acid/L is shown in **Table 2.** The sum of these values do not equal 100% because only the major fatty acids of interest are listed. As with phospholipids, linoleic acid also increased in the triglyceride fraction (nearly two-fold for 100 μmol/L, and two-fold for either 200 or 300 μmol/L) compared with control cultures. In contrast to linoleic acid-enrichment experiments, addition of supplemental linolenic acid to culture media did not cause any significant increases in endothelial cell triglyceride linolenic acid content.

Discussion

A number of studies suggest that damage to the endothelium and the resulting disturbance in endothelial integrity may be involved in the process of atherosclerotic lesion formation (4, 5). The endothelial cells in the arterial wall are exposed to chylomicrons derived from dietary fat or VLDLs of hepatic origin (17). Triglyceride-rich lipoproteins carrying dietary fat may be particularly rich in ω-6 and ω-3 fatty acids, especially if an often advocated increase in the consumption of vegetable and fish oils has occurred. When these triglyceride-rich lipoproteins are elevated, triglyceride hydrolysis by lipoprotein lipase occurs in proximity to the endothelial surface (7, 8). This may both expose the endothelium to an excessive local concentration of fatty acid anions and lead to an accumulation of fatty acids within the endothelial cells (5, 9). Furthermore, this excessive liberation of fatty

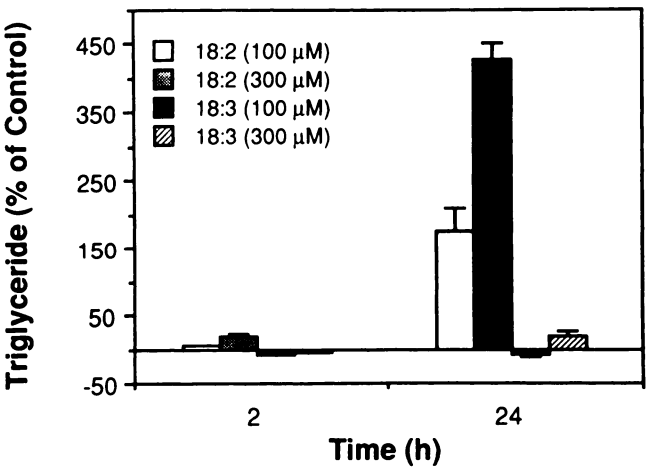

FIG 2. The effect of fatty acid enrichment on the triglyceride content of cultured endothelial cells.

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TABLE 1
Fatty acid composition of endothelial cell phospholipids after exposure to media supplemented with linoleic or linolenic acid*

		Fatty acid composition							
		Linoleic acid			Linolenic acid				
Fatty acid	Control	100 μmol/L	200 μmol/L	300 μmol/L	100 μmol/L	200 μmol/L	300 μmol/L	Pooled SEM	p
		area %			*area %*				
Palmitic (C16:0)	23.13[a]	22.50[a]	22.66[a]	18.66[c]	21.52[ab]	20.71[abc]	17.67[bc]	1.14	0.02
Stearic (C18:0)	19.96[a]	21.39[a]	21.54[a]	15.64[ab]	20.08[a]	19.92[a]	13.86[b]	1.82	0.05
Oleic (C18:1)	20.34	16.98	18.52	14.71	18.99	19.04	16.64	1.55	NS
Linoleic (C18:2ω6)	2.21[c]	8.10[c]	12.32[b]	18.39[a]	2.59[c]	3.06[c]	2.41[c]	1.49	0.0001
Linolenic (C18:3ω3)	0.80[d]	1.34[d]	0.61[d]	0.73[d]	8.18[c]	11.17[b]	16.07[a]	0.55	0.0001
Arachidonic (C20:4ω6)	4.45[a]	1.73[bc]	1.65[c]	2.81[abc]	3.81[ab]	2.61[abc]	2.85[abc]	0.62	0.05
Eicosapentaenoic (C20:5ω3)	0.03[c]	0.00[c]	0.00[c]	0.02[c]	1.09[a]	0.66[b]	0.79[b]	0.06	0.0001

* Each value represents the $\bar{x}$, $n = 3$. Means within rows with different superscripts are significantly different by Duncan's multiple range test.

acid anions during lipoprotein triglyceride hydrolysis may cause endothelial injury and in turn may allow increased penetration into the arterial wall of cholesterol-rich remnant lipoproteins derived from chylomicrons or VLDLs (5). In support of this hypothesis, one of us (18) demonstrated an increase in the transfer of albumin, a protein with a molecular weight of 67 000, across the endothelium after exposure of cultured cells to elevated concentrations of oleic acid (C18:1ω9) (10). In the present series of experiments we demonstrated that cell treatment with linoleic acid, but not with linolenic acid, decreased the barrier function of cultured endothelial cell monolayers. Because the more unsaturated linolenic acid had no effect on transendothelial movement of albumin, it is unlikely under the present experimental settings that the greater albumin transfer seen with linoleic acid was caused by peroxidative injury. In addition, the induced elevation in albumin transfer by linoleic acid was largely reversible within 8 h after removal of the medium supplemented with the fatty acid. Even though the free fatty acid concentrations we used were high from the physiological standpoint, they are still reasonable relative to values that can occur in humans. Plasma concentrations of free fatty acids can range from 180 to 1650 μmol/L (19). Assuming the normal plasma albumin concentration is ~600 μmol/L, the molar ratio of free fatty acids to albumin in human plasma will vary between 0.3 and 2.8. Metabolic studies (20) indicate that the molar ratio of free fatty acids to albumin is the main factor controlling free fatty acid availability to tissues. In the present studies these molar ratios ranged from 0 to 3. It is possible that these levels may be exceeded during hydrolysis of lipoprotein triglycerides in proximity to the endothelium.

Although our current data do not permit a definite conclusion about the exact mechanism causing increased albumin transfer across endothelial monolayers by linoleic but not linolenic acid treatment, this mecha-

TABLE 2
Fatty acid composition of endothelial cell triglycerides after exposure to media supplemented with linoleic or linolenic acid*

		Fatty acid composition							
		Linoleic acid			Linolenic acid				
Fatty acid	Control	100 μmol/L	200 μmol/L	300 μmol/L	100 μmol/L	200 μmol/L	300 μmol/L	Pooled SEM	p
		area %			*area %*				
Palmitic (C16:0)	24.35	23.70	25.59	27.81	25.52	24.30	24.98	1.15	NS
Stearic (C18:0)	26.40[ab]	26.75[a]	18.83[bc]	18.68[bc]	24.82[abc]	21.02[abc]	18.37[c]	2.32	0.05
Oleic (C18:1)	16.39	17.24	20.65	21.06	17.25	19.17	15.39	2.26	NS
Linoleic (C18:2ω6)	3.78[c]	6.22[abc]	8.50[a]	7.59[ab]	3.96[c]	4.67[bc]	4.15[bc]	1.05	0.03
Linolenic (C18:3ω3)	4.29	3.95	1.50	0.86	3.31	4.19	5.60	1.27	NS
Arachidonic (C20:4ω6)	0.11	0.11	0.15	0.06	0.08	0.07	0.18	0.07	NS

* Each value represents the $\bar{x}$, $n = 3$. Means within rows with different superscripts are significantly different by Duncan's multiple range test.

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nism is possibly related to altered lipid composition of both the cell membrane and cytosol. Cell enrichment with linoleic acid resulted both in increased incorporation of this fatty acid into phospholipids and triglycerides and in increased transfer of albumin across cultured endothelial monolayers. A more dramatic pattern of fatty acid enrichment into the phospholipid fraction of endothelial cells was observed when the media contained supplemental linolenic acid. In contrast to linoleic acid-enrichment experiments, cell exposure to supplemental linolenic acid neither caused a significant increase in the linolenic acid content of the triglyceride fraction of endothelial cells nor resulted in increased albumin transfer. Furthermore, total cell triglyceride content only increased following incubation with linoleic acid, but not with linolenic acid-enriched media. These data suggest that changes in endothelial cell triglyceride levels are related to monolayer permeability characteristics. However, this does not discount an effect of altered membrane phospholipid composition on endothelial cell monolayer permeability or a combination of these effects. It also should be noted that only linolenic acid enrichment resulted in the accumulation of its elongation products, such as eicosapentaenoic acid, in the cellular phospholipid fraction, suggesting both a high affinity of Δ^6-desaturase for linolenic acid (21) and the possible involvement of eicosanoid metabolism in functional properties of cultured endothelial cell monolayers (22).

The mechanism by which fatty acid compositional changes of endothelial cell lipids affect cell permeability is not understood completely. We speculate that these changes may result either in an increased transcellular movement of albumin through vesicles or transcellular channels (23, 24), or in the alteration of cell to cell adhesion and increased passage of macromolecules between cells. In summary, our data demonstrate an association between alterations in cell lipid composition and the integrity of the endothelium as a barrier to macromolecules. This may have implications toward the understanding of the etiology of atherosclerosis, especially with regard to injury to the endothelium and permeability to plasma components. These results suggest that the current popular recommendation of increasing dietary vegetable oils (eg, linoleic acid) may not be as prudent as the recommendation to increase dietary fish oils (eg, linolenic acid).

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