Electron Spin Resonance Studies of Fatty Acid-induced Alterations in Membrane Fluidity in Cultured Endothelial Cells

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Endothelial cell dysfunction has been implicated in the development of atherosclerosis. Of vital importance to the maintenance of endothelial cell integrity is the preservation of membrane functional and structural properties, such as membrane fluidity. The aim of this study was to develop a model for studying the relationship between endothelial cell integrity and membrane fluidity alterations in a well-defined cell culture setting. Alterations in membrane fluidity were assessed using electron spin resonance after labeling endothelial cells with the lipid-specific spin labels, CAT-16 and 12-nitroxide stearic acid. Endothelial cells were exposed to various 18-carbon fatty acids, i.e. stearic (18:0), oleic (18:1), linoleic (18:2), or linolenic (18:3), in addition to lipolyzed HDL (L-HDL) and benzyl alcohol. Membrane phospholipid fatty acid composition of endothelial cells supplemented with these fatty acids was analyzed using gas chromatography. All fatty acids, except 18:0, decreased membrane fluidity. A relationship between membrane fluidity and fatty acid compositional alterations in cellular phospholipids was observed. In particular, the arachidonic acid content decreased following exposure to 18:1, 18:2, or 18:3. Exposure of endothelial cells to L-HDL, lipoprotein particles which contain high levels of 18:1 and 18:2, also decreased membrane fluidity. The stabilization of cytoskeletal actin filaments by phalloidin partially prevented 18:2-induced increases in albumin transfer, thus implicating a cytoskeletal involvement in the 18:2-induced membrane fluidity changes involved in endothelial cell dysfunction. The present study shows that the exposure of endothelial cells to various lipids causes membrane fluidity alterations which may contribute to endothelial cell dysfunction and atherosclerosis.

Keywords: Endothelial cells Spin-labeling Electron spin resonance Membrane fluidity Fatty acids

INTRODUCTION

A number of studies suggest the involvement of endothelial dysfunction or injury in atherosclerosis (DiCorleto and Chisolm, 1986; Renkin and Curry, 1982). Therefore, the preservation of endothelial structure and function are important prerequisites for the maintenance of arterial integrity. Injury to the endothelium may result from exposure to various plasma components which alter vital cell functions through as yet unknown mechanisms. For
example, the endothelium is exposed to high concentrations of lipoproteins that are rich in triacylglycerols and cholesterol (Fielding, 1981). Hydrolysis of these lipoproteins by lipoprotein lipase occurs in proximity to the endothelial surface resulting in an excessive local concentration of fatty acid anions which have been hypothesized to cause endothelial injury (Zilversmit, 1976). An impairment in endothelial barrier function, as a result of cell injury/dysfunction, has been shown following the exposure of cultured endothelial cells to certain fatty acids, such as oleic and linoleic, as well as oxidation derivatives of linoleic acid (Hennig et al., 1984, 1985, 1986; Ramasamy et al., 1991). Much of this injury may be related directly or indirectly to the dysfunction of cell membranes. Lipid-induced disruption of endothelial barrier function may involve membrane compositional changes sufficient to alter either or both transcellular and paracellular passage of molecules across the endothelium.

The term membrane “fluidity” embodies the concept that the measured motion of a membrane bilayer-incorporated spin label is a function of the membrane lipid order, packing and motion. Thus, it has been demonstrated that there is a decrease in microsomal membrane fluidity prior to lesion formation in rabbits having cholesterol-induced atherosclerosis (Robinson and Gillies, 1986). Furthermore, Gillies and Robinson (1988) later reported that a similar decrease in membrane fluidity in cholesterol-fed rabbits was observed after 2 and 10 weeks of feeding, indicating that membrane fluidity changes occur early in the development of atherosclerosis. Even though there is evidence that alterations in membrane fluidity can be implicated in the etiology of atherosclerosis, little is known about alterations in membrane fluidity of the different types of cells in the vascular tissues (e.g. endothelial cells) in relation to the atherosclerotic disease process.

Clearly, further examination of endothelial cell membrane fluidity alterations in response to lipid exposure is important in understanding the development of atherosclerosis. In an effort to investigate this relationship, a major objective of the present study was to expose endothelial cells to lipids which are known to disrupt endothelial barrier function, and to measure membrane fluidity changes using electron spin resonance (ESR) spin labeling techniques.

MATERIALS AND METHODS

Endothelial cell culture

Endothelial cells were obtained from porcine pulmonary arteries and cultured as previously described (Hennig et al., 1984). The isolation of endothelial cells was confirmed by a “cobblestone” morphology using phase contrast microscopy (Nikon, Inc., Garden City, N.J.) and by the determination of angiotensin converting enzyme activity according to the procedure supplied by Ventrex Laboratories (Portland, ME). Experiments were conducted with cells from passages 5–16.

Endothelial barrier function determination

Albumin transfer across endothelial cells cultured on micropore filters was used as an indication of endothelial monolayer integrity or barrier function (Hennig et al., 1984). Cells were grown on polycarbonate filters (0.8 μm in pore size) commercially adhered to polystyrene chemotactic chambers (Millicell PCF, Millipore Corp., Bedford, Mass.). Endothelial cells were plated at a density of 3 × 10⁶ cells/filter in medium 199 (M199; Gibco Laboratories) +10% fetal bovine serum (FBS; Hyclone, Logan, Utah) and incubated at 37°C. Experimental media were composed of M199 +5% FBS (Control) and Control to which was added increasing concentrations of phalloidin. Following a 24-hr incubation period, the cells were incubated with experimental media, i.e. Control media and Control media to which were added 120 μM 18:2, phalloidin, or 18:2 plus phalloidin. Endothelial cells were incubated with the experimental media for another 24 hr after which albumin transfer was measured over a 1-hr period. Albumin concentrations were determined by measuring the absorbance at 630 nm following the addition of bromcresol green (Sigma Chemical Co.) to an aliquot of the media from the chamber and the well.

Spin labeling and ESR spectroscopy using CAT-16

A spin-labeling method established in erythrocytes (Wyse and Butterfield, 1988) was modified for use with cultured endothelial cells and published previously (Alvarado et al., 1994). Briefly, a 25 mM stock solution of 4-(N,N-dimethyl-1-n-hexadecyl) ammonium-2,2,6,6-tetramethylpiperidine-1-oxyl iodide (CAT-16; Molecular Probes, Eugene, OR) was prepared and endothelial cells were labeled with
prepared and endothelial cells were labeled with CAT-16 at a final concentration of 10 μM. Endothelial cells in suspension (5 × 10^7 cells/ml phosphate-buffered saline [PBS]) were incubated with CAT-16 for 30 min at 37°C. The cells were centrifuged and resuspended in 350 μl PBS and stored on ice until 30 min before obtaining the ESR spectra. All samples were coded in a blind manner and recorded. Each sample was contained in a 300 μl E-238 quartz flat cell for data acquisition. A Varian E-109 ESR spectrometer (Palo Alto, Calif.) with a TM mode cavity and computerized data acquisition was used to record the spectra at 20 ± 0.5°C at a 3365 G field set and 50 G field scan width with a modulation amplitude of 0.32 G at 100 kHz and a time constant of 0.128 sec. The acquisition time of a single scan was 16 min in order to keep the signal to noise ratio high. Spin labeling and ESR spectroscopy using 12-NS Endothelial cells were spin labeled with 12-nitroxide stearic acid (12-NS; Molecular Probes) and the resulting spectra were obtained in a fashion similar to that used with CAT-16. A 0.2 mM stock solution of 12-NS was prepared in chloroform and aliquots were dried under nitrogen gas. Endothelial cells were collected following exposure to trypsin (GIBCO Laboratories, Grand Island, N.Y.), washed 3 times in PBS and centrifuged for 10 min at 1200 rpm. The resulting cell pellet was resuspended in PBS and added to the dried spin label. Following a 30 min incubation period at room temperature, a Varian E-109 ESR spectrometer was used to obtain the spectra at 20 ± 0.5°C at a 3345 G field set and 100 G field scan width with a modulation amplitude of 0.32 G at 100 kHz and a time constant of 0.25 sec. The acquisition time of a single scan was 16 min in order to keep the signal to noise ratio high. All samples were coded in a blind manner and recorded. Data obtained from the CAT-16 and 12-NS spin labels are reported as the apparent rotational correlation coefficient (τ_r) in nsec and the half width at half height (A_{h1/2}) in Gauss, respectively. Preparation of experimental media Endothelial cells were exposed to fatty acid-enriched media as described by Hennig et al. (1984). Briefly, stock solutions of fatty acids (Nu Chek Prep, Elysian, MN) were prepared in hexane (Fisher Scientific, Pittsburgh, Pa.). After adding 1–2 drops 6 N sodium hydroxide (Fisher Scientific) to the desired amount of fatty acid, the material was dried under nitrogen gas, redissolved in approx. 0.5 ml of warm water and added to M199 + 10% FBS. The remainder of the volume consisted of M199, resulting in a final concentration of 5% FBS. All control media were composed of M199 + 5% FBS.

In some experiments, endothelial cells were exposed to dl-α-tocopherol (vitamin E; Sigma Chemical Co., St Louis, Mo.) as described by Hennig et al. (1989). A 25 mM stock solution of vitamin E was prepared in 100% ethanol (Midwest Solvent Co. of Illinois, Pekin, Ill.). To prepare the stock solution, one drop of vitamin E was weighed in a 25 ml Erlenmeyer flask and dissolved in the appropriate amount of ethanol. The desired amount of vitamin E stock solution then was added to M199 + 5% FBS. Vitamin E concentrations of 25 and 100 μM were used.

The lipolyzed lipoproteins were prepared at the University of Alabama as described by Hennig et al. (1992). Briefly, fresh hypertriglycerideremic (HTG; type IV and V) sera were obtained from volunteers at the Alabama Regional Blood Center, Birmingham, Ala. To achieve the lipolysis of triglyceride-rich lipoproteins in HTG serum, a portion of HTG serum was incubated in vitro with purified lipoprotein lipase (50 μl/ml serum) for 90 min at 37°C. The post-lipolysis samples of HTG sera then were subjected to single vertical spin density gradient ultracentrifugation as described by Chung et al. (1980) to fractionate the serum into lipolyzed VLDL, LDL, and HDL fractions. Since Hennig et al. (1992) have reported that the greatest alterations in endothelial barrier function resulted after exposure to lipolyzed HDL (L-HDL), the effect of this lipoprotein fraction on membrane fluidity was examined in the present study. The experimental media were composed of M199 enriched with 5% FBS plus 50 μg L-HDL cholesterol/ml culture medium.

Following the 24-h incubation with experimental media, the samples required several hours of preparation prior to and during the acquisition of the data. The issue of oxidation during this time was addressed by preparing the samples with PBS containing 0.5 mM ethylenediaminetetraacetic acid (EDTA). EDTA at this concentration prevents the oxidation of fatty acids and was used during procedures such as the determination of thiobarbituric reactive substances to prevent further fatty acid oxidation. A significant decrease in membrane fluidity was evident regardless of the presence of EDTA (data not shown).
Fatty acid analysis

Lipids from endothelial cells were extracted with chloroform:methanol (2:1, Vol:Vol). Polar lipids (phospholipids) were isolated by solid phase extraction (Hamilton and Comai, 1988). Methyl esters of fatty acids were prepared using boron trifluoride (14% in methanol) and analyzed by capillary gas-liquid chromatography. The methyl esters were extracted in hexane for chromatographic analysis by an HP 5890A gas chromatograph equipped with a flame ionization detector, autosampler and workstation (Hewlett-Packard Co., Avondale, Pa). A DB23 (50% cyanopropyl-50% methyl) fused silica capillary column of 30 m × 0.25 mm i.d. (J & W Scientific Co., Rancho Cordova, Calif.) was used with helium as the carrier gas. The initial oven temperature of 175°C was held for 10 min and increased at a rate of 1°C/min until the final temperature of 210°C was reached and held for 5 min. The total gas chromatographic run time was 50 min. An external standard mixture prepared from known amounts of methylated fatty acids (Nu Chek Prep) was used to obtain retention times and to develop the calibration table. Fatty acid values are presented as area percentages which represent the area under the chromatographic peaks identified as a particular fatty acid based on its retention time when compared to the external standard mixture.

Statistical analysis

All ESR measurements were performed in a blind fashion, i.e. the experimenter did not know the identity of each sample until all measurements and calculations were completed. Mean responses were compared among the treatments by a mixed model two way ANOVA with random effect due to replicates and fixed effect due to treatments. Post hoc comparison among the means were performed using Fisher's protective least significant difference procedure after accounting for the significance of the two way interactive effect. A statistical probability of $P < 0.05$ was considered significant.

RESULTS

As shown in Fig. 1A, CAT-16 contains a long, acyl chain which can intercalate into membranes and a head group consisting of a nitroxide radical and a quaternary amine which prevents the label from entering the other lipid bilayers within the cell. A typical ESR spectrum of the $M_I = +1$ low-field and $M_I = 0$ mid-field resonance lines of CAT-16 in endothelial cells was published previously from our laboratory (Alvarado et al., 1994). The precise location of CAT-16 within the membrane extracellular lamella of endothelial cells was verified by the total quenching of the ESR signal by membrane impermeable ascorbic acid (Alvarado et al., 1994). The measurements required to calculate the parameter which characterizes the motion of this label, the apparent rotational correlation time, $\tau_c$, are indicated in our previous publication (Alvarado et al., 1994). $\tau_c$ is defined as the time required for the label to rotate through an angle of one radian in space. The following equation was used to calculate $\tau_c$ from the resulting ESR spectrum of CAT-16.

$$\tau_c = 0.746(AG)[(A(0)/A(+1))]^{1/2} - 1$$

where $\Delta G$ is the peak-to-peak width of the center resonance line ($M_I = 0$) in Gauss; $A(0)$ is the peak-to-peak amplitude of the center resonance line; and $A(+1)$ is the peak-to-peak amplitude of the low-field resonance line ($M_I = +1$).

To examine the fluidity of the deeper regions of the membrane lipid bilayer, the 12-NS spin label was utilized. As shown in Fig. 1B, 12-NS is also amphipathic, containing a long, acyl chain which can intercalate into membranes, a polar head group and a nitroxide radical located on the 12th carbon atom. As opposed to CAT-16, 12-NS is distributed to all membranes within the cell (Butterfield, 1982). Since the reporter moiety on the 12-NS spin label is located on the 12th carbon (Fig. 1B), this spin label reports on the fluidity of this region of the membrane, i.e. 12 carbons into the membrane. It is well known that the further the nitroxide label is located from the polar head group in stearic acid spin-labeled membranes, the more fluid the probed environment will be and the less isolation can be
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**Fig. 2.** The low-field resonance line of 12-NS intercalated into endothelial cell membranes.

expected between the parallel and perpendicular components of the hyperfine coupling tensor (Hubbell and McConnell, 1971; Mason et al., 1977; Butterfield, 1982). Furthermore, the quantum mechanics of motionally restricted nitroxides are such that the high-field resonance lines are broader and, therefore, of lower amplitude than the low- and mid-field lines (Butterfield, 1982). Because of this and because the $M_s = 0$ mid-field parallel and perpendicular components of the hyperfine tensor overlap, the low-field side of the $M_s = +1$ low-field line provides the only usable, non-overlapping portion of the spectrum for measurements of the half-width at half height ($\Delta h_L$), an especially sensitive measure of membrane fluidity (Mason et al., 1977; Butterfield, 1982). Accordingly, the ESR spectrum of the low-field ($M_s = +1$) resonance line of 12-NS intercalated into endothelial cells is shown in Fig. 2. The $\Delta h_L$ is a parameter used to characterize the motion of this label since it primarily measures lipid motion and, in contrast to the order parameter, is independent of the polarity of its microenvironment (Butterfield, 1982; Mason et al., 1977). $\Delta h_L$ is considered to be a more sensitive measure of membrane fluidity than the order parameter, because the latter also depends on the polarity of the local microenvironment. Small alterations in membrane fluidity cause significant changes in $\Delta h_L$ before any measurable changes in the parallel and perpendicular components of the hyperfine coupling tensor, used in the calculation of the order parameter, are detected (Butterfield, 1982; Mason et al., 1977). Consistent with theoretical models and analogous to chemical exchange phenomenon, increased (decreased) lipid fluidity is associated with increased (decreased) values of $\Delta h_L$ (Butterfield, 1982).

In the present study, endothelial cell membranes labeled by CAT-16 in a blind manner exhibited a significant decrease in membrane fluidity (increase in rotational correlation time) following exposure to 150 $\mu$M 18:2 as compared to control cultures (Fig. 3). Because 120 $\mu$M fatty acid is at the upper limit of the physiologic range, no greater concentration was used in the subsequent experiments. The known membrane fluidizing agent, benzyl alcohol, was utilized in an effort to characterize the system and to provide a positive control. As shown in Table 1, increased membrane fluidity resulted in the region of the membrane surrounding the 12-NS spin label as indicated by an increase in the $\Delta h_L$.

To determine the effect of fatty acids on the fluidity near the center of the membrane, endothelial cells were exposed to media enriched with various fatty acids. Following a 24-hr exposure period, stearic acid (18:0; 120 $\mu$M) had no effect on membrane fluidity as determined by blind studies of the motion of 12-NS, whereas, oleic acid (18:1; 120 $\mu$M) significantly decreased membrane fluidity (Table 1). Endothelial cells exposed to linoleic (18:2; 120 $\mu$M) or linolenic (18:3; 120 $\mu$M) acids exhibited an even greater decrease in membrane fluidity within this region (Table 1).

**Fig. 3.** The effect of linoleic acid (18:2) on the apparent rotational correlation time of CAT-16. The experimental media were composed of M199 + 5% FBS (Control) and Control to which was added increasing concentrations of 18:2. Endothelial cells were incubated with experimental media for 24 hr prior to measuring the rotational correlation time. Values are mean ± SEM ($n = 4$). *Significantly different from 0 $\mu$M 18:2.
Table 1. The effect of benzyl alcohol, stearic (18:0), oleic (18:1), linoleic (18:2), linolenic (18:3) acids and lipolyzed-HDL (L-HDL) on the half width at half height of the 12-NS spectra

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzyl Alcohol</td>
<td>5.13 ± 0.10</td>
<td>5.58 ± 0.10*</td>
</tr>
<tr>
<td>18:0</td>
<td>5.31 ± 0.11</td>
<td>5.20 ± 0.11</td>
</tr>
<tr>
<td>18:1</td>
<td>5.31 ± 0.11</td>
<td>4.79 ± 0.12*</td>
</tr>
<tr>
<td>18:2</td>
<td>4.45 ± 0.11</td>
<td>3.65 ± 0.12*</td>
</tr>
<tr>
<td>18:3</td>
<td>4.53 ± 0.24</td>
<td>3.66 ± 0.16*</td>
</tr>
<tr>
<td>L-HDL</td>
<td>5.24 ± 0.14</td>
<td>3.38 ± 0.19*</td>
</tr>
</tbody>
</table>

*Significantly different from Control.

Endothelial cells were exposed to 25 mM benzyl alcohol for 10 min prior to measuring the half width at half height.

For lipid treatment, the experimental media were composed of M199 + 5% FBS (Control) and Control to which were added 120 μM of the respective fatty acids or 50 ng L-HDL cholesterol/ml media. Endothelial cells were incubated with experimental media for 24 hr prior to measuring the half width at half height.

In some experiments, endothelial cells were exposed to vitamin E for 24 hr prior to the incubation with 18:2-enriched media. The 18:2-induced decrease in membrane fluidity, i.e. a decrease in ΔHₐ, was not prevented by 25 μM (Fig. 4) or 100 μM (data not shown) vitamin E, suggesting that free radical lipid peroxidation was not responsible for the above results.

As determined by Hennig et al. (1992), the lipolytic remnant lipoprotein, L-HDL, caused the greatest increase in albumin transfer and had the highest concentrations of free fatty acids, in particular 18:1 and 18:2, as compared to other lipolyzed lipoprotein fractions. To examine the effect of L-HDL on membrane fluidity, endothelial cells were exposed to 50 μg L-HDL cholesterol/ml culture media for 24 hr. A highly significant decrease in membrane fluidity was observed in the region surrounding the 12-NS spin label (Table 1).

Since the membrane fatty acid composition may affect membrane fluidity, endothelial cell phospholipid fatty acid compositions were determined by gas chromatographic analysis. Endothelial cells supplemented with 120 μM 18:0, 18:1, 18:2, and 18:3 for 24 hr showed significant incorporation of the supplemented fatty acid into the phospholipid fractions (Fig. 5). However, a significant decrease in phospholipid 20:4 after exposure to 18:1, 18:2 and 18:3 was observed (Fig. 5), i.e. a decrease in polyunsaturated fatty acids directly parallels the effect of these 18-carbon fatty acids on membrane fluidity.

Cytoskeletal alterations may play an important role in increasing paracellular passage of molecules across the endothelium. Since the cytoskeleton is anchored in the membrane, any alteration in membrane fluidity could conceivably affect the cytoskeleton. The involvement of the cytoskeleton in 18:2-induced disruption of endothelial barrier function was examined by stabilizing actin with phalloidin. Partial protection against the 18:2-induced increase in albumin transfer was observed in the presence of phalloidin (Fig. 6).

DISCUSSION

Atherosclerosis is a response to injury with several lines of evidence implicating endothelial dysfunction as an initiating factor (DiCorleto and Chisolm, 1986; Renkin and Curry, 1982; Ross, 1986). Atherogenic high-fat diets contribute to elevated levels of circulating triglyceride-rich lipoproteins, i.e. chylomicrons and very low density lipoproteins. Products of triglyceride hydrolysis, such as free fatty acids and lipoprotein remnants, may cause cell injury/dysfunction. Under normal conditions, free fatty acids are bound to serum albumin with a molar ratio ranging from 0.15 to 4 under various conditions. However, the concentration of free fatty acids generated by the action of lipoprotein lipase near the endothelium may

Fig. 4. The effect of vitamin E on linoleic acid (18:2)-induced alterations in the half width at half height of the 12-NS spectra. The experimental media were composed of M199 + 5% FBS (Control) and Control to which was added 25 μM vitamin E. Following a 24-hr incubation period, the cells were incubated with Control media and Control media to which were added 25 μM vitamin E, 120 μM 18:2; or vitamin E and 18:2. Endothelial cells were incubated with the experimental media for another 24 hr prior to measuring the half width at half height. Values are the mean ± SEM (n = 3). *Significantly different from the corresponding Control.
Lipid-induced alterations in endothelial cells

Membrane Phospholipid Fatty Acid Composition

Fig. 5. The effect of 18-carbon fatty acids differing in degree of unsaturation on the fatty acid composition of endothelial cell phospholipids. The experimental media were composed of M199 + 5% FBS (Control) and Control to which were added the respective fatty acids: 120 μM 18:0, 18:1, 18:2, and 18:3. Endothelial cells were incubated with experimental media for 24 hr prior to measuring the fatty acid content. Values are the mean ± SEM (n = 4). *Significantly different from corresponding Control.

Fig. 6. The dose-dependent effect of phalloidin on linoleic acid (18:2)-induced alterations in albumin transfer. The experimental media were composed of M199 + 5% FBS (Control) and Control to which was added increasing concentrations of phalloidin. Following a 24-hr incubation period, the cells were incubated with experimental media, i.e. Control media and Control media to which were added 120 μM 18:2, phalloidin, or 18:2 plus phalloidin. Endothelial cells were incubated with the experimental media for another 24 hr prior to measuring albumin transfer. Values are the mean ± SEM (n = 6). *Significantly different from corresponding Control. †Significantly different from 18:2 supplement only.

exceed these levels significantly. Most fatty acid concentrations used in the present work resulted in a free fatty acid to albumin ratio of 4:1.

The mechanism of injury to endothelial cells caused by excessive concentrations of free fatty acids remains uncertain. The incorporation of exogenous fatty acids into endothelial membranes is likely to alter the membrane fluidity characteristics as has been reported in Ehrlich ascites cells (King and Spector, 1978) and monocytes (Kuo et al., 1990). As yet, there have been no published reports of membrane fluidity measurements in large vessel-derived endothelial cells using ESR. The results of the present series of experiments involving endothelial cell exposure to various agents support the hypothesis that the effects of certain plasma components may involve membrane fluidity changes. Specifically, the effects of free fatty acids and lipolyzed HDL on membrane fluidity using CAT-16 and 12-NS ESR spin labels were examined.

In addition to causing the greatest increases in albumin transfer (Hennig et al. 1990, 1993), 18:2 also caused significant changes in membrane fluidity. In the present study, 18:2 significantly decreased membrane fluidity both at the lipid/water interface (increased the rotational correlation time of CAT = 16; Fig. 3) and 12 carbons into the membrane (decreased the ΔHc of the 12-NS spectrum; Table 1). A decrease in membrane fluidity also was seen in 18:1- and 18:3-treated cells as compared with control cultures (Table 1). Alterations in the fatty acid composition of endothelial cells in response to media supplemented with selected fatty acids may, in part, contribute to the membrane fluidity changes which were observed. The results of the gas chromatographic analyses reported in this study of fatty acid supplemented endothelial cells are consistent with earlier reports (Spector et al., 1981; Hennig and Watkins, 1989). Specifically, a decrease in phospholipid 20:4 content following 18:2 supplementation was observed in the present gas chromatographic analyses (Fig. 5). In addition, various fatty acid supplementation decreased phospholipid 20:4 content in the following order:
18:3 > 18:2 > 18:1 > 18:0 (Fig. 5). Thus, the relative increase in saturated and decrease in highly unsaturated fatty acids paralleled the effect of these fatty acids on membrane fluidity.

Our results, however, are not in agreement with previous reports of increased membrane fluidity with an increase in membrane polyunsaturated fatty acids in incubated Ehrlich ascites cells (King and Spector, 1978). Ehrlich ascites cells enriched with 18:1, 18:2, or 18:3 exhibited greater membrane fluidity as compared to control cultures. Since the presence of oxidized lipids could explain this discrepancy, endothelial cells were enriched with vitamin E prior to lipid treatment. Vitamin E did not prevent the 18:2-induced decrease in membrane fluidity (Fig. 4). In addition, gas chromatographic analysis of the endothelial cell membrane fatty acids revealed that fatty acids isolated from endothelial cells had retention times similar to the retention times of a non-oxidized standard mixture of fatty acids. Therefore, it is unlikely, under the experimental conditions used in the present work, that lipid oxidation was a major cause of the decrease in membrane fluidity which was observed.

Endothelial cells are not able to synthesize significant amounts of 20:4 from 18:2 since Δ6 desaturase activity is very low compared to the activity of elongases and Δ5 and Δ9 desaturases (Spector et al., 1981). In addition, 18:2 also has been shown to compete with 20:4 for incorporation into endothelial cell phospholipids (Spector et al., 1981). From the results of the present study, such competition for incorporation into cellular phospholipids also could be occurring in 18:1-, 18:2-, and 18:3-supplemented endothelial cell cultures (Fig. 5). Furthermore, unsaturated fatty acids, more effectively than saturated fatty acids, compete with 20:4 for the R2 position of phospholipids (Lands and Hart, 1965; Bills et al., 1977). The replacement of 20:4, which is a long and highly unsaturated fatty acid, with shorter and more saturated fatty acids, i.e. 18:1, 18:2, and 18:3, could result in the decrease in membrane fluidity observed in the present study. Due to the structural differences between 20:4 and 18-carbon fatty acids, the primary effect of this substitution is likely to be most pronounced deep within the membrane bilayer. The results of the present study support this hypothesis, i.e. 120 μM 18:2 caused a significant decrease in membrane fluidity in the interior of the membrane near the region probed by the 12-NS spin label located 12 carbons into the membrane (Table 1), whereas, 150 μM 18:2 was necessary to decrease significantly the membrane fluidity at the lipid/water interface (Fig. 3).

The decrease in membrane fluidity following endothelial cell exposure to unsaturated fatty acids is in agreement with the changes which occurred following exposure to lipolyzed HDL (L-HDL; Table 1). In addition to albumin, lipoprotein remnants may be significant carriers of free fatty acids (Hennig et al., 1992; Spector and Fletcher, 1978; Chung and Segrest, 1986). The L-HDL used in this study contained high free fatty acid concentrations, in particular 18:1 and 18:2 and significantly increased albumin transfer (Hennig et al., 1992). In the present study, L-HDL markedly decreased membrane fluidity, which may have been a consequence of its high content of 18:1 and 18:2.

The present study provides evidence in support of the hypothesis that alterations in membrane fluidity directly or indirectly contribute to changes in endothelial barrier function. It is possible that the membrane fluidity alterations resulting from lipid supplementation cause a disruption of the junctions between cells thereby allowing enhanced penetration of albumin across the endothelial monolayer. In addition, alterations in membrane fluidity also may lead to various cellular changes which may account, at least in part, for the reported endothelial injury. For example, cytoskeletal components may be altered leading to cellular contraction which would facilitate the intercellular passage of plasma components. Such cytoskeletal rearrangements may be the result of alterations in the cellular signal transduction pathways. The data presented in this study indicate cytoskeletal involvement in 18:2-induced endothelial cell injury (Fig. 6). Since the increase in albumin transfer was not completely prevented by the cytoskeletal stabilizing agent, phalloidin, it is likely that the effect of 18:2 on albumin transfer is the cumulative result of various mechanisms of injury including alterations in endothelial cell membrane fluidity.

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