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DISRUPTION OF ENDOTHELIAL BARRIER FUNCTION: RELATIONSHIP TO FLUIDITY OF MEMBRANE EXTRACELLULAR LAMELLA

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Abstract—1. Endothelial cells were cultured in tissue culture flasks or on microcarrier beads and labeled with a lipid specific spin-label.

2. Exposure of endothelial cells to benzyl alcohol caused a dose- and time-dependent increase in membrane fluidity using electron spin resonance (ESR). Maximum fluidity was reached after a 5-min exposure to 100 mM benzyl alcohol.

3. Albumin permeability across endothelial cells cultured on micropore filters was used as an indication of endothelial monolayer integrity.

4. A significant increase in permeability occurred with 50 mM benzyl alcohol. Maximal albumin permeability was reached after a 5-min exposure to 100 mM benzyl alcohol.

INTRODUCTION

The motion of a membrane bilayer-incorporated spin label is a function of the membrane lipid order, packing and motion. These concepts are embodied in the term membrane "fluidity". Alterations in membrane fluidity have been reported in various pathologic states such as atherosclerosis (Holmes and Kummerow, 1985), cancer (Van Blitterswijk, 1985), and degenerative muscular diseases (Butterfield, 1985). Many of the hypotheses concerning the development of atherosclerosis primarily focus on the effect of cholesterol on membrane fluidity (Holmes and Kummerow, 1985; Gillies and Robinson, 1988; Gillies *et al.*, 1987). Moreover, membrane fluidity changes occurred prior to atherosclerotic lesion development in both cholesterol-fed rabbits and pigs (Gillies and Robinson, 1988; Gillies *et al.*, 1987) implicating alterations in membrane fluidity early in the development of atherosclerosis.

A number of studies suggest the involvement of endothelial dysfunction or injury in atherogenesis (Alavi *et al.*, 1983; DiCorleto and Chisolm, 1986). The endothelium serves as a selectively permeable barrier between the blood and subendothelial tissues, and a disruption of its integrity may allow accelerated deposition of plasma components such as cholesterol-rich lipoprotein remnants into the vessel wall (Zilversmit, 1976). Various plasma components are believed to be injurious to the endothelium by altering vital cell functions although their precise mechanisms of action remain unknown.

The examination of endothelial cell membrane fluidity alterations in response to injurious agents such as lipids, cytokines, and modified lipoproteins is important in the understanding of the development of atherosclerosis. The objective of the present study was to investigate the relationship between alterations in membrane fluidity and disruption of endothelial integrity, using a known membrane fluidizer. A model for studying this relationship was developed based on an established erythrocyte spin labeling procedure (Wyse and Butterfield, 1988) with modifications for use with cultured endothelial cells. The lipid specific, cationic spin label CAT-16 incorporates into the extracellular lamella of the endothelial cell plasma membrane, and its nitroxide moiety provides

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information on the lipid/water interface of the membrane. The motion of this spin label is primarily determined by the interactions of the transmembrane regions of proteins and the order and motion of the membrane lipid components (Wyse and Butterfield, 1988).

MATERIALS AND METHODS

Cell culture

Endothelial cells were obtained from porcine pulmonary arteries and cultured as previously described (Hennig *et al.*, 1984). Endothelial cells were identified 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).

Cells from passages 5 to 16 were used for the ESR experiments. Endothelial cell monolayers were grown to confluence in tissue culture flasks (Corning Laboratory, Corning, N.Y.) or on microcarrier beads (Pharmacia, Piscataway, N.J.). The culture media consisted of Medium 199 with Earle's salts (M199, GIBCO Laboratories, Grand Island, N.Y.) enriched with 10% fetal bovine serum (FBS, Hyclone Laboratories, Logan, Utah).

Membrane fluidity alterations

Tissue culture flasks were seeded with 3×10^6 cells which usually reached confluence in 3 days. The confluent monolayer was rinsed with Hanks' Balanced Salts Solution and exposed to benzyl alcohol (Aldrich Chemical Co., Inc., Milwaukee, Wis.) at 37°C for up to 30 min. Subsequently, the cells were harvested using trypsin and centrifuged at 150 g for 10 min. The cell pellet was resuspended in 0.1 M phosphate-buffered saline (PBS) and centrifuged three times to remove excess benzyl alcohol.

Endothelial cells were also grown on Cytodex 3 collagen-coated dextran microcarrier beads. Briefly, cells were grown on beads having a diameter of 150 μm and contained in culture vessels (Techne Inc., Princeton, N.J.) with attached stirring mechanisms to prevent the beads from settling. The entire apparatus was incubated at 37°C at an atmosphere of 95% air and 5% CO_2. Cell growth was monitored by direct microscopic observation of the beads. Endothelial cells were exposed to benzyl alcohol as described above.

In order to further characterize this model system, glutaraldehyde (Ted Pella Inc., Redding, Calif.) was used to induce a decrease in membrane fluidity. Furthermore, ascorbic acid was used to verify the location of CAT-16 within the plasma membrane. Since ascorbic acid is a free radical scavenger which is unable to enter the cell, the ESR signal generated by radicals at the outer surface of the plasma membrane is quenched. As a result, the degree to which the signal is eliminated is proportional to the amount of CAT-16 present in the outer leaflet of the plasma membrane.

Spin labeling and ESR spectroscopy

A spin-labeling method established in erythrocytes (Wyse and Butterfield, 1988) was modified for use with cultured endothelial cells. Briefly, a stock solution of 25 mM CAT-16 (Molecular Probes Inc., Eugene, Ore.) was prepared by dissolving 1 mg CAT-16 in 40 μl ethanol followed by 40 μl PBS. The final labeling solution of 10 μM CAT-16 consisted of 40 μl of the stock solution added to 100 ml PBS. Endothelial cells in suspension (5×10^7 cells/ml PBS) were incubated with 20 ml CAT-16 solution 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 and recorded in a blind manner. Each sample was contained in a 300 μl quartz flat cell for data acquisition. A Varian E-109 ESR spectrometer with 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.

Albumin permeability measurements

Albumin transfer across cultured endothelial cell monolayers was measured as described by Hennig *et al.* (1984). Briefly, cells were cultured on gelatin-impregnated micropore filters (Nuclepore Corp., Pleasanton, Calif.; 13 mm dia, 0.8 μm pore size). Before cell plating, treated filters were glued to polystyrene chemotactic chambers (ADAPS Inc., Dedham, Mass.) contained in a 24-well plate, sterilized with ethylene oxide and coated with fibronectin. Cells attached to filters were incubated at 37°C. After 3 days, the cells were rinsed with PBS and exposed to benzyl alcohol for up to 30 min prior to measuring albumin flux across the cell monolayers. M199 enriched with 200 μM albumin was added to the chemotactic chamber and only M199 was added to the well. After 1 hr, the albumin concentrations in the chamber and well

were determined by measuring the absorbance at 630 nm following the addition of bromcresol green (Sigma Chemical Co.).

Statistics

Mean responses were compared among the treatments by a mixed model two way analysis of variance 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. Statistical probability of $P < 0.05$ was considered significant.

RESULTS

The structure of CAT-16 and the spectrum resulting from its incorporation into endothelial cells are shown in Fig. 1. To verify its location within endothelial cells, membrane-impermeable ascorbic acid was used as an extracellular free radical scavenger. Exposure of endothelial cells for 1 min to 100 mM ascorbic acid completely quenched the ESR signal indicating the presence of CAT-16 in the plasma membrane in the outer lamella only (data not shown). The following equation was used to calculate the apparent rotational correlation time (τ) from the resulting spectrum:

$$\tau = 0.746(\Delta G)\ \{[A(0)/A(+1)]^{1/2} - 1\}$$

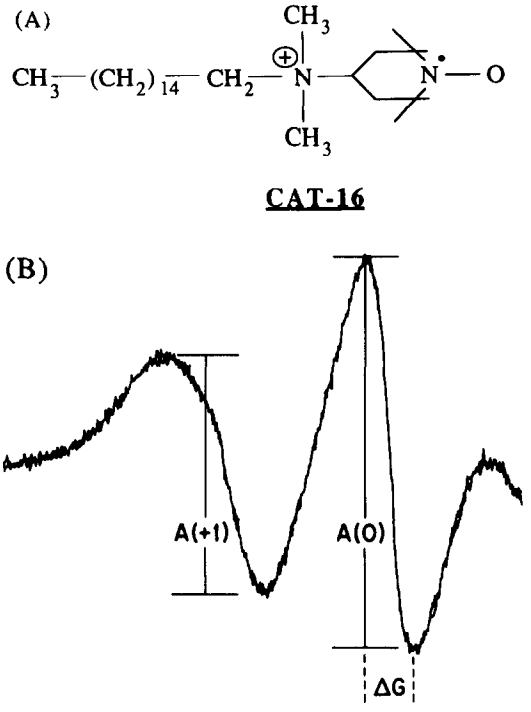

Fig. 1. (A) The chemical structure of the lipid-specific spin label, CAT-16 and (B) the resulting low-field and center resonance lines of its spectrum.

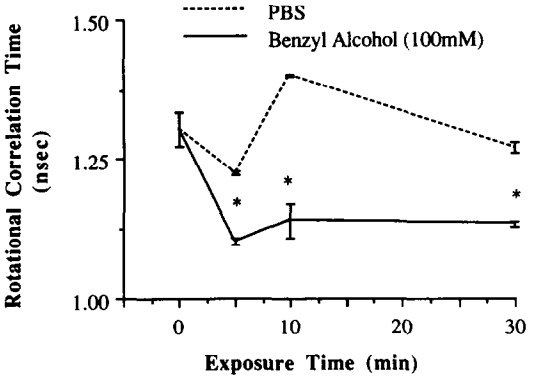

Fig. 3. The effect of increasing exposure time of PBS or benzyl alcohol on the apparent rotational correlation time of CAT-16. Values are mean ± SEM ($n = 3$). *Significantly different from PBS at each corresponding time point ($P < 0.001$).

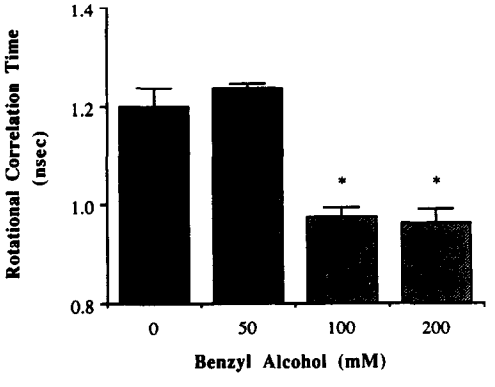

Fig. 2. The effect of increasing concentrations of benzyl alcohol on the apparent rotational correlation time of CAT-16. Values are mean ± SEM ($n = 3$). *Significantly different from 0 mM benzyl alcohol ($P < 0.002$).

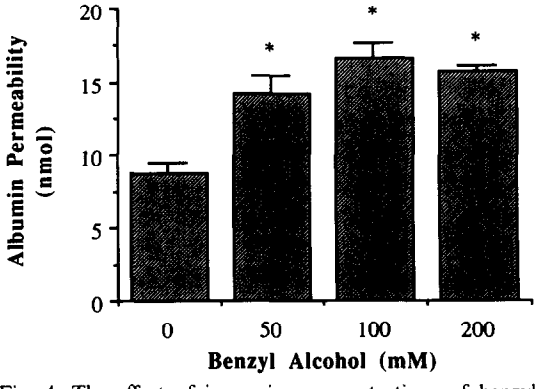

Fig. 4. The effect of increasing concentrations of benzyl alcohol on albumin permeability. Values are mean ± SEM ($n = 6$). *Significantly different from 0 mM benzyl alcohol ($P < 0.001$).

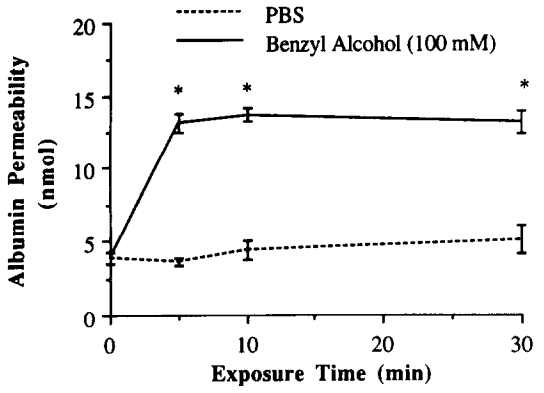

Fig. 5. The effect of increasing exposure time of PBS or benzyl alcohol on albumin permeability. Values are mean ± SEM ($n = 6$). *Significantly different from PBS at each corresponding time point ($P < 0.001$).

Where $\Delta G, A(0), A(+1)$ are, respectively, the peak-to-peak width of the center line and the peak-to-peak amplitude of the center and low-field resonance lines. The apparent rotational correlation time is a measure of the time required for the nitroxide moiety of CAT-16 to rotate one radian through space; therefore, a decrease in τ indicates an increase in the motion of the probe, i.e. an increase in membrane fluidity.

Endothelial cells cultured in flasks and on microcarrier beads were exposed to increasing concentrations of benzyl alcohol. The exposure of endothelial cells to 100 mM benzyl alcohol resulted in a significant decrease in the rotational correlation time (Fig. 2) indicating an increase in membrane fluidity. This increase in fluidity was evident after a 5 min exposure to 100 mM benzyl alcohol, as compared to the baseline control (Fig. 3). Conversely, 0.5% glutaraldehyde, known to cross link membrane components, decreased membrane fluidity (data not shown).

To determine the effect of benzyl alcohol on endothelial barrier function, endothelial cells were cultured on micropore filters and exposed to increasing concentrations of benzyl alcohol. As shown in Fig. 4, albumin permeability across endothelial monolayers increased significantly following exposure to 50 mM benzyl alcohol with a maximum effect after 100 mM benzyl alcohol. Figure 5 shows maximal albumin permeability after a 5 min exposure to 100 mM benzyl alcohol.

Phase-contrast micrographs of endothelial cells exposed to 0, 50, and 100 mM benzyl alcohol are shown in Fig. 6. Although dark, swollen nuclei were prominent (Fig. 6C), 100 mM benzyl alcohol did not result in the formation of intercellular gaps and perturbations of the cell cytosol.

DISCUSSION

Endothelial integrity is crucial in the maintenance of endothelial barrier function. The disruption of this barrier by plasma components has been proposed to occur in the development of atherosclerosis (Ross, 1986; Harrison *et al.*, 1991). Various lipids such as free fatty acids (Hennig *et al.*, 1984, 1992), oxysterols (Hennig and Boissonneault, 1987; Pettersen *et al.*, 1991) and modified lipoproteins (Steinberg *et al.*, 1989; Boissonneault *et al.*, 1991) have been shown to disrupt endothelial barrier function. It is likely that these agents alter membrane properties, e.g. possibly membrane fluidity, which results in the disruption of endothelial cell integrity. The purpose of this study was two-fold: (i) to develop a model system which allows for the examination of such membrane alterations in relation to endothelial cell injury and (ii) to expose endothelial cells to a known membrane fluidizer in order to study the relationship between membrane fluidity and endothelial barrier function.

Various spectroscopic techniques are used in the measurement of membrane fluidity, such as nuclear magnetic resonance, fluorescence, and electron spin resonance (ESR). These techniques provide information about interactions that are in part determined by the density of the liquid being studied. The term "fluidity" normally describes the ease of movement of a lipid or the inverse of viscosity (Lands and Davis, 1985). With respect to the membrane bilayer, "fluidity" refers to motion and order of bilayer lipids (Butterfield, 1985). ESR is a non-optical method and is therefore not susceptible to optical interference, a concern with other techniques such as fluorescence spectroscopy. The use of relatively small membrane probes, e.g. CAT-16 or 5-doxylstearic acid, in ESR provide a distinct advantage over larger, more bulky probes, e.g. diphenylhexatriene or 2-(9-anthroyl) stearic acid, which themselves may affect membrane fluidity due to their long, rigid structure.

Few studies have examined endothelial cells using ESR techniques. Recently, Phelan and Lange (1991) used ESR spin labels to examine the effect of cerebral ischemia and reperfusion on cerebral capillary endothelial cells. Ischemia and reperfusion resulted in a decrease in membrane fluidity. This membrane ordering effect

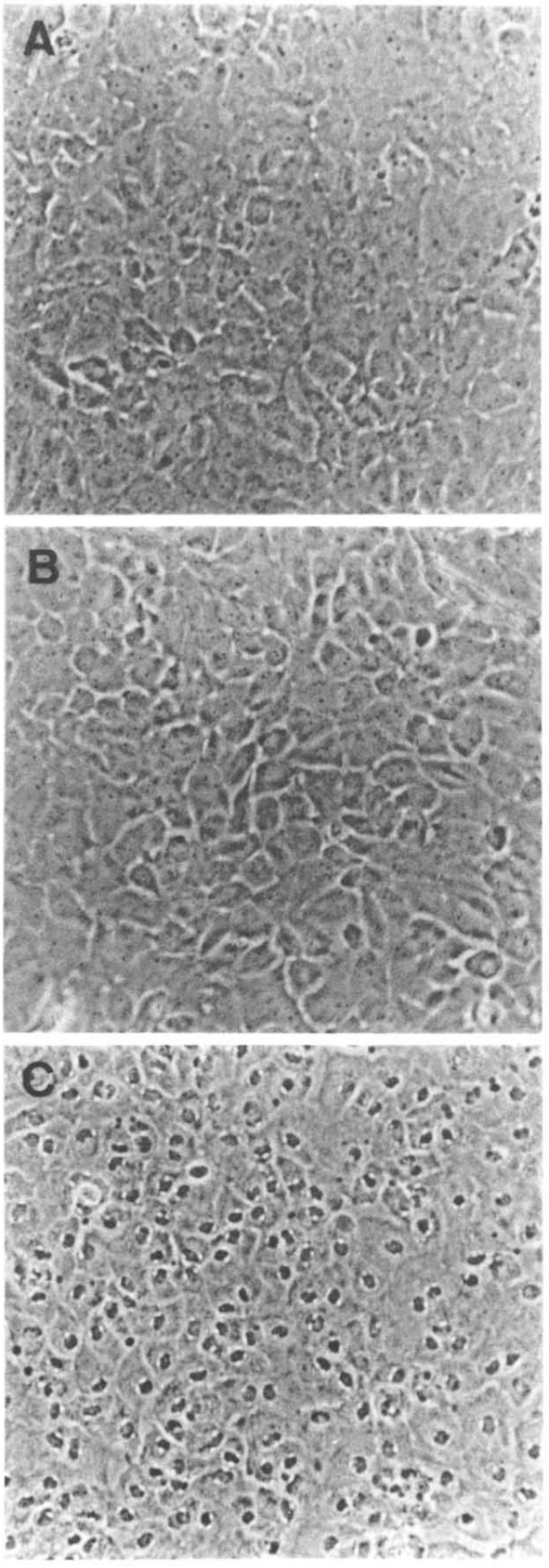

Fig. 6. Phase contrast micrographs of endothelial cells exposed to (A) 0 mM, (B) 50 mM, and (C) 100 mM benzyl alcohol for 10 min (original magnification $\times 200$).

was likely due to lipid peroxidation since the infusion of liposomal-incorporated superoxide dismutase prevented this membrane alteration. There have been no published reports of membrane fluidity measurements in large vessel-derived endothelial cells using ESR although studies utilizing other cell types such as erythrocytes (Wyse and Butterfield, 1988), tumor cells (Guffy *et al.*, 1982) and plant tissue (McKersie and Thompson, 1979) have been reported.

The present study describes membrane fluidity measurements in porcine pulmonary artery endothelial cells using the cationic spin label CAT-16. This label characterizes in particular the membrane extracellular lamella as indicated by the total quenching of the ESR signal by ascorbic acid. This system was further characterized using the anesthetic benzyl alcohol (Seeman, 1972) which is known to increase membrane fluidity (Wyse and Butterfield, 1988; Chabanel *et al.*, 1985). Using fluorescence polarization techniques, Cooper and Meddings (1991) concluded that benzyl alcohol acts primarily at the core of the bilayer in erythrocytes. Benzyl alcohol at 25 and 50 mM concentrations did not significantly alter erythrocyte membrane fluidity near the lipid/water interface of the bilayer as indicated by the 3-(9-anthroyloxy) stearic acid fluorescent probe. The results of the present study using endothelial cells confirm the non-significant effect with 50 mM benzyl alcohol and furthermore indicate that fluidization can be induced in endothelial cells at the lipid/water inferface with 100 and 200 mM benzyl alcohol. Similar effects of 100 mM benzyl alcohol on membrane order have been reported in erythrocytes spin-labeled with CAT-16 (Wyse and Butterfield, 1988).

There are various consequences of membrane fluidity alterations that may compromise cellular integrity. It is well established that membrane fluidity influences the activity of many membrane associated enzymes such as adenylate cyclase (Le Grimellec *et al.*, 1992) and recently, 5′-nucleotidase (Pieri *et al.*, 1991). In addition to altered enzyme activities, membrane fluidity changes have been associated with altered membrane function as measured by 5-hydroxytryptamine uptake and lactate dehydrogenase release (Block, 1991). Clearly, membrane fluidity can affect cell integrity and function; however, the relationship between membrane fluidity and cellular permeability characteristics has been studied only in a limited number of systems. For example, an increase in membrane fluidity induced by benzyl alcohol was associated with an increase in the "leakiness" of the tracheal epithelial cell membrane as measured by its permeability to water (Worman *et al.*, 1986). In the present study, it is not clear whether alterations in membrane fluidity are directly or indirectly affecting albumin permeability. It is possible that the fluidization induced by benzyl alcohol causes a disruption of the junctions between cells thereby allowing enhanced penetration of albumin across the endothelial monolayer. On the other hand, membrane fluidity also may lead to various cellular changes which may account for the increased albumin permeability. For example, cytoskeletal components may be altered leading to cellular contraction, followed by intercellular passage of albumin. Such cytoskeletal rearrangements may be the result of altered signal transduction pathways such as those involving eicosanoids and nucleotides. Our data suggest that there is no measurable change in membrane fluidity prior to permeability changes. This suggests that especially at low levels of benzyl alcohol, subtle disruptions of membrane integrity may be sufficient to trigger events leading to permeability changes. Since CAT-16 only characterizes membrane changes at the extracellular lamella of the plasma membrane, additional studies probing deeper within the membrane will be necessary to more fully understand the possible causal relationship between membrane fluidity and cellular permeability. These studies are currently in progress.

SUMMARY

The preservation of membrane functional and structural properties may be important prerequisites for the maintenance of endothelial integrity. To test this hypothesis, alterations in endothelial cell membrane fluidity were assessed using ESR and related to endothelial cell integrity. Endothelial cells were exposed to the known membrane fluidizer, benzyl alcohol, and labeled with the lipid-specific spin-label, 4-(*N*,*N*-dimethyl-1-*n*-hexadecyl) ammonium-2,2,6,6-tetramethylpiperidine-1-oxyl iodide (CAT-16) which localizes into the extracellular lamella of the endothelial cell plasma membrane. Benzyl alcohol caused a dose- and time-dependent decrease in the apparent rotational correlation time of CAT-16, i.e. an increase in membrane fluidity. The exposure of endothelial cells to benzyl alcohol also disrupted endothelial

integrity as measured by an increase in the passage of albumin across endothelial monolayers cultured on a micropore filter. A model for studying the relationship of endothelial membrane fluidity alterations and cell integrity has been developed in a well-defined cell culture setting. This model may be useful in determining mechanisms of action of plasma components known to disrupt endothelial barrier function, especially as this relates to changes in membrane integrity. Furthermore, membrane fluidity changes may contribute to the disruption of endothelial function in various diseases.

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